Growth Phase-Dependent Activation of the DccRS Regulon of *Campylobacter jejuni*[▽]

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Two-component systems are widespread prokaryotic signal transduction devices which allow the regulation of cellular functions in response to changing environmental conditions. The two-component system DccRS (Cj1223c-Cj1222c) of *Campylobacter jejuni* is important for the colonization of chickens. Here, we dissect the DccRS system in more detail and provide evidence that the sensor DccS selectively phosphorylates the cognate effector, DccR. Microarray expression profiling, real-time reverse transcription-PCR (RT-PCR), electrophoretic mobility shift assay, and primer extension analyses revealed that the DccRS regulon of strain 81116 consists of five promoter elements, all containing the consensus direct repeat sequence WTTCAC-N6-TTCACW covering the putative −35 promoter regions. One of these promoters is located in front of an operon encoding a putative macrolide efflux pump while the others are in front of genes coding for putative periplasmic or membrane proteins. The DccRS-regulated genes in *C. jejuni* strain 81116 are needed to enhance early *in vivo* growth of *C. jejuni* in 7-day-old chickens. The DccRS system is activated in the late stationary bacterial growth phase, probably by released metabolic products. Whole-genome mRNA profiling and real-time RT-PCR analysis under these conditions demonstrated that the system has no influence on the transcription of genes outside the DccRS regulon.

Eubacterial species typically harbor several dozen two-component systems, each responding to distinct cues and controlling the expression of discrete sets of genes. Usually they are composed of a histidine sensor protein that perceives environmental stimuli via its N-terminal input domain and a cognate response regulator. In the presence of the appropriate stimulus, the sensor protein autophosphorylates at a highly conserved histidine residue in the transmitter domain (13). Subsequently, the kinase activity of the histidine sensor transfers the phosphoryl group to an aspartic acid residue in the N-terminal receiver domain of the response regulator, resulting in a conformational change and activation of its C-terminal output domain, which frequently has DNA-binding properties. This ultimately leads to a bacterial phenotype that is optimally adapted to its ecological niche.

Campylobacter jejuni is the leading causative agent of bacterial enteritis worldwide (1). It is widespread throughout nature and can be isolated from many sources, including birds, domestic animals, and water. However, its most favored habitat appears to be the intestine of avian species, in which up to 10^9 CFU per gram of feces can be found (9). Ingestion of as few as 100 *Campylobacter* bacteria is sufficient to colonize the avian gut (12, 24). Little information regarding the molecular mechanisms regulating the expression of *Campylobacter* genes needed for optimal colonization of the host is available.

The relatively small genome (between 1,650 to 1,800 genes) of *C. jejuni* is regulated by only 37 putative transcription factors, of which 10 are response regulators (20). Therefore, many regulatory networks found in other bacterial species are missing in *C. jejuni*. Due to the lack of global stationary-phase regulatory elements, the physiological response to the conditions existing in stationary-phase cultures is restricted, and the transcription factors that might regulate this adaptation are largely unknown. One system shown to be important for the *C. jejuni* colonization of 1-day-old chickens is the response regulator DccR (8). This protein directly activates two hypothetical genes, Cj0200c and Cj1356c, but may regulate additional genes, as hypothesized from the presence of conserved DNA motifs (8). The role of the DccR/DccS system in chicken colonization as well as the signal that activates the system remains to be discovered.

In this study, we analyzed the DccR two-component system in more detail, and we provide evidence of the following in *C. jejuni* strain 81116: (i) DccR is the cognate response regulator for the sensor DccS, (ii) the two-component system is constitutively expressed under the conditions employed and not subject to autoregulation, (iii) the system upregulates seven genes through interaction of DccR with the promoter region, (iv) strong activation of the genes regulated by the DccRS system occurs in the late stationary phase, and (v) the system is not required for but accelerates colonization in the chicken.

MATERIALS AND METHODS

Strains and growth conditions. The strains and plasmids used in this study are listed in Table 1. *C. jejuni* 81116 and derivatives were routinely maintained at 37°C under microaerobic conditions (5% O_2, 10% CO_2, and 85% N_2) either on blood agar base II medium (Oxoid Ltd., London, United Kingdom) containing

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or relevant characteristic(s)	Source or reference
C. jejuni strains		
81116	Wild type	10
81116 *dccR*::Cm mutant	81116 derivative *dccR*::Cm	This study
E. coli strains		
PC2955	*relA1* φ80d*lacZ*ΔM15 *phoA8 hsdR17 recA1 endA1 gyrA96 thi-1 relA1 luxS glnV44*	NCCB
BL21(DE3)	F⁻ *ompT hsdS*$_B$(r$_B^-$ m$_B^-$) *gal dcm* (DE3)	Novagen Inc.
Plasmids		
PGEM-T Easy	PCR cloning vector; Amp^r	Promega Corp., Madison, WI
pAV35	pBluescript II SK containing *Campylobacter coli* Cm^r cassette	16
PGEM*dccR*	pGEM-T Easy containing *dccR* gene on a 2,009-bp fragment	This study
PGEM*dccR*::Cm	pGEM*dccR* with Cm^r inserted in *dccR*	This study
pMA1	*C. jejuni-E. coli* shuttle conjugative expression vector; Km^r	15
pMA1-1223	pMA1 containing DccRS operon	This study
pT7.7	Expression vector; Amp^r	14
pT7-1223-H6C	pT7.7 containing Cj1223c gene with C-terminal His tag	This study
pT7-PhosR-H6N	pT7.7 containing Cj0890 gene with N-terminal His tag	19
pT7-1491-H6N	pT7.7 containing Cj1491 gene with N-terminal His tag	This study
pT7-1222-H6N	pT7.7 containing Cj1222c gene with N-terminal His tag	This study
pT7-RacR-H6N	pT7.7 containing *racR* gene with N-terminal His tag	This study

5% horse blood lysed with 0.5% saponin (Sigma, St. Louis, MO) or in heart infusion (HI) broth (Oxoid). *Escherichia coli* was grown in Luria-Bertani medium at 37°C. When appropriate, growth media were supplemented with ampicillin (100 μg/ml) or chloramphenicol (20 μg/ml).

