JOURNAL OF BACTERIOLOGY, June 2005, p. 3873–3877 Vol. 187, No. 11
0021-9193/05/$08.00+0 doi:10.1128/JB.187.11.3873–3877.2005
Copyright © 2005, American Society for Microbiology. All Rights Reserved.

Osmotic Regulation of the *Escherichia coli bdm* (Biofilm-Dependent Modulation) Gene by the RcsCDB His-Asp Phosphorelay

Anne Francez-Charlot, Marie-Pierre Castanié-Cornet, Claude Gutierrez, and Kaymeuang Cam*

Laboratoire de Microbiologie et de Génétique Moléculaire, Centre National de la Recherche Scientifique, 118 Route de Narbonne, 31062 Toulouse, France

Received 30 September 2004/Accepted 22 February 2005

The RcsCDB His-Asp phosphorelay is shown to positively regulate the *bdm* (biofilm-dependent modulation) and *sra* (stationary-phase-induced ribosome-associated protein) genes in *Escherichia coli*. The regulation is direct and requires an RcsB box next to the *bdm* −35 element. In addition, *bdm* is shown to be activated by osmotic shock in an Rcs-dependent way.

The RcsCDB His-Asp phosphorelay system, initially identified as a positive regulator of the capsular exopolysaccharide (EPS) biosynthesis gene cluster (*wza-wca*) in *Escherichia coli* (8), is conserved in several γ-proteobacteria, including animal and plant pathogens. In *E. coli*, the Rcs system is required for recovery from chlorpromazine-induced stress (3). It is also involved in multidrug resistance (11) and participates in biofilm development (6). In *Salmonella enterica* serovar Typhimurium, it is involved in later stages of infection in mice and in cationic peptide resistance (5). The regulator RcsB is activated upon the transfer of a phosphoryl group from its cognate sensor, RcsC (19), via a *h*istidine-containing *p*hospho*t*ransmitter (Hpt) domain protein, RcsD (K. E. Rudd, http://bmb.med.miami.edu/EcoGene/EcoWeb/; formerly called YojN [21]). Activation of the RcsCDB pathway, usually observed by monitoring the expression of the *wza-wca* gene cluster and the development of a mucoid phenotype, occurs under some environmental conditions, such as dehydration and osmotic shock (15, 18). Curiously, among the well-characterized targets, only the EPS synthesis genes have so far been found to be regulated following osmotic shock through the Rcs system; in addition, this regulation seems to be strain dependent and can be rather modest (4, 21), suggesting that the outcome of the Rcs response to osmolarity is modulated by unidentified factors. Recently, Ferrières and Clarke (6) reported that interaction with a solid surface activates the Rcs pathway, an observation consistent with the involvement of the Rcs system in the development of biofilms.

Targets regulated by the Rcs system are of two types, depending on responsiveness to the RcsB-cofactor RcsA. For the RcsA-independent class of RcsB targets, the site required for RcsB activity (the RcsB box) is located next to the −35 sequence, centered at −41/−42 (1, 4). For the RcsA-dependent class, this site (the RcsAB box) is located either further upstream (23) or downstream from the promoter (7).

Transcriptome analyses indicated that up to 2.5% of the *E. coli* genome might be regulated by the Rcs system (6, 10; our unpublished results). Approximately half of the putative targets have no defined function, suggesting that the Rcs system might be involved in adaptation to environmental conditions not usually encountered in the laboratory. The vast majority of the remaining targets are involved in envelope composition or trafficking.

In the present study, we characterize the regulation by the RcsCDB His-Asp phosphorelay of the *b*iofilm-*d*ependent *m*odulation gene *bdm* as well as the *s*tationary phase-inducible *r*ibosome-*a*ssociated protein gene *sra*. We also show that *bdm* is activated by osmotic shock and that this osmoregulation requires the Rcs system.

