Cell cycle transition from S-phase to G1 in *Caulobacter* is mediated by ancestral virulence regulators

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Zinc-finger domain transcriptional regulators regulate a myriad of functions in eukaryotes. Interestingly, ancestral versions (MucR) from Alpha-proteobacteria control bacterial virulence/symbiosis. Whether virulence regulators can also control cell cycle transcription is unknown. Here we report that MucR proteins implement a hitherto elusive primordial S→G1 transcriptional switch. After charting G1-specific promoters in the cell cycle model *Caulobacter crescentus* by comparative ChIP-seq, we use one such promoter as genetic proxy to unearth two MucR paralogs, MucR1/2, as constituents of a quadripartite and homeostatic regulatory module directing the S→G1 transcriptional switch. Surprisingly, MucR orthologues that regulate virulence and symbiosis gene transcription in *Brucella*, *Agrobacterium* or *Sinorhizobium* support this S→G1 switch in *Caulobacter*. Pan-genomic ChIP-seq analyses in *Sinorhizobium* and *Caulobacter* show that this module indeed targets orthologous genes. We propose that MucR proteins and possibly other virulence regulators primarily control bacterial cell cycle (G1-phase) transcription, rendering expression of target (virulence) genes periodic and in tune with the cell cycle.
How S-phase cells instate the G1-phase transcriptional programme is poorly understood. The synchronizable Alpha-proteobacterium Caulobacter crescentus (henceforth Caulobacter) is the pre-eminent model system commonly used to dissect cell cycle transcription at the most fundamental level. Caulobacter divides into a smaller and motile swarmer cell and a larger and sessile stalked cell, residing in G1- and S-phase, respectively (Fig. 1a). Such asymmetric division has also been reported for related Alpha-proteobacterial pathogens/symbionts belonging to the genera Brucella, Agrobacterium or Sinorhizobium, some of which are also synchronizable. As Alpha-proteobacteria generally encode most known cell cycle regulatory proteins originally identified in Caulobacter, the underlying mechanisms for the G1-phase transcriptional programme seem to be conserved and perhaps serve to control expression of virulence/symbiosis functions as a function of the cell cycle. Indeed, virulence functions are typically present in non-replicative dispersal cell types of unrelated pathogens such as members of the Chlamydiaceae, and features of an underlying cytological and/or molecular asymmetry have also been reported for the pathogenic Gamma-proteobacteria Pseudomonas aeruginosa and Klebsiella pneumoniae and distantly related mycobacteria. This suggests that the implementation of daughter cell-specific transcriptional programs is pervasive among different prokaryotic lineages, and that lineage-specific mechanisms direct this re-programming.

In Caulobacter, the two daughter cell types can be conveniently discerned based on functional and morphological criteria: while the G1-phase cell harbours several adhesive pili and a single flagellar motor at the old pole, the S-phase cell harbours a stalk with an adhesive holdfast at the corresponding pole. In addition, these two cell types differ in their buoyancy, a feature that is exploited for the enrichment of a pure population of G1 cells on a density gradient. A hallmark of the Caulobacter G1→S transition is the loss of the flagellum and pili, the elaboration of a stalk and holdfast, as well as the switch in cellular buoyancy. In the ensuing S-phase, cells segregate the replicated DNA, activate motility genes and assemble the flagellar motor and pilus secretion apparatus at the pole opposite the stalk. As soon as the predivisional cell compartmentalizes, the G1-phase transcriptional programme is instated in the swarmer chamber, pili are extruded, the flagellum is energized and the cellular buoyancy is reversed. In the stalked chamber, DNA replication re-initiates and S-phase transcription resumes.

How the switch from S-phase to the G1-phase transcriptional programme (henceforth referred to as S→G1 transcriptional switch) is induced at compartmentalization is unresolved. PpilA, the promoter of the gene encoding the structural component of the pilus filament (pilA), is a target of this regulation and thus suitable as a genetic proxy. PpilA is activated in G1-phase11 by the conserved and essential cell cycle transcriptional regulator A (Ctra)12. Ctra can function either as an activator or repressor of transcription and also as an inhibitor of DNA replication by directly binding the TTAA-N(7)-TTAA target motif (Ctra box) in promoters and the origin of replication, Cori13. Binding of Ctra to its targets is enhanced by phosphorylation (Ctra→P) of aspartate 51 (D51) by way of a complex phosphorylating the conserved and essential hybrid histidine kinase Ccka13. Ctra is proteolytically removed during the G1→S transition, re-synthesized and again phosphorylated by the ClpXP protease in the nascent S-phase chamber upon compartmentalization1. Thus, Ctra is not a G1-specific regulator as it is already present and active before compartmentalization (that is, in late S-phase), for example, at promoters of early (class II) flagellar and other motility genes1,14. Determinants other than Ctra→P likely promote the switch from S→G1 phase transcription of Ctra-dependent genes during compartmentalization15,16.

A candidate for such an accessory role is the conserved helix-turn-helix motif protein SciP that accumulates in G1-phase17,18. SciP binds Ctra directly and impairs Ctra’s ability to recruit RNA polymerase (RNAP) holoenzyme to PpilA and other Ctra-activated promoters in vitro, apparently without establishing sequence-specific contacts to DNA18,19. An alternative view holds that SciP does not require Ctra to bind DNA. Instead, SciP was proposed to bind DNA directly at the 5′-TGTCGCG-3′ motif in the Ctra promoter in vitro20. Surprisingly, the occurrence of this motif (>1,460 predicted sites in the 4.01 Mbp GC-rich Caulobacter genome) vastly exceeds the number of previously predicted Ctra target promoters with ~40 (50). Also, mutation of the 5′-TGTCGCG-3′ motif did not affect binding of Ctra and SciP to PpilA in vitro20. As both models posit that SciP targets all Ctra-dependent promoters, further investigations on the S→G1 transcriptional switch and on the possible role of SciP in this event are warranted.

Here we report a system-level and forward genetic approach for the dissection of this transcriptional switch. We unearthed two uncharacterized ancestral zinc-finger domain proteins, MucR1 and MucR2, as key determinants of a novel quadrupartite and homeostatic regulatory module that together with Ctra and SciP turn on G1-phase genes and concomitantly shut off S-phase genes, respectively. Using pan-genomic ChIP-seq (chromatin immunoprecipitation coupled to deep-sequencing), we reveal MucR as a direct regulator of orthologous genes in Sinorhizobium that can direct cell cycle transcription in Caulobacter. Thus, a conserved genetic module uses an ancestral transcription factor fold, extensively researched in the eukaryotic domain of life, to integrate virulence, symbiosis and/or cell cycle transcription in a bacterial lineage from which eukaryotic organelles descended.

Results

Target promoters of the S→G1 transcriptional switch.

