The CtrA phosphorelay integrates differentiation and communication in the marine alphaproteobacterium Dinoroseobacter shibae

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Abstract

Background: Dinoroseobacter shibae, a member of the Roseobacter clade abundant in marine environments, maintains morphological heterogeneity throughout growth, with small cells dividing by binary fission and large cells dividing by budding from one or both cell poles. This morphological heterogeneity is lost if the quorum sensing (QS) system is silenced, concurrent with a decreased expression of the CtrA phosphorelay, a regulatory system conserved in Alphaproteobacteria and the master regulator of the Caulobacter crescentus cell cycle. It consists of the sensor histidine kinase CckA, the phosphotransferase ChpT and the transcriptional regulator CtrA. Here we tested if the QS induced differentiation of D. shibae is mediated by the CtrA phosphorelay.

Results: Mutants for ctrA, chpT and cckA showed almost homogeneous cell morphology and divided by binary fission. For ctrA and chpT, expression in trans on a plasmid caused the fraction of cells containing more than two chromosome equivalents to increase above wild-type level, indicating that gene copy number directly controls chromosome number. Transcriptome analysis revealed that CtrA is a master regulator for flagellar biosynthesis and has a great influence on the transition to stationary phase. Interestingly, the expression of the autoinducer synthase genes luxI2 and luxI3 was strongly reduced in all three mutants, resulting in loss of biosynthesis of acylated homoserine-lactones with C14 side-chain, but could be restored by expressing these genes in trans. Several phylogenetic clusters of Alphaproteobacteria revealed a CtrA binding site in the promoters of QS genes, including Roseobacters and Rhizobia.

Conclusions: The CtrA phosphorelay induces differentiation of a marine Roseobacter strain that is strikingly different from that of C. crescentus. Instead of a tightly regulated cell cycle and a switch between two morphotypes, the morphology and cell division of Dinoroseobacter shibae are highly heterogeneous. We discovered for the first time that the CtrA phosphorelay controls the biosynthesis of signaling molecules. Thus cell-cell communication and differentiation are interlinked in this organism. This may be a common strategy, since we found a similar genetic set-up in other species in the ecologically relevant group of Alphaproteobacteria. D. shibae will be a valuable model organism to study bacterial differentiation into pleomorphic cells.
Background

Bacteria have in the past been regarded as very simple organisms, dividing by binary fission into clones of identical daughter cells which can perform an unlimited number of cell divisions and thus were considered immortal in the absence of external killing events. This view has since been abandoned and replaced by the concept of asymmetric cell division in most if not all bacterial species, resulting in progeny of different cellular composition, different history, and different fate [1]. Asymmetric cell division has first been recognized in those bacterial species that undergo complex differentiation processes, e.g. formation of filaments, buds, spores, swarmer cells and stalked cells. However, even binary fission is asymmetric in many bacteria, including E. coli which produces morphologically identical daughter cells. Each daughter cell contains an old and a young cell pole, and this orientation is maintained in subsequent divisions, resulting in an increasing probability of death with increasing number of cell divisions [2,3]. Like in eukaryotic cells, the number of cell divisions that an individual cell is able to perform appears to be limited in prokaryotic cells, too [1]. Asymmetric cell division through growth from one cell pole has frequently been observed in Alphaproteobacteria and recognized as a possibility to create progeny with dissimilar age and cell fates [4].

The freshwater bacterium Caulobacter crescentus became the model organism to study asymmetric cell division due to its strictly dimorphic lifestyle. A sessile stalked cell gives birth to a flagellated motile cell that cannot divide until it loses its flagellum and develops a stalk again. The complex molecular mechanisms controlling the cell cycle of C. crescentus have been thoroughly analysed [5,6]. In brief, asymmetry of daughter cells is ensured through a phosphorylation gradient of the essential response regulator CtrA [7] between the two cell poles [8]. This gradient is maintained by the trans-membrane histidine kinase CckA that is localized at the poles in the dividing cell [9]. Different enzymatic complexes maintain its activities as a kinase at the swarmer pole and as a phosphatase at the stalked pole that are transmitted to CtrA via the diffusible phosphotransferase ChpT [10]. This phosphorelay system is conserved in most Alphaproteobacteria and has been found to control divergent traits in addition to cell division. The underlying functional principles however are only poorly understood outside of the specialized Caulobacter model system [11].

Polar growth is conserved in the order Rhizobiales [12] as are most of the cell-cycle regulating genes of C. crescentus [11]. CtrA has been demonstrated to be essential in the plant symbiont Sinorhizobium meliloti [13] and might play a role during differentiation from free-living, dividing cells into non-dividing, nitrogen fixing and endosymbiotic bacteroids [14]. There is strong evidence that CtrA is also essential for replication and cell division in the plant pathogen Agrobacterium tumefaciens [15,16] as well as in Brucella abortus, the causative agent for brucellosis in mammals including humans [17].

In contrast to the aforementioned examples, CtrA has not been found to be essential in the Rhodospirillales and Rhodobacterales strains examined so far. In both orders only a subset of C. crescentus cell-cycle genes is present [11]. The differentiation of the phototroph Rhodospirillum centenum from flagellated swarmer cells into non-motile aggregating cysts is controlled by a ctrA homologue [18]. Based on evidence from the literature and their finding that CtrA controls flagellar biosynthesis but not cell division in Magnetospirillum magneticum, Greene et al. suggested that regulation of motility is the primordial role of the CtrA phosphorelay and other functions like control of the cell cycle might have been acquired later during evolution [19]. Indeed, flagella and chemotaxis gene expression but not growth and cell division are impaired in a ctrA knock-out mutant of Rhodobacter capsulatus [20,21]. In this organism however, the CtrA phosphorelay also controls expression of a gene cluster coding for the gene transfer agent (GTA) mediating a virus-like exchange of DNA between organisms [21]. As only a small fraction of the population expresses the GTA gene cluster, regulation by CtrA might be heterogeneous or bistable in this case [22]. Another exciting new finding was that expression of ctrA depends on the quorum sensing (QS) system of R. capsulatus [23]. QS refers to a form of cell-to-cell communication that involves the production, excretion and detection of small diffusible signaling molecules called autoinducers (AI). Thus, the CtrA phosphorelay is integrated into the communication system of this organism.

