The Campylobacter jejuni PhoSs/PhosR operon represents a non-classical phosphate-sensitive two-component system

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Summary
The bacterial pathogen Campylobacter jejuni carries several putative two-component signal transduction systems of unknown function. Here we report that the PhoSs (Cj0889) and PhosR (Cj0890) proteins constitute a two-component system that is activated by phosphate limitation. Microarray analysis, real-time RT-PCR, and primer extension experiments indicated that this system regulates 12 genes (including the pstSCAB genes) present in three transcriptional units. Gel shift assays confirmed that recombinant PhosR protein bound DNA fragments containing the promoter regions upstream of these three transcriptional units. Although functionally similar, the PhosS/PhosR system does not exhibit sequence homology with the classical PhoBR systems, has a different pho box (5'-GTTTCNAAAANGTTTC-3') recognized by the C. jejuni response regulator, and is not autoregulated. Because of these atypical properties, we designated the Cj0889-Cj0890 operon as the C. jejuni PhoSs/PhosR system (phosphate sensor/phosphate response regulator) and the phosphate-regulated genes as the pho regulon of C. jejuni.

Introduction
Phosphorus (P) is an essential component for all living organisms. In bacteria, P typically is assimilated as inorganic orthophosphate (P\textsubscript{i}) that is transported into the cell via specific uptake systems. Due to the scarcity of P\textsubscript{i} in the natural world (Ozanne, 1980), organophosphates and phosphonates also may serve as P sources. These compounds are either degraded extracellularly where after the released P\textsubscript{i} is taken up, or imported as complex molecules by specific uptake systems and degraded inside the cells.

Limitation of inorganic phosphate forces bacteria to synthesize a number of proteins involved in the assimilation of P, from the environment. The genes encoding these proteins comprise the phosphate (pho) regulon that is controlled by the two-component signal transduction system PhoBR present in many bacterial species (named PhoP-PhoR in Bacillus subtilis) ( Winner, 1996; Hulett, 2002). In response to P\textsubscript{i} limitation, the sensor PhoR autophosphorylates at a highly conserved histidine residue. The phosphoryl group is transferred to an aspartic acid residue in the N-terminal receiver domain of the response regulator PhoB (PhoP). This results in a conformational change that activates the C-terminal output domain, which then changes the cell physiology by modulating transcription. Phosphorylated PhoB or PhoP transcriptional factors activate the expression of more than 30 different genes in Escherichia coli and B. subtilis, respectively, by binding to the pho boxes located upstream of the phosphate-regulated genes (Martin, 2004). Some of these E. coli genes code for a high-ATP-driven Pi transport system (pstSCAB), a phosphatase (phoA), a negative PhoBR regulator protein (phoU), a polyanion transporter (phoE) and systems for the uptake and degradation of organophosphates (ugpBAEC) and phosphonates (phnC- phnP) (Winner, 1996). Like in E. coli, the B. subtilis PhoPR system is autoregulated and activates the Pi transport system (pstSCA B; B\textsubscript{2}) and phophatases (phoA and phoB). In response to P\textsubscript{i} limitation, B. subtilis obtains phosphate by replacing its teichoic acid with teichuronic acid by activating the PhoPR-dependent genes phoD and tuaABCDFGH and repressing the tagABDEF genes (Hulett, 2002).

Campylobacter jejuni is the leading causative agent of bacterial enteritis worldwide (Altekruse et al., 1999). It is widespread throughout nature and can be isolated from most warm-blooded animals and from a wide variety of watery environmental sources (Rosef et al., 2001; Diergaardt et al., 2004). Analysis of the genome sequence of C. jejuni revealed the presence of 11 putative response regulators, six histidine sensor proteins and one
hybrid sensor response regulator protein (Parkhill et al., 2000). Five of the genes encoding C. jejuni response regulators, cheY, flgR, racR, dccR and cbrR, have been characterized to date (Yao et al., 1997; Bras et al., 1999; MacKichan et al., 2004; Wösten et al., 2004; Raphael et al., 2005). Based on amino acid sequence neither a PhoBR homologue is present, nor genes known to be regulated by the E. coli PhoBR or B. subtilis PhoPR two-component systems, except for the high-ATP-driven P, transport system (pstSCAB). This lack of homology suggests that the C. jejuni pstSCAB genes may be regulated by a different mechanism.

In the present study, we provide evidence that the C. jejuni PhosS (Cj0889) and PhosR (Cj0890) proteins constitute a two-component system that, in response to P, limitation activates (in a direct fashion) the pstSCAB genes and eight other genes. The PhosS/PhosR system shows no sequence homology with and lacks typical features of other phosphate-sensitive two-component systems. Our results suggest that the C. jejuni system may have evolved independently from thus far identified phosphate-sensitive systems in other prokaryotes.

**Results**

The PhosS and PhosR proteins represent a two-component system

The Campylobacter PhosS and PhosR proteins show structural similarities to the family of two-component sensor and response regulator proteins respectively (Parkhill et al., 2000). To confirm that PhosS and PhosR constitute a two-component system we performed phosphorylation assays. Hereto the PhosS and PhosR proteins were expressed in E. coli and isolated as His-tagged recombinant proteins. Phosphorylation assays with the purified proteins demonstrated that the cytoplasmic domain of the sensor PhosS was able to rapidly autophosphorylate itself in the presence of radioactive ATP (Fig. 1). Addition of recombinant regulator PhosR resulted in a rapid dephosphorylation of the sensor. After 4 min of incubation most of the phosphate was transferred from the sensor to the regulator. The total amount of labelled phosphate associated with the sensor and regulator decreased during the course of the experiment, indicating that one of the proteins might have phosphatase activity. This phosphate transfer shows that PhosS and PhosR communicate with each other and represent a two-component system.