Before surveying the extent of the S→G1 promoter switch on a genome-wide scale, we first characterized candidate promoters by quantitative ChIP (qChIP) following precipitation with polyclonal antibodies to Ctra and monoclonal antibodies to the RpoC (B) subunit of RNAP from chromatin of wild-type (WT) and Δplec cells. pleC encodes a histidine kinase/phosphatase that partitions with the G1-phase progeny (Fig. 1a) and is required for the accumulation of G1-specific transcripts, including pilA, and for maximal accumulation of Ctra mRNA peaks in late S-phase (84 min), that the pilA in WT cells compared with WT cells (Fig. 1b), consistent with the reduced PpilA activity (Fig. 1b–d). We also noted a similar reduction in Ctra occupancy at PpilA in WT cells (Fig. 1a). By contrast, Ctra abundance at PpilA in WT cells (Fig. 1a) was not noticeably affected (Fig. 1e). Knowing that fliL mRNA peaks in late S-phase ( ~84 min), that the pilA and tacA mRNAs surge in G1 (~120 min (ref. 16)) and that PilA accumulation is PleC-dependent (Fig. 1f), we hypothesized that PleC-dependent Ctra (PleC·Ctra) target promoters regulate G1-phase genes.

Next, we charted other PleC·Ctra target promoters on a genome-wide scale by comparative ChIP-seq of Ctra occupancy in WT and ΔpleC cells. Bioinformatic analyses predicted >100 Ctra target sites that, akin to PpilA, are bound substantially less efficiently by Ctra (that is, with log2 difference of < −0.8) in
Figure 1 | CtrA-bound promoters that are affected in \(\Delta\text{pleC}\) cells. (a) Schematic of the regulatory interactions between \(\text{ctrA}\), \(\text{scIP}\) and \(\text{mucR1}\) and \(\text{mucR2}\) (\(\text{mucR1/2}\)) during the \(C.\) crescentus cell cycle. Phosphorylated CtrA (CtrA~P) activates transcription of S- and G1-phase genes. In S-phase, MucR1/2 represses G1-genes such as sciP. The sciP gene is activated in G1 and the newly synthesized SciP translation product represses S-phase promoters. The antagonistic kinase/phosphatase pair, DivJ (yellow dot) and PleC (green dot) indirectly influence CtrA

\(\Delta \text{pleC}\) versus \(\text{WT}\) cells (Figs 1g and 2a; and Supplementary Data 1). To confirm that these sites indeed harbour PleC:CtrA target promoters, we constructed promoter-probe reporters of the top 18 PleC:CtrA target sites and measured promoter activities in \(\text{WT}\) and \(\Delta \text{pleC}\) cells (Supplementary Figs 1B and 2A,B).

All reporters were less active in \(\Delta \text{pleC}\) cells, showing that they indeed harbour PleC:CtrA target promoters. Since the transcripts produced from these promoters are restricted to G1-phase\(^{15,22}\), we conclude that these sites define a new class of G1-phase promoters that are activated by CtrA in a PleC-dependent manner.

\(\Delta \text{pleC}\) mutant cells harbouring a \(\text{pilA}\) (\(P\)\(\text{pilA}\)) promoter-probe reporter. Data are from three biological replicates. Error is shown as s.d. (f) Immunoblots showing PilA (lower band, approximately 6 kDa) and CtrA (upper band, approximately 26 kDa) steady-state levels in \(\text{WT}\) and \(\text{pilA}::\text{Tn}\) mutant cells harbouring \(\text{WT}\) \(\text{ctrA}\) or phosphomimetic \(\text{ctrA}(\text{D51E})\) expressed from a plasmid in the absence of \(\text{pilA}\) (\(P\)\(\text{pilA}\)) and \(\text{filL}\) promoter (\(P\)\(\text{filL}\)) in \(\text{WT}\) (NA1000), \(\Delta \text{pleC}\) and \(\Delta \text{pleC}:\text{mucR1::Tn5}\) mutant cells as determined by quantitative chromatin immunoprecipitation assays (qChIP) using antibodies to CtrA or RpoC, as well as \(\text{pilA}\) transcription measurements conducted using a \(\text{pilA}::\text{lacZ}\) promoter-probe reporter. Data are from three biological replicates. Error is shown as s.d. (f) Immunoblots showing PilA (lower band, approximately 6 kDa) and CtrA (upper band, approximately 26 kDa) steady-state levels in \(\text{WT}\) and \(\text{pilA}::\text{Tn}\) mutant cells harbouring \(\text{WT}\) \(\text{ctrA}\) or phosphomimetic \(\text{ctrA}(\text{D51E})\) expressed from a plasmid in the absence of chromosomally encoded CtrA (\(\text{ctrA}::\Omega\)). Molecular size standards are indicated on the left as blue lines with the corresponding values (blue) in kDa. (g) Comparative ChIP-seq performed with antibodies to CtrA on chromatin from \(\text{WT}\) and \(\Delta \text{pleC}\) cells. Boxed in grey are PleC:CtrA promoters that were verified as being PleC dependent (Supplementary Fig. 1B). Blue labels indicate PleC:CtrA promoters that are bound by MucR2 as determined by the ChIP-seq experiments (Fig. 2). Blue arrowheads point to promoters for which the ChIP-seq traces are shown in Fig. 5. The colour key at the bottom indicates the degree by which the occupancy of CtrA is altered by the \(\Delta \text{pleC}\) mutation, expressed as \(\log_2\) ratio (Supplementary Data 1).
Importantly, the promoter of the G1-phase gene sciP also falls into this class (Supplementary Fig. 1B; see below).

SciP preferentially binds S-phase target promoters of CtrA. ChIP-seq experiments (Fig. 2b) with a polyclonal antibody to SciP revealed that SciP does not associate with all CtrA target sites (Fig. 2a) in vivo. In fact, we observed a clear promoter preference for SciP, targeting the S-phase promoters, but not G1-phase promoters activated by CtrA. Bioinformatic analyses predicted 76 SciP-binding sites in vivo (see Methods section and Supplementary Data 2) upstream of CtrA-activated genes whose transcripts all peak in late S-phase, such as flagellar genes.
for example, flmG, pleA, fliQ, fliX, fliB, fliL, fliI and fliJ), pilus secretion genes (for example, cpaB and others, see below) and chemotaxis gene orthologues (for example, CC_2317, CC_2281 and CC_1655). If ScIP binds DNA only through a direct association with CtrA, then all CtrA targets, including Cori and CtrA-repressed promoters, should be efficiently precipitated with the ScIP antibody. These CtrA target sites as well as those of PleC-CtrA (G1) promoters such as P_{pilA} were not enriched in the ChIP sample, indicating that they are not preferred targets of ScIP in vivo (Supplementary Discussion). A MEME-based motif search using 50 of the top ScIP target sites predicted S'-G(A/J)TTAACCAT(A/G)-3' as possible ScIP consensus motif (Supplementary Data 2), a motif having half a CtrA box (underlined, see above) and often overlapping with the CtrA target site in promoters (Supplementary Data 2). These results suggest that CtrA and ScIP compete for binding to these promoters, or that repression by ScIP involves a cooperative binding mode between ScIP and CtrA at this site, perhaps through an ‘extended’ CtrA box that includes a CtrA half site harboring a ScIP consensus motif. Interestingly, combinatorial promoter control by two regulators has been described during sporulation in the unrelated Delta-proteobacterium Myxococcus xanthus.\(^{23}\)

