# *Campylobacter jejuni* BumSR directs a response to butyrate via sensor phosphatase activity to impact transcription and colonization

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***Campylobacter jejuni*** **monitors intestinal metabolites produced by the host and microbiota to initiate intestinal colonization of avian and animal hosts for commensalism and infection of humans for diarrheal disease. We previously discovered that *C. jejuni* has the capacity to spatially discern different intestinal regions by sensing lactate and the short-chain fatty acids acetate and butyrate and then alter transcription of colonization factors appropriately for in vivo growth. In this study, we identified the *C. jejuni* butyrate-modulated regulon and discovered that the BumSR two-component signal transduction system (TCS) directs a response to butyrate by identifying mutants in a genetic screen defective for butyrate-modulated transcription. The BumSR TCS, which is important for infection of humans and optimal colonization of avian hosts, senses butyrate likely by indirect means to alter transcription of genes encoding important colonization determinants. Unlike many canonical TCSs, the predicted cytoplasmic sensor kinase BumS lacked in vitro autokinase activity, which would normally lead to phosphorylation of the cognate BumR response regulator. Instead, BumS has likely evolved mutations to naturally function as a phosphatase whose activity is influenced by exogenous butyrate to control the level of endogenous phosphorylation of BumR and its ability to alter transcription of target genes. To our knowledge, the BumSR TCS is the only bacterial signal transduction system identified so far that mediates responses to the microbiota-generated intestinal metabolite butyrate, an important factor for host intestinal health and homeostasis. Our findings suggest that butyrate sensing by this system is vital for *C. jejuni* colonization of multiple hosts.**

*Campylobacter jejuni* | butyrate | short-chain fatty acids | BumS | two-component signal transduction system

A complex ecosystem develops in the intestines of humans and animals that is influenced by the physiology and diet of the hosts and the metabolic capacities of diverse members of the microbiota (1, 2). As a result, specific biogeographies form in each intestinal tract region that are composed of metabolic byproducts and the microbiota that feed upon and contribute to these metabolites (1, 3). Short-chain fatty acids (SCFAs) such as butyrate, propionate, and acetate are important metabolites produced by an established intestinal microbiota that also contribute to the health of the host (4, 5). SCFAs are generated predominantly by clostridial species that ferment dietary fibers and sugars and can reach ∼130 mM in the lumen of the lower intestines (4–6). While all SCFAs have roles in host physiology, butyrate is particularly important for lower intestinal homeostasis as a primary energy source for colonocytes and its functions in inflammatory processes (7, 8). In contrast, the organic acid lactate generated by *Lactobacillus* species in large quantities is more constrained to the upper intestinal tract regions (9–11). Lactate is an energy source for commensal bacteria and pathogens while also promoting immune modulatory and intestinal barrier maintenance properties for the host (12–14).

Due to the abundance and intestinal distribution of SCFAs and lactate, bacterial pathogens can use these metabolites as biogeographical cues to discriminate among different regions of the host intestines (15–19). Furthermore, some pathogenic bacteria sense and exploit these metabolites for expansion and consequently induce inflammation during dysbiosis (12, 20–22). This phenomenon is particularly apparent among foodborne intestinal pathogens, including *Salmonella enterica* serovar Typhimurium and enterohemorrhagic *Escherichia coli* that sense SCFAs through a two-component signal transduction system (TCS) composed of the sensor kinase BarA and its cognate response regulator (annotated as UvrY in *E. coli* and SirA in *Salmonella*) to regulate virulence (23–25). In *E. coli*, BarA may directly sense formate to influence virulence gene expression and metabolism (25). However, acetate influences signal transduction independently of BarA by its conversion to acetyl-phosphate (AcP) through acetogenesis, thereby serving as a phosphodonor for UvrY to alter gene expression (25). Similarly, acetate activates *Salmonella* SirA through AcP synthesis to promote

## Significance

**Microbiota-derived short-chain fatty acids influence processes important for the intestinal health of hosts and the activities of resident bacteria. Herein, we identified the *Campylobacter jejuni* BumSR TCS as an indirect sensor of butyrate. Because the BumSR TCS is important for infection of humans for diarrheal disease and optimal commensal colonization of avian hosts, a butyrate response by BumSR likely promotes molecular eavesdropping for *C. jejuni* recognition of lower intestinal niches across hosts. Further analysis suggests a noncanonical mechanism for signal transduction in which BumS functions mainly as a phosphatase, rather than as a kinase, to control activity of the cognate BumR response regulator. Our findings indicate a complex and multifactorial mechanism for enacting butyrate-dependent responses in a bacterium.**

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Data deposition: RNASeq data are publicly available for download at the National Center for Biotechnology Information Gene Expression Omnibus (NCBI GEO) at GEO accession GSE142852.

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expression of invasion genes (23). Butyrate and propionate inhibit *Salmonella* invasion gene expression independent of BarA and SirA (23, 26). Propionate, via conversion to propionyl-CoA, leads to posttranslational regulation of the HilD response regulator (23, 24). However, identification of a butyrate sensor in these bacteria and others has remained elusive.

Another prominent cause of diarrheal disease in humans is *Campylobacter jejuni*, but little is known regarding how *C. jejuni* senses the intestinal biogeography (19, 27, 28). Upon oral ingestion, *C. jejuni* establishes infection mainly in the colon and rectum, causing a mild, watery to inflammatory, bloody diarrhea (29). In contrast, *C. jejuni* is a commensal organism in the intestinal tract of many avian species and other important animals in agriculture. In the natural avian host, infection can occur within days after hatch with *C. jejuni* establishing a prolonged, asymptomatic commensal colonization primarily in the ceca and large intestines of the lower intestinal tract with little resulting inflammation (30–33). Consequently, zoonotic cases of campylobacteriosis are often attributed to handling or consumption of contaminated retail chicken meats (34, 35).

To establish commensalism in avian hosts and cause disease in humans, we hypothesized that *C. jejuni* recognizes intestinal niches that support growth, which include the mucus layer lining the crypts of the lower intestinal epithelium, by sensing molecular cues produced by the microbiota. We previously acquired evidence that *C. jejuni* spatially discriminates among different regions of the avian intestinal tract by modulating gene expression in response to microbiota-derived metabolites (19). We discovered that *C. jejuni* induces transcription of genes encoding nutrient acquisition systems, energy generation pathways, and other colonization determinants in the presence of physiologic concentrations of the SCFAs butyrate and acetate. As such, regions of the avian lower intestinal tract where *C. jejuni* preferentially colonizes and grows abundantly are enriched in these SCFAs (19, 36, 37). In contrast, transcription of beneficial colonization factors are repressed in the presence of the organic acid lactate, which is enriched in the upper intestinal tract where *C. jejuni* does not robustly colonize (19, 38, 39). Since the spatial gradients of microbiota-generated SCFAs and lactate are oriented similarly in the avian and human host and at similar concentrations (3, 6, 40–42), we propose that *C. jejuni* senses these same metabolites to impact gene expression in the intestinal tract of humans to promote infection and diarrheal disease.

In this work, we discovered that *C. jejuni* senses the microbiota-derived SCFA butyrate via the noncanonical BumSR TCS. This system, encoded by *Cjj81176_1484* and *Cjj81176_1483* in the *C. jejuni* strain 81-176 genome (here annotated as BumS and BumR, respectively), was previously analyzed, although the signals detected by this TCS remained unknown (43). Additionally, BumR has been shown to be important for promoting infection of humans, suggesting that directing a response to butyrate is important for colonization of hosts (44). Our data suggest that the predicted BumS sensor kinase of this TCS appears to lack natural kinase activity and instead functions as a phosphatase. We propose that BumS likely senses butyrate indirectly to influence BumS phosphatase activity to ultimately control the function of the cognate BumR response regulator and modulate transcription of colonization factors necessary for infection of humans and avian species.

## Results

**The *C. jejuni* BumSR TCS Mediates Butyrate-Dependent Responses.** We previously identified a collection of genes from *C. jejuni* strain 81-176 whose expression was altered in the presence of SCFAs (19). We investigated this gene set to identify a promoter that we could link to a promoterless *astA* gene (encoding arylsulfatase) to function as a quantitative and qualitative transcriptional reporter for analyses (45–49). We identified the promoter of *peb3*, encoding a glycoprotein that may function as a transporter for phosphate-containing metabolites, as a robust transcriptional reporter when fused to *astA* (50, 51). When wild-type (WT) *C. jejuni* was grown in *Campylobacter*-defined media (CDM), which contains amino acids and keto acids as the primary carbon sources for *C. jejuni* growth (52), supplemented with 12.5 mM butyrate, and buffered to pH 7.0, expression of the *peb3*::*astA* transcriptional reporter was reduced 10.2-fold compared to growth in CDM without butyrate (Fig. 1*A*). This butyrate concentration is within the physiological range present in the ceca and lower intestinal regions of chickens and humans (10 to 34 mM) (6, 36, 37, 40, 42).