Construction of recombinant protein overexpression plasmids. To overexpress the complete response regulators DccR (Cj1223c), RacR (Cj1261), and Cj1491 and the cytoplasmic domain of sensor protein DccS (Cj1222c), chromosomal DNA of *C. jejuni* 81116 was amplified by PCR using the primer combinations Cj1223start/Cj1223Hisstop, RacRPstI/RacRNHISNdeI, HisCj1491start/Cj1491stop, and HisCj1222start/Cj1222stop, respectively (Table 2). The resulting PCR products of 683, 670, 680, and 678 bp were digested with NdeI and PstI and cloned into NdeI and PstI sites of plasmid pT7.7 to form pT7-DccR-H6C, pT7-RacR-H6N, pT7-1491-H6N, and pT7-DccS$_c$-H6N (where H6 is an N-terminal [H6N]or C-terminal [H6C] His$_6$ tag and where DccS$_c$ is the cytoplasmic domain of DccS), respectively. The nucleotide sequence of the cloned PCR products was verified by sequencing using an ABI Prism 310 genetic analyzer (Applied Biosystems Inc., Foster City, CA) and an ABI dye terminator cycle sequencing kit.

Purification of recombinant proteins. Histidine-tagged DccS, DccR, PhosR, Cj1491, RacR, and FlgR proteins were expressed in *E. coli* BL21(DE3) containing either plasmid pT7-DccS-H6C, pT7-DccR-H6N, pT7-PhosR-H6N, pT7-1491-H6N, pT7-RacR-H6N, or pT7-FlgR-H6N.Protein expression and purification were performed as described previously (21). Protein concentrations were determined by the Bradford method (3).

Phosphorylation assays. Recombinant DccS$_c$-H6C (5.2 pmol) and DccR-H6N (0.85 pmol) proteins were incubated at room temperature for 15 min, with 10 μCi of [γ-^{32}P]ATP (MP Biomedicals Netherlands) in 15 μl of phosphorylation buffer (50 mM Tris-HCl, 75 mM KCl, 2 mM MgCl$_2$, 1 mM dithiothreitol [DTT], pH 8.3). Phosphorylation of 0.85 pmol of DccR-H6 was achieved by the addition of 5.2 pmol of autophosphorylated DccS$_c$-H6 in 15 μl of phosphorylation buffer. The reaction was stopped with SDS loading buffer after 0.5, 2, 4, 8, 16, or 32 min. Cross-phosphorylation experiments were performed by incubating 0.85 pmol of DccR-H6N, 0.8 pmol of PhosR-H6N, 1.5 pmol of Cj1491-H6N, 1.0 pmol of RacR-H6N, or 0.5 pmol of FlgR-H6N protein with 5.2 pmol of phosphorylated DccS$_c$-H6C protein for 15 min at room temperature in 15 μl of phosphorylation buffer. The reaction was stopped with loading buffer. Samples were run on 12.5% SDS-polyacrylamide gels. After electrophoresis, the gel was dried and autoradiographed.

Construction of the *dccR* mutant. A 1,990-bp DNA fragment containing the complete *dccS* and *dccR* genes was amplified from the *C. jejuni* 81116 chromosome using the primers Cj1223CF and Cj1223R (Table 2). The product was cloned into pGEM-T Easy, resulting in plasmid pGem1223. This plasmid was subsequently amplified with primers Cj1223RBamHI and Cj1223FBamHI. The resulting PCR product was digested with BamHI and ligated to a 0.7-kb BamHI fragment containing a chloramphenicol resistance gene (Cm^r) of pAV35, result-

ing in the knockout construct pGEM*dccR*::Cm. Natural transformation was used to introduce the knockout constructs in *C. jejuni* 81116 (18). Homologous recombination resulting in double-crossover events was demonstrated by PCR.

Construction of a DccR complementation plasmid. To complement the *dccR*::Cm mutant, the complete DccRS operon located on a 2.1-kb SphI-SacI fragment of plasmid pGem1223 was cloned into the *E. coli-C. jejuni* shuttle conjugative plasmid pMA1 (15). In the resulting complementation plasmid, pMA1-1223, the *dccR* and *dccS* genes are regulated by their own promoters. Finally, pMA1-1223 was introduced in the *dccR*::Cm mutant via conjugation (7).

RNA isolation. Precultures of *C. jejuni* were grown at 32°C, 37°C, or 42°C in HI broth, diluted to an A_{550} of 0.02 in 5 ml of HI broth, and incubated on a gyratory shaker (150 rpm) under microaerobic conditions at 32°C, 37°C, or 42°C, respectively.

Total RNA was extracted from cultures of early logarithmic (EL), late logarithmic (LL), and late stationary (LS) phases (A_{550} of 0.1 to 0.3, 0.7 to 0.9, and 1.0 to 1.4, respectively) with an RNA-Bee kit (Tel-Test, Inc.) according to the manufacturer's specifications.

RNA was also isolated from early logarithmic cell cultures in which the medium had been replaced at 2 h before RNA extraction by fresh HI broth, phosphate-buffered saline (PBS), or spent filtrated (0.4 μm) stationary-phase culture medium.