Dinoroseobacter shibae is a representative of the Roseobacter clade, an ecologically important phylogenetic cluster of marine Rhodobacterales [24]. It was isolated from the dinoflagellate Proorocentrum lima [25] and lives in symbiosis with marine algae [26]. D. shibae relies on acylated homoserine-lactones (AHL) for communication like many Proteobacteria [24]. Genome analysis revealed the presence of three LuxI type AHL synthase genes (termed luxI1-3). luxI1 and luxI2 are located on the chromosome down-stream of a gene encoding a LuxR-type transcriptional regulator, whereas luxI3 is on the 86-kb plasmid down-stream of a gene encoding an autoinducer but no DNA binding domain [26]. Homologs to the genes ctrA, chpT and cckA have been identified, too [27].

Recently we found that QS induces differentiation of D. shibae into pleomorphic cells utilizing distinct types of cell division [27]. A luxI1 knock-out led to a complete loss of AHL biosynthesis, homogeneous cell morphology and faster rate of cell division. Expression of the genes ctrA, chpT and cckA was strongly reduced in this strain. Here we test the hypothesis that the QS induced differentiation of D. shibae is mediated by the CckA-ChpT-CtrA phosphorelay system.
Results

Nine homologs of C. crescentus cell cycle control genes can be found in the genome of D. shibae (Table 1). Like in most other Rhodobacterales, the replication initiator dnaA, the proteases clpP/X, the DNA-methylase ccrM, the transcription factor gcrA and the divL–cckA–chpT-ctrA phosphorelay are present. Only the latter four genes showed reduced expression in the QS defect mutant [27]. As the putative divL-homolog was only weakly conserved and other putative homologs could be found in the genome of D. shibae, we concentrated on the analysis of the genes ctrA, chpT and cckA. We constructed single knockout mutants by replacing these three genes with a gentamicin resistance cassette and compared these strains to the wild-type and the QS deficient strain.

Single gene knockouts of ctrA, cckA and chpT reduce the morphological heterogeneity of D. shibae

All deletion strains were viable and as predicted by our hypothesis their phenotypes were comparable to those of the QS defective mutant. All strains showed a reduced lag phase and increased growth rate in minimal sea water medium supplemented with 5 mM succinate (Figure 1A). Interestingly, the doubling time of the ΔchpT mutant was almost identical to that of ΔluxI1. For ΔctrA and ΔcckA it was slightly longer but still reached only 63% and 65% of the wild type doubling time, respectively (Table 2). Microscopic investigation confirmed the absence of strongly enlarged and elongated cells in all three mutants during exponential growth (Figure 1B). Like in ΔluxI1 cells were very homogeneous in size, although a few slightly longer cells were found in the ΔctrA and ΔcckA strains. Scatter-plots of flow cytometric analysis of cultures in the mid-exponential phase revealed a reduction of cells with a large forward and sideward scatter – both parameters increase with cell size [28] – in all three mutants (Figure 1C). The number of cells in the upper right quadrant of the plot, representing highly enlarged cells, reached 6%, 23% and 34% of the wild-type for ΔchpT, ΔctrA and ΔcckA, respectively. These data provide evidence that the CtrA phosphorelay is indeed involved in the control of morphological heterogeneity of D. shibae.

Replicative diversification is controlled by ctrA, cckA and chpT

Next we asked whether these mutants had an altered chromosome content as it was revealed for the ΔluxI1 strain [27]. We determined the number of chromosome equivalents per cell by stoichiometric DNA staining and subsequent flow cytometric analysis. The strains ΔctrA (Figure 1D) and ΔcckA (Figure 1E) displayed a similar distribution of chromosome equivalents per cell. In both cases the fraction of cells with more than two chromosome equivalents was reduced although not completely absent as in the ΔluxI1 strain. In contrast to this finding, the distribution of chromosome equivalents per cell of the ΔchpT strain was almost identical to that of the QS deficient mutant (Figure 1F). Interestingly, the distribution of the wild-type could not be restored by complementation of any of the mutant strains with the respective gene in trans (Figure 2A). Expression of ctrA on a low copy plasmid resulted in a larger fraction of cells with three chromosome equivalents than in the wild-type. In the case of cckA no changes in the chromosome content distribution compared to the mutant were observed. Expression of chpT increased the number of cells with three chromosome equivalents compared to the wild-type. Cells with even higher chromosome content were present, too. Thus, the CtrA phosphorelay is involved in the control of replication in D. shibae. In particular it seems to induce replication without cell division, resulting in cells with increased number of chromosomes. Next we compared the expression level of all three genes in the complemented strains using RT-qPCR. Whereas ctrA and chpT showed an expression level only 1.5 fold higher than in the wild-type, cckA was

Table 1 Homologs of cell cycle related genes from Caulobacter crescentus NA1000 in D. shibae DFL-12

| Locus Tag D. shibae | Gene Symbol | Description | Locus Tag C. crescentus | Query coverage | E value | Max. identity |
|---------------------|-------------|-------------|------------------------|----------------|---------|---------------|
| Dshi_1644           | cckA        | Integral membrane sensor histidine kinase | CCNA_01132 | 50% | 2E-103 | 49% |
| Dshi_1470           | chpT        | Histidine phosphotransferase | CCNA_03584 | 89% | 2E-16 | 29% |
| Dshi_1508           | ctrA        | Two component transcriptional regulator | CCNA_03130 | 94% | 1E-126 | 74% |
| Dshi_3433           | divL        | Hypothetical protein | CCNA_03598 | 46% | 3E-25 | 34% |
| Dshi_1387           | clpX        | ATP-dependent Clp protease, ATP-binding subunit | CCNA_02039 | 97% | 0E + 00 | 81% |
| Dshi_1388           | clpP        | Endopeptidase | CCNA_02041 | 96% | 1E-104 | 69% |
| Dshi_2616           | gcrA        | Cell cycle regulator | CCNA_02328 | 98% | 1E-32 | 40% |
| Dshi_0024           | ccrM        | DNA methylase N-4/N-6 domain protein | CCNA_00382 | 94% | 2E-179 | 68% |
| Dshi_3373           | dnaA        | Chromosomal replication initiator protein | CCNA_00008 | 98% | 1E-115 | 42% |
six fold over-expressed (Figure 2B). Thus, the differences in chromosome content between wild-type and complemented mutant could be explained by the slightly higher expression level of ctrA and chpT in trans. For the failure of overexpressed cckA to influence the chromosome content of the mutant strain other explanations have to be found.
Comparative transcriptome analysis revealed substantial overlaps of gene expression in ctrA, cckA and chpT-deficient strains