RcsB positively regulates the *bdm* gene independently of RcsA. In order to define the Rcs regulon, the transcriptome of an MG1655 derived-strain was analyzed following overproduction of RcsB (data not shown). Among the targets identified in this analysis, the gene *bdm* (*yddX*), encoding a putative 71-amino-acid protein, attracted our attention for several reasons. First, *bdm* is located at 33.5 min in the *E. coli* genetic map, next to the osmoregulated gene *osmC* but oriented opposite to it (Fig. 1). We have previously shown that the *osmC* gene is directly activated by RcsB, and it was therefore of interest to investigate the mechanism of activation of two divergent transcripts by RcsB (4, 20). Second, *bdm* was also identified as a putative target of the Rcs system in the transcriptome analysis of Hagiwara et al. (10) following activation of the phosphorelay at a low temperature in the presence of glucose and Zn^{2+}, but not in that of Ferrières and Clarke (6), in which activation of the Rcs system was followed by overexpression of the membrane protein gene *djlA* (13). Third, *bdm* was shown to be down-regulated in biofilms (16), and the Rcs system was shown to be required for efficient biofilm development (6). In order to characterize the role of the Rcs system in the regulation of *bdm*, several transcriptional fusions between the putative *bdm* promoter region and *lacZ* were constructed and installed in monocopy on the chromosome (17). The activities of these fusions were monitored following the overexpression of RcsB from the pHRcsB plasmid (1). As shown in Fig. 1, a fusion with a region extending from −339 to +127 relative to the transcription start point (determined in this study [Fig. 2]) was activated 19.3-fold upon overexpression of RcsB (λ*bdm1*). Therefore, *bdm* is positively regulated by RcsB. When the fusion λ*bdm1* was tested after overexpression of RcsA from

* Corresponding author. Mailing address: Laboratoire de Microbiologie et de Génétique Moléculaire, Centre National de la Recherche Scientifique, 118 Route de Narbonne, 31062 Toulouse, France. Phone: (33) 561335963. Fax: (33) 561335886. E-mail: cam@ibcg.biotoul.fr.