Primer extension. Localization of the 5′ mRNA start site of DccR-regulated promoters was performed by primer extension analysis using RNA extracted from wild-type strain 81116 and the *dccR*::Cm mutant grown to late logarithmic phase at 42°C and the primers Cj0200prex, Cj0606prex, Cj1004prex, Cj1356prex, and Cj1626prex (Table 2). cDNA synthesis was performed in a 25-μl volume with 20 μg of total RNA, 2 pmol of primer labeled with T4 polynucleotide kinase and 50 μCi [γ-^{32}P]ATP, and 50 units of SuperScriptII RNase H⁻ reverse transcriptase (Life Technologies, Inc.) according to the manufacturer's instructions. Extension products were analyzed by electrophoresis on a 6% polyacrylamide–7.5 M urea gel and compared with sequence ladders initiated with primer extension primers.

Real-time RT-PCR. Real-time reverse transcription-PCR (RT-PCR) analysis was performed as previously described (21). The calculated threshold cycle (C_T) for each gene amplification was normalized to the C_T value for the *gyrA* gene amplified from the corresponding sample before calculating fold change using the arithmetic formula $2^{-\Delta\Delta C_T}$, where $\Delta\Delta C_T = [(C_{T \text{ target}} - C_{T \text{ gyrA}})_{\text{phasex}} - (C_{T \text{ target}} - C_{T \text{ gyrA}})_{\text{phasey}}]$ or $[(C_{T \text{ target}} - C_{T \text{ gyrA}})_{\text{mutant}} - (C_{T \text{ target}} - C_{T \text{ gyrA}})_{\text{wild type}}]$ (11) and where target is the gene of interest, x is EL, LL, or LS, and y is EL. 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.

Microarray hybridization and analysis. Microarray hybridization and analysis were performed as previous described (19) but with the use of total RNA

TABLE 2. Primers used in this study

Primer function and name	Sequence (5′–3′)
Mobility shift assay	
Cj200RDig	GTTTTAGACTATCTGCAAAA
CJ201F	TTTCATCTTCAATATACTCTAA
Cj605FDig	ATGTGATGATGGCTTTAGAATT
CJ606R2	GAGTTAAATAGCTGATTTTTT
Cj1003cRDig	GATATTGTAATAATCATAATCATAAGAAA
Cj1004F	TTTAAAGATAAGCTTAAAATAGTAA
Cj1223FDig	ATGATCTCACTCAAGCTCAA
Cj1223CR	TGAAAGGATTATAGATAAAA
Cj1356RDig	AGAACAAACTAAGATAGATAT
Cj1357F	AGCTTGCTCCTTATAATTAT
Cj1626RDig	ATCTTTAGCTAAAAGCACCG
CJ1627F	AAGATGGAAAAGAAATAAAATTT
Cj1723RDig	AGAAATTGTATTTGCACTTG
CJ1724F	CGGAAATGTACACACAGTCA
Real-time RT-PCR	
Cj0200Ftaq	CAACAAAACCTTGCTTATTGACTCTATT
Cj0200Rtaq	CACAATCATCCATTTCTATTTCGGTAT
Cj0606Ftaq	ATGATTTTTTGCGTATTGGTATGAGT
Cj0606Rtaq	TTAATTGCGTATTGGTATGAGT
Cj0607Ftaq	CATACTTGCACACAAACTTCGTTCT
Cj0607Rtaq	GCCTAATCCTAAAGCTACAACACAA
Cj0608Ftaq	CAGATGTGAGATCTAAACTCAATAGCTTAAA
Cj0608Rtaq	TCCCACTTAATGCTCCACCTAAA
Cj1004Ftaq	TCCTTCCAAGCTCCATCTATATCAA
Cj1004Rtaq	CGTGCAAAAAGACAAAAATTCCT
Cj1356Rtaq	TCTTTACTTGGAGATATGATTATGCTTCAT
Cj1356Ftaq	CAAACAAAGTAATCATAAGCCCTGAAA
Cj1626Rtaq	TTCCTTGCATATCTGTATAAACTTCCA
Cj1626Ftaq	GCTTTGCCACATACTATCTTACCTAATG
Cj1222Ftaq	AGCCTTGTTTTAAATCATTTGTTGTG
Cj1222Rtaq	AGAATTAAGAAAAAGCGGAAAGCA
Protein expression	
Cj1223HisPstI	TTTACTGCAGTTAATGGTGATGGTGATGGTGGCCATAGCAATATCCCCTG
Cj1223NdeI	TATACATATGGCTGCTAAAATTTTACTTTT
Cj1222HisNdeI	ATTCATATGCACCATCACCATCACCATAAAATTGCTCTAAAGCCTTTG
Cj1222PstI	TGACTGCAGTTACCAATTTAATTTAAAAAC
RacRPstI	TTCTGCAGTCATCCTATCAGTTTATAT
RacRNHISNdeI	TACATATGCATCACCATCACCATCACATTAATGTGTTGATGATAGA
Cj1492HisC	CTGCAGCTAATGGTGATGGTGATGGTGCTCTACCCTCTCTTTTAGTTTTA
Cj1492EcoRI	AAGAATTCTTAGCGATATAGCACATCAATGG
Primer extension	
Cj1223prex	ATGATCTCACTCAAGCTCAA
Cj0200R	GTTTTAGACTATCTGCAAAA
Cj1356R	AGAACAAACTAAGATAGATAT
Cj1626R	ATCTTTAGCTAAAAGCACCG
prex1004	CAGAAATTATTATATCAGCA
prex0606	AATAAGCCCCAACACTTCCA
Cloning	
Cj1223CF	AATATCTGCTCTAACCTCTT
Cj1223R	TGAAAGGATTATAGATAAAA
Cj1223RamHI	TCAGGATCCAAGCTTTGCTTAAAAGAGCT
Cj1223FamHI	GCAGGATCCTAACCCTGATAGATAACTCG

extracted from wild-type and *dccR*::Cm mutant bacteria grown at late stationary phase.