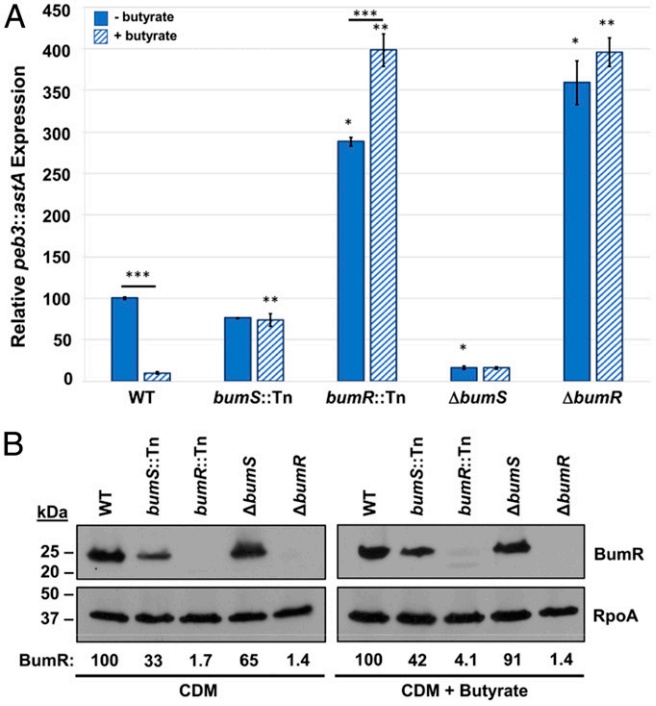


**Fig. 1.** The BumSR TCS is required for butyrate-modulated expression of *peb3*. (*A*) Expression of the *peb3*::*astA* transcriptional reporter in WT *C. jejuni* Δ*astA* and isogenic *bumS* and *bumR* mutants. The *bumS* and *bumR* mutants examined include a representative *bumS*::Tn and *bumR*::Tn mutant isolated in our genetic screen to identify Tn mutants defective for butyrate-dependent repression of *peb3*::*astA* expression and previously constructed *C. jejuni* Δ*bumS* and Δ*bumR* mutants with in-frame deletion of the coding sequence of each gene. *C. jejuni* strains were grown for 16 h in CDM alone (solid blue bars) or CDM supplemented with 12.5 mM butyrate (hatched blue bars) as indicated. The level of *peb3*::*astA* expression in each mutant is relative to the level of expression in WT *C. jejuni* grown without butyrate, which was set to 100 units. Results from a representative assay with each sample analyzed in triplicate are shown. Error bars indicate SDs of the average level of expression from three samples. Statistical significance was calculated in GraphPad Prism by ANOVA with Tukey's test: * indicates the mutant grown in CDM alone had significantly increased or decreased expression relative to the WT strain grown in CDM alone; ** indicates the mutant grown with butyrate had a significantly increased or decreased expression relative to the WT strain grown with butyrate; *** indicates a strain showed a significantly different level of expression when grown in the presence of butyrate compared to growth in CDM alone ($P < 0.05$). (*B*) Production of BumR in WT and isogenic *bumS* and *bumR* mutants. Immunoblot analysis of the level of BumR in whole-cell lysates of WT and mutant strains after growth in CDM alone or CDM supplemented with 12.5 mM butyrate. RpoA served as a control for protein loading. Immunoblots were performed in triplicate, and values underneath the immunoblots represent quantified BumR mean signals relative to RpoA and normalized to WT, which was set to 100.

WT *C. jejuni peb3*::*astA* appears as a light blue colony when grown on CDM with 12.5 mM butyrate containing X-S (the chromogenic substrate for arylsulfatase). We conducted a genetic screen to identify genes required for butyrate sensing by performing transposon (Tn) mutagenesis with WT *C. jejuni peb3*::*astA* and examining *darkhelmet* Tn mutants on CDM with butyrate and X-S. We identified 12 out of over 10,000 Tn mutants with a shift to a darker blue colony phenotype relative to WT on CDM with butyrate. Six transposons were located within *Cjj81176_1484* and the remaining six were in the gene immediately downstream, *Cjj81176_1483*, in the 81-176 genome. Due to our findings presented below, we annotated *Cjj81176_1484* as *bumS* and *Cjj81176_1483* as *bumR* for their requirement in butyrate-modulated gene expression. Repression of *peb3*::*astA* expression in the presence of butyrate was abolished in *bumS*::Tn and *bumR*::Tn mutants (representative Tn mutants are shown in Fig. 1*A*). *C. jejuni bumS*::Tn and *bumR*::Tn exhibited a 7.6-fold and 40.9-fold increase in *peb3*::*astA* expression, respectively, in the presence of butyrate compared to WT with butyrate (Fig. 1*A*).

We verified that the BumSR TCS was responsible for butyrate-modulated responses by examining *C. jejuni* Δ*bumS* and Δ*bumR* from our strain collection that contained in-frame deletions of large portions of the coding sequences of each gene (43). In comparison to the *bumS*::Tn mutant, Δ*bumS* demonstrated a 4.6-fold reduction in *peb3-astA* expression regardless of butyrate supplementation. *C. jejuni* Δ*bumR* behaved similarly as the *bumR*::Tn mutant with increased *peb3*::*astA* expression compared to WT regardless of butyrate supplementation (Fig. 1*A*). Immunoblot analysis revealed that BumR levels were mostly similar in WT *C. jejuni* and Δ*bumS*, but were further reduced in *bumS*::Tn likely because of polar effects on *bumR* expression due to insertion of the transposon in *bumS* (Fig. 1*B*). These different BumR levels in *bumS*::Tn and Δ*bumS* likely contributed to the differences in *peb3*::*astA* expression between the mutants (Fig. 1*B*). Butyrate did not cause alterations of BumR levels in WT *C. jejuni*, suggesting that *bumSR* expression is independent of butyrate (Fig. 1*B*). Our findings that mutation of *bumS* and *bumR* result in contrasting effects suggest that signal transduction through the BumSR TCS resulted in BumS influencing the activity of BumR as a transcriptional repressor, at least for *peb3* expression.

*In cis* complementation of Δ*bumS* and Δ*bumR* with native promoters and the respected genes inserted into *rdxA* on the chromosome reversed the phenotypes of each mutant (*SI Appendix*, Fig. S1) (53, 54). Butyrate supplementation reduced *peb3* transcription in each complemented strain, similar to the butyrate-dependent repression of *peb3* transcription in WT *C. jejuni*. These results indicate that the BumSR TCS is responsible for sensing butyrate either directly or indirectly and mediating butyrate-modulated transcription.

**BumR Receiver Domain Mutants Are Impaired for Butyrate-Modulated Gene Expression.** Previous analyses revealed that D58 of the BumR receiver domain is the site of phosphorylation with AcP as a phosphodonor (43). Whereas the D58E or D58N mutation of BumR prevented autophosphorylation, a D58A mutation destabilized the protein (43). In a study of *C. jejuni* infection of human volunteers, BumR was discovered to be important for infection (44). In this previous study, the inoculum for infection of humans unknowingly contained a mixed population of WT *C. jejuni* and a mutant with a 9-base pair in-frame deletion in *bumR* that altered a KFKKFK sequence at position 46 to 51 in the BumR receiver domain to a single KFK unit ($BumR_{\Delta KFK}$), which is near the site of BumR phosphorylation at D58 (43). This mixed inoculum given to human volunteers was discovered by SNP analysis after the infection study was completed. *C. jejuni* $bumR_{\Delta KFK}$ was outcompeted by WT *C. jejuni* and not recovered from any human volunteers, demonstrating that WT BumR was important for infection of humans (44). However, a mechanistic reason for why and how the $bumR_{\Delta KFK}$ mutation affected processes in *C. jejuni* was not determined.

We recreated $bumR_{\Delta KFK}$ in WT *C. jejuni* strain 81-176 and examined this mutant along with the $bumR_{D58E}$ and $bumR_{D58N}$ mutants for butyrate-modulated *peb3* transcription. In whole-cell lysates, $BumR_{\Delta KFK}$ levels were greatly reduced likely due to instability of the protein, with $BumR_{D58E}$ and $BumR_{D58N}$ only slightly or modestly reduced compared to WT BumR (Fig. 2*A*), suggesting that $bumR_{\Delta KFK}$ might have the same phenotype as Δ*bumR*. Analysis of *peb3* transcription in WT and mutant strains grown with or without 12.5 mM butyrate revealed that mutation of D58 affects BumSR activity as *peb3* transcription was not repressed by butyrate in $bumR_{D58E}$ and $bumR_{D58N}$ (Fig. 2*B*). Thus, D58, as the phosphorylation site of BumR, is important for BumSR TCS signal transduction in response to butyrate. As expected, $bumR_{\Delta KFK}$ like Δ*bumR* showed augmented levels of *peb3* transcription relative to WT *C. jejuni* that was not repressed by butyrate. These results suggest that butyrate modulation of gene expression is defective with the $bumR_{\Delta KFK}$ allele. Since this mutation also abolished the ability of *C. jejuni* to infect humans, our findings suggest that butyrate sensing and alteration of transcription via BumSR TCS signal transduction is highly important for in vivo growth in humans and progression to diarrheal disease.