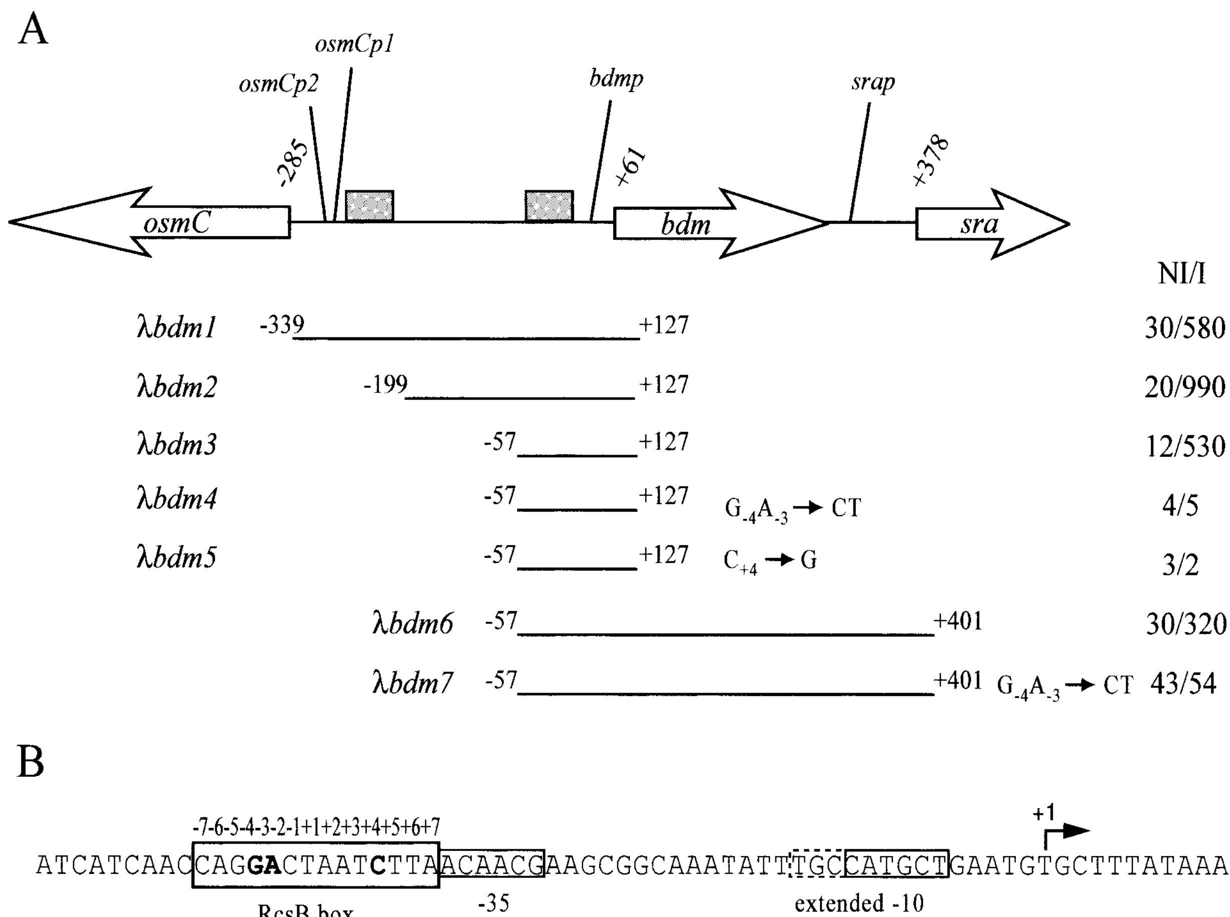

FIG. 1. Identification of the *bdm* RcsB box. (A) The genetic organization of the *osmC-sra* region is presented at the top of the figure. Grey boxes represent the *osmC* and the putative *bdm* RcsB boxes. Coordinates of *osmC*, *bdm*, and *sra* translational start sites are given and refer to the *bdm* transcriptional start site (+1). Below are indicated the coordinates of the fragments used in the different transcriptional fusions with *lacZ* (λ*bdm1* to -7). (B) The relevant sequences are shown. The *bdm* −35 and extended −10 sequences are boxed, the transcriptional start point is indicated (+1), and the three most conserved nucleotides of the RcsB box are in boldface. On the right-hand side of the figure are indicated the β-galactosidase-specific activities of the fusions expressed in Miller units 90 min after the induction (I) or noninduction (NI) of the expression of *rcsB* from the pHRcsB plasmid (1) in MG1655 Δ*lacIZ*(MluI).

the pHRcsA plasmid (4), no effect was observed, indicating that *bdm* is not regulated by the RcsB auxiliary factor RcsA (data not shown).

Identification of the *bdm* transcription start site. To confirm the transcriptional regulation of *bdm* by RcsB and to localize the concerned promoter, mapping of the *bdm* transcription start site was performed by reverse transcription. RcsB production from pHRcsB was induced or left uninduced in cultures of a strain containing the plasmid version of the λ*bdm1* fusion. Total RNA isolated from these cells was used as templates in extension reactions with two different primers. As shown in Fig. 2, both primers identified the same RNA species, which started with a T residue. The transcript was barely visible except in samples in which *rcsB* was overexpressed, in agreement with the positive transcription regulation of the promoter by RcsB. A putative −10 sequence, 5′-CATGCT-3′ with three conserved nucleotides, including the two most conserved at positions 2 (A) and 6 (T), is found 6 nucleotides upstream of the T residue. The likelihood that this is a promoter is strengthened by the presence of a 5′-TGN-3′ motif characteristic of extended −10 promoters (14) at the 5′ end of the −10 hexamer (5′-TGCCATGCT-3′). A poorly conserved −35 element is found 17 nucleotides upstream of the −10 sequence (5′-ACAACG-3′), with only the fourth and fifth nucleotides matching the canonical sequence 5′-TTGACA-3′. However, strong deviations from the consensus of the −35 sequence are not unusual with extended −10 promoters (14) and with activated promoters.