Gel mobility shift assays. The promoter regions upstream of the genes Cj0200c, Cj0606, Cj1004, *dccR*, Cj1356c, Cj1626c, and Cj1723c were amplified by PCR using *C. jejuni* 81116 chromosomal DNA as a template while the region upstream of Cj1723c was amplified using Cj11168 chromosomal DNA. Primers Cj1003cRDIG, Cj605FDIG, Cj200RDIG, Cj1626RDIG, Cj1723RDIG, and Cj1356RDIG were digoxigenin (DIG) labeled (Isogen). The Cj1004 (104 bp), Cj605 (240 bp), Cj200c (250 bp), Cj1626c (200 bp), Cj1723c (300 bp), and Cj1356c (232 bp) promoter regions were amplified

using primer sets Cj1003cRDIG/Cj1004R, Cj605FDIG/Cj606R, Cj200RDIG/ Cj201F, Cj1626RDIG/Cj1627F, Cj1723RDIG/Cj1724F, and Cj1356RDIG/ Cj1357F, respectively (Table 2). Approximately 35 pmol of labeled DNA and 0, 7.5, 37.5, or 187.5 pmol of DccR-H6 protein 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, 5 mM MgCl₂, 50 mM KCl, 50 μg of bovine serum albumin, 1.0 μg of poly(dI-dC), and 5% glycerol, pH 7.4. Samples (20 μl) were run on a 4% nondenaturing Tris-glycine polyacrylamide gel at 4°C. Following electrophoresis, DNA was transferred to nylon membranes (Amersham) and UV cross-linked. DIG-labeled DNA was detected with anti-DIG antibodies according to the manual of the manufacturer (Roche).

Chicken experiments. Challenge experiments were performed with Ross 308 broiler chickens housed in isolators as described previously (5). At day 7 after hatching, two groups of 25 animals were inoculated with an overnight HI broth culture of 10^5 CFU of either wild-type *Campylobacter* or *dccR*::Cm mutant bacteria. At days 2, 4, 7, and 14 after inoculation, five animals from each group were sacrificed, and *C. jejuni* was reisolated from cecal contents. The cecal contents were serially diluted and plated onto charcoal cefoperazone deoxycholate agar plates ([CCDA] item CM0739; Oxoid, Basingstoke, United Kingdom).

Microarray data accession number. Microarray data were deposited in the Gene Expression Omnibus (GEO) database under accession number GSE19803.

RESULTS

Identification of the DccS/DccR regulon. In *C. jejuni* strain 81-176, the DccRS system directly regulates the transcription of at least two genes encoding putative periplasmic and membrane proteins. These genes share a conserved repeat sequence (TTCAC) in their promoter regions (8). Analysis of all 14 *C. jejuni* genome sequences available in the public databases revealed that they contain either five or six promoter elements with the TTCAC motif that might be directly regulated by the DccR/DccS system (Fig. 1A). These elements were located upstream of the genes Cj0200c, Cj0606, Cj1004, Cj1356c, Cj1626c, and Cj1723c in strain 11168, which are annotated as putative periplasmic or membrane proteins. To obtain experimental evidence that these promoter elements are directly regulated by the DccR/DccS system, we performed electrophoretic mobility shift assays (EMSAs) (Fig. 1B). The DccR response regulator protein was isolated as a His-tagged recombinant protein (DccR-H6N) and incubated with DNA fragments containing the promoter regions of Cj0200c, Cj0606, Cj1004, Cj1356c, Cj1626c, and Cj1723c and of the *dccR* (Cj1223c) gene itself. DNA mobility shifts were seen for the Cj0200c, Cj0606, Cj1004, Cj1356c, Cj1626c, and Cj1723c promoter fragments, indicating that all of these promoters bind DccR. The *dccR* (Cj1223c) promoter fragment, used as a negative control, did not shift.

To determine that the identified promoter regions are indeed regulated by the DccR/DccS system *in vivo*, we performed primer extension experiments. Total RNA was isolated from cultures of wild-type 81116 grown overnight and from a constructed *dccR* mutant. We did not perform primer extension on gene Cj1723c as it is not present in *C. jejuni* strain 81116. Primer extension assays yielded a single cDNA product for the genes Cj0200c, Cj0606, Cj1004, Cj1356c, Cj1626c, and *dccR* when wild-type RNA was used as a template (Fig. 1C). However, when total RNA of the *dccR* mutant was used, a cDNA product was detected for only the *dccR* gene. As the identified DccR-dependent transcription start sites were all located just downstream of the putative −10 region (Fig. 1A), these results clearly show that the transcription of the Cj0200c, Cj0606, Cj1004, Cj1356c, and Cj1626c genes is dependent on a functional DccR protein and, in addition, that the *dccR* gene is not autoregulated. These results are in agreement with the results of EMSAs.

Selective phosphorylation of DccR by DccS. Components of two-component systems communicate with each other via phosphate transfer, but phosphate transfer may also occur between different two-component systems, thus expanding the DccS regulon. To investigate whether the sensor DccS is able to autophosphorylate and to transmit the phosphate group to

the DccR response regulator, phosphorylation experiments were carried out. The DccR protein and the cytoplasmic domain of DccS were isolated as His-tagged labeled recombinant proteins and incubated with [γ-^{32}P]ATP. The cytoplasmic domain of the sensor DccS showed rapid autophosphorylation in the presence of radioactive ATP (Fig. 2A). A rapid dephosphorylation of the sensor was seen when recombinant regulator DccR was added. After 16 min all the phosphate from the sensor was transferred to the regulator. In the absence of the sensor, phosphorylation of the response regulator was not observed.