**BumR Is Required for Commensal Colonization of Avian Hosts at Early Time Points of Infection.** Although BumR is important for infection of human volunteers (44), we previously analyzed the commensal colonization capacity of *C. jejuni* Δ*bumS* and Δ*bumR* for the ceca of day-of-hatch chicks at day 7 postinfection and found that the mutants colonized as well as WT *C. jejuni* (43). For more thorough investigation of the BumSR TCS in commensal colonization of chicks, we compared the ability of WT *C. jejuni* and isogenic Δ*bumS* and Δ*bumR* mutants to colonize different regions of the avian intestinal tract over time. Day-of-hatch chicks were orally gavaged with *C. jejuni* strains and the levels of *C. jejuni* in the proximal and distal small intestines, ceca, and large intestines from day 1 to 14 postinfection were determined. BumR was required for optimal colonization throughout the intestinal tract at days 1 and 4 postinfection, as Δ*bumR* showed 3- to 782-fold reductions in colonization compared to WT *C. jejuni* (Fig. 3 *A*–*D*). In contrast, Δ*bumS* only showed a 6-fold lower colonization capacity in the large intestines at day 1 postinfection (Fig. 3 *A*–*D*). Similar to our previous analysis, we did not observe reductions of colonization by the mutants at day 7 and 14 postinfection of day-of-hatch chicks (43). We did observe enhanced colonization by Δ*bumS* and Δ*bumR* relative to WT in some regions of the intestinal tract, most noticeably in the large intestines at day 14 postinfection (Fig. 3 *A*–*D*). Our results, combined with the previous human volunteer study, indicate that BumR is required for infection of the human host and optimal colonization of the avian host, at least in young chicks. Our findings also suggest that BumS and BumR possibly have modulatory effects during prolonged commensal colonization.

**The *C. jejuni* BumSR TCS Impacts Expression of Colonization Determinants.** To understand the impact of butyrate on *C. jejuni* gene expression, we performed a transcriptome analysis of WT *C. jejuni* grown with or without a physiological concentration of butyrate to identify the butyrate-modulated regulon. Because we observed differences in *peb3* expression and requirements for commensal colonization between Δ*bumS* and Δ*bumR*, we hypothesized that the transcriptomes of the two mutants likely differ. Therefore, we also performed RNA sequencing (RNAseq) analysis to compare the transcriptomes of WT *C. jejuni*, Δ*bumS*, and Δ*bumR* to fully reveal the BumSR regulon.

RNAseq analysis of the transcriptomes of WT *C. jejuni* with or without exposure to 12.5 mM butyrate revealed at least 11 potential genes with significantly altered transcription (*SI Appendix*,

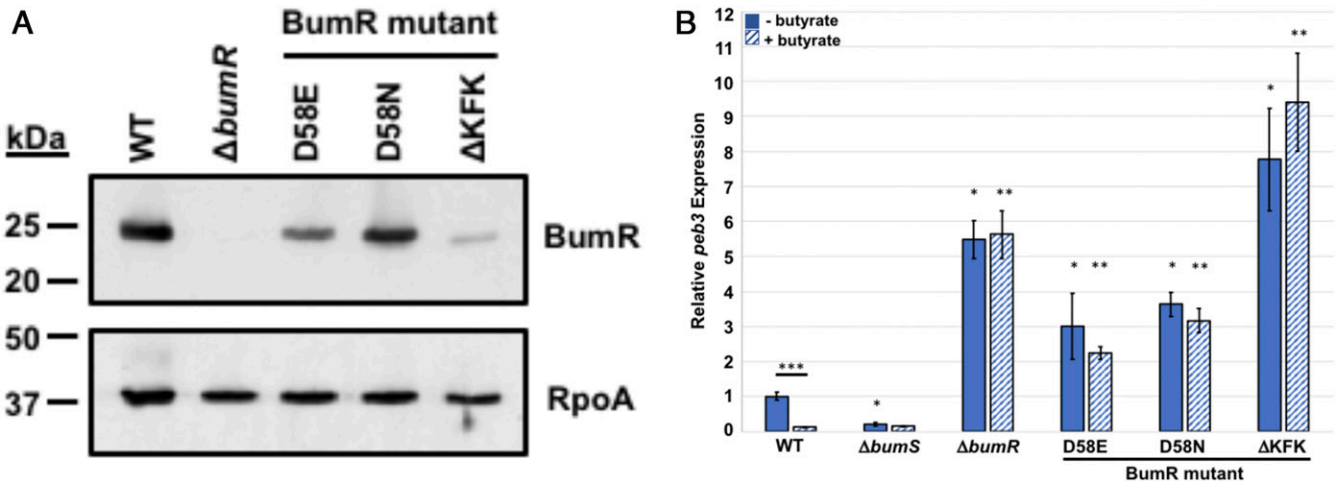


**Fig. 2.** Effect of BumR receiver domain mutations on butyrate-modulated transcription. (*A*) Production of BumR in WT *C. jejuni* and *bumR* mutants. Immunoblot analysis of the level of BumR in whole-cell lysates of WT and mutant strains after growth in Mueller–Hinton media. RpoA served as a control for protein loading. (*B*) qRT-PCR analysis of *peb3* transcription in WT *C. jejuni* and *bumR* mutants grown in CDM alone or CDM supplemented with 12.5 mM butyrate. The expression of *peb3* in CDM alone (solid blue bars) and CDM with butyrate (hatched blue bars) in WT *C. jejuni* and mutant strains are shown. The level of *peb3* transcription in WT grown in CDM alone as measured by qRT-PCR was set to 1. Expression of *peb3* in WT grown in CDM with butyrate or mutants grown with or without butyrate is shown relative to the WT strain grown in CDM alone. Results from a representative assay with each sample analyzed in triplicate are shown. Error bars indicate SDs of the average level of expression from three samples. Statistical significance of $\Delta C_T$ values relative to *secD* reference gene was calculated in GraphPad Prism by ANOVA with Tukey's test: * indicates the mutant grown in CDM alone had significantly increased or decreased expression relative to the WT strain grown in CDM alone; ** indicates the mutant grown with butyrate had a significantly increased or decreased expression relative to the WT strain grown with butyrate; *** indicates a strain showed a significantly different level of expression when grown in the presence of butyrate compared to growth in CDM alone ($P < 0.05$).

Table S1 and Dataset S1). Validation of RNAseq analysis by qRT-PCR revealed significant repression of some genes (and some cotranscribed operonic genes) in the presence of butyrate (Fig. 4*A*). Supplementation with butyrate led to 14.5-fold decreased expression of *peb3* (the reporter gene in our genetic screen to identify the BumSR TCS), 3.4-fold reduction of *cetAB* (encoding an energy taxis system), and 4.1-fold reduction of *Cjj0211* (the first gene of an operon encoding an iron acquisition system) (Fig. 4*A*). Note, for simplicity in describing genes from the 81-176 genome, we simplified the locus tags so that "*Cjj0211*" represents "*Cjj81176_0211*," for example. We also found *Cjj1656* and *Cjj1657* showed 2.6- and 4.2-fold reductions in expression in the presence of butyrate, respectively. We confirmed that other genes were induced upon butyrate exposure (Fig. 4*A*). Transcription of *Cjj0438* and *Cjj0439*, which compose a gluconate dehydrogenase complex required for colonization of chicks that we previously found to be regulated by the BumSR TCS (43, 55), was induced 3.5-fold upon butyrate supplementation (Fig. 4*A*).

Comparison of the transcriptomes of WT *C. jejuni* and isogenic Δ*bumS* and Δ*bumR* mutants grown in CDM without butyrate revealed 46 potential genes with significantly altered transcription (*SI Appendix*, Table S2 and Datasets S2–S5). Four potential genes were differentially expressed between WT *C. jejuni* and Δ*bumS* and 33 potential genes were differentially expressed between WT *C. jejuni* and Δ*bumR*. Moreover, nine potential genes were significantly altered in expression in both Δ*bumS* and Δ*bumR* compared to WT *C. jejuni* (*SI Appendix*, Table S2 and Datasets S2 and S3). We confirmed the results of the RNAseq analysis via qRT-PCR, and by combining the data from both experiments, genes could be grouped into specific categories based on their expression profiles. Transcription of some genes (class I) including *peb3* and *Cjj0580* were repressed by BumR as transcription was increased in Δ*bumR* (Fig. 4*B*). However, Δ*bumS* exhibited an opposing phenotype with decreased expression of these genes compared to WT. Another set of genes (class II) that includes the *Cjj0438* and *Cjj0885* operons were increased in expression in both Δ*bumS* and Δ*bumR* compared to WT, suggesting that BumR represses transcription of these genes (Fig. 4*B*). A third set of genes (class III), which includes *cetAB*, *herA*, and *rpsU*, were repressed in Δ*bumS* with either slight repression or no change in expression in Δ*bumR* (Fig. 4*B*). The BumSR regulon did contain genes modulated by butyrate supplementation in WT *C. jejuni*, but not originally identified to be differentially expressed in our RNAseq analysis of WT grown with or without butyrate (Fig. 4*C* and Datasets S1–S5). We were able to expand the butyrate-modulated regulon as *cgpD* and *herA* were reduced in expression in WT *C. jejuni* in the presence of butyrate, and *Cjj1485* expression was induced with butyrate supplementation (Fig. 4*C*). Other BumSR regulon members showed only modest-to-no significant alterations in expression with butyrate supplementation (Fig. 4*C*). Our findings for how Δ*bumS* and Δ*bumR* differently impact expression of genes such as *peb3*, the *Cjj0438* operon, and *cetAB* suggest complexity in molecular mechanisms of signal transduction through the BumSR TCS and for different modes of regulating transcription of genes within its regulon.