The gene pstS (Cj0613) is regulated by the two-component system PhosS/PhosR

To address the role of the PhosS/PhosR two-component system in C. jejuni, we made a mutation in the response regulator PhosR, by insertion of a chloramphenicol resistance (Cm\(^\text{R}\)) cassette. To identify the genes regulated by the PhosS/PhosR system, RNA of logarithmic grown [Heart Infusion (HI) broth] wild-type and phosR::Cm mutant bacteria was isolated and RNA levels were compared using microarray analysis. The microarrays used cover 94% of the predicted open reading frames (ORFs) of the C. jejuni strain 11168 and an additional 276 C. jejuni genes not present in the C. jejuni 11168 genome. Thirteen genes were more than twofold up- or downregulated in the phosR::Cm mutant compared with the parent strain (data not shown). The differences in mRNA levels for these genes were also examined by real-time RT-PCR, normalized to the level of gyrA transcript. Most of the genes identified by the microarray analysis (like Cj1184) could not be confirmed by real-time RT-PCR; however, the pstS (Cj0613) gene was clearly downregulated in the phosR::Cm mutant (Fig. 2A).

Phosphate limitation promotes the transcription of the pstS gene

Transcription of pstS in other bacteria is inducible by phosphate limitation (Allenby et al., 2004). To investigate whether this is true for the C. jejuni pstS gene, we performed real-time RT-PCR on RNA isolated from wild-type and phosR::Cm mutant bacteria grown in low phosphate-containing medium. Transcription of the pstS gene in the
wild type and phosR::Cm mutant was similar when bacteria were grown in defined medium containing 1.6 mM [Pi] (Fig. 2B). In contrast, the pstS transcription levels differed more than 800-fold when the wild-type and mutant strains were grown in defined media with 0.016 mM [Pi] due to upregulation of pstS transcription in the wild-type strain but not in the mutant.

The PhosS/PhosR system is required for optimal growth in phosphate-limited media

To examine whether phosphate limitation influenced the growth kinetics of the phosR::Cm mutant compared with the wild-type strain, we measured the optical densities of the wild-type and mutant bacteria grown under reduced phosphate concentrations. Phosphate limitation clearly resulted in a lower optical density for all strains, indicating that [Pi] was the limiting factor in the media (Fig. 3). Growth curves of the strains were similar in defined media containing 1.6 mM [Pi]. In media containing 0.08 or 0.016 mM [Pi], the initial growth rate of the strains was also the same; however, the phosR::Cm mutant reached a final lower optical density faster. To verify that the disruption of the phosR gene was responsible for the lower final optical density, we complemented the phosR::Cm strain. Hereto plasmid pCP890, containing the complete sensor phosS and response regulator phosR gene, was introduced into the phosR::Cm mutant. The growth curve of the complemented strain was similar to that of the wild type in all tested media, indicating that the mutation of PhosR is responsible for the lower final optical density under phosphate-limiting conditions.

Twelve genes are regulated by the PhosS/PhosR system

To identify the complete regulon belonging to the PhosS/PhosR system, we repeated the microarray analysis, but this time using RNA isolated under PhosS/PhosR inducing conditions (0.08 mM [Pi]). Twenty-five genes were more than 2.5-fold up- or downregulated in the wild type compared with the phosR::Cm mutant. Of these 25 genes, five genes, Cj0145, pstS, pstC, Cj0728 and Cj0729, were more than 70-fold upregulated (Fig. 4A). Genome analysis showed that the pstS and Cj0728 genes were the first of a series of three and five downstream genes respectively, that were also shown to be dependent on a functional PhosS/PhosR system. Most likely, the genes pstSCAB (Cj0613-Cj0616) and Cj0728-Cj0733 form two PhosS/PhosR-regulated operons. All transcripts that were more than 4.5-fold up- or downregulated by microarray were verified by real-time RT-PCR. As a result, three operons Cj0145, pstSCAB and Cj0728-Cj0733 appeared to be regulated by PhosS/PhosR system (Fig. 4B). Based on the gene organization however, we expected Cj0727 rather than Cj0728 to be the first gene of the Cj0727-Cj0733 operon. Unfortunately, Cj0727 was missing from the microarray. Therefore, we also performed real-time RT-PCR on this gene. Transcription of the Cj0727 gene was strongly reduced in the phosR::Cm mutant as was observed for the Cj0728 gene. From these results we conclude that 12 genes, Cj0145, pstSCAB and Cj0727-Cj0733, located in three putative operons, depend on a functional PhosS/PhosR system, forming the pho regulon of C. jejuni.
PhosS/PhosR regulates an alkaline phosphatase

