JOURNAL OF BACTERIOLOGY, Apr. 1996, p. 1813–1820 Vol. 178, No. 7
0021-9193/96/$04.00+0

In Vitro Binding of the *Salmonella dublin* Virulence Plasmid Regulatory Protein SpvR to the Promoter Regions of *spvA* and *spvR*

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Received 19 September 1995/Accepted 19 January 1996

The *spv* regulon of *Salmonella dublin* is essential for virulence in mice. SpvR, a LysR-type regulator, induces the expression of the *spvABCD* operon and its own expression in the stationary phase of bacterial growth and in macrophages. We constructed fusion proteins to the maltose-binding protein (MBP) and a His tag peptide (His) to overcome the insolubility and to facilitate purification of SpvR. We demonstrated that both fusion proteins, MBP-SpvR and His-SpvR, were able to induce *spvA* expression in vivo. MBP-SpvR was produced as soluble protein, whereas His-SpvR was only marginally present in the soluble cell fraction. Affinity chromatography resulted in at least 95% pure MBP-SpvR protein and in an enrichment of His-SpvR. Gel mobility shift assay revealed that the SpvR fusion proteins were able to bind to 125- and 147-bp DNA fragments of the *spvA* and *spvR* promoter regions, respectively. DNase I footprint experiments showed that the fusion proteins protected DNA regions of 54 and 50 bp within the *spvA* and *spvR* promoter regions, respectively.

The plasmid-encoded *spv* genes of certain *Salmonella* serovars greatly enhance virulence in experimental infections of mice, and strains containing virulence plasmids are strongly associated with extraintestinal disease in domestic animals and humans (14). Studies with mice have shown that the *spv* genes are required neither for the invasion and colonization of Peyer's patches nor for the transfer of bacteria to the spleen or liver, but the *spv* locus is crucial for growth in the liver and spleen (18). The ability to grow inside host cells has been shown to correlate with the virulence phenotype (12, 16).

The *spv* regulon consists of the positive regulator *spvR* and four structural genes, *spvABCD* (14, 15). The *spvABCD* gene products show no significant overall homology to other known proteins and appear to be unique for salmonellae. Nevertheless, a portion of the SpvB protein has sequence similarity to the recently described accessory cholera toxin Ace (43). mRNA analyses indicated that the *spvABCD* genes constitute an operon, and primer extension experiments revealed two closely positioned transcriptional start sites, both upstream of *spvA* (24, 28).

The first gene in the regulon, *spvR*, encodes a protein (33.8 kDa) with homology to the LysR-type transcriptional regulators (25, 33). These regulator proteins show several common features (37, 38). They are of similar size, contain a DNA binding region (helix-turn-helix motif) in the conserved N-terminal domain, and are proposed to require an inducing compound for activation. Such a signal has not been identified for SpvR, and there is at least one protein of this family, Nac, which does not need a coinducer molecule (39). Although most of the LysR-type regulators are divergently transcribed from the operons that they regulate, *spvR* differs in this respect and has the same orientation as *spvABCD*. Recently, primer extension experiments revealed the transcriptional start site 17 nucleotides upstream of the *spvR* start codon (1).

Expression of the *spv* structural genes is induced as bacteria enter the stationary phase or are grown under carbon starvation (7, 9, 24). Deprivation of essential nutrients appears to be the primary stimulus for *spv* synthesis, although other stress factors may have additional roles (44). Also, the expression of *spvABCD* and the synthesis of SpvB are rapidly induced after ingestion of salmonellae by macrophages and epithelial cells (11, 35).

The SpvR protein is essential for expression of the *spvABCD* operon (3, 9, 24). Gel mobility shift assays showed that SpvR present in crude cell extracts binds to the promoter region upstream of *spvA* (23, 28). Mutations in the helix-turn-helix motif of SpvR abolish DNA binding in vitro and *spvA* expression in vivo (23). Expression of *spvR* itself is also positively regulated by its own product but only by a factor of 3- to 10-fold, compared with 100-fold for *spvA* induction (2, 5, 22, 40). In addition, the expression of *spvR* and *spvABCD* is dependent on *rpoS* (2, 5, 10, 22, 32). RpoS is a class II sigma factor (σ^S) that has been identified as a central regulator of a large number of starvation and stationary-phase genes in *Escherichia coli* (19, 26). SpvR acts together with RpoS for maximal transcription of the *spvR* and *spvABCD* genes (5, 22). Moreover, there appears to be a basal level of *spvR* expression in the absence of both *rpoS* and *spvR*, while *spvA* expression is absolutely dependent on a functional SpvR (5). SpvR is the first transcriptional regulator to be described that interacts with RNA polymerase containing σ^S.