Given that we found BumR to be important for the initial colonization of chicks, we analyzed whether some butyrate-modulated genes within the BumSR regulon were involved in colonization in a natural chick model of commensalism. For this experiment, day-of-hatch chicks were orally gavaged with WT *C. jejuni* or an isogenic mutant and the levels of *C. jejuni* in the proximal and distal small intestines, ceca, and large intestines were determined at days 4 and 7 postinfection (*SI Appendix*, Fig. S2). Mutants lacking a specific BumSR regulon member demonstrated a range of colonization capacities for the avian intestinal tract. Mutants in *peb3* and *Cjj0580,* which have similar expression profiles in WT *C. jejuni* supplemented with butyrate and in the Δ*bumS* and Δ*bumR* mutants, exhibited similar colonization defects with 68- to 688-fold reductions in the ceca and large intestines at an early time point postinfection (*SI Appendix*, Fig. S2 *C* and *D*). The *peb3* mutant colonization defect continued

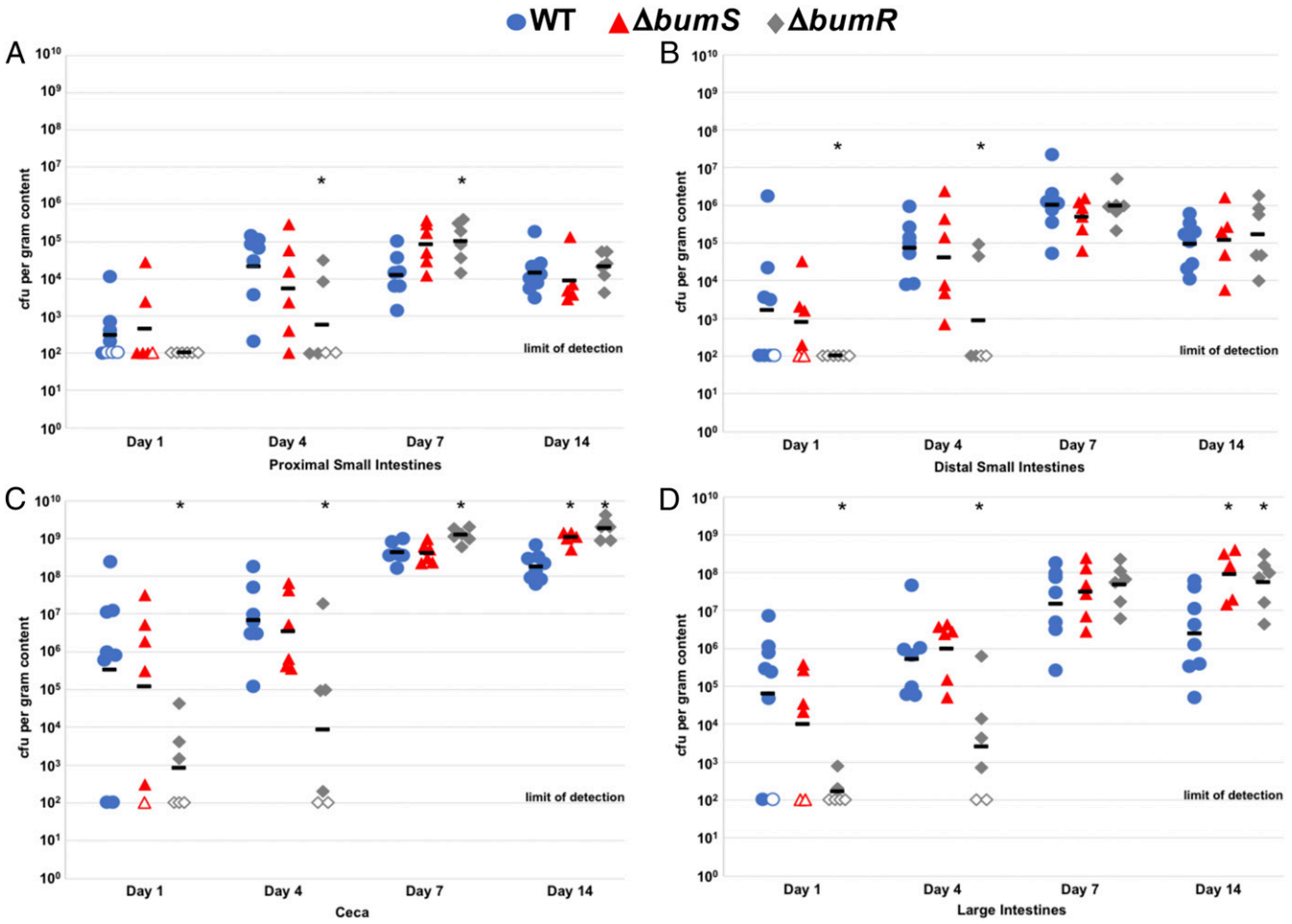


**Fig. 3.** Colonization dynamics of WT *C. jejuni* and isogenic Δ*bumS* and Δ*bumR* mutants over time in the avian intestinal tract. Day of hatch chicks were orally infected with ∼100 colony-forming units (CFU) of WT *C. jejuni* (blue circles), isogenic Δ*bumS* (red triangles), and Δ*bumR* (gray diamonds) mutants. Chicks were killed at days 1, 4, 7, or 14 postinfection and the levels of each *C. jejuni* strain in *A* the proximal small intestine, (*B*) distal small intestines, (*C*) ceca, and (*D*) large intestines was determined (reported as CFU per gram of content). Each closed symbol represents the level of *C. jejuni* in a single chick. Open symbols represent chicks with *C. jejuni* levels below the limit of detection (<100 CFU per gram of content). Horizontal bars represent geometric mean for each group. Statistical analysis was performed using the Mann–Whitney *U* test (**P* < 0.05).

for the large intestines and became more prominent in some other intestinal areas at day 7. Mutants lacking Cet energy taxis system components (CetA, CetB, or CetC; refs. 56–58), which generally depend on both BumS and BumR for WT level of expression, were not defective for cecal colonization, but were lower in other areas of the intestinal tract especially at day 7 postinfection (*SI Appendix*, Fig. S2 *A–D*). In contrast, the mutant lacking *herA* encoding a hemerythrin (59), which is mainly dependent on BumS for expression, was not defective for colonization in any intestinal areas. Considering that deletion of *bumS* or *bumR* results in lack of expression for some genes and derepression and overexpression of others, linking any one BumSR regulon member to general colonization defects or enhancements observed with Δ*bumS* and Δ*bumR* is hindered. Rather, the BumSR TCS controls expression of butyrate-modulated determinants, of which many can influence commensal colonization in different areas of the intestinal tract over time.

**The BumSR TCS Specifically Responds to Butyrate but Not Acetate or Lactate.** We previously discovered that *C. jejuni* senses and responds to physiological levels of intestinal acetate and L-lactate in addition to butyrate (19). In this prior study, we also analyzed whether *C. jejuni* senses and responds to propionate, but only detected a transcriptional change to propionate at a concentration ∼10-fold greater than physiological levels present in the lower intestinal tract of humans and avian species. We analyzed whether BumSR sensing is specific for butyrate or if it has an expanded repertoire for acetate and lactate. Expression of two butyrate-modulated genes of the BumSR regulon, *peb3* and *Cjj0438*, was also modulated when WT *C. jejuni* was grown in CDM with a physiological concentration of 100 mM acetate (Fig. 5*A*). The reported levels of acetate in the lower intestinal regions of chickens ranges for 47 to 124 mM (36, 37). In the presence of acetate, *peb3* transcription was reduced 28.5-fold and *Cjj0438* was increased 7.9-fold in WT *C. jejuni*. In Δ*bumS* and Δ*bumR*, we continued to observe significant repression of *peb3* transcription and induction of *Cjj0438* expression in the presence of acetate (Fig. 5*A*), suggesting that a system other than the BumSR TCS is responsible for sensing and responding to acetate.

Given that transcription of several genes within the BumSR regulon were not affected by L-lactate, we analyzed expression of *Cjj0683* and *ggt*, which we previously found were modulated by L-lactate (19). Reported levels of lactate in the upper intestinal regions of chickens range from 21 to 72 mM (38, 39). In WT *C. jejuni* grown in CDM with 25 mM L-lactate, significant transcriptional repression of both genes was observed (Fig. 5*B*).