To confirm that the observed regulation of transcription can be extrapolated to the protein level, one of the regulated genes was studied in more detail. In *E. coli* and other bacteria, limitation of [P] induces a PhoB/PhoR-dependent alkaline phosphatase PhoA (Wanner, 1996; Hulett, 2002). Although a homologue of the *E. coli* PhoA appears to be absent in the *C. jejuni* genome, we investigated whether the PhosS/PhosR system is regulating an unknown alkaline phosphatase. To examine this, the wild type and the *phosR::Cm* mutant were grown in defined medium with 1.6, 0.4, 0.08 or 0.016 mM [P] and subjected to an alkaline phosphatase assay. A clear increase in phosphatase activity was observed for the wild-type bacteria as the phosphate concentration decreased (Fig. 5). No phosphatase activity was measured for the *phosR::Cm* mutant, indicating that the PhosS/PhosR is regulating a phosphatase. Of the 12 genes regulated by the PhosS/PhosR system, gene Cj0145, annotated as hypothetical protein in the database, showed some similarity with the alkaline phosphatase of *Vibrio cholerae*. To determine if the gene Cj0145 is coding for the phosphatase, we inactivated this gene. Alkaline phosphatase assays of the Cj0145 mutant revealed that this gene is responsible for the alkaline phosphatase activity in the wild type (Fig. 5). No phosphatase activity was measured for the *phosR::Cm* mutant, indicating that the PhosS/PhosR is regulating a phosphatase. Of the 12 genes regulated by the PhosS/PhosR system, gene Cj0145, annotated as hypothetical protein in the database, showed some similarity with the alkaline phosphatase of *Vibrio cholerae*. To determine if the gene Cj0145 is coding for the phosphatase, we inactivated this gene. Alkaline phosphatase assays of the Cj0145 mutant revealed that this gene is responsible for the alkaline phosphatase activity in the wild type (Fig. 5). These phosphatase assays confirm the mRNA induction seen for the PhosS/PhosR-regulated genes in response to phosphate starvation.

Complementation in trans of the *phosR::Cm* mutant results in a wild-type mRNA profile

To verify that disruption of *phosR* was responsible for the transcription defect of the above-mentioned 12 genes, we complemented the *phosR::Cm* strain. Hereto plasmid pCP890, containing the complete sensor *phosS* and response regulator *phosR* genes, was introduced into the *phosR::Cm* mutant. Transcripts of the Cj0145, pstS and Cj0727 genes in the wild type, mutant and complemented mutant grown under phosphate rich (1.6 mM) or poor (0.08 mM) conditions were determined by real-time RT-PCR (Fig. 6). Only minor transcript fold differences were observed between wild type and mutant or the complemented strain in phosphate-rich defined medium. However, transcript differences of more than 200-fold between the wild-type and mutant strains for the Cj0145, pstS and Cj0727 genes were seen at low [P] while less than 30-fold in the case of the wild-type and complemented strain. Taken together, these results demonstrate that plasmid pCP890 is able to complement the defect in the *phosR* gene and thus that PhosR is involved in the regulation of the *C. jejuni* pho regulon.

Mapping of the transcriptional start sites of promoters located upstream Cj0145, pstS and Cj0727 genes

To identify the PhosR-dependent transcription start sites in the Cj0145, pstS and Cj0727 promoter regions, we performed primer extension experiments using total RNA isolated from the wild-type and the *phosR::Cm* mutant strains grown under phosphate starvation conditions. Primer extension products were obtained only when RNA isolated from the wild-type cells was used (Fig. 7A), consistent with the real-time RT-PCR data for these genes. Two adjacent PhosR-dependent transcription start points were obtained for the Cj0145 promoter corresponding to a T or G residue 30 or 29 bp upstream of the Cj0145 translational start site (Fig. 7A and F). Single PhosR-dependent transcription start sites were identified for the pstS and Cj0727 promoters, corresponding to a C residue 23 bp upstream of the pstS translation start codon and to
an A residue 21 bp upstream of the Cj0727 translation start codon. Upstream the transcription start sites of these genes, a -10 promoter region is located displaying similarity to the C. jejuni σ⁰ -10 promoter region (Wösten et al., 1998); however, no conserved σ⁰ -35 promoter region could be identified (Fig. 7F).

Identification of the C. jejuni pho box located in front of the Cj0145, pstS and Cj0727 genes by gel mobility shift assays

To investigate whether PhosR is able to bind to the promoter regions upstream the Cj0145, pstS and Cj0727 genes, the Cj0145 translation start codon was used as a probe in gel mobility shift assays (Fig. 4B). A putative Pho box located 117 bp upstream of the Cj0145 translation start codon was identified. The Pho box consists of two separate motifs that are not conserved within the C. jejuni genome. A Pho box was also identified upstream of the Cj0727 translation start codon, which consists of two separate motifs that are conserved within the C. jejuni genome. The Pho boxes upstream of the Cj0145 and Cj0727 translation start codons were used as probes in gel mobility shift assays (Fig. 4C). The Pho boxes upstream of the Cj0145 and Cj0727 translation start codons were found to be recognized by PhosR, which is consistent with the results of the microarray analysis.
genes, we performed gel mobility shift assays with PhosR. The PhosR response regulator protein was isolated as his-tagged recombinant protein (PhosR-His) and incubated (in non-phosphorylated form) with DNA fragments containing the promoter regions upstream of \( \text{pstS} \), Cj0145 or Cj0727. The PhosR-His protein was able to shift the DNA fragments \( \text{pstS-F1} \) and \( \text{pstS-F2} \) containing the \( \text{pstS} \) promoter with or without the \(-10\) region respectively (Fig. 7B and C). The formation of the PhosR-His protein and \( \text{pstS-F1} \) DNA complex was efficiently inhibited in the presence of an excess of unlabelled \( \text{pstS-F1} \) fragment, indicating specificity of the interaction (Fig. 7D). No band shifts were detected when a DNA fragment \( \text{pstS-F3} \) lacking the \(-10\) and \(-35\) regions of the \( \text{pstS} \) promoter was used (Fig. 7C). The difference between the DNA fragments \( \text{pstS-F2} \) and \( \text{pstS-F3} \) is only 33 bp comprising the putative \(-35\) promoter region of the \( \text{pstS} \) gene. Based on the gel shift and primer extension experiments, the results suggest that the PhosR-His recombinant protein binds to the DNA sequence overlapping the putative \(-35\) region. The promoter regions of Cj0145 and Cj0727 could not be shifted by PhosR-His recombinant protein alone, indicating that the PhosR-His recombinant protein has a higher affinity for the \( \text{pstS} \) promoter region than for the Cj0145 and Cj0727 promoter regions (Fig. 7E). The recombinant sensor PhosS-His protein together with ATP is needed to observe a band shift for the Cj0145 and Cj0727 promoter regions (Fig. 7E). Alignment of the three promoter regions showed a strong conserved region around the \(-35\) putative promoter regions resembling the sequence GTTTC NAAAANGTTTC (Fig. 7F). This sequence is located in the 33 bp extra DNA present in \( \text{pstS-F2} \) compared with \( \text{pstS-F3} \) which we designated as the \( \text{pho} \) box of \( C. \text{jejuni} \).