Our results also reveal that ScIP binds neither P_{fliF} (the promoter of the class II flagellar gene fliF that is activated by CtrA in S-phase\(^{16}\)) nor P_{pilA} efficiently in vivo. We therefore suggest that the ability of ScIP to interfere with the recruitment of RNAP to CtrA-activated promoters such as P_{fliF} and P_{pilA} in vitro\(^{18,19}\) likely reflects a secondary, later-acting mechanism to silence CtrA-dependent transcription. It is conceivable that this mechanism comes into play later in G1-phase once CtrA activation of sciP (and other genes that is, after compartmentalization) has led to a build-up of a threshold in ScIP. Since our ChIP-seq experiments failed to reveal ScIP at all CtrA-activated promoters, it seems that ScIP binds the bipartite CtrA target complex weakly and/or only very transiently in vivo. The simplest interpretation of our ChIP-seq data is that ScIP stably associates with promoters that CtrA activates in S-phase, while promoters that CtrA activates in G1-phase or represses (such as the podJ or fliS z promoter) are not preferred targets of ScIP in vivo. ScIP also does not target the CtrA-bound Cori site or the CtrA-activated promoter P_{fliF} efficiently in vivo. P_{fliF} is repressed by FliB, the $\sigma^{54}$-dependent transcriptional activator of class III/IV and repressor of class II flagellar promoters\(^{14}\). Our ChIP-seq experiments showed that FliB binds P_{fliF} and several other class III/IV flagellar promoters (Fig. 2c and Supplementary Fig. 3). FliB also targets, in a mutually exclusive fashion with ScIP, class II flagellar promoters (Fig. 2c and Supplementary Discussion), but neither P_{pilA} nor other PleC-CtrA target promoters.

**MucR1/2 binds promoters of G1-phase genes.** Our observation that phosphomimetic variants of CtrA cannot elevate P_{pilA} activity in ΔpleC cells (Supplementary Fig. 1C) and PilA protein accumulation, regardless of whether WT CtrA is present or not (Fig. 1f), predicted an unknown repressor(s) that prevents CtrA-mediated activation at P_{pilA}, P_{sciP} and other G1-promoters in ΔpleC cells. To identify this repressor, we mutated ΔpleC cells harbouring a P_{pilA}::pilII transcriptional reporter (integrated at the chromosomal pilA locus; pilA::pilA::pilII) with a mini-Tn5-Gm\(^{5}\) (encoding gentamycin resistance, Gm\(^{5}\)). Selecting for kanamycin-resistant transposon mutants in which P_{pilA}::pilII expression had been restored (Fig. 3a), we isolated one such mutant (mucR1::Tn5) and determined the Tn5 insertion to be in the middle (codon 74) of the mucR-like gene CC_0933 (CCNA_00982, henceforth mucR1; Supplementary Fig. 1D). MucR1 belongs to the conserved MucR/Ros family of transcriptional regulators that harbour a zinc-finger-type fold\(^{24}\) and control virulence, symbiosis and/or motility in the human pathogen Brucella suis\(^{25}\), the plant pathogen Agrobacterium tumefaciens\(^{26}\) and the plant symbiont Sinorhizobium fredii NGR234 (ref. 24).

Unexpectedly, and in contrast to the mucR1::Tn5 allelic, an in-frame deletion of mucR1 (ΔmucR1) did not mitigate the defect in P_{sciP}::lacZ and P_{pilA}::lacZ transcription (Fig. 1c and Supplementary Fig. 2A,B) and PilA expression (Fig. 3b) of ΔpleC cells. However, when an in-frame deletion of mucR1 was introduced along with a deletion in the gene encoding the MucR2-paralog CC_0949 (CCNA_0098, henceforth mucR2), the P_{sciP}::lacZ and P_{pilA}::lacZ activity in the ΔpleC ΔmucR1ΔmucR2 double mutant even exceeded that of the ΔpleC mucR1::Tn5 cells (Supplementary Fig. 2A,B). By contrast, reporter activity was hardly altered after deletion of either mucR1 or mucR2 from ΔpleC cells (Supplementary Fig. 2A,B and Table 1).

**Trans-dominance of mucR1::Tn5 on mucR2.** The results above suggest that the mucR1::Tn5 insertion not only disrupts mucR1 but also causes trans-dominance on mucR2. Several findings support this conclusion. First, a multi-copy plasmid carrying the coding sequence of truncated MucR1 from mucR1::Tn5 (pMT335-mucR1::Tn5, see Methods section) alleviates the PilA (Fig. 3b) and P_{pilA}::lacZ (Supplementary Fig. 2C) expression defect of ΔpleC ΔmucR1 double-mutant cells, while an analogous plasmid with WT mucR1 (pMT335-mucR1) does not. Second, MucR1 and MucR2 directly associate with P_{pilA} and P_{sciP} in vitro (as determined by electrophoretic mobility shift assays (EMSA); Supplementary Fig. 2D,E) and in vivo (qChIP experiments conducted using polyclonal antibodies to MucR1 or to MucR2; Fig. 3c,d). Third, mucR1::Tn5 impairs binding of MucR2 at P_{pilA} in vivo (Fig. 3d) and antibodies to MucR1 no longer precipitate P_{pilA} from chromatin of mucR1::Tn5 cells (Fig. 3c). As immunoblotting revealed that mucR1::Tn5 encodes a C-terminally truncated form of MucR1 (Fig. 3e) that does not affect MucR2 steady-state levels (Fig. 3f), we conclude that the mucR1::Tn5 mutation not only removes codons required for DNA binding but also interferes with MucR2 binding to its targets. Finally, and most importantly, pull-down experiments using extracts of ΔmucR1 cells expressing a MucR1 derivative carrying C-terminal tandem affinity purification (TAP) tag revealed that MucR2 interacts with MucR1-TAP and MucR1ΔmucR2-TAP (Fig. 4a).

These findings along with the facts that MucR proteins can dimerize\(^{24,27}\) and that eukaryotic zinc-finger transcriptional regulators form heterodimers\(^{20,28}\) suggest that the trans-dominance of truncated MucR1 on MucR2 is due to the formation of inactive heterodimers. To map the residues in MucR1 that promote such trans-dominance on MucR2, we conducted random mutagenesis of mucR1 to isolate missense mutants that restore kanamycin resistance to ΔpleC pilA::pilA::pilII cells, akin to the mucR1::Tn5 mutation. We isolated four mucR1 alleles encoding different single amino-acid substitutions (R85C, L87P, Y97C or Y97H; Supplementary Fig. 1D; see Methods section) that disrupt one or both of the two conserved $\alpha$-helices (residues 82-89 and 94-101) in the C-terminal DNA-binding domain of A. tumefaciens MucR/Ros determined by nuclear magnetic resonance spectroscopy (NMR)\(^{24}\). The residue corresponding to R85 of MucR1 is situated within the DNA wraps around DNA and establishes specific base contacts\(^{24}\), suggesting that loss of this basic residue in MucR1 disturbs DNA
binding. As pull-down assays showed that MucR1(Y97C)-TAP still interacts with MucR2 (Fig. 4a), we suggest that a MucR1(Y97C) homo-dimer and a MucR1(Y97C)-MucR2 heterodimer are non-functional, and that the other mutations are also loss-of-function mutations that can still interact with MucR2 and thus exert trans-dominance.