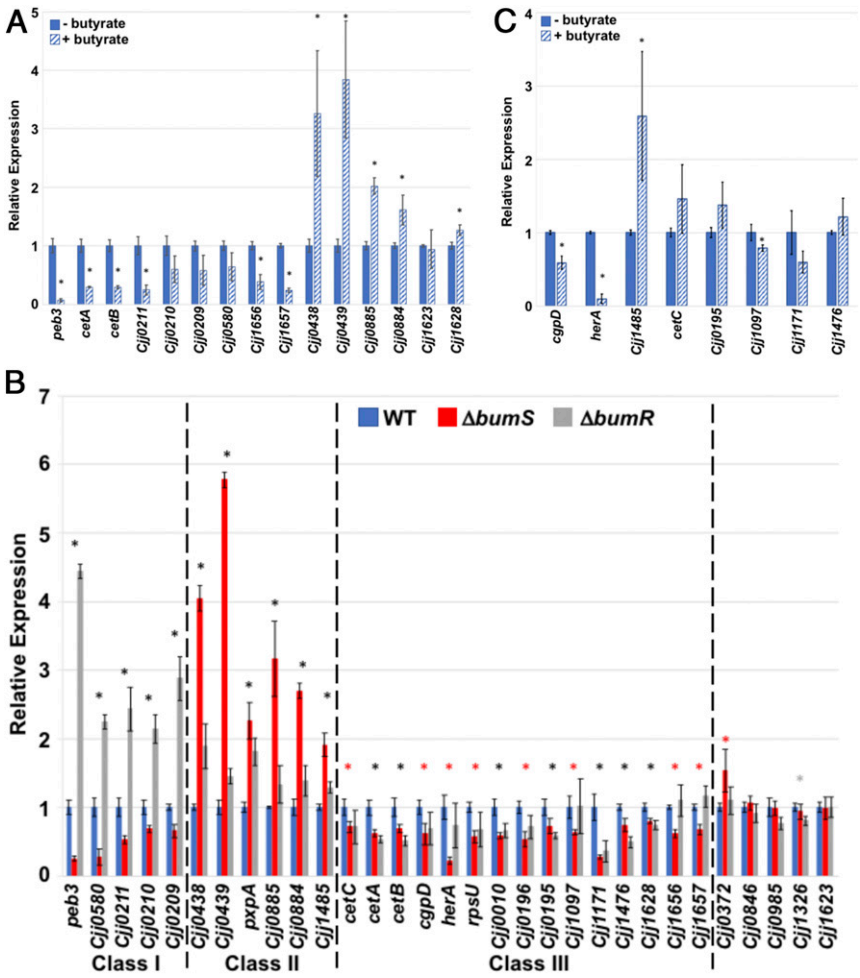


**Fig. 4.** Analysis of *C. jejuni* butyrate-modulated genes and the BumSR regulon. (*A*) qRT-PCR analysis of transcription of genes initially identified by RNAseq analysis of WT *C. jejuni* to be modulated in expression by 12.5 mM butyrate during growth in CDM. The expression of each gene in WT *C. jejuni* grown in CDM alone (solid blue bars) as measured by qRT-PCR was set to 1. Expression of each gene in WT *C. jejuni* grown in CDM supplemented with 12.5 mM butyrate (hatched blue bars) is shown relative to WT grown in CDM alone. (*B*) qRT-PCR analysis of transcription of genes initially identified by RNAseq analysis to be altered in *C. jejuni* Δ*bumS* and/or Δ*bumR* relative to WT *C. jejuni*. The expression of each gene in WT *C. jejuni* grown in CDM alone (solid blue bars) as measured by qRT-PCR was set to 1. Expression of each gene in Δ*bumS* (red bars) and Δ*bumR* (gray bars) is shown relative to WT. Dotted lines indicates divisions between gene sets (classes I to III) show similar effects in expression due to deletion of *bumS* and/or *bumR*. (*C*) Analysis of the ability of BumSR TCS regulon members to be modulated by butyrate for transcription. Select genes that were altered in expression in *C. jejuni* Δ*bumS* or Δ*bumR* were analyzed for butyrate-modulated expression in WT *C. jejuni* grown in CDM alone (solid blue bars) and in CDM supplemented with 12.5 mM butyrate (hatched blue bars) by qRT-PCR. The expression of each gene in WT *C. jejuni* grown in CDM alone was set to 1. Expression of each gene in WT *C. jejuni* grown in CDM supplemented with 12.5 mM butyrate is shown relative to WT grown in CDM alone. For (*A*–*C*), results from a representative assay with each sample analyzed in triplicate are shown. Error bars indicate SDs of the average level of expression from three samples. Statistically significant differences in gene expression between WT *C. jejuni* grown with or without butyrate is indicated by * in *A* and *C* ($P < 0.05$). For (*B*), different colored asterisks indicate statistically significant differences ($P < 0.05$) in gene expression between: WT *C. jejuni* and both Δ*bumS* and Δ*bumR*, black *; WT *C. jejuni* and Δ*bumS* only, red *; and WT *C. jejuni* and Δ*bumR* only, gray * ($P < 0.05$). Statistical analysis of $\Delta C_T$ values relative to *secD* reference gene was calculated by the Student's *t* test.

Transcriptional repression was also evident in Δ*bumS* and Δ*bumR* when grown in L-lactate (Fig. 5*B*). Therefore, we conclude that the BumSR TCS specifically directs a response to butyrate and not other SCFAs or organic acids such as acetate or L-lactate.

**Evidence for BumS Controlling BumR Activity Primarily through Phosphatase Activity.** Canonical bacterial TCSs respond to environmental stimuli and mediate outputs by first sensing a specific signal via the sensory domain of the kinase, which leads to autophosphorylation on a conserved histidine residue (60, 61). The phosphohistidine is a substrate for autophosphorylation of a cognate response regulator, which causes a conformational change in the effector domain to alter the activity of the protein (60). For response regulators that are transcription factors, phosphorylation affects the ability of these proteins to bind DNA and function as activators or repressors of gene expression. Many sensor kinases function as phosphatases to control the level of phosphorylation and activity of a cognate response regulator when not functioning as an autokinase (62). This phosphatase activity contributes to signaling fidelity and elimination of crosstalk between signal transduction systems in bacteria (63).

Most bacterial sensor kinases contain transmembrane domains to localize to the cytoplasmic membrane. However, BumS is predicted to be a cytoplasmic sensor histidine kinase as no transmembrane domains are predicted in its protein sequence. Based on the canonical TCS signal transduction model, H195 in the H box of BumS is the best candidate as the site of autophosphorylation. We previously confirmed D58 in the receiver domain of BumR as the site of phosphorylation (43). Further sequence analysis of BumS reveals potential alterations in two regions

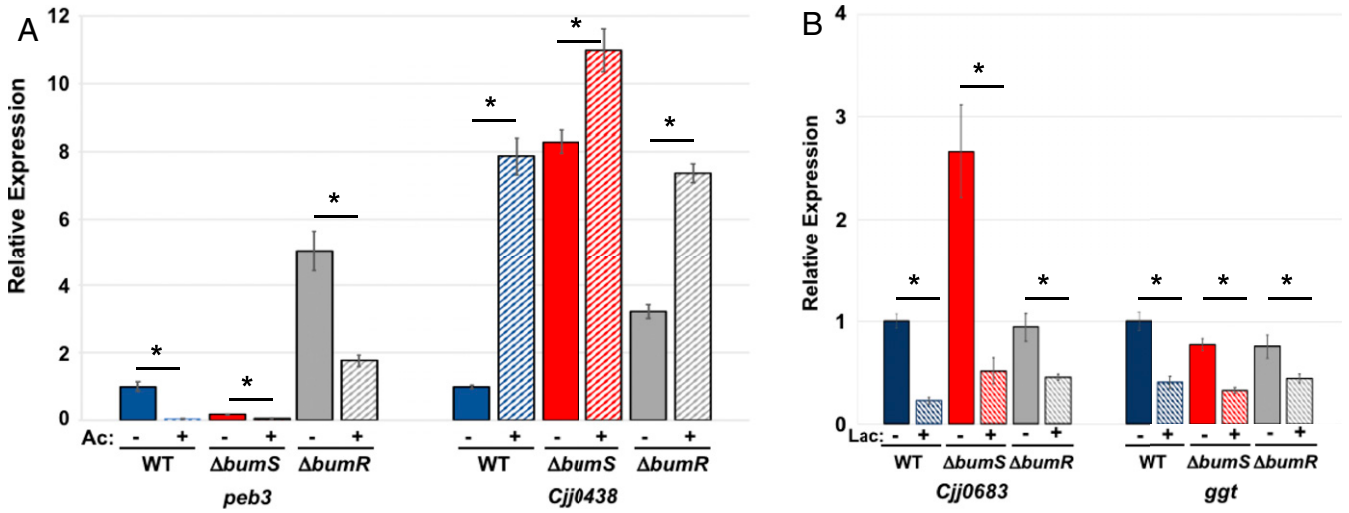


**Fig. 5.** The BumSR TCS is not required for acetate- or lactate-modulated gene expression. (*A*) qRT-PCR analysis of transcription of *peb3* and *Cjj0438* in WT *C. jejuni* and Δ*bumS* or Δ*bumR* mutants grown in CDM alone or CDM supplemented with 100 mM acetate. The expression of *peb3* and *Cjj0438* in WT *C. jejuni* grown in CDM alone (sold blue bars) as measured by qRT-PCR was set to 1. Expression of genes in WT grown in CDM supplemented with acetate (hatched blue bars), Δ*bumS* without (solid red bars), or with acetate (hatched red bars), or Δ*bumR* without (solid gray bars) or with acetate (hatched gray bars) is shown relative to WT grown in CDM alone. (*B*) qRT-PCR analysis of transcription of *Cjj0683* and *ggt* in WT *C. jejuni* and Δ*bumS* or Δ*bumR* mutants grown in CDM alone or CDM supplemented with 25 mM L-lactate. The expression of *Cjj0683* and *ggt* in WT *C. jejuni* grown in CDM alone (sold blue bars) as measured by qRT-PCR was set to 1. Expression of genes in WT grown in CDM supplemented with L-lactate (hatched blue bars), Δ*bumS* without (solid red bars) or with L-lactate (hatched red bars), or Δ*bumR* without (solid gray bars) or with L-lactate (hatched gray bars) is shown relative to the WT strain grown in CDM alone. Results from a representative assay with each sample analyzed in triplicate are shown. Error bars indicate SDs of the average level of expression from three samples. Statistical significance of $\Delta C_T$ values relative to *recA* reference gene (for acetate analysis in *A*) or *secD* reference gene (for lactate analysis in *B*) was calculated in GraphPad Prism by ANOVA with Sidak's test: * indicates statistically significant differences in gene expression between a strain grown in CDM alone and in CDM supplemented with acetate or lactate ($P < 0.05$).

that could affect autokinase activity. One region includes the H box, where the histidine that is the site of autophosphorylation is usually followed by an aspartate or glutamate and a hydrophobic residue (most often a leucine or isoleucine) (64, 65). In BumS, H195 is followed by a glutamine and tryptophan. The second potentially altered domain is the DxGxG motif in the D box of the histidine kinase and GHL ATPase superfamilies that coordinates a magnesium ion to bind ATP (65). The sequence of the D box in BumS is DxAxG; this glycine-to-alanine alteration eliminated autokinase activity in CheA sensor kinases (66, 67).