**Discussion**

Bacteria monitor changes in their environment to be optimally adapted to their ecological niches. The sensing and responding to external stimuli is usually mediated by two-component signal transduction systems. Here we provide evidence that (i) the bacterial pathogen \( C. \text{jejuni} \) PhosS and PhosR proteins form a two-component system, (ii)
Fig. 7. Characterization of the promoter regions upstream the Cj0145, pstS and Cj0727 genes.
A. Primer extension experiments were performed to determine the transcription start sites upstream of the Cj0145, pstS and Cj0727 genes. Primer extension products were generated using total RNA isolated from wild type (lane 1) or phosR::Cm mutant (lane 2) bacteria grown in defined medium containing 0.08 mM [Pi]. The primer extension products were run on a 6% sequencing gel against dideoxy sequencing reactions primed with the same primer as used for the extension reactions. The sequence spanning the transcription start site is shown, and the transcription start site is marked with an arrow.
B. DNA sequence of the intermediate region between the genes cft and pstS. Coding sequence, ribosomal binding sequence (RBS) and primers used for gel mobility shift assays are indicated.
C. Gel mobility shift assays of pstS promoter fragments bound by PhosR-H6N protein. Dig-labelled PCR fragments pstS-F1 (232 bp), pstS-F2 (163 bp) and pstS-F3 (130 bp) made with primers sets CFTDig/pstSR1, CFTDig/pstSR2 and CFTDig/pstSR3 respectively, and incubated with 0, 1, 5, 25 or 50 pmol of PhosR-H6N protein. The concentration of PhosR-H6N protein used in the reactions is indicated above the lanes.
D. Competition gel mobility shift assays were performed by incubation of 25 pmol PhosR-H6N protein with 25 pmol labelled pstS-F1 and different indicated concentrations of unlabelled pstS-F1* DNA fragments.
E. Gel mobility shift assays of the Cj0145, pstS and Cj0727 promoter regions bound by PhosR-H6N. Dig-labelled PCR fragments containing the Cj0145, pstS and Cj717 promoter region were incubated with 25 pmol PhosR-H6N and 44 pmol PhosS-H6N with or without 2 mM ATP.
F. Alignment of the Cj0145, pstS and Cj0727 promoter DNA sequences. Start codon (Met), ribosomal binding sequence (RBS) and experimental determined transcription startpoints (small capital) and putative -10 regions (-10) as well as the putative PhosR binding sequence (PhosR) are shown.

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this system is activated during phosphate starvation, (iii) this system is required for optimal growth under phosphate limitation, (iv) this system regulates 12 genes involved in the acquisition of Pᵢ, (v) (GTTTC NAAAANGTTTC) is likely to be the PhosR binding sequence and (vi) the Cj0145 gene of unknown function is coding for an alkaline phosphatase (Fig. 8).

Evidence that the PhosS and PhosR genes comprise a two-component system was provided by the autophosphorylation and phosphate transfer assays with purified PhosS and PhosR recombinant proteins (Fig. 1). The rapid phosphorylation of PhosS in the presence of ATP indicated that the protein carried autokinase activity, which is typical for two-component sensor kinase proteins. In many other two-component systems, phosphorylated response regulators are less stable due to phosphatase activity of the regulator or sensor protein (Stock et al., 2000; Wösten et al., 2004). Our results suggest that the PhosS/PhosR two-component system also may possess phosphatase activity. Because both the sensor PhosS and response regulator PhosR do not show any obvious sequence similarities with other known members of the family of two-component proteins (Stock et al., 2000), we decided to perform microarray analysis to identify the regulon that is activated by this system. The identification of the gene annotated as pstS and supposedly coding for a protein involved in phosphate uptake led us to investigate whether low phosphate levels could activate this system. We were able to demonstrate that the PhosS-PhosR system was activated and required for bacterial growth during phosphate starvation and that its induction resulted in the expression of 12 genes, representing the pho regulon of C. jejuni. In many other bacterial species, genes involved in the assimilation of P, from various phosphorus compounds are under the control of the PhoBR two-component signal transduction system (Wanner, 1996; Hulett, 2002). Although functionally similar (i.e. regulated by phosphate), the PhosS/PhosR and classical PhoRB systems exhibit major differences: (i) in contrast to PhoR, the sensor PhosS contains a large periplasmic domain but no PAS domain (Koretke et al., 2003), (ii) the DNA motif (pho box) recognized by the C. jejuni response regulator does not resemble that of PhoB, although they both do recognize a direct repeat, (iii) PhosR-regulated promoters contain only one pho box, (iv) the PhosS/PhosR system is not autoregulated as no difference in the amount of PhosS transcript were observed between the wild type and phosR::Cm mutant grown at low [Pᵢ] (data not shown) and (v) in addition, the negative PhoBR regulator PhoU appears absent in C. jejuni as deduced from BLAST analysis and the microarray results. Based on these differences we proposed to rename Cj0889 as PhosS (phosphate sensor) and Cj0890 as PhosR (phosphate response regulator) instead of using the symbols PhoB/PhoR.