To investigate whether DccS cross-phosphorylates other response regulators and thus regulates more regulons than DccR, we incubated phosphorylated DccS with other purified *C. jejuni* response regulators, e.g., FlgR, PhosR, RacR, and Cj1491 (Fig. 2B). DccS was able to phosphorylate only DccR. Conversely, the sensors FlgS, PhosS, RacS, and Cj1492 were not able to phosphorylate DccR (data not shown). These results indicate that the interaction of DccS and DccR is highly specific and that no cross-communication exists (at least at the level of phosphorylation) between the DccS/DccR, PhosS/PhosR, RacR/RacS, FlgS/FlgR, and Cj1491/Cj1492 two-component systems.

Environmental regulation of the activity of the DccRS system. The DccRS system of strain 81-176 is required for the optimal colonization of 1-day-old chicks (8). We monitored, over time, the colonization of our 81116 *dccR* knockout strain in 7-day-old chickens (Fig. 3). Similar final colonization levels in the cecum were observed for the wild-type and mutant strains. However, the colonization was delayed for the mutant strain: maximum colonization levels were achieved at day 3 for the wild type but only at day 7 for the DccR mutant. This may indicate that the DccRS system is particularly relevant in the initial adaptation to the gastrointestinal milieu rather than for persistent colonization of the cecum.

In the search for environmental cues that activate the DccRS system, we tested whether temperature influenced the transcription of the DccR regulon. Real-time RT-PCR on RNA isolated from cultures grown at 32°C, 37°C, and 42°C showed that transcript levels for the DccR-regulated genes Cj0200c and Cj1004 were independent of the growth temperature while *dccR* mRNA levels were almost 6-fold higher when bacteria were grown at 32°C than when they were grown at 42°C (Fig. 4A). These data indicate that the relatively high ambient body temperature of chickens of 42°C is not likely the cause of the DccRS activation.

The putative DccRS homolog in *Helicobacter pylori*, the CrdRS two-component system, is required for copper-mediated induction of the copper resistance determinant CrdA (17). Growth curves of the *C. jejuni* 81116 wild-type strain and the *dccR* mutant in the presence of different concentrations of copper (0 to 0.5 μM) were identical. Real-time RT-PCR analysis confirmed that the transcript levels for the DccRS-dependent genes were not regulated by copper (data not shown).

Next, we investigated whether the bacterial growth phase influenced the transcription of the DccR regulon. Comparison of mRNA levels at different stages of bacterial growth revealed a clear increase in Cj0200c and Cj1656 mRNA levels after prolonged growth while *dccR* mRNA levels remained constant (Fig. 4B). To investigate whether the increase in mRNA for
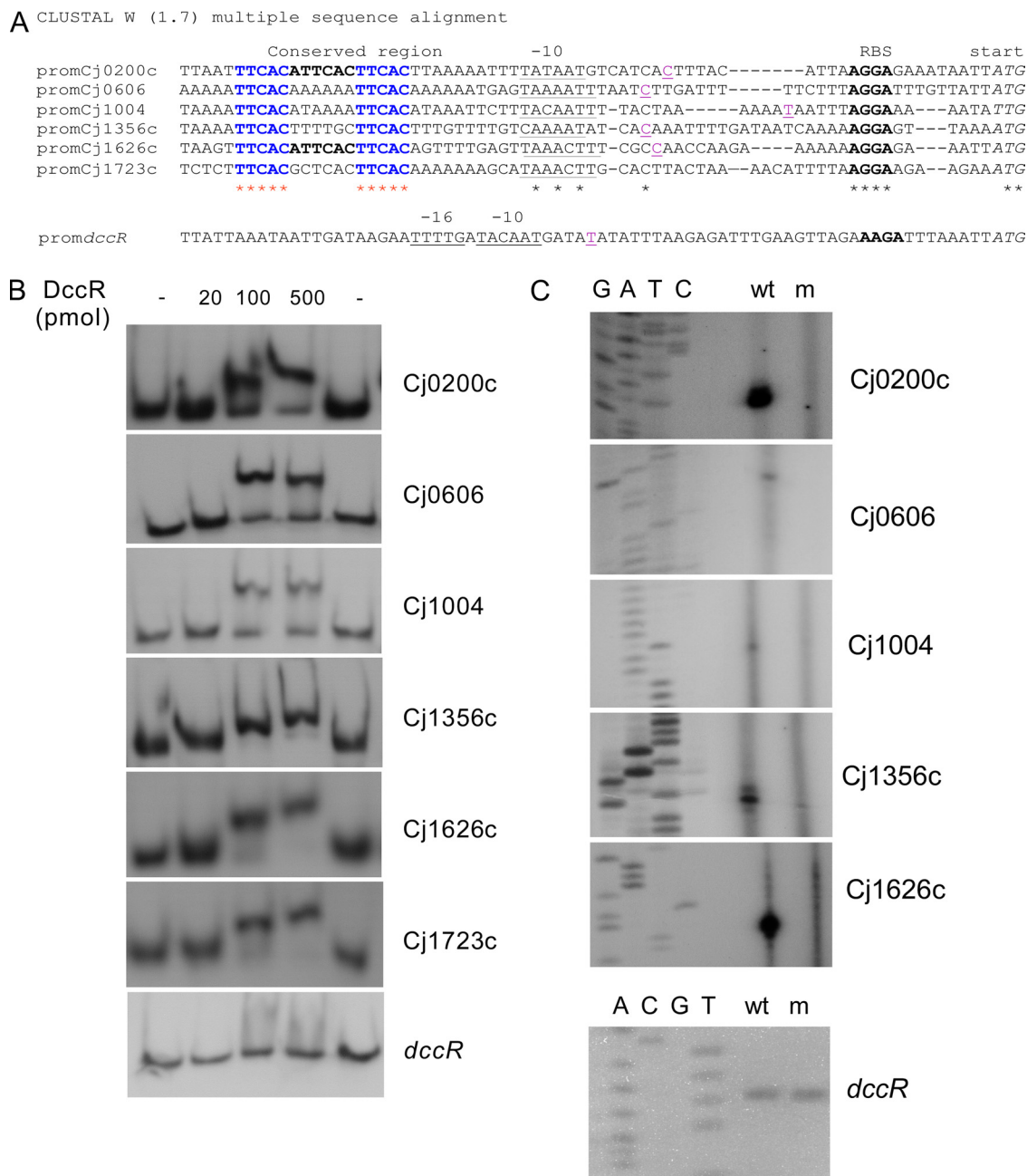
FIG. 1. Identification of the DccS/DccR regulon. (A) Alignment of promoter DNA sequences containing a TTCAC repeat sequence which might be directly regulated by the DccRS system. Start codon (italic), ribosomal binding sequence (bold), putative −10 and −16 regions (underlined), transcription start points (pink underlined), and direct TTCAC repeats (blue) are indicated. (B) EMSAs with the DccR-H6N protein and the promoter regions in front of gene Cj0200c, Cj0606, Cj1004, Cj1356c, Cj1626c, Cj1723c, or *dccR*. The concentration of DccR-H6N in each reaction mixture is indicated at the top of each lane. (C) Mapping of the transcription start site of *dccR* promoter and DccR-regulated promoters by primer extension. Primer extension products were generated after reverse transcription of total RNA isolated from late-logarithmic phase wild-type (wt) strain or *dccR* mutant (m) grown in HI broth at 37°C. The primer extension product was run on a 6% sequencing gel against dideoxy sequencing reactions primed with the same primer.