**Developmental control by MucR1 and MucR2.** ChIP-seq analyses with antibodies to MucR1 and to MucR2 predicted 162 and 227 target sites for MucR1 and MucR2 (Fig. 2d,e; and Supplementary Data 4 and 5), respectively. Cluster analyses of MucR1/2-, SciP- and CtrA target sites revealed a large overlap between CtrA, MucR1 and MucR2 targets (red set ‘a–c’, Fig. 3g). Importantly, MucR1 and/or MucR2 bind G1-phase (PleC:CtrA) target promoters in vivo, but not SciP targets (Supplementary Data 1, as summarized in Fig. 3h). The occupancy of CtrA and MucR1/2 over four selected promoter regions (including P\_pilA and P\_sciP; Fig. 5a–d) revealed overlapping or proximal peaks. Promoter-probe experiments with several of these target promoters confirmed their dependency on MucR1/2 in vivo (Table 1).

In support of the notion that MucR1/2 regulates G1-phase promoters, simultaneous deletion of \( mucR1 \) and \( mucR2 \) from WT cells (AmucR1/2) imparts multiple developmental defects, disturbing the acquisition of motility, the buoyancy switch and
holdfast gene expression (see below). These deficiencies are corrected when ΔmucR1/2 cells are complemented with a pMT335-derived plasmid harbouring mucR1 or mucR2, but neither by a derivative harbouring mucR1Δ5 (Fig. 6a,b) nor by the distantly related mucR-paralog CC_1356 (CCNA_01418). Moreover, the developmental defects are attenuated by mutations in ctrA or sciP that suppress the ΔmucR1/2 motility defect (see motile flare in Fig. 6b and Supplementary Fig. 3A,B; see below).

### Table 1 | Activity of MucR1/2-bound C. crescentus promoters.

| Promoters | WT | ΔR1/R2 | mucR1/Tn5 |
|-----------|----|--------|-----------|
| perP      | 100.0 ± 0.0 | 144.7 ± 17.6 | 129.8 ± 6.2 |
| CC_0420   | 102.7 ± 3.5 | 156.0 ± 14.6 | 224.6 ± 21.9 |
| CC_0430   | 107.0 ± 8.3 | 155.1 ± 4.9 | ND ± ND |
| CC_2810   | 100.7 ± 3.7 | 222.1 ± 31.8 | 206.2 ± 7.0 |
| CC_3001   | 94.0 ± 7.0 | 312.2 ± 23.7 | 186.6 ± 12.3 |
| fliM      | 93.5 ± 8.0 | 52.5 ± 4.0 | 85.8 ± 1.7 |
| CC_2819   | 100.0 ± 0.7 | 47.0 ± 2.0 | 75.8 ± 6.1 |
| flaF      | 95.6 ± 7.8 | 86.5 ± 3.1 | 75.4 ± 3.7 |
| pilA      | 100.0 ± 0.7 | 118.7 ± 7.9 | 119.5 ± 8.9 |
| pilA (synUTR) | 100.6 ± 2.7 | 131.7 ± 2.8 | 129.2 ± 7.8 |

- **Strains**
- **ΔmucR1**
- **ΔmucR2**

| Promoters | ΔmucR1 | ΔmucR2 |
|-----------|--------|--------|
| CC_0420   | 120.8 ± 4.1 | 116.3 ± 2.1 |
| CC_2810   | 97.3 ± 2.6 | 129.0 ± 2.9 |
| CC_3001   | 105.5 ± 3.8 | 161.5 ± 11.8 |
| CC_2819   | 94.6 ± 5.1 | 90.8 ± 4.4 |
| flaF      | 101.1 ± 1.1 | 95.9 ± 0.8 |

*ND*: not determined; UTR, untranslated region; WT, wild-type.

β-Galactosidase activity measurements of extracts from WT, mucR1/Tn5, ΔmucR1 and ΔmucR2 single- and double-mutant cells (ΔR1/R2) harbouring various lacZ-based promoter-probe plasmids. The pilA(synUTR)-reporter is a modified version of pilA in which the 74 nt UTR has been replaced by a synthetic UTR from E. coli (see Supplementary Note 1 for sequence). Error is shown as s.d.

The **mucR1/2-sciP-ctrA regulatory module.** Promoter-probe and immunoblotting experiments revealed that the activity of flagellar promoters (Supplementary Fig. 3E) and the abundance of class III and class IV flagellar proteins are diminished in ΔmucR1/2 cells relative to WT (Fig 6c,d). Since class II flagellar promoters are bound by SciP (but not MucR1/2, see above) in vivo and required for expression of class III and IV flagellar genes \(^\text{14}^{14}\), and since transcription of sciP is negatively regulated by MucR1/2 (Supplementary Fig. 2B), we hypothesized that SciP represses S-phase promoters ectopically in ΔmucR1/2 cells (Fig. 6e). Four lines of evidence support this conclusion. First, the promoters that are most efficiently bound by SciP in vivo (Supplementary Data 2, including P_plac, P_fliB, P_CC_3676 and P_PCC_3439) are the most downregulated in ΔmucR1/2 cells (Supplementary Figs 3E and 4A). Second, as predicted, CtrA also targets these four promoters (Supplementary Fig. 4) and induces a peak in transcript accumulation in S-phase \(^\text{14}^{14}\). Third, suppressor mutations in either sciP or ctrA augment the activity of these promoters in ΔmucR1/2 cells (Supplementary Fig. 4A). Fourth, comparative ChIP-seq of WT and ΔmucR1/2 cells revealed an increase in abundance of SciP at its preferred target sites in ΔmucR1/2 versus WT cells (for example, with a log2 difference of < −0.6 for P_CC_3676, P_PCC_3439, P_PLac, P_PCC_3676 and P_Pfla, Supplementary Data 6). Thus, while the mucR1/2-sciP-ctrA module maintains the correct balance in motility gene expression, deletion of mucR1/2 introduces an imbalance in regulation through SciP.