We identified three transcriptional units that are regulated by the PhosS/PhosR system by microarray analysis, real-time RT-PCR analysis, and primer extension. The response regulator PhosR binds to the sequence overlapping the −35 promoter regions upstream of these units. The DNA shift experiments suggest that the protein has a higher affinity for the pstS than for the Cj0145 and Cj0727 promoter regions (Fig. 7C). It is known that phosphorylation of response regulators increases the binding affinity to their target promoters (Cho et al., 2001). While the three promoter regions share a common small direct repeat (GTTTC) separated by a conserved polyA region,
the direct repeat in the \textit{pstS} promoter sequence is much larger: AATGTTTCCAA, which might explain the affinity difference for the target promoter elements. Scanning the \textit{C. jejuni} genome for PhosR-binding sequence (GTTTCNAAAANGTTTC) did not lead to the discovery of additional PhosR-dependent promoter elements. Based on the microarray results and genome search for PhosR-binding sequences, we assume that the identified phosphate-sensitive genes constitute the complete \textit{C. jejuni} \textit{pho} regulon.

Further analysis of the genes comprising the \textit{C. jejuni} \textit{pho} regulon revealed that the gene Cj0145 is coding for an alkaline phosphatase (Fig. 5). This protein is 52% identical at the amino acid level to the \textit{V. cholerae} PhoA\textsuperscript{BC}. This protein is a monomeric alkaline phosphatase that is different from the well-studied PhoA of \textit{E. coli} (Majumdar \textit{et al.}, 2005). To avoid confusing symbols we have named gene Cj0145, \textit{phoA}\textsuperscript{C}. The four genes Cj0613-Cj0616 code for the high-ATP-driven \textit{P}i transport system \textit{PstSCAB} (Wanner, 1996) that can be found in many bacterial species (Yuan \textit{et al.}, 2000). Although the predicted amino acid sequences of the remaining seven genes in the \textit{pho} regulon (Cj0727-Cj0733) are all highly conserved among other bacterial species, their function still needs to be determined (Fig. 4A) (Parkhill \textit{et al.}, 2000). Four of these genes, however, appear to encode ABC transporters that might be part of an unknown uptake system.

Where does \textit{C. jejuni} encounter low phosphate conditions? The natural habitat of \textit{C. jejuni} is the intestine of warm-blooded animals and a wide variety of watery en-

\begin{table}
\centering
\begin{tabular}{|l|l|l|}
\hline
\textbf{Strain or plasmid} & \textbf{Genotype or relevant characteristics} & \textbf{Source or reference} \\
\hline
\textit{C. jejuni} strains &  &  \\
81116 & Wild type & Palmer \textit{et al.} (1983) \\
\textit{phosR}:Cm & 81116 derivative \textit{phosR}:Cm & This study \\
Cj0145::Cm & 81116 derivative Cj0145::Cm & This study \\
\textit{E. coli} strains &  &  \\
PC2955 & relA \textit{F}80lacZM15 \textit{phoA8} hsdR17 recA1 endA1 gyrA96 thi-1 \textit{luxS} \textit{glnV44} & NCCB \\
BL21(DE3) & ompThadS dcm \textit{Tet} \textit{gal(DE3)} endA1 het & Novagen \\
\textbf{Plasmids} &  &  \\
PGEM-T Easy & PCR cloning vector, \textit{Amp'} & Promega Corporation, Madison, WI \\
pAV35 & pBluescript II SK containing \textit{C. coli} \textit{Cm'} cassette & van Vliet \textit{et al.} (1998) \\
pGEM890-889 & pGEM-T Easy containing the \textit{phosR} and \textit{phosS} genes on a 3580 bp fragment & This study \\
pGEM890::Cm & pGEM890 with \textit{Cm'} inserted in \textit{phosR} & This study \\
pGEM145 & pGEM-T Easy containing gene Cj0145 on a 3230 bp fragment & This study \\
pGEM145::Cm & pGEM145 with \textit{Cm'} inserted in Cj0145 & This study \\
pT7.7 & Expression vector, \textit{Amp'} & Tabor and Richardson (1985) \\
pT7-889c-H6N & pT7.7 containing the truncated \textit{phosS} gene with N-terminal His-taq & This study \\
pT7-890-H6N & pT7.7 containing \textit{phosR} gene with N-terminal His-taq & This study \\
pGEM1491 & pGEM-T Easy containing the Cj1493, Cj1492 and Cj1491 genes on a 2549 bp fragment & This study \\
pWM1007Pr1492 & pWM1007 containing the Cj1492 promoter & This study \\
pCP890 & \textit{phosS} and \textit{phosR} genes downstream the 1492 promoter & This study \\
\hline
\end{tabular}
\caption{Bacterial strains and plasmids used in this study.}
\end{table}