Repeated attempts to observe in vitro autophosphorylation of recombinant BumS purified from *E. coli* were unsuccessful, even

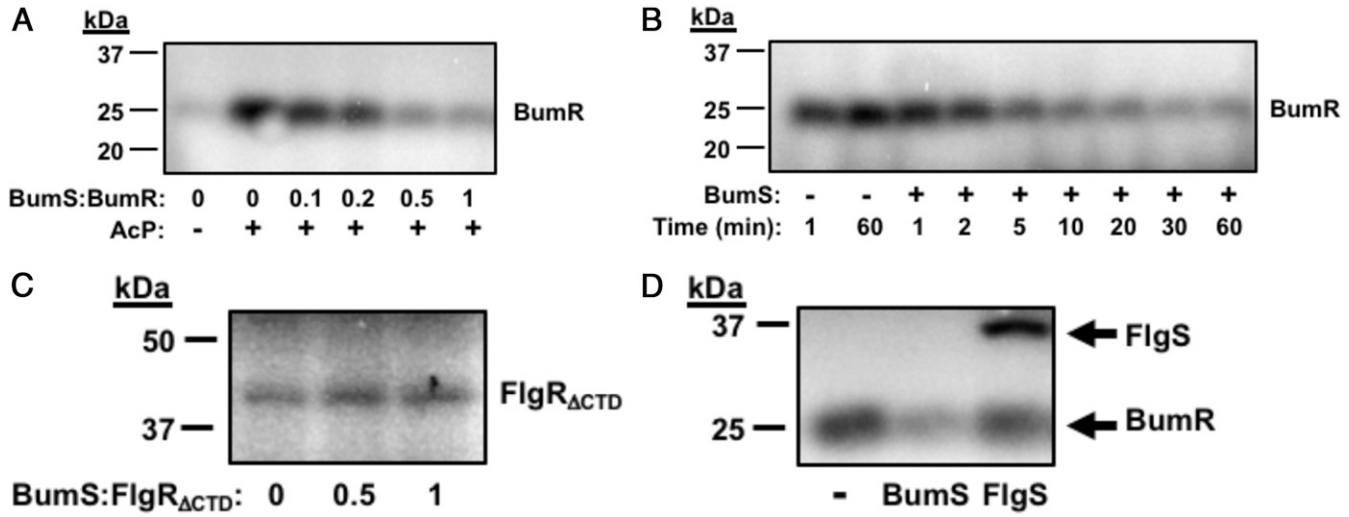


**Fig. 6.** BumS phosphatase activity is specific for phospho-BumR. (*A*) Dephosphorylation of phospho-BumR by BumS. After autophosphorylation of BumR by Ac[$^{32}$P], increasing concentrations of BumS were added as indicated by BumS:BumR molar ratios and then incubated for 10 min. (*B*) Dephosphorylation of phospho-BumR by BumS over time. After autophosphorylation of BumR by Ac[$^{32}$P], mock treated or BumS at a 1:2 BumS:BumR molar ratio was added and incubated for up to 60 min. Reactions were stopped at various times indicated. (*C*) Dephosphorylation of phospho-FlgR$_{\Delta CTD}$ by BumS. After autophosphorylation of FlgR$_{\Delta CTD}$ by Ac[$^{32}$P], BumS was added at 1:2 or 1:1 BumS:FlgR$_{\Delta CTD}$ molar ratio for 20 min. (*D*) Comparison of dephosphorylation of phospho-BumR by BumS and FlgS. After autophosphorylation of BumR by Ac[$^{32}$P], BumS or FlgS were added at 1:2 molar ratio to BumR and the reactions were incubated for 10 min.

in the presence of physiological concentrations of butyrate or with recombinant BumR (*SI Appendix*, Fig. S3 *A* and *B*). As a control, we observed both in vitro autophosphorylation of another *C. jejuni* sensor histidine kinase, FlgS, and phosphorylation of a mutant form of its cognate response regulator, $FlgR_{\Delta CTD}$, which is more promiscuous for autophosphorylation by AcP, as previously described (*SI Appendix*, Fig. S3 *A* and *B*; refs. 49, 68, and 69). These findings suggest that WT BumS does not possess observable in vitro autokinase activity at least by these conditions employed and may not be responsible for the phosphorylation of BumR in *C. jejuni*.

We then investigated whether BumS functions as a phosphatase with phospho-BumR as a substrate. For these analyses, we added Ac[$^{32}$P] to promote autophosphorylation of BumR and then observed whether levels of phospho-BumR were reduced upon addition of BumS. We observed that BumS caused robust dephosphorylation of BumR that was dependent on the concentration of BumS and time (Fig. 6 *A* and *B*). BumS phosphatase activity was specific for BumR as BumS was unable to dephosphorylate the noncognate phosphorylated $FlgR_{\Delta CTD}$ response regulator (Fig. 6*C*). Furthermore, FlgS was unable to dephosphorylate BumR (Fig. 6*D*), also indicating that dephosphorylation of BumR is an activity specific for BumS. In the course of this experimentation, we observed that BumR is naturally more readily autophosphorylated with Ac[$^{32}$P] than is $FlgR_{\Delta CTD}$ (~65-fold more; *SI Appendix*, Fig. S3*C*).

We next tested whether butyrate might be sensed by BumS and impact BumS phosphatase activity for BumR. Titration of butyrate up to 12.5 mM, which we observed to impact butyrate-modulated gene expression, did not influence the phosphatase activity of BumS (*SI Appendix*, Fig. S3*D*). These findings suggest that butyrate-modulated gene expression likely involves indirect sensing of butyrate by BumS to alter BumS phosphatase activity to control the level of phosphorylation of BumR and its activity as a response regulator. Due to the lack of detectable in vitro BumS kinase activity, BumR phosphorylation in *C. jejuni* is hypothesized to occur by other noncognate kinases or metabolite phosphodonors.

As discussed above, WT BumS contains alterations in the H and D boxes that could impair its ability to function naturally as an autokinase to ultimately serve as a direct phosphodonor for BumR. BumS sequences across many *C. jejuni* strains also conserve these alterations in the H and D boxes found in WT BumS in *C. jejuni* strain 81-176, which was used throughout this study. We tested whether kinase activity could be restored to BumS by altering residues in the H and D boxes to consensus residues present in a majority of active kinases. As such, we purified two recombinant BumS proteins, $BumS_{QW196\text{-}7EL}$ and $BumS_{A338G}$ ("restored" H and D boxes, respectively) and a third protein that combined the three mutations ($BumS_{HD\ box}$). None of the mutations alone or combined restored autokinase activity to BumS (*SI Appendix*, Fig. S4). We then assessed whether BumS phosphatase activity was affected by these mutations. Addition of WT BumS to phospho-BumR at a 1:2 ratio resulted in 50% dephosphorylation of BumR between 5 and 10 min of incubation (Fig. 7 *A* and *B*). A slightly enhanced level of BumR dephosphorylation was noted for $BumS_{A338G}$, indicating that A338 was not required for phosphatase activity. In contrast, $BumS_{QW196\text{-}7EL}$ exhibited significantly decreased phosphatase activity toward BumR with no more than a 20% reduction in phospho-BumR over time (Fig. 7 *A* and *B*). These findings suggest that BumS may have evolved these mutations to primarily serve as a phosphatase rather than a kinase to mediate control of BumR activity.

**BumR Binding to Target Promoters Is Modulated by Phosphorylation.** Since BumR activates and represses expression of different genes in its regulon, we examined the effect of BumR phosphorylation on promoter binding via electrophoretic mobility shift assays (EMSAs). We tested *peb3*, *Cjj0580*, and *cetA* promoter DNA for binding by BumR; a *recA* promoter probe served as a negative control to ensure specificity of BumR binding. Unphosphorylated BumR bound all promoters except *recA* at the highest BumR concentration tested (1.5 μM; Fig. 8*A*). Pretreatment of BumR with AcP increased binding at lower concentrations of BumR to all promoters except the *recA* negative control probe, suggesting that phosphorylated BumR augments DNA-binding activity to activate or repress transcription at different promoters. Competition assays with excess unlabeled promoter DNA or *recA* promoter DNA indicated that BumR specifically interacted with all target promoters (Fig. 8*B*). These findings are consistent with our previous results that control of the level of phosphorylation of BumR influences direct interactions with target promoter and the level of transcription of genes in the BumSR regulon (43).