To understand the global contribution of the CtrA phosphorelay to gene regulation in D. shibae, we performed microarray analysis to examine gene expression profiles of the three mutant strains compared to the wild-type in both mid-exponential and stationary phase of growth. Differential expression was assumed for genes with an absolute log2 fold change higher or equal to one and a Benjamini-Hochberg-adjusted p-value smaller than 0.05. The log2 fold-changes and p-values for all genes present on the microarray can be found in Additional file 1: Table S1, also including the microarray data for ΔluxI1 versus wild-type [27] for comparison. The differential expression of four genes was also confirmed by quantitative reverse transcription PCR (qRT-PCR). This data can be found in supplementary Additional file 2: Figure S1.

During the exponential growth phase 196 genes (28 up, 168 down) were differentially expressed in the ΔctrA mutant in comparison to the wild-type strain. In the ΔcckA mutant 104 genes (6 up, 98 down), and in ΔchpT mutant 152 genes (17 up, 135 down) were differentially expressed (Figure 3A). A total of 100 genes were differentially expressed in all three mutant strains, among which only 5% with increased, but 95% with decreased expression. Thus, the CtrA phosphorelay acts as an activator of gene expression during exponential growth.

In the stationary phase, the number of genes that were differentially expressed increased remarkably (Figure 3A). 526, 417 and 517 genes were differentially expressed in the ΔctrA, ΔcckA and ΔchpT mutant, respectively. In all three cases around half of the genes were up-regulated compared to the wild-type. 349 genes were differentially expressed in all three mutants. The large overlap in gene expression of all three mutant strains suggests that CckA, ChpT and CtrA indeed act in a common pathway in D. shibae.

Differentially regulated genes were categorized by Clusters of Orthologous Groups (COG) designations, and the numbers of genes differentially regulated both in exponential and stationary phase in each category are shown in Figure 3B. Categories that showed a notable over-representation of down-regulated genes in both growth phases included those related to cell motility, intracellular trafficking and secretion, and transcription and signal transduction. Specific groups of the CtrA regulon will be discussed in later sections. The CtrA binding site was well conserved in D. shibae and could be found in 101 promoter regions (Figure 3C and Additional file 3: Table S2).

The ctrA phosphorelay influences the adaptation of D. shibae to stationary phase

The enormous increase in the number of differentially expressed genes in the stationary phase was mainly due to the higher expression of genes falling in the categories primary metabolite biosynthesis, transport and catabolism as well as genes with a function in transcriptional control and signal transduction (Figure 3B). In particular many

### Table 2 Growth rate and doubling time of D. shibae wild-type and mutant strains

| Strain   | Growth rate ± s.d. (h⁻¹) | Doubling time ± s.d. (h) |
|----------|--------------------------|-------------------------|
| wild-type| 0.16 ± 0.00              | 4.33 ± 0.07             |
| ΔluxI1   | 0.27 ± 0.02              | 2.61 ± 0.14             |
| ΔctrA    | 0.25 ± 0.01              | 2.72 ± 0.08             |
| ΔcckA    | 0.25 ± 0.01              | 2.80 ± 0.09             |
| ΔchpT    | 0.27 ± 0.01              | 2.57 ± 0.09             |

Values represent means and standard deviations of ten biological replicates.

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genes involved in amino acid biosynthesis and transport showed a stronger expression in all three mutants. Remarkably genes encoding the cytochrome c complex, a cytochrome bd ubiquinol oxidase as well as two other cytochromes were higher expressed indicating that the redox state of mutant cells may be altered and they may adapt to
stationary phase in a different way. The activation of various genes predicted to encode heat- and cold-shock proteins as well as chaperones indicates a stress response in the mutant strains (Additional file 1: Table S1). We asked next which regulatory genes might be involved in the different adaptation strategies. Two sigma factors (\(rpoH_2\) and a putative sigma-24 factor) and one sigma/anti sigma factor pair (\(rpoE\) and \(chrR\)) showed up-regulation in all three mutants exclusively in stationary phase (Figure 3D). These \textit{bona fide} regulators of the oxidative stress response [29] therefore represent likely candidates for the observed induction of gene expression in the stationary phase. None of them had a CtrA binding site in their promoter region. The only sigma factor with a respective binding site was \(rpoH_1\) (Figure 3E) which showed reduced expression in all three mutants during both growth phases (Figure 3D). Thus, the differences between wild-type and mutants in the stationary phase might not be a result of direct regulation by the CtrA phosphorelay.

Could phenotypic observations help to interpret these data? We found that the wild-type cells were still heterogeneous in the stationary phase, although cell size was on average lower than in the exponential growth phase. In contrast, mutant strain cells – small rods in the exponential phase – were more coccoid in the stationary phase (Additional file 4: Figure S2A). Flow cytometric analysis confirmed the overall size reduction of all strains (Additional file 4: Figure S2B). Determination of the number of chromosome equivalents per cell revealed a shift of the peaks to 20% lower intensity levels (Additional file 4: Figure S2C). As this shift was not stoichiometric we assume that it was caused not by a reduction of chromosome equivalents but rather by a reduction of the RNA content in the cells. RNA has not been removed prior to staining and is also stained by SybrGreen. Furthermore the fraction of cells with only one chromosome equivalent was substantially higher for all strains. It was highest for the \(\Delta luxI_1\) mutant, followed by \(\Delta chpT\), \(\Delta cckA\), \(\Delta ctrA\) and the wild-type. The latter was also the only strain for which cells with more than two chromosome equivalents could still be found. Interestingly, some cells of the \(\Delta ctrA\) and \(\Delta cckA\) mutant strains formed long chains indicating that they could not divide properly (Additional file 4: Figure S2A). Both, transcriptome and phenotypic data suggest that the QS induced and CtrA phosphorelay mediated pleomorphism substantially alters the adaptation of \textit{D. shibae} to nutrient limitation.