Experimental procedures

\textbf{Strains and growth conditions}

The strains and plasmids used in this study are listed in Table 1. \textit{C. jejuni} 81116 and derivatives were routinely maintained at 37°C under microaerobic conditions (5\% \textit{O}2, 10\% \textit{CO}2 and 85\% \textit{N}2) either on blood agar base II medium (Oxoid, London, UK) containing 5\% horse blood lysed with 0.5\% saponin (Sigma, St Louis, MO) or in HI broth (Oxoid). \textit{E. coli} were grown in Luria–Bertani medium at 37°C. When appropriate, growth media were supplemented with ampicillin (100 \textmu g ml\textsuperscript{-1}), chloramphenicol (20 \textmu g ml\textsuperscript{-1}) or kanamycin (50 \textmu g ml\textsuperscript{-1}).
**Construction of the phosR mutant**

A 3580 bp DNA fragment, containing the complete phosR and phosS genes, was amplified from the C. jejuni 81116 chromosome using the primers Cj0890F and Cj0890C (Table 2). This fragment was cloned into pGEM-T Easy (Promega Corporation, Madison, WI) resulting in plasmid pGEM890-889. Plasmid pGEM890-889 was subsequently amplified with primers Cj0890FBamHI and Cj0890RBamHI to introduce a BamHI restriction site. The PCR product was digested with BamHI and ligated to a 0.7 kb BamHI fragment containing a Cmr gene of pAV35, resulting in the knockout construct pGEM890:Cm. This knockout construct containing the phosR gene with a 10 bp deletion and the Cmr gene in the same orientation as the Cj0145 gene was introduced in C. jejuni containing a Cmr gene of pAV35, resulting in the knockout construct pGEM890::Cm. This knockout construct containing the phosR gene was introduced by natural transformation in C. jejuni 81116 (Wassenaar et al., 1993). Homologous recombination resulting in double-crossover event was verified by PCR.

**Construction of the Cj0145 mutant**

The Cj0145 gene with flanking regions was amplified from the 81116 genome by PCR using the primers Cj144F2 and trxB (Table 2). The resulting 3230 bp fragment was cloned into pGEM-T Easy to form plasmid pGEM145. This plasmid was digested with XbaI and ClaI (resulting in a Cj0145 deletion of 174 bp) and ligated to a 0.7 kb XbaI-AccI fragment containing a Cmr gene of pAV35. The resulting knockout construct pGEM145:Cm containing the Cmr cassette in the same orientation as the Cj0145 gene was introduced in C. jejuni 81116 by natural transformation.

**Construction of PhosR and PhosS overexpression plasmids**

To overexpress the complete response regulator PhosR and the cytoplasmic domain of sensor protein PhosS, chromosomal DNA (strain 81116) was amplified using the primer combinations HisCj0890NdeI/Cj0890PstI and HisCj0890NdeI/Cj0890PstI respectively (Table 2). The resulting PCR fragments (662 and 728 bp long respectively) were digested with Ndel and PstI and cloned into the Ndel and PstI sites of pT7.7 to form pT7-890-H6N and pT7-889c-H6N respectively. The nucleotide sequence of the cloned PCR products was verified by sequencing both strands.

**Construction of a PhosR complementation plasmid**

The Cj1492 promoter, present on a 2549 bp PCR product made with primers Cj1491cF and Cj1491cR (Table 2), was isolated from plasmid pGEM1491 by digesting this plasmid with Sall and Muni. The resulting 1414 bp promoter fragment was ligated into the Campylobacter shuttle vector pWM1007 digested with Sall and EcoRI to obtain plasmid pWM1007Pc1492. The intact sensor and regulator genes phosS and phosR were cloned downstream the Cj1492 promoter by digesting the plasmids pGEM890-889 and pWM1007Pc1492 with SstI and NotI followed by ligation to obtain the complementation plasmid pCP890.

**Purification of recombinant proteins**

Histidine-tagged PhosS and PhosR proteins were expressed in E. coli BL21(DE3) containing plasmid pT7-890-H6N or pT7-889-H6N. Protein expression and purification were performed as described previously (Wösten et al., 2004). Protein concentrations were determined using the Bradford method (Bradford, 1976).

**Phosphorylation assays**

Recombinant PhosSc-H6N (22 pmol) and PhosR-H6N (4.3 pmol) proteins were incubated at room temperature for 15 min, with 10 μCi of [γ-32P]ATP in 40 and 10 μl phosphorylation buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 2 mM MgCl2, and 1 mM DTT) respectively. Phosphorylated PhosSc-H6N was separated from [γ-32P]ATP by ultrafiltration (Microcon YM10, Millipore Corporation, Bedford, MA). Phosphorylation of 4.3 pmol PhosR-H6N was accomplished by adding 22 pmol of autophosphorylated PhosSc-H6N in 80 μl phosphorylation buffer. After 0, 0.1, 0.5, 1, 2, 4, 8 and 16 min, a 10 μl sample was taken and the reaction of this sample was stopped with SDS-loading buffer. Samples were run on 12.5% SDS-polyacrylamide gels. After electrophoresis the gel was dried and autoradiographed.

**Growth curve**

Overnight cultures of Campylobacter grown in defined medium (1.6 mM [P]) (Leach et al., 1997) were diluted to A620 of 0.05 in 10 ml of defined medium containing either 1.6, 0.08 or 0.016 mM [P] and incubated at 37°C, 150 rpm under microaerobic conditions (Brazier and Smith, 1989). Throughout the growth cycle, flasks for each time point were removed from their incubation conditions, and 1 ml samples of the cultures were obtained for analysis.