Sequences required for regulation of *bdm* and *osmC* do not overlap. In order to determine the sequence involved in the regulation of *bdm* by RcsB, a 5′-end deletion analysis was performed. As shown in Fig. 1, no sequence beyond −57 is required for the regulation of *bdm* by RcsB, as a deletion removing regions beyond that coordinate left a sequence still sensitive to the overexpression of RcsB (λ*bdm3*). As expected, a deletion removing regions beyond −199 was also sensitive to RcsB (λ*bdm2*). Both deletions in λ*bdm2* and λ*bdm3* fusions remove the RcsB box, which is required for the regulation of *osmC* by RcsB (4), indicating that sequences required for the

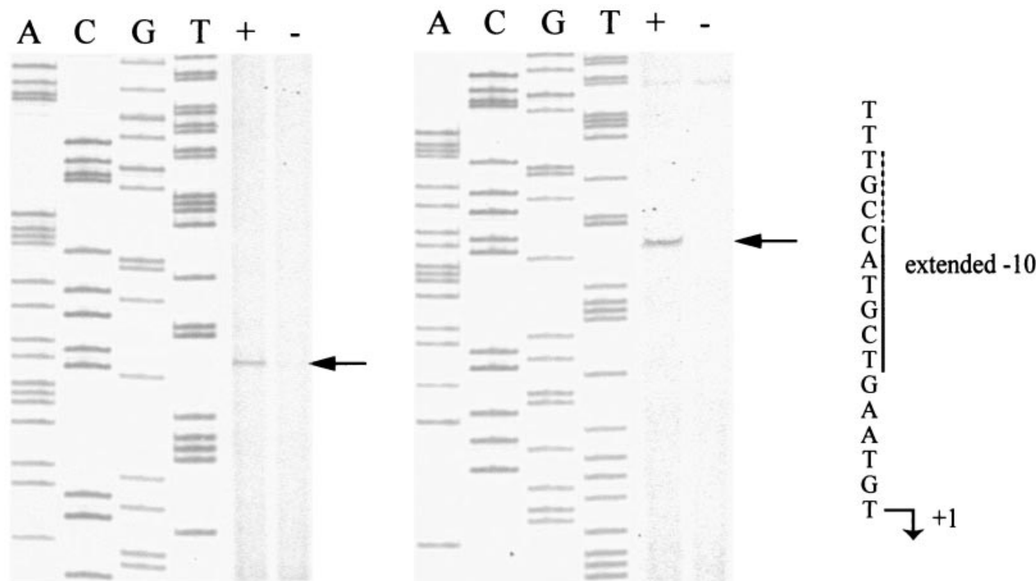

FIG. 2. Determination of the *bdm* transcriptional start point. Reverse transcription was performed with total RNA purified from the MG1555 Δ*lacIZ*(MluI) strain containing the plasmid version of the λ*bdm1* fusion and overproducing (+) or not overproducing (−) RcsB from the pHRcsB plasmid (1). Two different primers were used. The relevant sequences are highlighted.

regulation of *bdm* can function independently of those required for the regulation of *osmC*.

Inspection of the sequence in the vicinity of the promoter revealed a putative RcsB box next to the *bdm* −35 element, notably with the conserved motif GA-5N-C (1, 4, 7), suggesting that *bdm* might be directly regulated by RcsB (Fig. 1 and 3). The relevance of this sequence to RcsB regulation was tested by changing either the conserved dinucleotide GA to CT or the conserved C to a G in the λ*bdm3* fusion. As shown in Fig. 1, regulation by RcsB was lost in both fusions (λ*bdm4* and λ*bdm5*, respectively). Thus, *bdm* regulation by RcsB requires a region containing a characteristic RcsB box upstream of the promoter −35 element.

Identification of the RcsB box in vitro. The identification of the *bdm* RcsB box was confirmed by DNase I protection assays. The experiment was performed with a purified mutant form of RcsB, $RcsB_{D56E}$, in which the conserved Asp residue was replaced by a Glu residue. This mutation makes the protein more active, probably by mimicking the phosphorylated state of the protein (4, 9). A protected region was observed next to the bdm_p −35 element in both the template and the nontemplate strand (Fig. 3). In agreement with the genetic data, the protected region includes the RcsB box. The probe used in the assay also contains the *osmC* RcsB box, and as expected, the profile of the protected region matches that reported in the work of Sturny et al. (20) at the *osmC* nontemplate strand. We note that the protected region for *bdm* was visible at lower concentrations of $RcsB_{D56E}$ than for *osmC*, suggesting that $RcsB_{D56E}$ has a higher affinity for the *bdm* RcsB box than for that of *osmC* (Fig. 3).