The eleven suppressor mutations in sciP that we isolated are scattered throughout the entire (93-residue) coding region (Supplementary Fig. 3B), suggesting that reduced SciP function (or abundance) can be beneficial in the absence of MucR1/2. Importantly, the T65A mutation lies in a residue previously implicated in SciP function \(^\text{17}^{17}\), and we observed that derivatives of pMT335 harbouring either sciP(T24I) or sciP(T65A) are less efficient than the WT sciP version in inhibiting motility (of WT cells; Supplementary Fig. 3F). The three suppressor mutations that we found in ctrA (encoding T168A, T170A and T170P; Supplementary Fig. 3A) all map to the predicted DNA-binding domain. Interestingly, a related mutation (T170I) encoded by the ctrA401ts allele \(^\text{12}^{12}\) impairs motility and prevents growth at 37°C, while allowing growth at 28°C. By contrast, WT strains harbouring ctrA(T170A) in place of WT ctrA exhibited no such temperature sensitivity. Thus, unlike the hypomorphic ctrA401ts allele, ctrA(T170A) acts as a hypermorphic allele. In support of this conclusion, motility is not inhibited upon mild overexpression of SciP from pMT335 in ctrA(T170A) cells. By contrast, ctrAΔ2 cells harbouring pMT335-sciP are non-motile (Fig. 6f; and Supplementary Fig. 3C,D). These findings support the conclusion that transcription of sciP in G1-phase prevents activation of motility and other S-phase genes by CtrA (Fig. 6e), and that inappropriate expression of sciP from a multi-copy plasmid or by the ΔmucR1/2 mutation prevents activation of these promoters in S-phase. Hypomorphic mutations in SciP (for example, T65A) or hypermorphic mutations in the CtrA DNA-binding domain (for example, T170A) mitigate these effects, presumably because the T170A substitution enhances CtrA’s ability to compete against repression by SciP in vivo.

The ΔmucR1/2 mutation also results in a diminished promoter activity (67% reduction versus WT) of the hsf/holdfast gene (CC_0095) \(^\text{29}^{29}\) and a buoyancy defect (Fig. 6e). Although the genetic basis of the Caulobacter buoyancy has not yet been determined, we found that the ΔmucR1/2 buoyancy defect is reversed by the suppressor mutations in sciP and ctrA (Fig. 6e). Thus, the mucR1/2-sciP-ctrA module appears to act on an unknown buoyancy gene(s). Intriguingly, our ChIP-seq experiments revealed that the 26-kb genomic island that is located circa 480-kb clockwise from C0 and encodes at least one unknown buoyancy determinant \(^\text{30}^{30}\) harbours the preferred binding sites of MucR1 and target sites of MucR2 (Figs 2d–f; and Supplementary Data 4,5 and 7). To explore this defect further, we conducted a himar1 transposon (Tn) mutagenesis experiment in mucR1/2 mutant cells and uncovered five buoyancy pseudo-reversion mutants, each harbouring a Tn insertion in the same gene (CCNA_04006, encoded on the aforementioned 26-kb genomic island, Fig. 2f) whose predicted translation product resembles the putative N-acetyl-L-fucosamine transferase WbuB from Escherichia coli \(^\text{31}^{31}\). An in-frame deletion in CCNA_04006 (ACCNA_04006) recapitulates the buoyancy pattern of the Tn mutation in either ΔmucR1/2 or WT cells (Fig. 2g) and is corrected when CCNA_04006 is expressed from a plasmid (pMT335-CCNA_04006), indicating that deletion of mucR1/2 affects CCNA_04006-dependent buoyancy switch.

### Direct and positive auto-regulation of mucR1/2 and ctrA

Above we reported the functional and regulatory interactions between mucR1/2-sciP and between mucR1/2-ctrA. Interestingly, MucR1/2 bind P_sciP and the ChIP-seq data traces in Fig. 4b indicate that MucR1 also binds the ctrA promoter. This notion was confirmed by EMSAs showing that His6_SUMO-MucR1, but not His6_SUMO-MucR2 or His6_SUMO, binds a ctrA promoter probe (Fig. 4d), albeit with somewhat lower affinity than the
Figure 4 | Heterodimerization and auto-regulation by MucR1/2. (a) Immunoblots showing that MucR2 co-purifies with MucR1-TAP before (that is, in cell lysates, lysate) or after the first TAP purification step (cleavage with TEV protease, TAP). The upper panel shows a blot probed with antibodies to MucR1 (α-R1). The asterisk indicates untagged MucR1 in wild-type (NA1000) cells. The arrowheads indicate MucR1-TAP before (black) or after (red) cleavage of the protein-A moiety in the TAP tag with the TEV protease, eluting the proteins from the IgG agarose beads. The two lowest panels shows a cleavage of the protein-A moiety in the TAP tag with the TEV protease, eluting the proteins from the IgG agarose beads. The two lowest panels shows a blot probed with antibodies to MucR2 (α-R2) revealed at two different exposures. The lower amount of MucR2 that co-purifies with WT MucR1-TAP compared with MucR1T97C or MucR1Tns proteins is likely because of a reduction in the total MucR2 levels when MucR1 is overexpressed. Molecular size standards are indicated on the left as blue lines with the corresponding values (blue) in kDa. (b) Traces of the occupancy of various transcription factors at the ctrA promoter based on ChIP-seq data. (c) β-Galactosidase measurements in extracts of WT (dark blue) and ΔR1/2 (light blue) cells harbouring a P<sub>ctrA</sub>-lacZ promoter-probe plasmid. Data are from three biological replicates. Error is shown as s.d. (d) Electrophoretic mobility shift assay (EMSA) showing the binding of Hist-SUMO-MucR1 or -MucR2 to P<sub>ctrA</sub>. The white arrows indicate the unbound probe. The black arrows indicate the shifted ctrA probes bound by MucR1. Hist-SUMO-MucR2 or Hist-SUMO does not retard the probe.

P<sub>pilA</sub> or P<sub>sciP</sub> probes (Supplementary Fig. 2D,E). Moreover, transcription from a ctrA promoter reporter plasmid (P<sub>ctrA</sub>-lacZ) is reduced by 62% in ΔmucR1/2 cells (Fig. 4c). Thus, MucR can also act positively on its target promoters as observed for other MucR proteins<sup>25,32</sup>, and a direct regulatory and functional relationship exists between mucR and ctrA.

ChIP-seq also indicated a direct auto- and cross-regulatory relationship between MucR1 and MucR2 (Supplementary Data 4 and 5). P<sub>mucR1</sub>-lacZ and P<sub>mucR2</sub>-lacZ promoter-probe reporters are reduced by 62% and 72%, respectively, in ΔmucR1/2 cells relative to WT (Fig. 4c). Although the steady-state levels of MucR1 and MucR2 remain fairly constant during the cell cycle (Supplementary Fig. 5A) and MucR1 occupancy at P<sub>pilA</sub> in ΔpleC cells is near the occupancy seen in WT cells, we found that a Tn5 insertion in the cell cycle kinase genes divI or divL (that act in the PleC-signaling pathway<sup>15</sup>) increases the occupancy of MucR1 at P<sub>pilA</sub> in ΔpleC cells (Supplementary Fig. 5B). This divI- or divL-dependent increase of MucR binding is not because of diminished CtrA occupancy at P<sub>pilA</sub>, P<sub>lacA</sub> or P<sub>phi</sub> (Supplementary Fig. 6A–C). Moreover, the recruitment of RNAP to P<sub>pilA</sub> (Supplementary Fig. 6D) and the production of PilA is restored (Supplementary Fig. 1D in ref. 33). Finally, the abundance of CtrA~P is not reduced by these mutations in divI and divL (Supplementary Fig. 6E). Thus, components of the cell cycle regulatory circuitry such as DivI and DivL can modulate the occupancy of MucR1 on its targets. Conversely, MucR1 directly regulates expression of the master cell cycle regulator CtrA.