In this study, we report the construction and purification of SpvR fusion proteins and show that they function in vivo to induce *spvA* expression. We show that the SpvR fusion proteins are able to bind to the promoter regions of *spvA* and *spvR*, demonstrating the direct involvement of SpvR in *spvA* and *spvR* regulation. Furthermore, we define both the *spvR* and *spvA* promoter binding sites, using DNase I protection assays.

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TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Relevant characteristic(s)[a]	Reference or source
E. coli		
DH5α	*endA1 hsdR17* (r_K^- m_K^+) *supE44 thi-1 recA1 gyrA* (Nal^r) *relA1* Δ(*lacZYA-argR*)*U169*	17
BL21	deoR [ϕ80*dlac*Δ(*lacZ*)M15] *hsdS gal*	41
S. dublin		
Lane	Wild-type virulent strain containing pSDL2	6
LD842	Plasmid-cured Lane strain	6
Plasmids		
pBluescript II-KS(+)	Cloning vector, Pen^r	Stratagene, La Jolla, Calif.
pBS-spvR	*spvR* in pBluescript II-KS(+), downstream of P_{lac}, Pen^r	23
pFF14	*spvRAB′-′lacZ* fusion on pACYC184, Cm^r	9
pFF18	*spvR* with 1.16-kb upstream DNA on pUC19, Pen^r	9
pMal-c2	Vector for constructing *malE* fusions leading to cytoplasmic expression, *lacI*^{q}, P_{tac} *malE′-′lacZ′*α, Pen^r	New England Biolabs
pPG1	267-bp 5′ fragment of *spvR* carrying a *Bsa*I site cloned into pBluescript II-KS(+), Pen^r	This work
pPG2	1.04-kb *Sca*I-*Eco*RI fragment of pFF18 containing the 3′ end of *spvR* cloned into the partial *Sca*I-*Eco*RI-digested pPG1, Pen^r	This work
pPG5	2,081-bp *Eco*RI-*Bam*HI fragment of pFF14 containing *spvA* and the 5′ end of *spvB* cloned into pUC19, Pen^r	This work
pPG6	1,176-bp *Bsa*I-*Xho*I fragment of pPG2 cloned into pMal-c2, resulting in *malE′-′spvR* fusion, Pen^r	This work
pPG9	414-bp *Nru*I-*Sca*I fragment of pFF18 containing the *spvR* promoter cloned into the *Sma*I site of pBluescript II-KS(+), Pen^r	This work
pPG10-1	197-bp fragment containing the *spvR* promoter region cloned into pBluescript II-KS(+), Pen^r	This work
pPG10-2	Same as pPG10-1, but the insert has the other orientation, Pen^r	This work
pPG13	925-bp *Nde*I-*Eco*RI fragment of pBS-spvR cloned into pTrcHisA, resulting in *his′-′spvR*, Pen^r	This work
pPG14-1	317-bp *Eco*RI-*Apa*I fragment of pPG5 containing the promoter region of *spvA* cloned into pBluescript II-KS(+), Pen^r	This work
pPG14-2	Same as pPG14-2, but the insert has the other orientation, Pen^r	This work
pSpvA-Z_{mutR}	*spvRA′-′lacZ* translational fusion on pACYC184, but *spvR* carries a termination linker insertion, Cm^r	24
pTrcHisA	Vector for constructing His fusion proteins, *lacI*^{q}, P_{trc} *his′*, Pen^r	Invitrogen
pUC19	Cloning vector, Pen^r	Pharmacia, Uppsala, Sweden

[a] Pen^r and Cm^r, resistance to penicillin and chloramphenicol, respectively.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. *Salmonella dublin* LD842 is an avirulent strain cured of the virulence plasmid pSDL2 derived from *S. dublin* Lane (6). Plasmid pSpvA-Z_{mutR} carries a translational *spvA′-′lacZ* fusion and a termination linker insertion in *spvR* (24). *E. coli* and *S. dublin* strains were grown in Luria-Bertani (LB) broth medium, and 1.5% Bacto Agar was added for solid media (36). The following antibiotics concentrations were used: penicillin, 200 μg/ml; and chloramphenicol, 20 μg/ml.

DNA manipulations. Recombinant DNA techniques were performed according to established protocols as compiled by Sambrook et al. (36). Vent DNA polymerase for PCR, Klenow enzyme, and restriction endonucleases were purchased from New England Biolabs (Beverly, Mass.) and were used according to the manufacturer's recommendations.