the DccR-regulated genes was due to the activation of the DccRS system, we performed real-time RT-PCR on the DccRS-regulated genes and the *dccR* gene itself by using total RNA of the wild-type strain, the *dccR* mutant, and the *dccR* mutant containing complementation plasmid pMA1-1223 isolated at different growth phases. No difference in *dccR* mRNA

levels was observed, confirming the primer extension results that the *dccR* gene is not autoregulated (Fig. 4C). The differences in mRNA levels of different DccRS-upregulated genes were most prominent at the late stationary phase. This effect was less pronounced for Cj0606. Plasmid pMA1-1223 was able to complement the defect in the *dccR* gene as no transcript
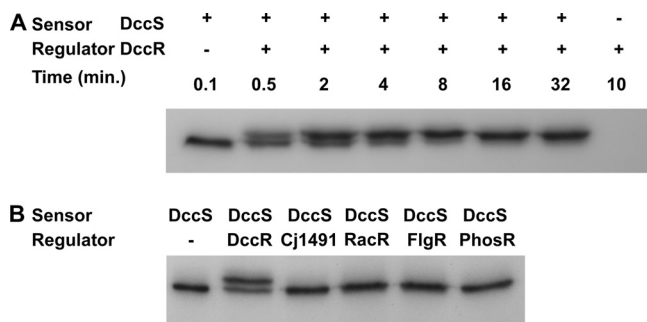
FIG. 2. Phosphorylation assays of the cytoplasmic domain of the sensor DccS and *C. jejuni* response regulators *in vitro*. Autophosphorylation of the truncated sensor DccS$_c$-H6C was accomplished by incubation of DccS$_c$-H6C with [γ-^{32}P]ATP for 15 min at room temperature. (A) Time course of phosphotransfer from ^{32}P-labeled DccS$_c$-H6C to DccR-H6N. The DccR protein was incubated for 20 min with [γ-^{32}P]ATP. (B) Phosphotransfer specificity of the sensor DccS. In this assay ^{32}P-labeled DccS$_c$-H6C was incubated with His-tagged recombinant DccR-H6N, Cj890-H6N, RacR-H6N, Cj1491-H6N, or l FlgR-H6N protein *in vitro*. Samples were run on a 12% SDS-polyacrylamide gel. Autophosphorylation and phosphotransfer were visualized by autoradiography.

differences were observed between the wild-type and the complemented *dccR* mutant strain. Taken together, these results indicate that prolonged *C. jejuni* growth generates a signal that activates the DccRS system.

To determine if depletion of the medium or the accumulation of factors in the medium is the signal that activates the DccRS system at late stationary phase, we replaced the medium of wild-type bacteria grown to logarithmic phase with either PBS (no nutrients), spent filtrated stationary-phase culture medium (lacking nutrients and containing accumulated secreted factors), or fresh medium. Two hours after the medium replacement, RNA was extracted, and transcript levels were determined by real-time RT-PCR. After a 2-h incubation, the optical density levels at 600 nm (OD$_{600}$s) of the PBS and spent filtrated medium were equal, indicating that *C. jejuni* was
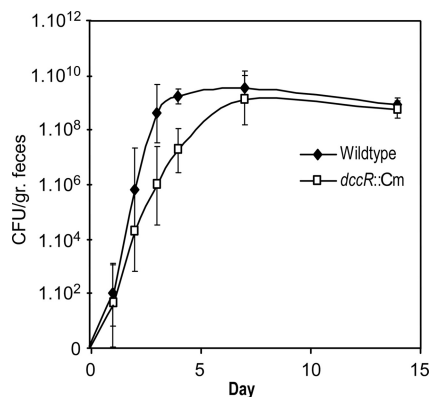