Taken together, we conclude that mucR1/2 is a critical component of an integrated and homeostatic (auto)regulatory module, mucR1/2-ctrA-sciP, in which MucR not only engages in a double-negative regulation (MucR and SciP), but also in a double-positive one (MucR and CtrA) that likely helps reinforce and/or synchronize the molecular events underlying the S→G1 transcriptional switch.
Pan-genomic ChIP-seq reveals conserved target regulation. To investigate whether the link of MucR with the cell cycle circuitry has been maintained during evolution, we first assessed the extent of the functional conservation in the symbiont S. fredii NGR234 (henceforth S. fredii) that also contains two mucR-like genes: a00320 on the symbiotic plasmid pNGR234a and c07580 on the chromosome. Both a00320 and c07580 can substitute for Caulobacter mucR1/2 in motility control (Fig. 7a), as is also the case for the unique mucR homologues encoded in the genomes of the animal pathogen B. suis and the plant pathogen A. tumefaciens (Fig. 7a), indicating that expression of sciP and the downstream motility target genes are sufficiently controlled by the heterologous MucR proteins to support motility. Because of this functional conservation, we asked if direct regulatory interactions were also maintained. To this end, we conducted pan-genomic ChIP-seq analyses using the antibodies to Caulobacter MucR1 and MucR2 to identify promoters bound by MucR-like proteins in S. fredii (Fig. 7b; Supplementary Fig. 7A; and Supplementary Data 4 and 5). We then used these promoters to show the converse genetic dependency, that is, that these MucR-bound S. fredii promoters are regulated by Caulobacter mucR1/2. Indeed, promoter-probe experiments with Caulobacter WT and ΔmucR1/2 cells revealed that six S. fredii promoters are regulated negatively and two positively by MucR1/2 (out of 10 promoters tested; Table 2).

Next, we attempted to compute pan-genomic consensus motif for MucR from the top 50 MucR target sites of Caulobacter and S. fredii (Fig. 7c) using the MEME algorithm (see Methods section). As proof-of-principle, we also computed a pan-genomic consensus motif of the top 50 CtrA-bound sites in the Caulobacter and S. fredii genomes determined by ChiP-seq using antibodies to Caulobacter CtrA (Supplementary Data 7). The deduced motif is remarkably similar to the CtrA target sequence determined biochemically for Caulobacter (TTAA-N7-TTAA; Fig. 7c), showing that this strategy can accurately predict consensus motifs. The pan-genomic consensus motif that was deduced for MucR1 is fairly degenerate, revealing a preference for AT-rich DNA substrates in GC-rich genomes (65% for Caulobacter and 61% for S. fredii). Indeed, synthetic reporters (T5_mucRb-lacZ and two attenuated derivatives; Supplementary Note 1) in which two MucR1 motifs were placed in tandem, downstream of a heterologous strong promoter from E. coli T5 phage driving lacZ expression are MucR-dependent in Caulobacter (Supplementary Fig. 8A,B), thereby validating our predicted pan-genomic MucR1-consensus sequence as a MucR1 target.

Finally, we compared the target sites of CtrA and MucR in S. fredii using the consensus sequences. This revealed a suite of overlapping (or proximal) targets as for Caulobacter, albeit fewer in number (Fig. 7b). Importantly, several of these target sites in S. fredii are linked to orthologous genes that we determined to be under MucR and CtrA control in Caulobacter (including flaF- and sciP-like genes that are cell cycle-regulated and perform developmental functions in Caulobacter; Fig. 7d)3,4. Thus, the functional and direct regulatory relationships between MucR proteins and the cell cycle are (at least partly) conserved during evolution.

Discussion

Through forward genetics and (pan)genomic promoter occupancy of key cell cycle transcriptional regulators, we unearthed a conserved regulatory module, mucR1/2/sciP-ctrA, that implements the S→G1 switch in Caulobacter. The ancestral zinc-finger transcription factor paralogs MucR1/2 repress G1-phase promoters that are activated by the essential master regulator CtrA including that controlling the negative regulator SciP that turns off S-phase promoters activated by CtrA. Through this double-negative wiring, the induction of G1-promoters occurs concomitantly with the repression of S-phase promoters. Superimposed on this double-negative regulatory wiring is a double-positive circuit in which MucR1/2 promotes expression of CtrA. Thus, MucR has both negative and positive roles in reinforcing the S→G1 transcriptional switch in Caulobacter.

Remarkably, G1-phase-specific transcripts were recently also detected in synchronized S. melliloti3, raising the intriguing possibility that the underlying regulatory mechanisms that direct G1-phase transcription in Caulobacter also operate in symbiotic or virulent relatives, and that the mucR-scip-ctrA regulatory module coordinates virulence/symbiosis and cell cycle transcription. Several findings are consistent with this notion. First, MucR proteins were previously described as regulators of virulence and symbiosis gene expression in Brucella, Sinorhizobia and Agrobacteria that also divide asymmetrically3,25,35. Second, we showed that MucR1/2 are required for proper implementation of the cell cycle transcriptional programs in dividing Caulobacter cells. Third, we found that MucR from Brucella, Sinorhizobia and Agrobacteria can confer motility to Caulobacter ΔmucR1/2 mutants, a function that is dependent on proper cell cycle-dependent expression of SciP in G1-phase, while mis-expression of SciP impairs motility gene expression and leads to non-motile cells in soft agar (Fig 6b,f). Fourth, MucR-bound promoters of S. fredii NGR234 are also under MucR1/2 control in Caulobacter.
cαrriyng mucR1 inoculated with DΔR1/2 expression in agar. (WT)

MucR controlled in morphology are both critical for virulence and known to be muc polysaccharides (responsible for the in the host. Indeed, motility and cell envelope-associated necessary transcripts to establish virulence or symbiosis a G1-phase-arrested daughter cell in Brucella specific cell cycle stage. For example, MucR proteins could endow cycle transcription that restrict virulence gene expression to a virulence/symbiosis should be re-classified as regulators of 4 transcription and virulence/symbiotic functions in mind, it is tempting to propose that certain transcriptional regulators previously thought to function exclusively as regulators of virulence/symbiosis should be re-classified as regulators of cell cycle transcription that restrict virulence gene expression to a specific cell cycle stage. For example, MucR proteins could endow a G1-phase-arrested daughter cell in Brucella, Sinorhizobia, Agrobacteria and likely other Alpha-proteobacteria with the necessary transcripts to establish virulence or symbiosis in the host. Indeed, motility and cell envelope-associated polysaccharides (responsible for the mucoid colony morphology) are both critical for virulence and known to be MucR controlled in Sinorhizobia and Brucella.

Interestingly, the fact that heterologous MucR proteins control cell cycle transcription indicates that they are properly regulated in Caulobacter, implying that MucR control is conserved in the Alpha-proteobacteria. MucR1/2 protein levels remain relatively constant during the Caulobacter cell cycle, suggesting that MucR is regulated post-translationally. Indeed, the ability of heterologous MucR proteins to support function in another host is easier to reconcile with regulation by a small molecule that is produced by most Alpha-proteobacteria, rather than by a very promiscuous factor that must recognize the different MucR proteins.