The \textit{ctrA} phosphorelay regulates the expression of a gene transfer agent gene cluster

Gene transfer agents (GTAs) are phage-like particles that have first been discovered in \textit{Rhodobacter capsulatus} [30]. In \textit{D. shibae}, we have found an approximately 15 kb GTA gene cluster (Dshi_2162-2178) with identical organization like in \textit{R. capsulatus}. Our microarray results showed a slightly but consistently reduced expression of the complete GTA gene cluster in all three mutant strains. Two genes of this cluster (Dshi_2174, Dshi_2175) were significantly down-regulated in all mutants (Additional file 1: Table S1). In \textit{R. capsulatus}, CckA and CtrA have been shown to positively regulate the expression of the GTA structural genes [31]. However, only 0.15% of all cells actively expressed this cluster [22]. Assuming a similar frequency in \textit{D. shibae}, it is likely that down-regulation of the GTA cluster might not result in a strong signal for transcriptome analysis of the whole culture.

\textbf{CtrA is a master regulator of flagellar biosynthesis}

The most striking group of differentially regulated genes that were significantly changed in both growth phases were genes associated with cell motility (Figure 3B). In the \textit{D. shibae} genome 37 predicted flagellar genes are present. The majority of them are located in three gene clusters (Dshi_3246-3267, Dshi_3358-3365, Dshi_3376-3380). Two additional genes (Dshi_1409 and Dshi_1845) are located at two separate positions in the chromosome. Microarray analysis showed that all the flagellar genes were down-regulated in the \(\Delta ctrA\) mutant compared to the wild-type strain (Figure 4). Flagella genes are categorized into four functional classes which are regulated in a hierarchical way [32]. Class I genes encode the master regulators. In \textit{C. crescentus} the \textit{ctrA} gene belongs to class I which is known to activate class II flagellar genes, whose products are required for the expression of later flagellar genes [33]. To investigate if CtrA might similarly be a class I flagellar regulator in \textit{D. shibae}, we carried out prediction of CtrA binding sites in the \textit{D. shibae} genome using the well described consensus sequence (TTAA-N_1-TTAC) from \textit{C. crescentus} [11]. The results revealed that five flagellar genes possess a CtrA binding motif. They are \textit{flil} (Dshi_3246) and \textit{flir} (Dshi_3257) of the class II flagellar genes, as well as \textit{flgB} (Dshi_3247) and \textit{flgE} (Dshi_3379) of the class III flagellar genes and one gene encoding for a motor switch protein (Dshi_3380). These findings indicate that like in \textit{C. crescentus} \textit{ctrA} could belong to the class I flagellar gene and appears to be a master regulator of flagella gene expression in \textit{D. shibae}. Microarray analysis also showed reduced gene expression of all flagellar genes in the \(\Delta cckA\) and \(\Delta chpT\) mutants compared to the wild-type strain. The histidine kinase CckA and the phosphotransferase ChpT are required for phosphorylation of CtrA. These results suggest that phosphorylated CtrA is essential for the expression of flagellar genes.

\textbf{Plasmid gene expression in the \(\Delta ctrA\) mutant strain}

Genes with a significant change in expression in all three mutants were located on the chromosome, with four exceptions, among them the \textit{luxI_3} gene. This picture
changed when only those genes were taken into consideration that were regulated exclusively in the ΔctrA strain. Two of the five plasmids of *D. shibae*, pDshi01 (NC_009955) and pDshi03 (NC_009957), are in large parts syntenic (103 kb shared with 90% identity) and therefore regarded as sister plasmids of common origin [26]. Despite this strong similarity, they differ with respect to their replication operons which are from different compatibility groups [34]. We found 43 genes on pDshi01 being down-regulated only in *D. shibae* ΔctrA. In particular, the replication and toxin/antidote system showed a strong loss in the microarray signal (Additional file 5: Figure S3A). Plotting the expression changes of all genes against a theoretical normal distribution showed that the log2 fold changes were biased towards lower expression of pDshi01 (Additional file 5: Figure S3B). The observed bias in the expression changes of this plasmid leaves room for two different explanations. It could mean that CtrA acts exclusively as an activator of pDshi01 gene expression which is highly unlikely given the high degree of identity between pDshi01 and pDshi03. More likely, this bias could also be explained by a modulation of the copy number or loss of this plasmid in this mutant. PCR using pDshi01 specific primers and genomic DNA as a template demonstrated that indeed the plasmid has been lost in the ΔctrA knockout strain (Additional file 5: Figure S3C). Given the strong overlap in gene expression changes between and consistent phenotypic alterations of all three mutants we assumed that plasmid loss has no great impact on the experimental results regarding the ΔctrA mutant. However, as plasmid loss can also occur randomly, it should not be ascribed to the loss of CtrA activity.

**Regulation of *C. crescentus* cell-cycle homologs by the CtrA phosphorelay**

Transcriptome data for the nine homologs to the *C. crescentus* cell cycle regulators are summarized in Figure 5A. Deletion of *ctrA* did not affect the expression of *cckA* and *chpT* in the exponential growth phase whereas *cckA* expression was reduced in this mutant in the stationary phase. Deletion of *cckA* did not affect the expression of *ctrA* and *chpT* in both growth phases. Deletion of *chpT* caused reduced expression of *ctrA* in the exponential phase and of *cckA* in the stationary phase. Thus, the self-regulation of the CtrA phosphorelay genes seems to be dependent on the growth phase. Of the other homologs of *C. crescentus* cell cycle regulators only the putative *divL* homolog showed a reduced expression in all three mutants. Binding sites for CtrA were found in the promoter of *ctrA* itself, suggesting an auto-regulatory loop for this gene. Binding sites were also identified in the promoters of *cckA*, *divL* and *clpP*. The latter showed no response in any of the knock-outs, indicating that additional mechanisms are involved in the regulation of this gene.