FIG. 3. Kinetic study of *dccR* mutant recovered from the cecum following oral inoculation of chickens. Ross 308 broiler chickens were inoculated with the wild type or *dccR*::Cm at a dose of approximately 1×10^5 CFU per chick. At various times postinoculation up to 14 days, five chickens per strain were sacrificed, and the number of CFU per gram of cecum content was determined. Each symbol represents the average value from five animals.
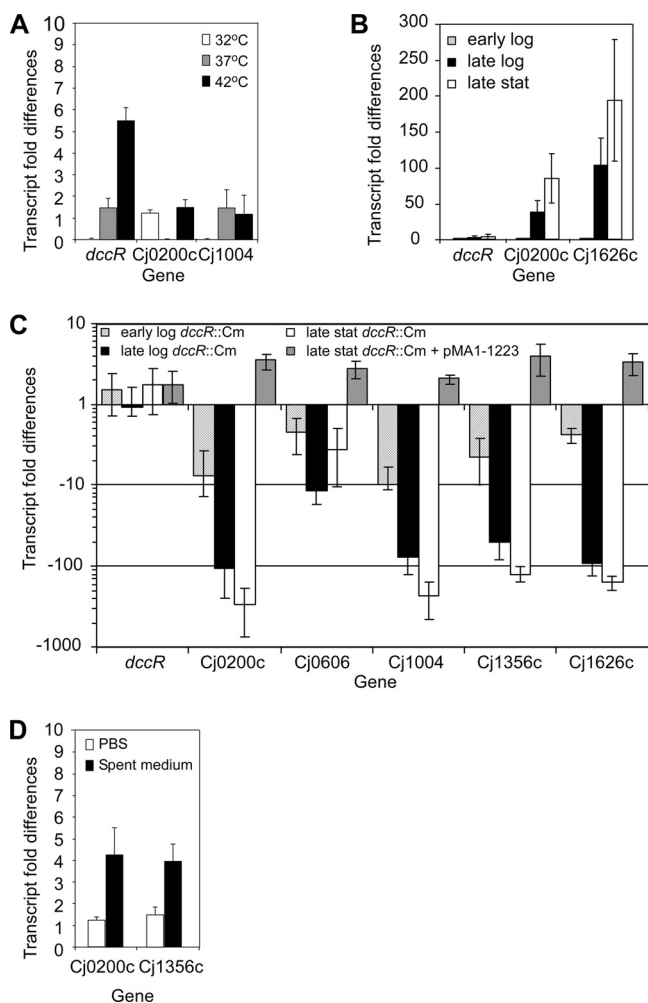

FIG. 4. Activation of the DccRS two-component system. (A) Real-time RT-PCR data showing the transcript fold difference of the *dccR*, Cj0200c, and Cj1004 mRNAs in wild-type *C. jejuni* grown at 32°C, 37°C, or 42°C. Fold change relative to the transcription levels was calculated using the arithmetic formula $2^{-\Delta\Delta CT}$, where $\Delta\Delta C_T = [(C_{T\ \text{target}} - C_{T\ gyrA})_{\text{temperature}\ x} - (C_{T\ \text{target}} - C_{T\ gyrA})_{\text{temperature}\ y}]$, where *x* is 42, 37, or 32°C and *y* is 32 or 37°C. (B) Growth-phase-dependent transcription of the *dccR*, Cj0200c, and Cj1656 genes as estimated for wild-type bacteria by real-time RT-PCR. Total RNA was extracted from cultures of early logarithmic, late logarithmic, and stationary phases (A_{550} of 0.1 to 0.3, 0.7 to 0.9, and 1.0 to 1.4, respectively) (C). Real-time RT-PCR data showing the transcript fold difference of the DccRS-regulated genes (Cj0200c, Cj0606, Cj1004, Cj1356c, and Cj1626c) in wild-type *C. jejuni* compared to the *dccR* mutant or to the *dccR* mutant complemented with plasmid pMA1-1223 at different phases of growth at 32°C. (D) Change in the transcript levels of the Cj0200c and Cj1004 genes in early-logarithmic-phase wild-type bacteria in response to the replacement of the medium by PBS or spent filtrated stationary-phase medium relative to the addition of fresh medium, as estimated by real-time RT-PCR. Data represent the mean values and standard deviations of four independent experiments with two independent preparations of RNA.

unable to grow in both media. Transcript levels for the DccR-regulated genes Cj0200c or Cj1004 were 5-fold higher for bacteria kept in spent medium than in for bacteria in fresh medium while no difference in their mRNA levels was detected for bacteria kept in PBS (Fig. 4D). These results suggest that

TABLE 3. Gene expression data given as transcript fold reduction
for the *C. jejuni* 81116 *dccR* mutant

Gene	Transcript fold reduction in stationary-phase cells (mean ± SD)	
	Microarray	Real-time RT-PCR
Cj0200c	99.8 ± 9.7	601.0 ± 221.3
Cj0606	2.4 ± 0.38	7.4 ± 3.7
Cj0607	2.4 ± 0.25	7.2 ± 3.2
Cj0608	1.7 ± 0.31	7.2 ± 2.5
Cj1004	5.0 ± 1.5	289.2 ± 155.3
Cj1356c	11.6 ± 1.1	301.7 ± 151.6
Cj1626c	18.4 ± 2.9	244.3 ± 120.3
Cj1080c	2.2 ± 1.2	1.2 ± 0.2
Cj0988c	2.6 ± 1.4	1.0 ± 0.2

the DccRS system may be activated by a factor that accumulates in the stationary phase rather than by a lack of nutrients.