The *bdm* and *sra* genes form an operon which is regulated by RcsB. *bdm* is located upstream of *sra*, a gene encoding a ribosome-associated protein whose expression is induced in stationary phase (Fig. 1) (12). A promoter responsible for *sra* induction in the stationary phase was identified in the intergenic region between *bdm* and *sra*. However, deletion analysis indicated that a second region located between positions −455 and −325 from the *sra* ATG initiation codon also contributes to *sra* expression (12). This region corresponds to −77 to +13 relative to the *bdm* transcription start site and therefore encompasses the *bdm* promoter and the *bdm* RcsB box. These observations suggest that *bdm* and *sra* constitute an operon in which *bdm* and *sra* are coregulated by RcsB. In agreement with this hypothesis, a fusion extending from the *bdm* regulatory region to the *sra* 5′ end is activated 12.5-fold when *rcsB* is overexpressed, whereas the same fusion with a mutated RcsB box was completely insensitive to RcsB (Fig. 1, λ*bdm6* and λ*bdm7*, respectively). Both fusions were still activated at the stationary phase, in agreement with the presence in the constructions of the previously reported specific *sra* stationary-phase-inducible promoter (12). This activation by stationary phase was not observed with fusions containing only the *bdm* promoter (data not shown). The conservation of the genetic organization of *bdm*/*sra* in several members of the *Enterobacteriaceae* might suggest that both genes are involved in the same biological function. Unfortunately, no phenotypes that might have indicated the functions of those genes have yet been associated with *bdm* or *sra* mutants (12; our unpublished results).

The osmotic induction of *bdm* requires RcsB. Contradictory results on the effect of changes in osmolarity on the expression of *bdm* have been reported. Using a *bdm::lacZ* fusion, Prigent-Combaret et al. (16) showed that *bdm* was repressed by high osmolarity in either biofilms or liquid medium. In contrast, Weber and Jung (22) reported a Northern analysis showing that *bdm* was activated following an osmotic upshift. A similar result was also obtained in the microarray-based study of Cheung et al. (2). We found that the expression of the λ*bdm3*