**The CtrA phosphorelay induces autoinducer biosynthesis**

Recently we found that knock-out of the autoinducer synthase *luxI1* led to a reduced expression of *ctrA*, *cckA*,

![Figure 4 Regulation of flagellar biosynthesis.](image-url)
as well as the QS genes \textit{luxR}_{2I2} and the AHL synthase \textit{luxI}_3 during exponential growth and stationary phase, resulting in a complete loss of autoinducer biosynthesis [27]. Here, we found that the latter QS genes were also down-regulated in the CtrA phosphorelay mutants in both growth phases (Figure 5B). Expression of \textit{luxR}_{2I2} was most strongly reduced in the \textit{Δ}ctrA strain and for all three strains expression was lower in the exponential phase. By contrast, the down-regulation of the AHL synthase \textit{luxI}_3 was most strongly increased in the \textit{Δ}luxI2 strain and for all three strains expression was lower in the exponential phase. By contrast, the down-regulation of the \textit{luxI}_3 expression in the \textit{Δ}luxI2 and \textit{Δ}luxI3 reduced by LuxI2 or LuxI3 contributed most to the response of the sensor strain \textit{P. putida} F117 pKR-C12. The luxR transcriptional regulator of this sensor strain is derived from the \textit{las}-operon of \textit{P. aeruginosa} and is most sensitive for 3-oxo-C12-HSL. GC-MS analysis of the extracts confirms this hypothesis (Table 3 and Additional file 6: Figure S4). C18en- and C18dien-HSL as well as C14en- and 3-oxo-C14-HSL could be detected in extracts derived from a culture of wild-type \textit{D. shibae}. However, only the AHLs with a C18 side-chain could be detected in all three mutant strains. 3-oxo-C14-HSL could only be detected in the complemented \textit{Δ}ctrA and \textit{Δ}chpT strains but the concentration was below the detection limit of the GC-MS analysis.

**Integration of the CtrA phosphorelay and quorum sensing might be present in other Alphaproteobacteria**

Although the control of \textit{ctrA} expression by a LuxR-type transcription factor has recently been demonstrated in \textit{R. capsulatus} [23] no case in which CtrA regulates QS genes has been reported yet. We wondered if this regulatory relationship might also be present in other Alphaproteobacteria. We selected 89 finished genomes of Alphaproteobacteria analysis [36]. However, LuxI1 is the only one producing the C18en-HSL and C18dien-HSL which are the most powerful AI in \textit{D. shibae} and which also trigger expression of \textit{luxI}_2 and \textit{luxI}_3. Since the transcription of \textit{luxI}_1 was unchanged in the mutants, but transcription of \textit{luxI}_2 and \textit{luxI}_3 was reduced, we hypothesize that the AHLs produced by LuxI2 or LuxI3 contributed most to the response of the sensor strain \textit{P. putida} F117 pKR-C12. The luxR transcriptional regulator of this sensor strain is derived from the \textit{las}-operon of \textit{P. aeruginosa} and is most sensitive for 3-oxo-C12-HSL. GC-MS analysis of the extracts confirms this hypothesis (Table 3 and Additional file 6: Figure S4). C18en- and C18dien-HSL as well as C14en- and 3-oxo-C14-HSL could be detected in extracts derived from a culture of wild-type \textit{D. shibae}. However, only the AHLs with a C18 side-chain could be detected in all three mutant strains. 3-oxo-C14-HSL could only be detected in the complemented \textit{Δ}ctrA and \textit{Δ}chpT strains but the concentration was below the detection limit of the GC-MS analysis.
that harbor both the ctrA gene and at least one gene coding for an autoinducer synthase. We searched for CtrA binding sites in the promoters of all genes that have either an autoinducer synthesis (pfam00765) or an autoinducer binding (pfam03475) domain. We constructed a neighbor joining tree of the identified CtrA proteins in order to test whether there is a relationship between the evolution of CtrA and the evolution of its integration into QS systems. We found that – among the selected genomes – CtrA binding sites in front of QS genes were restricted to the orders Rhizobiales and Rhodobacterales and also scattered in these orders (Figure 6A). In particular, this constellation was found in several (Brady-)Rhizobiaceae strains but absent from the Methylobacteriaceae family and the genus Rhodopseudomonas. Inside the order Rhodobacterales, putative CtrA regulation of QS was restricted to some marine Roseobacter strains. Examples of the genome environment are depicted in Figure 6B. One outstanding example is Rhizobium etli CFN42 with identified CtrA binding site in front of four QS genes. Three of them are located on plasmids, and the autoinducer synthase gene is located upstream of the repABC-type partitioning system. Another binding site was found in a bidirectional promoter between an autoinducer binding transcription factor and a chemotaxis protein encoding gene. The same constellation was also found for Rhizobium leguminosarum bv. trifolii CB782. It has already been shown that QS controls plasmid replication in Rhizobiales [37]. The marine Roseobacter species Octadecabacter antarcticus 307 has two luxR/luxI pairs encoded in its genome. The first one has one binding site in front of the luxI gene, the second has two binding sites in front of the luxR gene. In contrast Ruegeria pomeroyi and Roseobacter litoralis have only one luxR/luxI pair with two and one CtrA binding site, respectively. The scattered occurrence and the different ways how CtrA is integrated into QS control suggest that this regulatory mechanism has evolved several times independently in Alphaproteobacteria.

**Discussion**

17 years of thorough research have brought to us a comprehensive understanding of how the CtrA phosphorelay tightly controls cell cycle progression and differentiation in the strictly dimorphic Caulobacter crescentus. Here we studied the role of this regulatory system in a marine bacterium that represents the antithesis to this well-studied model organism. Growth and cell-division of *D. shibae* seem to be totally chaotic – as long as the cells are able to communicate. Cell size and morphology are very heterogeneous in the wild-type and cells divide by binary fission as well as budding from one or both cell poles. Heterogeneity in cell-size and type of cell division is lost in a QS deficient strain [27]. We now have demonstrated that the QS induced pleomorphic lifestyle is controlled by the CtrA phosphorelay. The regulated traits are summarized in Figure 7 and will be discussed in the following sections.