Several Alpha-proteobacteria including Caulobacter and S. fredii encode multiple MucR paralogs, and we show that the Caulobacter MucR paralogs can heterodimerize akin to eukaryotic zinc-finger transcription factors. Although a single endogenous or a heterologous MucR paralog can support cell cycle transcription in Caulobacter, it is conceivable that heterodimerization serves to fine-tune MucR activity and cell cycle transcription. With the eukaryotic mitochondrion having descended from an Alpha-proteobacterium and ancestral zinc-finger transcription factors being primarily represented in the

Figure 6 | Regulatory interplay between mucR1/2, sciP and ctrA in the control of motility. (a,b). The motility defect ofΔmucR1ΔmucR2 double-mutant cells (ΔR1/2) can be rescued by expression of mucR1 or mucR2 from a plasmid or by point mutation in sciP or ctrA. (a) Motility plates (0.3% agar) inoculated with ΔR1/2 cells containing the empty plasmid or derivatives with mucR1 (long form, original annotation of CC_0933), mucR1Δns or the plasmids carrying mucR1 (CCNA_00982) or mucR2 (CCNA_00998). (b) The motility ofΔR1/2 (c) cells harbouring point mutation in sciP (d) or in ctrA (e) relative to WT cells (a). The yellow arrow points to the emergence of motile suppressors from a non-motile inoculum of ΔR1/2 cells (c). With this close interplay of cell cycle transcription and virulence/symbiotic functions in mind, it is tempting to propose that certain transcriptional regulators previously thought to function exclusively as regulators of virulence/symbiosis should be re-classified as regulators of cell cycle transcription that restrict virulence gene expression to a specific cell cycle stage. For example, MucR proteins could endow a G1-phase-arrested daughter cell in Brucella, Sinorhizobia, Agrobacteria and likely other Alpha-proteobacteria with the necessary transcripts to establish virulence or symbiosis in the host. Indeed, motility and cell envelope-associated polysaccharides (responsible for the mucoid colony morphology) are both critical for virulence and known to be MucR controlled in Sinorhizobia and Brucella.

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Alpha-proteobacterial lineages, it is conceivable that these transcription factors originated in an Alpha-proteobacterium that had undergone a duplication of the mucR gene.
Strain constructions. The \( \Delta \)mucR1 and \( \Delta \)mucR2 marker-less single and double deletions were introduced into WT (NA1000) and \( \Delta \)pC\( \delta \)C\( \delta \) cells using the standard two-step recombinant-protoplasts reverse-counter-selection procedure by the pNP\( \delta \)TS138 derivatives. The resulting strains are the \( \Delta \)mucR1 and \( \Delta \)mucR2 single mutants, the \( \Delta \)pC\( \delta \)C\( \delta \)\( \Delta \)mucR1 and \( \Delta \)mucR2 double mutants, and the \( \Delta \)pC\( \delta \)C\( \delta \)\( \Delta \)mucR1 \( \Delta \)mucR2 triple mutant. Deletions were confirmed by PCR using outside primers that do not hybridize within the mucR deletion alleles carried on pNP\( \delta \)TS138.

The mucR1::Tn5 mutation (confering gentamycin resistance and encoding a truncated MucR1 derivative that is dominant negative on MucR2) was transduced from strain NRS13 (see below) into the WT (NA1000) and \( \Delta \)pC\( \delta \)C\( \delta \) strains by qPCR-directed generalised transduction, yielding strains in which endogenous mucR1 is replaced by mucR1::Tn5.

Protein purification and antibody production. For antibody production, His\( \delta \)-MucR1 or His\( \delta \)-SUMO-Ci\( \delta \)t trained from pCW350 and pCG\( \delta \) in E. coli Rosetta (DE3)/pLysS (EMD Millipore, Billerica, MA), respectively, and purified the recombinant proteins purified under standard native conditions using Ni\( \delta \) chelate chromatography. They were used to immunize New Zealand white rabbits (Josman LLC, Napa, CA). His\( \delta \)-SUMO-MucR2 was expressed from pUG\( \delta \)2 in E. coli Rosetta (DE3)/pLysS and purified using Ni\( \delta \) chelate chromatography in phosphate-buffered 8 M urea (Qiagen, Hilden, Germany). The protein was excised with a 13 SDS-PAGE gel and used to immunize rabbits.

For EMSAs, soluble His\( \delta \)-SUMO-MucR1, His\( \delta \)-SUMO-MucR2 or His\( \delta \)-SUMO was purified from E. coli Rosetta (DE3)/pLysS containing pUG\( \delta \)0, pUG\( \delta \)41 or pET28a-His\( \delta \)-SUMO, respectively, under native conditions using Ni\( \delta \) chelate chromatography. In brief, cells were pelleted, resuspended in lysin buffer (200 mM Tris, 500 mM NaCl, 2 mM Mg\( \delta \), pH 8.0), supplemented with 0.1 mg/ml RNase, 0.1 mg/ml DNase, 50 mM L-arginine, 50 mM L-idocitrate, pH 8.8) and lysed by three passages through a French pressure cell at 20,000 PSI. After centrifugation at 100,000 g for 1 h at 4°C, the supernatant was loaded onto a 1 ml HisTrap column (GE Healthcare, Fairfield, CT) and eluted with a linear gradient using the same buffer containing 500 mM imidazole.

Electrophoretic mobility shift assays. His\( \delta \)-SUMO-MucR1, His\( \delta \)-SUMO-MucR2 or His\( \delta \)-SUMO were always freshly diluted into the binding buffer (25 mM Tris, 100 mM KCl, 5 mM Mg\( \delta \), 5% glycerol and 0.5% dodecyl maltoside, pH 7.5) to test the binding to the different Cy3-labelled DNA fragments. They were mixed with BSA (0.5 mg/ml), sonicated salmon sperm DNA (0.05 mg/ml), Inuvitro Carlsbad, CA) and the Cy3-labelled DNA fragments (25 nM) in a total volume of 15 µl. The samples were incubated at room temperature for 10 min, then 5 µl of loading dye (4X binding buffer in 40% glycerol) was added, the samples separated for 3 h through a Typhoon FLA 7000 imager (GE Healthcare). Gel-purified 5’ Cy3-labelled PCR fragments (Macherey-Nagel, Bethlehem, PA) were used as probes.

qChIP assays. Mid-log phase cells were cross-linked in 10 mM sodium phosphate buffer (pH 7.4) and 1% formaldehyde at room temperature for 10 min and on ice for 30 min thereafter, washed three times in phosphate-buffered saline (PBS) and lysed in a Ready-Lyse lysome solution (EPtech Executive Technologies) according to the manufacturer’s instructions. Lysates were sonicated (Sonifier Cell Disruptor B-30, Branson Sonic Power Co., Danbury, CT) on ice using 10 bursts of 20 s at output level 4.5 to shear DNA fragments to an average length of 0.3–0.5 kb and cleared by centrifugation at 14,000 rpm for 30 min at 4°C. Lysates were normalized by protein content, diluted to 1 ml using ChIP buffer (0.01% SDS, 1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl (pH 8.1), 167 mM NaCl plus protease inhibitors (Roche, Switzerland) and pre-cleared with 80 µl of protein-A agarose (Roche) and 100 µg BSA. Ten percent of the supernatant was removed and used as total chromatin input DNA as described before.