Identification of indirectly DccR-regulated genes. Knowledge of the growth phase condition that activates the DccRS system provided the opportunity to test whether additional genes/transcription factors are indirectly regulated by this system. To identify all DccR-dependent genes of strain 81116, mRNA profiles were compared between the parent and *dccR* mutant by microarray analysis. Total RNA isolated from stationary-phase cells of the parent or *dccR* mutant was used. Microarray analysis indicated that no genes were ≥2-fold upregulated in the *dccR* mutant compared to the wild type. Six of the seven (Cj0200c, Cj0606, Cj0607, Cj1004, Cj1356c, and Cj1626c) directly DccR-regulated genes in strain 81116 were found among the eight genes which were more than 2-fold downregulated in the *dccR* mutant compared to the wild type (Table 3). The maximum change in transcription of indirectly DccR-regulated genes was 2.6-fold while that of directly regulated genes was more than 99-fold. None of the changes in indirectly DccR-regulated gene transcripts identified by microarray analysis could be confirmed by real-time RT-PCR (Table 3). These results suggest that mutation of DccR affects only directly regulated genes and thus that the complete regulon of the DccR has been elucidated.

DISCUSSION

In the present study, we provide experimental evidence that the *C. jejuni* proteins DccS and DccR form a classical two-component system that is activated in the late stationary growth phase. The system is not autoregulated but, upon activation, upregulates the activity of five or six *Campylobacter* promoters (depending on the *C. jejuni* strain) through direct binding of a specific DNA sequence located in front of these promoters. The DccS/DccR system neither communicates with other two-component systems nor influences transcription of genes outside the DccR regulon. The system was found to facilitate rapid colonization of 7-day-old chickens but was not essential for colonization of the cecum.

Previous genome analysis, DNA binding studies, and comparable phenotypes of Cj1223c and Cj1222c mutants indicated that DccS and DccR proteins likely form a two-component system (8). Our findings for the first time demonstrate that the DccS protein, indeed, autophosphorylates in the presence of

ATP and that the phosphate is rapidly transferred to the cognate effector, DccR. This event was shown to be highly specific as DccS was unable to transfer its phosphoryl group to other effector molecules. Similarly, the sensor proteins FlgS, PhosS, RacS, and Cj1492 were unable to transfer the phosphoryl group to the DccR protein (data not shown). This specificity indicates that the cross talk between different two-component systems in *C. jejuni* may be limited. In other bacterial pathogens, promiscuity between two-component systems has been observed (23), allowing bacteria to combine signals to achieve beneficial cross-regulation among pathways (2). The lack of cross talk between DccS/DccR and other two-component systems indicates that the DccR-regulated genes are important only in response to the signal that activates the sensor DccS.

Our microarray results, real-time RT-PCR, and primer extension analysis revealed that the *C. jejuni* strain 81116 response regulator, DccR, activates five promoter elements located upstream of the genes Cj0200c, Cj0606, Cj1004, Cj1356c, and Cj1626c. These putative σ⁷⁰-dependent promoters are all preceded by a WTTCAC-N6-TTCACW direct repeat located around the −35 region. Searching all available finished *Campylobacter* genome sequences for the single TTCAC sequence or direct repeat revealed that the gene Cj1723c in *C. jejuni* strain NCTC 11168 (and its homolog in other *C. jejuni* strains) is the only other gene that might be directly regulated by the DccRS system. EMSA confirmed binding of the DccR protein to all repeat-containing fragments. This suggests that the complete regulon of the DccRS has been identified. It should be noted that the pattern of transcription activation of the Cj0606 gene did not entirely follow that of other genes directly regulated by DccRS. This may indicate that the promoter of this gene is controlled by not only DccR but also other regulatory-mechanism factors. The absence of genes indirectly regulated by DccR confirms the observed lack of cross talk between DccS/DccR and other two-component systems and provides further evidence that this system is dedicated to a specific function.

Thus far, all *C. jejuni* two-component regulator mutants tested have been involved in the optimum colonization of chickens (4, 6, 8, 21) although the specific role of these systems has not been determined. MacKichan et al. (8) noted that a *dccR* mutant of strain 81-176 poorly colonized the cecum of 1-day-old chickens. Our *in vivo* challenge experiments, in which we monitored over time the colonization with the *dccR* mutant of strain 81116 in 7-day-old chickens, indicated that the two-component system is not essential but, rather, accelerates the colonization of the chicken cecum. The reason for this different result is unclear but may be related to the age of the animals, the experimental setup, and/or the use of different *C. jejuni* strains. Our data suggest that the genes activated by the DccR system may serve in the primary adaptation of *C. jejuni* to the chicken environment rather than in conferring bacterial persistence in the chicken. Identification of the specific signals that activate this system may explain the behavior of the bacterium under different environmental conditions and the specific functions associated with the genes present in DccR regulon.

We were able to eliminate several potential signals that might activate the DccRS system. Chickens have a relatively high body temperature of 42°C, yet we showed that temperature is not the signal that actives the DccRS system. Based on

amino acid sequence homology, the DccRS system appears most closely related to the copper-induced CrdRS two-component system of *H. pylori* (17). Like temperature, the availability of copper did not influence the transcription of the DccR-regulated genes. However, our results show that the genes that are directly regulated by the DccRS system are upregulated in the late stationary phase. This indicates that the DccRS system is activated by a specific signal that is present in the late stationary phase. *C. jejuni* undergoes numerous gene expression changes and substrate switching when it enters the stationary phase (22). Obvious stationary-phase signals, like pH, oxygen or nitrogen radicals (data not shown), starvation, or depletion of ions, however, did not influence the activation of the DccRS system. Based on the findings that the DccRS-regulated genes are activated in the stationary phase, together with the reduced initial chicken colonization of the *dccR* mutant, we speculate that the signals that activate the two-component system in the stationary phase are also present in the upper intestinal tract rather than in the cecum.

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