Knock-out of *ctrA, cckA* and *chpT* resulted in a similar reduction of morphological heterogeneity and cell size as in the QS mutant confirming that they act in a common pathway controlling cell growth. We further demonstrated that all three genes are involved in the control of replication as their loss led to reduction of cells with a larger number of chromosomes in the population. This was not unexpected as cell size and replication are coordinated in bacteria [38]. In contrast to *ctrA* and *chpT* genetic complementation of Δ*cckA* failed to shift the population towards cells with higher chromosome content. As the gene was transcribed and autoinducer synthesis could be restored in trans, it is unlikely that the introduced plasmid was not functional at all. The sensor histidine kinase is a trans-membrane protein. In *C. crescentus* it is specifically localized at the cell poles and creates a gradient of phosphorylated CtrA [39]. In *D. shibae*, the six fold over-expression of cckA might lead to a mislocation of the protein and therefore a gradient that might be necessary for differentiation into cells with multiple chromosomes could not be formed.

The phenotype of the ΔchpT mutant – in contrast to ΔctrA and ΔcckA – is almost identical to that of the ΔluxI1 mutant. However, comparison of the transcriptome data from ΔchpT and ΔluxI1 [27] reveals only few genes that are differentially expressed in both strains but not in ΔctrA and ΔcckA (Additional file 1: Table S1). Among them are genes encoding parts of an ATP synthase (*Dshi_3028-3031*) that are up-regulated and T4SS (*Dshi_3975/3976*) that are down-regulated in both mutant strains as well as few hypothetical genes. However, these data offer no explanation for the physiological differences between the aforementioned strains. In *C. crescentus*, CtrA is not the only target of phosphorylation by ChpT [10] and the differences between ΔchpT and ΔluxI1 (in the latter the whole phosphorelay is down-regulated) on one side and ΔctrA and ΔcckA on the

| Strain | 14en-HSL | 3-oxo-14HSL | 18en-HSL | 18dien-HSL |
|--------|----------|-------------|----------|------------|
| Wild-type | x | x | x | x |
| ΔctrA | | x | | x |
| ΔctrApHW1 | | x | | x |
| ΔcckA | | | x | x |
| ΔcckApHW2 | | x | | x |
| ΔchpT | | | | x |
| ΔchpTpHW3 | | | | x |
other might be a result of altered phosphorylation states rather than gene expression.

The remarkable variety of cell shapes and sizes as well as chromosome copy numbers of *D. shibae* naturally raises three questions: How is this heterogeneity induced, are there other traits that are heterogeneously regulated as well and finally, how does the population benefit from such heterogeneity? At the moment we can only speculate on the mechanisms underlying the development of heterogeneous cells. It might be stochastic gene expression or a bistable regulatory feedback loop (reviewed in [40] and [41]). Single cell techniques have to be developed to answer this question. We showed that the control of flagellar biosynthesis by CtrA is also conserved in *D. shibae*. Recently it has been demonstrated that under the same growth conditions that have been used for experiments in this manuscript, only a fraction of *D. shibae* cells are motile. Thus flagellar biosynthesis seems to be heterogeneously distributed, too [42]. Furthermore it seems reasonable that the GTA cluster in *D. shibae* is heterogeneously activated like in *R. capsulatus* [32].

Differentiation clearly represents a burden for population growth of *D. shibae*, as the doubling time is longer and the maximum number of cells is reduced [27]. In this context one might ask if it also represents a burden during adaptation to stationary phase. Our observations suggest that *D. shibae* reduces its cell size in response to nutrient depletion like marine Vibrios [43]. This cell-size reduction is of course easy to achieve if the cells are already small on entry to stationary phase, and should reduce the need for costly cellular reprogramming. However, our data also suggest that the fast dividing mutant cells might be subjected to higher oxidative stress than the slower growing wild-type population. Thus, fast growth in the exponential phase could also represent a burden during adaption to stationary phase. The role of the CtrA phosphorelay for adaptation to nutrient depletion clearly needs more in depth analysis.

Our recent paper on the role of the LuxI1 autoinducer synthase [27] together with the present work suggest a strong integration of QS and the CtrA phosphorelay. Our hypothesis was that LuxR1 and LuxI1 are at the top of a hierarchical regulatory system that activates expression of CtrA phosphorelay genes, the luxR3 luxI3 operon and the plasmid encoded luxI3. The present findings suggest that CtrA actually activates luxR2 luxI2 expression; consequently the phosphorelay is not only regulated by but actually integrated into the QS system. Thus, the CtrA mediated differentiation leads to enhanced autoinducer production. This has not been reported for any other organism so far, but our promoter analysis in *Alphaproteobacteria* predicts that this kind of integration of these two regulatory systems might indeed be widespread. The question how the cell or the whole population benefits from this regulatory constellation will be addressed in future research.

**Conclusion**

It is now recognized that QS is not only employed to coordinate the behavior of a bacterial population but often...
induces heterogeneous responses towards signaling molecules. This has for example also been demonstrated for the marine pathogen *Vibrio harveyi* [44,45] and the marine symbiont *Vibrio fischeri* [46]. *D. shibae* is an outstanding organism as its heterogeneity is manifested as a pronounced morphological pleomorphism. The fact that loss of morphological heterogeneity results in faster growth will be beneficial for screening libraries of mutants in order to identify genes that are involved in this differentiation process. Studying this organism in more detail will also enhance our understanding of the evolution of CtrA phosphorelay mediated control of cellular polarity, which in the case of *C. crescentus* leads to a tightly controlled cell cycle and in the case of *D. shibae* leads to high heterogeneity of replication and cell division.

**Methods**

**Bacterial strains and growth conditions**

The strains and plasmids used for this study are listed in Additional file 7: Table S3. *D. shibae* DFL-12 strains were grown at 30°C and 160 rpm in half-concentrated Marine Broth or on the same medium solidified with 1.5% agar. Alternatively, cells were grown in a chemically defined sea water medium (SWM) supplemented with 5 mM succinate, prepared as described previously [29]. When appropriate, 150 μg/ml of gentamicin or 500 μg/ml of kanamycin was added. *Escherichia coli* strains were grown at 37°C and 180 rpm in Luria-Bertani (LB) broth or on LB agar supplemented with ampicillin (100 μg/ml), gentamicin (20 μg/ml), kanamycin (50 μg/ml), and/or aminolevulinic acid (50 μg/ml) if necessary.