Plasmid constructions. Construction of the *malE-spvR* fusion was based on a protein purification system from New England Biolabs (Beverly, Mass.) and used their plasmid pMal-c2 as the vector. Two hundred fifty-nine base pairs of the 5′ end of *spvR* were amplified from pFF18 by PCR, using oligomers of *spvR* (5′ CGGTCTC[G/A]TGGATTTCTTGATTAATAAAAAA 3′ and 5′ TACCCGT AGGTCCGATTTCCTG 3′). The 5′ oligomer contained 8 nucleotides at the 5′ end to create a *Bsa*I restriction site upstream of the start codon of *spvR*, permitting the in-frame fusion of *spvR* to *malE*. The 267-bp fragment was isolated from a 0.7% agarose gel, filled in with Klenow enzyme, and ligated into *Sma*I-digested pBluescript II-KS(+), resulting in pPG1. To verify the correct amplification, the insert of pPG1 was sequenced. Plasmid pPG1 was partially digested with *Sca*I and completely cut with *Eco*RI, and the 3.04-kb fragment was isolated from a 0.7% agarose gel. This fragment was ligated with the 1.04-kb *Sca*I-*Eco*RI fragment from pFF18 containing the remaining part of *spvR*, yielding pPG2. This plasmid was cleaved with *Bsa*I, Klenow treated, and digested with *Xho*I. The 1,176-bp *spvR*-containing fragment was ligated into *Xmn*I-*Sal*I-digested pMal-c2. The resulting plasmid, pPG6, carries the *malE-spvR* gene fusion under the control of the *tac* promoter. The in-frame fusion between *malE* and *spvR* was confirmed by sequence analysis.

Vector pTrcHisA (Invitrogen, La Jolla, Calif.) was used to construct a plasmid encoding a His-SpvR fusion protein. Plasmid pBS-spvR, containing the *spvR* gene with an engineered *Nde*I site just upstream of the start codon, was cleaved with *Nde*I, Klenow treated, and digested with *Eco*RI (23). The 925-bp DNA fragment was ligated into the *Nhe*I-cleaved, Klenow filled-in, and *Eco*RI-cut pTrcHisA, resulting in plasmid pPG13. The correct fusion was confirmed by sequence analysis. This plasmid harbors the *trc* promoter, which controls the expression of the His-SpvR fusion protein harboring 14 extra amino acids at the N-terminal end of SpvR. This additional peptide sequence contains six consecutive histidine residues (His tag sequence) and has high affinity for Ni^{2+}-charged resin (20).

Plasmids were constructed for use in gel mobility shift or DNase I protection assays performed with either the *spvR* or *spvA* upstream region. Plasmid pFF18 was cleaved with *Nru*I and *Sca*I, and the 414-bp DNA fragment containing the *spvR* upstream region was ligated into *Sma*I-digested pBluescript II-KS(+), resulting in pPG9 (see Fig. 3B). The 2,081-bp *Eco*RI-*Bam*HI fragment of pFF14 was ligated into *Eco*RI-*Bam*HI-cleaved pUC19. The resulting plasmid, pPG5, was digested with *Eco*RI and *Apa*I and T4 DNA polymerase treated, and the 317-bp fragment carrying the *spvA* promoter region was ligated into *Eco*RV-cut pBluescript II-KS(+), generating pPG14-1 and pPG14-2 (see Fig. 3A). A 197-bp DNA region containing the 5′ end of *spvR* and 133 bp of upstream DNA was amplified with the oligomers 5′ TCGCCCATAATCCTATCCAGTAAC 3′ and 5′ TGAAGGAACCTGTTTCCATCAGTG 3′ and cloned into the *Eco*RV site of pBluescript II-KS(+), resulting in plasmids pPG10-1 and pPG10-2 (see Fig. 3B). The difference between plasmids pPG14-1 and pPG14-2 and plasmids pPG10-1 and pPG10-2 is the orientation of the insert.

β-Galactosidase activity. Quantitative determination of β-galactosidase activity was performed as previously described (31). Each experiment was repeated at least three times.

Purification of fusion proteins. One liter of LB medium containing 200 μg of penicillin per ml was inoculated with a 10-ml overnight culture of *E. coli* DH5α(pPG6) and incubated with vigorous aeration at 37°C. When the optical density at 600 nm of the culture reached 1.0 to 1.2, isopropylthiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM. After an additional 2 h of incubation, the cells were centrifuged, washed with 40 ml of Mal buffer (20 mM Tris-HCl, 200 mM NaCl, 10 mM β-mercaptoethanol), and resuspended in 10 ml of Mal buffer containing 1 mM phenylmethylsulfonyl fluoride. Cells were lysed by sonication with a Branson Sonifier 450 on ice-ethanol (four cycles of 20 s, level 8, 50% duty cycle). The lysate was centrifuged in a Sorvall SS34 centrifuge at 27,000 × *g* for 20 min, and the supernatant was applied to a column (1 by 3 cm) containing amylose-conjugated agarose (New England Biolabs) and washed with 10 to 15 volumes of Mal buffer at 4°C. Fusion protein was eluted with Mal buffer supplemented with 10 mM maltose and visualized by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE).