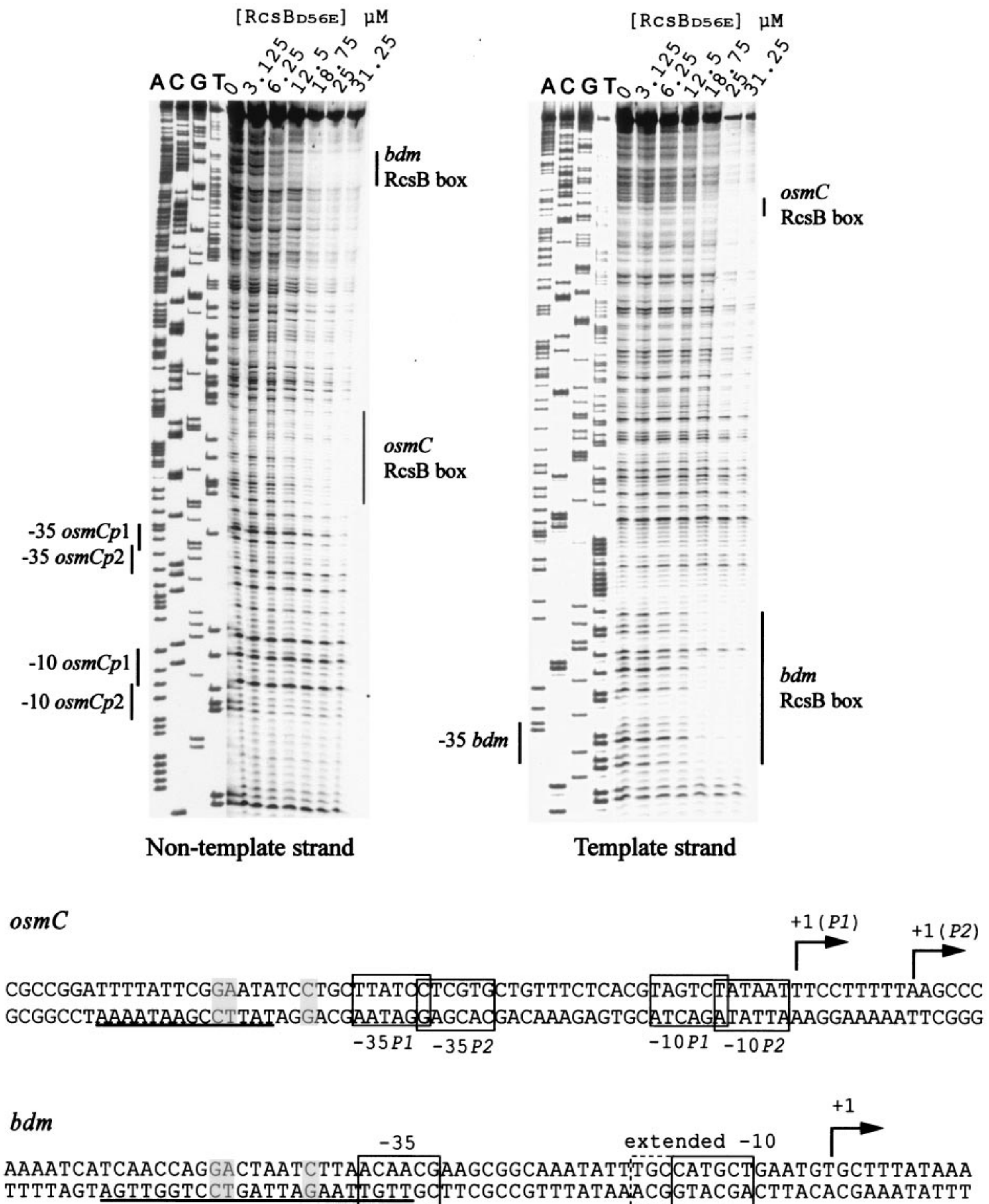

FIG. 3. DNase I protection assay by RcsB at the *bdm* region. Reactions were performed as described before (7). Final concentrations of the constitutive form of RcsB ($RcsB_{D56E}$) are indicated. Vertical lines show the protected regions. The sequences of the *osmC* and *bdm* promoter regions are shown. The three most conserved nucleotides of the RcsB boxes are shaded. The protected regions are underlined.

fusion in minimal medium following an osmotic shock increased 90 min after the addition of NaCl to 0.5 M (Fig. 4). Similar results were obtained with λ*bdm2* and λ*bdm1* fusions (data not shown). Therefore, in minimal medium *bdm* is activated by osmotic shock, in agreement with studies of Weber and Jung (22) and Cheung et al. (2). In addition, the osmotic induction was not observed either with the λ*bdm5* fusion in which the RcsB box was mutated (Fig. 4) or in the *rcsB* mutant

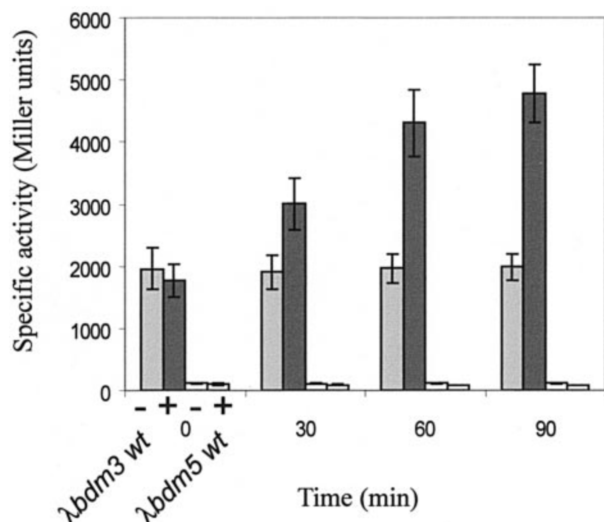

FIG. 4. Osmotic regulation of *bdm* expression by RcsB. Cultures were performed at 37°C in M63 supplemented with glucose and shocked at time zero with 0.5 M NaCl (+) or not shocked (−). Strains are MG1655 Δ*lacIZ*(MluI) containing either λ*bdm3* or λ*bdm5* fusions (Fig. 1).