Two microcolonies of polyclonal antibodies to CitrA\( \delta \), Citr\( \delta \) were prepared using the Cy3-labeled DNA fragments (25 nM) in a total volume of 50 µl with 80 µl of protein-A agarose beads pre-saturated with BSA, washed once with low salt buffer (0.1% SDS, 1% Triton X-100, 200 mM EDTA, 20 mM Tris-HCl (pH 8.1) and 150 mM NaCl), high salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1) and 500 mM NaCl and LiCl buffer (0.2 M LiCl, 1% NP-40, 1% sodium deoxycholate (1 mg/ml) and 0.5% Tween 80 (pH 8.1)), and twice with TE buffer (10 mM Tris-HCl (pH 8.1) and 1 mM EDTA). The protein DNA complexes were eluted in 500 µl freshly prepared elution buffer (1% SDS and 0.1 M NaHCO3), supplemented with NaCl to a final concentration of 300 mM and incubated overnight at 65°C to reverse the crosslinks. The samples were treated with 2 µg of Proteinase K 2 h at 45°C in 40 mM EDTA and 40 mM Tris-HCl (pH 6.5). DNA was extracted using phenolchloroformisoamylalcohol (25:2:1), ethanol precipitated using 20 µg of glycogen as carrier and resuspended in 100 µl of water. For deep sequencing (ChIP-seq), total chromatin input DNA was not reduced. To determine the specificity of MucR antibodies, samples were prepared and treated as for the ChIP, but after the washing steps the beads were resuspended in SDS loading buffer and boiled. Data are from three biological replicates.

Pull-down of MucR2 with MucR1-TAP. Overnight cultures were used to inoculate 80 ml of PYE (containing 50 µM vanillic acid to induce expression of MucR1-TAP
proteins) and cells were grown to an OD_{600} of 0.5–0.6. Sodium phosphate (pH 7.6) and formaldehyde were added to the cultures to a final concentration of 10 μM and 1%, respectively. The membrane cells were incubated for 10 min at room temperature followed by 30 min on ice. Cells were harvested by centrifugation (6500 rpm, 10 min at 4°C) and washed twice in PBS. Cell pellets were resuspended in 900 μl of resuspension buffer (50 mM sodium phosphate, pH 7.4, 1 mM EDTA, 50 mM NaCl, 10 mM MgCl₂, 0.5% 5-Dodecyl-β-D-maltoside, 1× protease inhibitors (Complete™ EDTA-free, Roche)) and lysed at room temperature for 15 min with a Ready-Lyse lysozyme solution (Epitect Technologies). Cell lysates were sonicated (Bioruptor Pico, Diagenode) at 4°C using 10 cycles of 30 s and cleared by centrifugation at a relative centrifugal force of 18,188 g for 2 min at 4°C. The supernatant was incubated with 1 ml of Sepharose beads (GE Healthcare Bio-Sciences, Sweden) for 2 h at 30 min at 4°C, washed six times with IPP150 buffer (10 mM Tris-HCl pH 8, 150 mM NaCl and 0.1% NP-40) and four times with TEV buffer (IPP150 buffer plus 0.5 mM EDTA and 1 mM DTT), and incubated overnight at 4°C with TEV buffer containing 100 μl of Proteinase K. The resulting mixture was then treated with RNase A (Promega) to release the tagged complex. The eluate was used for SDS-PAGE and immunoblotting.

**Real-time PCR.** Real-time PCR was performed using a Step-One Real-Time PCR system (Applied Biosystems, Foster City, CA) using 5% of each cDNA sample (5 μl) and 12.5 μl of SYBR green PCR master mix (Quanta Biotecnologies, Gaithersburg, MD), 0.5 μl of primers (10 μM) and 6.5 μl of water per reaction. Standard curve generated from the triplicate measurements done per PCR, the final data was generated from three independent cultures. The DNA regions analysed by real-time PCR were from nucleotide −287 to −91 relative to the start codon of pilA, −313 to +32 of pilL, −226 to +30 of tacA and −191 to +14 of scfL.

**EMSA probe preparation.** The different promoter regions were amplified in a PCR using plasmids pSP70 (plac290-based PprA-lacZ transcriptional fusion), pCC_0903-lac290 (sciP) or chromosomal DNA of NA1000 (ctra) as templates and with 5′-Cy3 labelled oligonucleotides piliCy3fw, pilACy3rev, sciPCy3rev, ctrACy3fwshort and ctrACy3revshort, or CtrACy3fwlong and CtrACy3revlong (see Supplementary Table 1 for sequences of oligonucleotides). The 5′-Cy3 labelled PCR fragments were gel purified (Macherey-Nagel).

**β-Galactosidase assays.** β-Galactosidase assays were performed at 30°C as described earlier. Fifty microlitres of cells at OD_{600nm} = 0.1–0.6 were lysed with chloroform and mixed with 750 μl of Z buffer (60 mM Na_{2}HPO_{4}, 40 mM NaH_{2}PO_{4}, 10 mM KCl and 1 mM MgSO_{4}·7H_{2}O) and 0.1% NP-40) and four times with TEV buffer (IPP150 buffer plus 0.5 mM EDTA and 1 mM DTT), and incubated overnight at 4°C with TEV buffer containing 100 μl of Proteinase K. The resulting solution was then treated with RNase A (Promega) to release the tagged complex. The eluate was used for SDS-PAGE and immunoblotting.

**Antibodies used for immunoblotting and ChIP.** Polyclonal antibodies were used to detect cell cycle-regulated protease.

**In vivo phosphorylation measurements.** In vivo P^{32} labelled following by immunoprecipitations (see Supplementary Fig. 1D in ref. 33) were done from cultures of a single colony of cells picked from a PYE agar plate that was washed with M5G medium lacking phosphate and was grown overnight in M5G with 0.05 mM phosphate to an optical density of 0.3 at 660 nm (ref. 47). The strains used were as described in Radhakrishnan et al. One millilitre of culture was labelled for 4 min at 28°C using 30 μCi of γ^{32}P-ATP. Following lysis, proteins were immunoprecipitated with 3 μl of antisera to CtaA. The precipitates were resolved by SDS-PAGE and gel purified (Macherey-Nagel).

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C.F., S.A., S.K.R., J.N., S.Ab., U.J. and P.H.V. conceived and designed the experiments. C.F., S.Ar., S.K.R., J.N., S.Ab., U.J. and P.H.V. contributed analytical tools. C.F., S.Ar., S.K.R., J.N., U.J. and P.H.V. wrote the paper.

**Additional information**

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**Supplementary Information**

accompanies this paper at http://www.nature.com/naturecommunications

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