**RNA isolation**

Precultures of Campylobacter grown in HI or defined media (1.6 mM [P]) were diluted to A550 of 0.05 in 5 ml HI or defined medium (containing either 1.6, 0.4, 0.08 or 0.016 mM [P]) respectively, and incubated at 37°C, 150 rpm under microaerobic conditions. Total RNA was extracted from logarithmic phase grown cultures (A550, 0.4–0.62) with the RNA-BeeTM kit (Tel.Test) according to the manufacturer’s specifications. Size chromatography of RNA was done with an Agilent 2100 Bioanalyser.

**Construction of the C. jejuni DNA microarray**

DNA fragments of individual ORFs were amplified using ORF-specific primers for those present in strain NCTC 11168 (Sigma Genosys, The Woodlands, TX) and for strain RM1221 using primers from Operon Technologies (Alameda, CA) designed with ArrayDesigner 2.0 (Premier Biosoft, Palo Alto, CA). Additionally, lipoooligosaccharide (LOS) genes were amplified from LOS locus classes A, B, C, D, E and F as described previously (Parker et al., 2005). Each PCR reac-
Table 2. Primers used in this study.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>DNA sequence (5'-3')</th>
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Real-time RT-PCR

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**Microarray hybridization and analysis**

For the expression profiling, an indirect comparison of gene expression was performed (Yang and Speed, 2002). In this microarray experimental design, each labelled cDNA was combined with a common reference (labelled genomic DNA), as described in previous studies (Eriksson et al., 2003; Lucchini et al., 2005). The labelled genomic DNA also served as a quality control for all the spots in the array. Twenty micrograms of total RNA from each *C. jejuni* isolate was labelled during reverse transcription to cDNA with Cy3-dUTP using Stratascript (Stratagene, Palo Alto, CA). Following 16 h labelling, RNA was degraded by the addition of NaOH to 0.3 M and incubation at 70°C for 10 min, followed by neutralization with an equimolar amount of HCl. The labelled cDNA was purified using Qiagen Qiaquick PCR columns according to manufacturer's directions. Genomic DNA from strain 81116 was labelled with Cy5-dUTP. Approximately 2 μg of DNA was mixed with 5 μl 10× NEBiot labelling buffer containing random sequence octamer oligonucleotides (New England Biolabs, Beverly, MA) and water to a final volume of 41 μl. This mixture was heated to 95°C for 10 min and then stored for 5 min on ice. After this incubation, the remainder of the labelling reaction components were added: 5 μl of 10× dNTP labelling mix (1.2 mM each dATP, dGTP, dCTP; 0.5 mM dTTP in 10 mM Tris pH 8.0; 1 mM EDTA), 3 μl of Cy5-dUTP (Amersham Biosciences, Piscataway, NJ) and 1 μl of Klenow fragment. The labelling reactions were incubated overnight at 37°C. Fluorescently labelled DNA was purified using Qiagen Qiaquick PCR columns according to manufacturer's directions.

For each gene expression hybridization, Cy5-labelled reference DNA from strain 81116 was mixed with Cy3-labelled test cDNA in 45 μl of Corning hybridization buffer (Acton, MA) and heated to 95°C for 5 min. Then 15 μl of the hybridization reaction (total reaction volume, 100 μl) consisted of 1× MasterAmp Taq PCR buffer, 1× MasterAmp Taq Enhancer, 2.5 mM MgCl₂, 200 μM each deoxynucleoside triphosphate, forward and reverse primers at 0.2 μM each, 0.5 U of MasterAmp Taq DNA polymerase (Epicentre Madison, WI), and approximately 50 ng of genomic DNA from strain NCTC 11168. Thermal cycling was performed using a Tetrad thermal cycler (MJ Research, Waltham, MA) with the following amplification parameters: 30 cycles of 25 s at 94°C, 25 s at 52°C, and 2 min at 72°C and a final extension at 72°C for 5 min. PCR products were analysed by gel electrophoresis in a 1% (wt/vol) agarose gel (containing 0.5 μg of ethidium bromide ml⁻¹) in 1× Tris-acetate-EDTA buffer. DNA bands were examined under UV illumination. We successfully amplified a total of 1530, 227 and 40 PCR products from strain NCTC 11168, RM1221 and the LOS genes respectively, covering 94% and 12% of the predicted ORFs of strains 11168 and RM1221. They were purified on a Qiagen 8000 robot using a Qiagen 96-well Biorobot kit (Qiagen, Valencia, CA), dried and resuspended to an average concentration of 0.1–0.2 μg ml⁻¹ in 20 μl of 50% dimethyl sulphoxide (DMSO) containing 0.3× saline sodium citrate (SSC). All of the PCR probes were then spotted in duplicate on UltraGAPS slides (Corning) using an OmniGrid Accent (GeneMachines, Ann Arbor, MI) producing a final array containing a total of 3594 features.
mixture was put onto the microarray slide and sealed with a coverslip in a Corning hybridization chamber and incubated at 42°C for 18 h. Following hybridization, microarray slides were washed for 2 min in 2× SSC, 0.1% SDS at 42°C to remove the coverslip and then washed twice for 5 min each time in each of the following buffers: (a) 2× SSC, 0.1% SDS at 42°C, (b) 0.2× SSC, and finally (c) 0.01× SSC. Microarray slides were dried by centrifugation at 300 g for 15 min before scanning.