**Construction of *D. shibae* deletion mutants**

For deletion of *ctrA* (Dshi_1508), *cckA* (Dshi_1644), and *chpT* (Dshi_1470), each full-length ORF with flanking regions (approximately 800 bp on either side) was amplified by PCR using *D. shibae* DFL12 genomic DNA and appropriate primers (Additional file 8: Table S4). These fragments were cloned into the multiple cloning site of pEX18Ap, a suicide plasmid carrying an ampicillin resistance cassette. From the resulting plasmids, the target ORFs were replaced with a gentamicin resistance cassette from pBBR1MCS-5 [47] using the CloneEZ PCR Cloning Kit (GenScript, Piscataway, USA) according to the manufacturer’s instructions. The resulting constructs were transferred to *D. shibae* DFL12 using conjugation with *E. coli* ST18 as a helper strain [48] and integrated into the wild-type strain by double-homologous recombination. The transconjugants were selected on half-concentrated Marine agar plates containing gentamicin. All mutant strains constructed in this study were confirmed by PCR and sequencing.

**Complementation of mutants**

Complementation of the deleted genes under the control of their native upstream sequences was performed with the broad-host-range vector pBBR1MCS-2 [47]. The complementing fragments were amplified by PCR using appropriate primers (Table 2) and cloned into pBBR1MCS-2, generating plasmids pHW1, pHW2 and pHW3. These plasmids were transferred into the respective mutant strain via conjugation using *E. coli* ST18 [49]. All complementations were also confirmed by PCR and sequencing.

**Microscopy**

Cell morphology was observed with an Olympus BX60 microscope with a Plan 100×/1.25 oil immersion objective. All images were acquired using the Olympus OM-4 Ti camera and the cell B image acquisition software (Olympus, Japan).

**Growth curves**

For growth curves, cell material from half-concentrated MB plates was inoculated into 20 ml of SWM + 5 mM succinate and incubated overnight at 30°C and 160 rpm to obtain a pre-culture. Optical density at 600 nm (OD\text{600}) was measured with spectrophotometer Ultrospec 3100 pro (Biochrom Ltd, Cambridge CB4 0FJ England). Cultures were diluted to 0.01 OD\text{600} and then 200 μl were placed into each well of a Honeycomb 2 plate (100 wells each plate, Oy Growth Curves Ab Ltd, Helsinki, Finland). OD\text{600} was monitored every 30 min for 36 hours in the automated microbiology growth analysis system Bioscreen C (Oy Growth Curves Ab Ltd, Helsinki, Finland). Growth curves were plotted in ln scale using the R program.

**Sampling**

Cultures for the flow cytometry and microarray analysis were inoculated into 100 ml medium in 300 ml Erlenmeyer flasks with an initial OD\text{600} of 0.01 from overnight cultures and incubated for 36 hours. Samples of the wild-type and all mutant strains were taken in the middle of the exponential phase at an OD\text{600} of approximately 0.4 and in the stationary phase 6 h after OD\text{600} had reached a maximum.

**Flow cytometry**

Samples for flow cytometry analyses were fixed at a final concentration of 2% glutaraldehyde and stained with SYBR Green I (Molecular Probes, Leiden, The Netherlands) by diluting the stock reagent 1: 10,000 into the samples. For each experiment, the DNA content was measured in a population of 50,000 cells using the BD FACS Canto analyzer. The data were analysed using the ‘flowCore’ package [50] of the R BioConductor project.
**RNA isolation**

Cells were collected by centrifugation at 12,000 × g for 1 min at 4°C, covered with 1 ml Trizol reagent (Ambion, Germany), immediately frozen in liquid N2 and stored at −70°C until processing. For RNA extraction, cells were homogenized with ~0.3 g of glass beads in the FastPrep-24 instrument (MP Biomedicals, California, USA) at 6.0 m/s for 3 min and then incubated for 5 min at room temperature. Samples were centrifuged at 12,000 × g for 10 min at 4°C and the supernatants were transferred to fresh tubes, followed by the addition of 100 μl of 1-bromo-3-chloropropane (BCP, Sigma, Germany) and incubation for 10 min at room temperature. Samples were centrifuged at 12,000 × g for 10 min at 4°C, after which the aqueous phase was transferred to new tubes and mixed with 500 μl of absolute ethanol. Extracts were applied to RNeasy spin columns (RNeasy mini kit, Qiagen, Hilden, Germany) and processed according to the manufacturer’s instructions. In addition, samples were treated with DNase I (Qiagen, Hilden, Germany) and processed according to the manufacturer’s instructions. Removal of genomic DNA was verified via PCR. The concentration of RNA was quantified using a NanoDrop spectrophotometer (Peqlab, Erlangen, Germany) and the RNA integrity was assessed using Bioanalyzer 2100 (Agilent, Santa Clara, USA).

**Microarray experiment**

2 μg of total RNA was labeled with Cy3 or Cy5 using the ULS-system (Kreatech, Amsterdam, The Netherlands) according to the manufacturer’s protocol. 600 ng of labeled RNA was fragmented and hybridized to the microarray according to Agilent’s two-color microarray protocol. Two biological replicates were applied in this experiment.

**Microarray data analysis**

Microarray slides were scanned using the Agilent DNA Microarray Scanner. Median foreground and background signals of the Cy3 and Cy5 channel were loaded into the R environment and processed using the LIMMA package [51]. Spots were down-weighted if 2 or more quality flags were set by the scanner software. Background signals were subtracted using the normexp method [52]. Cy3 and Cy5 signals were Loess normalized and finally quartile normalization was performed on all microarrays from one dataset. Signals from replicate probes for single genes were averaged. A linear model was fitted for each comparison set. Signals from replicate probes for single genes were Loess normalized and finally quantile normalized. The whole extraction process was repeated three times. The combined DCM fractions were concentrated to 2 ml using a rotary evaporator (Heidolph VV2001, Schwabach, Germany) and stored at −20°C until the bioassay was conducted. For bioassays, 20 μl of the concentrated extract was applied to each well of a 96 well polypropylene micro-titer plate (PlateOne, Starlab, Hamburg, Germany). After air-drying, the micro-titer plate was overlaid with 100 μl of medium and 100 μl of the sensor strain and then incubated at 30°C with gentle agitation for 24 hours. Green fluorescence and optical density at 620 nm were determined in a Victor3 1420 Multilabel counter (PerkinElmer, Waltham, USA). The presence of AHLs in the strains was calculated by dividing the specific fluorescence (gfp535/OD620) of extracts by that of the negative control (DCM).