strain (data not shown). Therefore, the osmotic activation of *bdm* is RcsB dependent. In contrast, with the λ*bdm1* fusion, osmotic induction was still observed in an *rpoS* background, indicating that RcsB induction is not mediated by the stationary-phase sigma factor RpoS (data not shown). No effect of the osmotic shock on *bdm* expression was observed when a Luria-Bertani broth-derived rich medium was used, either at 30°C or 37°C (data not shown). This is the second example, the first being the EPS biosynthesis *wza-wzc* gene cluster, of RcsB targets being up-regulated by osmolarity through the Rcs system (18). However, whereas induced expression of EPS genes was reported to be transient, with a maximum between 30 and 75 min, expression of *bdm* continued to increase beyond 280 min, suggesting that different mechanisms are involved in the RcsB-dependent osmoregulation of the *bdm* and the *wza-wzc* genes. Further studies will be required in order to understand these differences as well as to explain why the other characterized targets of the Rcs system, including *osmC*, are not osmoregulated through the Rcs pathway.

Finally, the Rcs system was shown to be required for efficient biofilm development and to be activated by contact with solid surfaces (6), suggesting that the system is activated in biofilms. This proposal seems to be in contradiction with the observation that *bdm* is down-regulated in biofilms (16). This implies that, in biofilms, *bdm* might be subject to repression by a second regulator.

We are grateful to D. Lane for helpful discussions on the manuscript and to M. Cashel for strain CF6343 [MG1655 Δ*lacIZ*(MluI)].

This work was supported in part by the Université Paul Sabatier and the French Ministère de l'Enseignement Supérieur et de la Recherche (Programme de Recherche Fondamentale en Microbiologie, Maladies Infectieuses et Parasitaires) and by grants from the Institut Universitaire de France and from the Fondation pour la Recherche Médicale to C.G. and A.F.-C., respectively.