## Discussion

In this report, we identified a system and potential mechanism for a bacterium to sense and respond to the microbiota-generated SCFA butyrate, a metabolite with important activities in intestinal health and homeostasis. The *C. jejuni* BumSR TCS altered gene expression that impacted host colonization in the presence of butyrate. This work, combined with our previous analyses (19), demonstrate that *C. jejuni* senses and responds to multiple microbiota-generated metabolites, including the SCFAs butyrate and acetate and the organic acid lactate. As the BumSR TCS specifically responds to butyrate but not acetate or lactate, we propose *C. jejuni* employs different sensing systems in vivo to collectively monitor spatial gradients of specific metabolites and process this information to identify lower intestinal regions ideal for supporting growth and then altering gene expression appropriately. Continued exploration will reveal how butyrate sensing by the BumSR TCS likely converges into a complex network of signaling systems each possibly specific for different metabolites to result in optimal responses to ensure colonization of human and avian hosts.

A previous study with human volunteers found that a BumSR TCS mutant, due to an in-frame deletion within *bumR* that we found to make the respective protein unstable, is important for *C. jejuni* to infect humans (44). However, the activity of the TCS and how it contributed to in vivo growth and pathogenesis of disease in humans was not determined. Our findings suggest that directing a response to butyrate by the BumSR TCS and the resultant signal transduction that culminates in modulation of transcription of colonization and virulence factors is likely important for *C. jejuni* to infect humans. We also found that WT BumR was required for optimal levels of colonization of chicks at early time points after infection, but not at later time points. As our colonization assay ended at day 14 postinfection of chickens, we have yet determined whether the BumSR TCS may be important for a persistent infection lasting many weeks or for transmission from one host to another. Assuming that the BumSR TCS responds to butyrate in humans, butyrate sensing seems to be more important for *C. jejuni* to promote infection of humans than colonization of avian hosts. If so, we speculate that evolution of the BumSR TCS along with other virulence determinants potentially encoded within the *C. jejuni* BumSR regulon may have allowed the bacterium to extend its host range from a commensal in the avian host to a pathogen of humans. Thus, a number of members of the BumSR regulon that we identified whose expression is modulated upon sensing butyrate may be important for promoting infection of humans to result in diarrheal disease.

To our knowledge, the *C. jejuni* BumSR TCS is the only bacterial TCS identified thus far to be responsible for sensing and responding to butyrate. A search for bacterial species that contain a cognate pair of BumS and BumR orthologs only revealed hits in other *C. jejuni* strains and the closely related diarrheal pathogen *Campylobacter coli*.

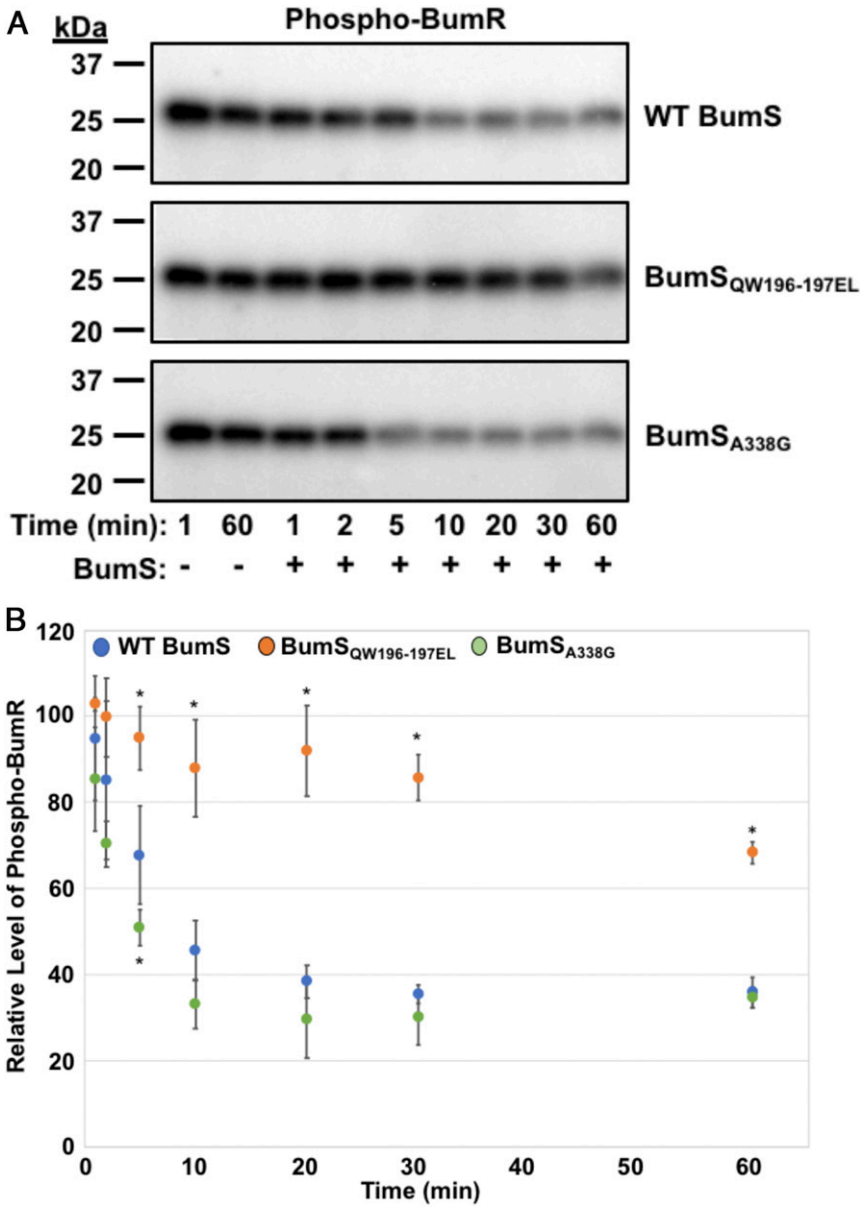


**Fig. 7.** BumS phosphatase activity is dependent on specific H box residues. (*A* and *B*) In vitro phosphatase activities WT BumS, BumS$_{QW196-7EL}$ (an H box mutant), and BumS$_{A338G}$ (a D box mutant) over time. After autophosphorylation of BumR by Ac[$^{32}$P], BumS proteins were added at 1:2 BumS:BumR molar ratios for up to 60 min. Reactions were stopped at various times indicated. (*A*) Representative phosphatase assay of WT BumS or BumS mutants for phospho-BumR. (*B*) Quantification of three independent phosphatase assays for WT BumS, BumS$_{QW196-7EL}$, and BumS$_{A338G}$. The level of phospho-BumR in each assay was normalized to phospho-BumR at 1 min of mock sample, which was set to 100%. No appreciable decrease in phospho-BumR in the absence of BumS was observed for the duration of the experiment (up to 60 min) as observed in *A*. Error bars indicate SDs of the relative level of phospho-BumR at indicated time after addition of BumS from three independent experiments. Statistical significance to compare WT to mutant proteins was calculated in GraphPad Prism by ANOVA with Dunnett's test (*$P < 0.05$).

Butyrate has been shown to repress transcription of *Salmonella* virulence genes to cause reduced invasion of intestinal epithelial cells (23, 26). However, the only mechanism linked to how butyrate affects *Salmonella* so far has been the reduced transcription of *hilD*, encoding a transcription factor that derepresses expression of HilA, which is a positive activator of SPI1 gene expression (26). It is not known how butyrate is sensed directly by *Salmonella* to alter expression of the HilD-HilA pathway. Butyrate can also influence expression of the *E. coli* locus of enterocyte effacement through the Lrp master regulator, but it is mechanistically unclear how butyrate is sensed (17).

A question that remains from our analyses presented is whether BumS directly or indirectly senses butyrate. Data supporting that BumS directly senses butyrate are that simply supplementing *C. jejuni* with exogenous butyrate in vitro resulted in transcriptional changes that were abolished by mutation of *bumS* and *bumR*. However, butyrate did not influence the in vitro kinase or phosphatase activity of BumS in our assays. BumS lacks predicted transmembrane domains and is presumed to be a cytoplasmic sensor kinase with a predicted PAS domain as a major feature of its sensor region. This composition implies that BumS may detect a cytoplasmic signal. If butyrate is the direct cue sensed by BumS, then we would expect butyrate to be transported into the cytoplasm

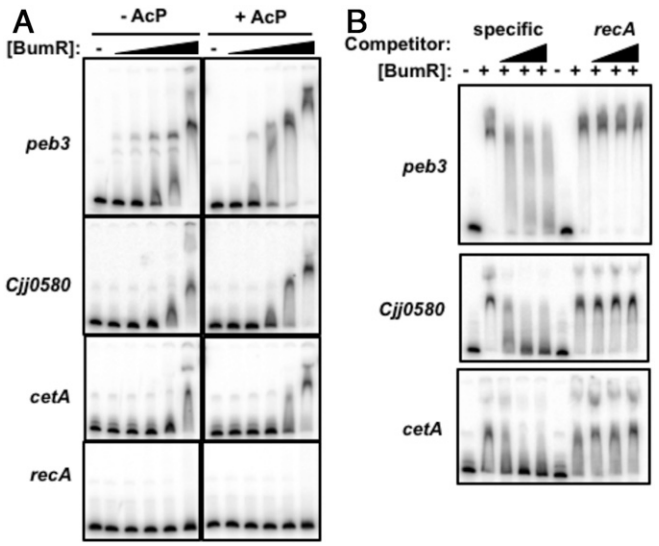


**Fig. 8.** Electrophoretic mobility shift assays for analysis of DNA-binding activity of BumR. (*A*) Comparison of BumR binding to target promoter with or without phosphorylation. Recombinant BumR alone or after autophosphorylation with Li-AcP was added to *peb3*, *Cjj0580*, *cetA*, or *recA* promoter DNAs at concentrations ranging from 0 to 1.5 μM. (*B*) Binding of 1.5 μm BumR after autophosphorylation with Li-AcP to radiolabeled promoter DNAs for *peb3*, *Cjj0580*, or *cetA* in the presence of increasing concentrations of the same unlabeled specific competitor DNA or unlabeled noncompetitor DNA (*recA* promoter).