**Validation of microarray data by qRT-PCR**

Reverse transcription of the same RNA samples as those used for microarray analysis was performed with the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany), according to the manufacturer’s protocol. qPCR analysis was performed using the LightCycler 480 (Roche, Mannheim, Germany) with the QuantiTect SYBR Green PCR Kit (Qiagen, Hilden, Germany). Primer sequences are listed in Additional file 8: Table S4. Relative expression was calculated by the ΔΔCt method and normalized to the dapB gene coding for dihydrodipicolinate reductase. dapB was selected as reference gene based on the microarray result, as it shows similar expression in all strains. The experiment was performed in duplicate.

**Identification of transcription factor binding sites (TFBS)**

For the identification of binding sites for the transcription factor CtrA, promoter-regions ranging from 400 bp upstream to 50 bp downstream of the translation start were searched for matches with a position weight matrix (PWM) obtained from a comparative phylogenetic analysis of cell cycle regulation in Alphaproteobacteria [11]. A TFBS was considered as identified if its score reached at least 85% of the maximum weight of the PWM.

**Extraction and detection of AHLs**

The production of AHLs was detected using the biosensor strain P. putida F117 pKR-C12 as previously described [24] with the following modifications. All D. shibae strains were grown in 100 ml of SWM with 5 mM succinate and 4% of adsorber resins (Amberlite XAD-16, Rohm & Haas) at 30°C and 160 rpm for 48 hours. Adsorber resins were harvested after 2 days by filtration and transferred to a separating funnel together with 50 ml distilled water, and then those resins were extracted with 25 ml of dichloromethane (DCM). The mixture was shaken vigorously for 1 min and the phases were allowed to separate. The DCM fraction was removed and another 25 ml of DCM was added. The whole extraction process was repeated three times. The combined DCM fractions were concentrated to 2 ml using a rotary evaporator (Heidolph VV2001, Schwabach, Germany) and stored at −20°C until the bioassay was conducted. For bioassays, 20 μl of the concentrated extract was applied to each well of a 96 well polypropylene micro-titer plate (PlateOne, Starlab, Hamburg, Germany). After air-drying, the micro-titer plate was overlaid with 100 μl of medium and 100 μl of the sensor strain and then incubated at 30°C with gentle agitation for 24 hours. Green fluorescence and optical density at 620 nm were determined in a Victor3 1420 Multilabel counter (PerkinElmer, Waltham, USA). The presence of AHLs in the strains was calculated by dividing the specific fluorescence (gfp535/OD620) of extracts by that of the negative control (DCM).
GC/MS analysis
A volume of 1 μL of the extract was injected into an Agilent GC 7890A gas chromatograph connected to a 5975C mass-selective detector (Agilent) fitted with a HP-5 MS fused silica capillary column (30 m × 0.25 mm i.d., 0.22 μm film; Hewlett-Packard, Wilmington, USA). Conditions were as follows: inlet pressure: 67.5 kPa, He 24.2 ml min⁻¹; injection volume: 1 μL; injector: 250°C; transfer line: 300°C; electron energy: 70 eV. The GC was programmed as follows: 50°C (5 min isothermal), increasing at 5°C min⁻¹ to 320°C, and operated in splitless mode; carrier gas (He): 1.2 ml min⁻¹. Retention indices were determined from a homologous series of n-alkanes (C8-C32).

Distribution of CtrA-binding sites in QS gene promoters in Alphaproteobacteria
Finished genomes of Alphaproteobacteria were analyzed for at least one gene with an autoinducer synthesis domain (pfam00765) and a CtrA homolog present were selected from IMG (http://img.jgi.doe.gov/). Promoter regions ranging from 400 bp up to 50 bp downstream of the start codon were downloaded for all genes with an autoinducer synthesis (pfam00765) or an autoinducer binding (pfam03475) domain (Additional file 9: Dataset S1). These regions were searched for putative CtrA binding sites the same way as described for the D. shibae genes.

CtrA phylogeny in Alphaproteobacteria
The evolutionary history of CtrA protein sequences found in Additional file 10: Dataset S2 was inferred using the Neighbor-Joining method [54]. The optimal tree with the sum of branch length = 3.83257521 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method [55] and are in the theoretical normal distributed dataset demonstrates the bias towards down-regulation in the ΔctrA strain. ctrA red; cckA green; chpT blue. (C) Gel electrophoresis testing the existence of pDs1h01 in the three mutant strains using two primer combinations. Plasmid DNA from the wild-type strain was used as positive control and DNA of ΔpDs1h01 strain as well as water were used as negative controls.

Additional files

Additional file 4: Figure S2. Phenotypic characterization of stationary phase cell extracts. (A) Phase contrast microscopy of D. shibae DFL12 wild-type and mutant strains in the stationary phase. Scale bar represents 20 μm. (B) Flow cytometric representation of morphological differences between mutant and wild-type strains in the stationary phase based on size (FSC, forward scatter) and granularity (SSC, side scatter). Numbers in plots (top right quadrant) indicate percent larger cells in this area. (C) Comparing the chromosome equivalent profiles of the corresponding strains in the exponential (grey area) and stationary phases (black line) by flow cytometry of SYBR Green I-stained cells (50,000 events counted). The x and y axes are in log, scale and represent fluorescence intensity and cell density. (D) Boxplot showing the differences in cell size of indicating strains in the exponential and stationary phase.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
HW, LZ and VM performed experiments, HW, LZ, OF, MM, JP, SS and JT analyzed the data, HW, LZ, OF, IWD and JT wrote the manuscript. All authors read and approved the final manuscript.

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