REFERENCES

1. **Carballès, F., C. Bertrand, J. P. Bouché, and K. Cam.** 1999. Regulation of *Escherichia coli* cell division genes *ftsA* and *ftsZ* by the two-component system *rcsC-rcsB*. Mol. Microbiol. **34:**442–450.
2. **Cheung, K. J., V. Badarinarayana, D. W. Selinger, D. Janse, and G. M. Church.** 2003. A microarray-based antibiotic screen identifies a regulatory role for supercoiling in the osmotic stress response of *Escherichia coli*. Genome Res. **13:**206–215.
3. **Conter, A., R. Sturny, C. Gutierrez, and K. Cam.** 2002. The RcsCB His-Asp phosphorelay system is essential to overcome chlorpromazine-induced stress in *Escherichia coli*. J. Bacteriol. **184:**2850–2853.
4. **Davalos-Garcia, M., A. Conter, I. Toesca, C. Gutierrez, and K. Cam.** 2001. Regulation of *osmC* gene expression by the two-component system *rcsB-rcsC* in *Escherichia coli*. J. Bacteriol. **183:**5870–5876.
5. **Detweiler, C. S., D. M. Monack, I. E. Brodsky, M. Hanza, and S. Falkow.** 2003. *virK*, *somA* and *rcsC* are important for systemic *Salmonella enterica* serovar Typhimurium infection and cationic peptide resistance. Mol. Microbiol. **48:**385–400.
6. **Ferrières, L., and D. J. Clarke.** 2003. The RcsC sensor kinase is required for normal biofilm formation in *Escherichia coli* K-12 and controls the expression of a regulon in response to growth on a solid surface. Mol. Microbiol. **50:**1665–1682.
7. **Francez-Charlot, A., B. Laugel, A. Van Gemert, N. Dubarry, F. Wiorowski, M. P. Castanie-Cornet, C. Gutierrez, and K. Cam.** 2003. RcsCDB His-Asp phosphorelay system negatively regulates the *flhDC* operon in *Escherichia coli*. Mol. Microbiol. **49:**823–832.
8. **Gottesman, S., P. Trisler, and A. Torres-Cabassa.** 1985. Regulation of capsular polysaccharide synthesis in *Escherichia coli* K-12: characterization of three regulatory genes. J. Bacteriol. **162:**1111–1119.
9. **Gupte, G., C. Woodward, and V. Stout.** 1997. Isolation and characterization of *rcsB* mutations that affect colanic acid capsule synthesis in *Escherichia coli* K-12. J. Bacteriol. **179:**4328–4335.
10. **Hagiwara, D., M. Sugiura, T. Oshima, H. Mori, H. Aiba, T. Yamashino, and T. Mizuno.** 2003. Genome-wide analyses revealing a signaling network of the RcsC-YojN-RcsB phosphorelay system in *Escherichia coli*. J. Bacteriol. **185:** 5735–5746.
11. **Hirakawa, H., K. Nishino, J. Yamada, T. Hirata, and A. Yamaguchi.** 2003. Beta-lactam resistance modulated by the overexpression of response regulators of two-component signal transduction systems in *Escherichia coli*. J. Antimicrob. Chemother. **52:**576–582.
12. **Izutsu, K., C. Wada, Y. Komine, T. Sako, C. Ueguchi, S. Nakura, and A. Wada.** 2001. *Escherichia coli* ribosome-associated protein SRA, whose copy number increases during stationary phase. J. Bacteriol. **183:**2765–2773.
13. **Kelley, W. L., and C. Georgopoulos.** 1997. Positive control of the two-component RcsC/B signal transduction network by DjlA: a member of the DnaJ family of molecular chaperones in *Escherichia coli*. Mol. Microbiol. **25:**913–931.
14. **Mitchell, J. E., D. Zheng, S. J. Busby, and S. D. Minchin.** 2003. Identification and analysis of 'extended −10' promoters in *Escherichia coli*. Nucleic Acids Res. **31:**4689–4695.
15. **Ophir, T., and D. L. Gutnick.** 1994. A role for exopolysaccharides in the protection of microorganisms from desiccation. Appl. Environ. Microbiol. **60:**740–745.
16. **Prigent-Combaret, C., O. Vidal, C. Dorel, and P. Lejeune.** 1999. Abiotic surface sensing and biofilm-dependent regulation of gene expression in *Escherichia coli*. J. Bacteriol. **181:**5993–6002.
17. **Simons, R. W., F. Houman, and N. Kleckner.** 1987. Improved single and multicopy *lac*-based cloning vectors for protein and gene fusions. Gene **53:**85–96.
18. **Sledjeski, D. D., and S. Gottesman.** 1996. Osmotic shock induction of capsule synthesis in *Escherichia coli* K-12. J. Bacteriol. **178:**1204–1206.
19. **Stout, V., and S. Gottesman.** 1990. RcsB and RcsC: a two-component regulator of capsule synthesis in *Escherichia coli*. J. Bacteriol. **172:**659–669.
20. **Sturny, R., K. Cam, C. Gutierrez, and A. Conter.** 2003. NhaR and RcsB independently regulate the *osmCp1* promoter of *Escherichia coli* at overlapping regulatory sites. J. Bacteriol. **185:**4298–4304.
21. **Takeda, S. I., Y. Fujisawa, M. Matsubara, H. Aiba, and T. Mizuno.** 2001. A novel feature of the multistep phosphorelay in *Escherichia coli*: a revised model of the RcsC→YojN→RcsB signalling pathway implicated in capsular synthesis and swarming behaviour. Mol. Microbiol. **40:**440–450.
22. **Weber, A., and K. Jung.** 2002. Profiling early osmostress-dependent gene expression in *Escherichia coli* using DNA macroarrays. J. Bacteriol. **184:** 5502–5507.
23. **Wehland, M., and F. Bernhard.** 2000. The RcsAB box. Characterization of a new operator essential for the regulation of exopolysaccharide biosynthesis in enteric bacteria. J. Biol. Chem. **275:**7013–7020.