to function as a signal. We have not yet analyzed whether butyrate is transported by an active or passive process into the *C. jejuni* cytoplasm. Alternatively, microbiota-generated intestinal butyrate might be sensed indirectly by the BumSR TCS by being converted into another factor that is a cytoplasmic cue directly sensed by BumS. Bioinformatic analysis of the *C. jejuni* genome, however, does not identify any encoded canonical metabolic pathways for utilization of butyrate or its conversion to another metabolite. In a preliminary experiment, we attempted to detect changes in concentration of potential butyrate-derived metabolites after growth of *C. jejuni* in the presence of butyrate, but detection of these metabolites was difficult, requiring further experimentation and analysis development; therefore, our analysis was inconclusive. Alternatively, exogenous butyrate may influence the activity of another metabolic pathway in *C. jejuni* that creates a factor directly sensed by BumS. Our genetic screen to identify *C. jejuni* mutants with defects in butyrate-modulated responses only identified mutants with transposon insertions in *bumS* or *bumR*, and not other such auxiliary components. It is possible that our transposon mutagenesis screen may not have been saturating to identify every factor required for the indirect sensing of butyrate. Alternatively, if butyrate is converted by *C. jejuni* into a cytoplasmic cue or influences a metabolic system to generate such a cue that is sensed directly by BumS, this system could be essential for viability, providing a reason for why it was not identified in our genetic screen. Because butyrate is not required for normal *C. jejuni* growth, we assume that such an auxiliary system has a butyrate-independent primary activity essential for viability and a secondary butyrate-dependent activity to create a cue directly sensed by BumS.

Based on our findings, we propose a model for BumSR signal transduction influenced by exogenous butyrate (Fig. 9). A salient feature of our model is BumS functioning prominently as a phosphatase rather than as a kinase to control the activity of BumR as a transcriptional regulator, with butyrate influencing a cytoplasmic signal that inhibits the BumS phosphatase activity to impact the level of phosphorylation of BumR. Using *peb3* as an example, we can highlight aspects of our model. Deletion of *bumR* increases *peb3* transcription, indicating that BumR serves as a transcriptional repressor in this context. Phosphorylation of BumR increased binding of BumR to the *peb3* promoter, indicating that high levels of phospho-BumR are required for full repression of *peb3*. Since Δ*bumS* demonstrated high repression of *peb3* transcription, BumR likely exists in a mostly phosphorylated form in Δ*bumS* to cause full repression. Addition of butyrate to WT *C. jejuni* decreased *peb3* expression, suggesting that butyrate sensed by BumS inhibits its phosphatase activity to increase phospho-BumR levels and repress *peb3* transcription. In contrast, *Cjj0438* transcription was influenced in the opposite manner; both butyrate exposure and deletion of *bumS* stimulated transcription. BumR also showed increased binding to the *Cjj0438* promoter upon phosphorylation (43). For this scenario, inhibiting the phosphatase activity of BumS (either with butyrate supplementation or deletion of the gene) leads to increased levels of phospho-BumR to activate transcription of *Cjj0438*.

Our proposed model for signal transduction through the BumSR TCS and how BumS functions is unusual as most sensor histidine kinases of bacterial TCSs studied to date sense a signal that influences its autokinase activity for phosphotransfer to its cognate response regulator as the primary mechanism to impact the activity of the regulator. Instead, we propose that butyrate sensing regulates the phosphatase activity of BumS for BumR, with BumS possibly lacking innate kinase activity. It is known that many bacterial sensor histidine kinases serve as phosphatases for their cognate response regulators when not functioning as kinases in the absence of their specific signals. Our model suggests that crosstalk with other *C. jejuni* sensor histidine kinases or high-energy phosphodonors such as AcP contributes to endogenous BumR phosphorylation on D58. In our cursory analysis, BumR did seem to be more readily phosphorylated by AcP than another *C. jejuni* response regulator ($FlgR_{\Delta CTD}$) in vitro, suggesting that BumR may be fairly susceptible to phosphorylation by noncognate phosphodonors in *C. jejuni*. Thus, the BumSR TCS is apparently unique on two fronts—in sensing and

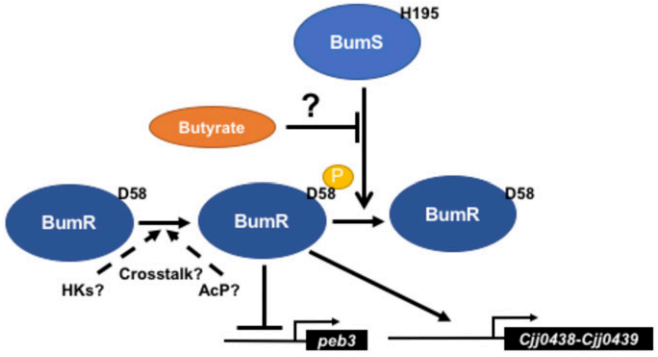


**Fig. 9.** Model for butyrate-modulated transcription of *C. jejuni* genes via the BumSR TCS. BumR is hypothesized to be endogenously phosphorylated by other kinases or high-energy phosphodonors in the cytoplasm of *C. jejuni*. BumS is proposed to primarily function as a phosphatase to control the level of phosphorylation of BumR and its activity as a transcriptional regulator. BumR shows increased affinity for target promoter DNA in its phosphorylated form and can function as a repressor or activator depending on the promoter. Exogenous butyrate produced by the intestinal microbiota is either sensed directly by BumS after transport to the cytoplasm, sensed indirectly by its conversion to another metabolite that is sensed by BumS, or sensed indirectly by its influence on another pathway that alters the level of a factor directly sensed by BumS. In any case, increased levels of exogenous butyrate are expected to have a negative influence on BumS phosphatase activity, which results in increased phospho-BumR levels to influence transcriptional outcomes.

responding to butyrate and in employing signal-dependent sensor phosphatase activity to exert control of its response regulator to modulate gene expression.

Examination of the amino acid sequence of BumS and motifs conserved in the protein histidine kinase family support our biochemical assays for BumS functioning as a phosphatase rather than a kinase. Although we did not perform ATP binding assays with recombinant BumS, the natural alteration in its D box from the canonical DxGxG motif to DxAxG motif present in BumS may prevent BumS from binding ATP and functioning as a kinase. Furthermore, many histidine kinases possess an H box in which the conserved autophosphorylated histidine residue is followed by an aspartate or glutamate and then a hydrophobic residue (65). In BumS, the predicted histidine that would normally be autophosphorylated, H195, is followed by a glutamine and the aromatic amino acid tryptophan. Of note, five of the six *C. jejuni* sensor histidine kinases of other TCSs all conserve the normal residues following the histidine that is the site of phosphorylation in the H box; the exception is CheA, whose orthologs in other bacterial species also do not have an H box (65). This H box alteration in BumS could have also caused BumS to no longer possess autokinase activity. We attempted to potentially restore BumS as a kinase by converting A338 to glycine to repair the DxGxG motif in the D box, along with converting Q196 and W197 to glutamate and leucine in the H box, respectively, which are the more standard amino acid residues at these positions in other kinases (65). However, combinations of single, double, and triple point mutants did not restore BumS kinase activity, which could suggest that BumS may have evolved multiple alterations besides those examined to more efficiently function as a phosphatase than a kinase. We actually demonstrated that mutation of Q196 and W197 to the canonical amino acids found in most histidine kinase greatly diminished BumS phosphatase activity without restoring kinase activity. Thus, these two noncanonical residues may represent a few evolutionary adaptations that have occurred to enhance the phosphatase activity of BumS that now drives the major mechanism by which BumS controls BumR activity, which is influenced by sensing exogenous butyrate via direct or indirect means. Overall, our work indicates a noncanonical, complex, and multifactorial mechanism for a bacterium to sense microbiota-derived butyrate in the intestines of hosts to promote infection of humans for diarrheal disease and colonization of avian hosts for commensalism.

## Materials and Methods

Materials and methods describing the growth conditions, plasmid construction, *C. jejuni* mutant construction, transposon mutagenesis and genetic screening procedures, arylsulfatase assays, immunoblotting, chick colonization assays, RNAseq and qRT-PCR analysis, protein expression and purification, in vitro kinase and phosphatase assays, and EMSAs are described in detail in *SI Appendix*. All bacterial strains and plasmids constructed and used in this work are included in *SI Appendix*, Tables S3 and S4, respectively. All use of animals in experimentation has been approved by the institutional animal care and use committee at the University of Texas Southwestern Medical Center.

**Data Availability.** All protocols and data discussed in the paper are available in the main text and *SI Appendix*. All strains and plasmids generated in this report will be made promptly available to readers.

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