DNA microarrays were scanned using an Axon GenePix 4000B microarray laser scanner (Axon Instruments, Union City, CA) and the data for spot and background intensities were processed using the GenePix 4.0 software. Poor features were excluded from analysis if they contained abnormalities or a reference signal lower than background plus three standard deviations. As described by Eriksson et al. (2003), fluorescence ratios were calculated after local background was subtracted from spot signals. To compensate for any effect of the amount of template and uneven Cy-dye incorporation, data normalization was performed as previously described (Anjum et al., 2003; Eriksson et al., 2003).

Normalized data that passed the quality controls were analysed using GENESPRING 7.2 software (Agilent). For the comparison of C. jejuni strains 81116 and 81116 phoR::Cm gene expression, six hybridization measurements were generated per biological experiment (three technical replicate arrays and two replicate features per array) and the experiment was repeated two times (biological replicates). Significance of the centred data at \( P \geq 0.05 \) was determined using a parametric-based statistical \( t \)-test adjusting the individual \( P \)-value with the Benjamini and Hochberg false discovery rate multiple test correction within the GeneSpring analysis package.

**Real-time RT-PCR**

Real-time RT-PCR analysis was performed as previously described (Wösten et al., 2004). Primers used in this assay are listed in Table 2. The calculated threshold cycle (CT) for each gene amplification was normalized to the CT of the gyrA gene amplified from the corresponding sample before calculating fold change using the arithmetic formula (2−ΔΔCT), where \( \Delta \Delta CT = \left[ \left( CT_{target} - CT_{gyrA} \right)_{mutant} - \left( CT_{target} - CT_{gyrA} \right)_{wild-type} \right] \) (Schmittgen, 2001), where target = gene. Each sample was examined in four replicates and was repeated with at least two independent preparations of RNA. Standard deviations were calculated and are displayed as error bars.

**Gel mobility shift assays**

The promoter region upstream of the genes Cj0145, pstS and Cj0727 were amplified by PCR using C. jejuni 81116 chromosomal DNA as template. Primers CFTDig, Cj144FDig and corAFD dig were digoxigenin labelled (Isogen). The Cj0145 (246 bp), pstS-F1 (232 bp), pstS-F2 (163 bp), pstS-F3 (130 bp) and Cj0727 (163 bp) promoter regions were amplified using primer sets Cj144FDig/Cj0145R, CFTDig/pstSR1, CFTDig/pstSR2, CFTDig/pstSR3 and corAFD/Cj0727R respectively. Approximately 25 pmol of digoxigenin-labelled pstS-F1, pstS-F2, pstS-F3 DNA fragments and 0, 1, 5, 25 or 50 pmol of PhosR-H6N protein in a 15 μl volume were incubated at room temperature for 15 min. The Cj0145, pstS-F1 and Cj0727 DNA fragments (25 pmol) were incubated with 25 pmol PhosR-H6N and 44 pmol PhosS-H6N with or without 2 mM ATP. For competition gel mobility shift assays 25 pmol PhosR-H6N protein, 25 pmol pstS-F1 DNA and 30 or 180 pmol unlabelled pstS-F1 DNA in a 15 μl volume were incubated at room temperature for 15 min. The binding buffer used for protein–DNA incubations contained 20 mM Tris, pH 7.4, 5 mM MgCl₂, 50 mM KCl, 5 μg bovine serum albumin, 1.0 μg poly (dl-dC), and 5% glycerol. Samples (20 μl) were run on a 4% non-denaturing Tris-glycine polyacrylamide gel at 4°C. Following electrophoresis, DNA was transferred to nylon membranes (Amersham) and UV cross-linked. Digoxigenin-labelled DNA was detected with anti-digoxigenin antibodies according to the manual of the manufacturer (Roche).

** Primer extensions**

Analysis of the 5′ ends of the Cj0145, pstS and Cj0727 mRNA transcripts was performed by primer extension using \( [\gamma \sp{32}P]ATP \) labelled primers. A total of 5 pmol of primer Cj0145Prex, pstSPrex or Cj0727Prex (Table 2) was annealed to 20 μg of RNA extracted from Campylobacter grown in defined medium with 0.08 mM [P]. Synthesis of cdNA was performed using SuperScript™II RnaseH – reverse transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. The extension products were analysed by electrophoresis on a 6% polyacrylamide, 7.5 M urea gel and compared with sequence ladders initiated with primers Cj0145Prex, pstSPrex or Cj0727Prex.

**Alkaline phosphatase assay**

Optical density of the culture (1 ml) was measured at 600 nm before pelleting. The pellet was resuspended in 0.5 ml of 10 mM Tris/HCl pH 8, 1 mM EDTA, 0.1% SDS and 5 μg lysozyme and incubated for 10 min at room temperature. Alkaline phosphatase activity was assayed by monitoring the release of \( \text{p}-\text{nitrophenol (PNP)} \) from 2 mM \( \text{p}-\text{nitrophenyl phosphate (PNPP)} \) at 37°C and stopped by adding 0.1 M KH₂PO₄. The units of alkaline phosphatase were calculated using the formula \( \text{OD}_{400} \times V \times t \)/\( \text{OD}_{600} \times 10^{9} \times A_{420} - (1.75 \times A_{550}) \)/\( A_{550} - A_{650} \), where \( A_{420} \) and \( A_{650} \) are the absorbencies of the reaction mix after an incubation time \( t \) (min), \( \text{OD}_{600} \) is the optical density of the culture and \( V \) is the volume (ml) of the culture used in the assay (Brickman and Beckwith, 1975)

**Acknowledgements**

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**References**