A 40-ml overnight culture of *E. coli* DH5α(pPG13) was used to inoculate 4 liters of LB medium containing 200 μg of penicillin per ml. At an optical density at 600 nm of 1.0 to 1.2, IPTG was added to a final concentration of 0.5 mM, and the cultures were incubated for an additional 2 h. The cells were washed with 50 ml of His-7.8 buffer (50 mM sodium phosphate [pH 7.8], 300 mM NaCl, 5 mM β-mercaptoethanol), resuspended in 25 ml of His-7.8 buffer containing 1 mM phenylmethylsulfonyl fluoride, and lysed by sonication (see above). The resulting extract was centrifuged first in a Sorvall SS34 centrifuge at 27,000 × *g* for 30 min and then in a Beckman 50Ti centrifuge at 134,000 × *g* for 1 h, and the supernatant was applied to a column (1 by 3 cm) containing Ni^{2+}-nitrilotriacetic acid (NTA) agarose (Qiagen, Chatsworth, Calif.). The column was washed first with 25 volumes of His-7.8 buffer, then with 20 volumes of His-6.0 buffer (50 mM sodium phosphate [pH 6.0], 300 mM NaCl, 10% glycerol), and finally with 25 volumes of His-Im20 buffer (50 mM sodium phosphate [pH 7.8], 300 mM NaCl, 20 mM imidazole). Fusion protein was eluted with a 30-ml gradient of 20 to 500 mM imidazole in His-7.8 buffer. Fractions were tested for binding to the *spvA* promoter in gel mobility shift assays. The positive fractions were pooled and concentrated in Centriplus-30 concentrators (Amicon, Beverly, Mass.).

Gel mobility shift assays. For gel mobility shift assays, a protocol modified from that described by Carey (4) was used. The double-stranded target DNA fragments were end labeled with [α-^{32}P]dCTP or [α-^{32}P]dATP, using Klenow fragment. The binding reaction was carried out in a final volume of 20 μl containing the fusion protein, labeled DNA fragments, 1 μg of poly(dI-dC) · poly(dI-dC) DNA, and 1× DNA binding buffer (5 mM Tris-HCl [pH 7.5], 25 mM KCl, 50 mM $CaCl_2$, 1 mM dithiothreitol, 2.5% glycerol, 125 μg of bovine serum albumin [BSA] per ml). After 15 min of incubation at room temperature, the samples were assayed on 5% native polyacrylamide gels which were prerun at 4°C (10 V/cm) in 50 mM Tris-glycine (pH 9.4) containing 1 mM EDTA. During and after loading, the gels were run at 27.5 V/cm until the bromphenol blue dye front had entered the gel and then were run at 10 V/cm for 2 h. Gels were dried for 1 h and autoradiographed. For quantification of the bound and unbound DNA, the autoradiographs were scanned with a densitometer and analyzed with IPLab Gel software version 1.5 (Molecular Dynamics, Sunnyvale, Calif.).

DNase I protection assays. The inserts of pPG14-1 and pPG10-1 or of pPG14-2 and pPG10-2 were used to analyze the protected region on the top or bottom strand, respectively. Plasmids pPG10-1 and pPG10-2 were digested with *Hin*dIII and *Pst*I, and plasmids pPG14-1 and pPG14-2 were digested with *Hin*dIII and *Sac*II. The 219-bp *Hin*dIII-*Pst*I and the 379-bp *Hin*dIII-*Sac*II DNA fragments were gel purified, labeled with [α-^{32}P]dCTP by treatment with Klenow fragment, and used in the DNase I protection assays.

The binding reaction was carried out as described above. After incubation for 15 min at room temperature, the volume was increased to 50 μl with 1× DNA binding buffer and 5 μl of a 10 mM $MgCl_2$–5 mM $CaCl_2$ solution was added. Subsequently, 0.15 to 0.6 U of DNase I was added, and the mixture was incubated for exactly 1 min at room temperature. The reaction was stopped with 140 μl of stop solution (192 mM sodium acetate, 32 mM EDTA, 0.14% SDS, 64 μg of yeast tRNA per ml). The DNA in the reaction mixtures was extracted once with phenol-chloroform (1:1), ethanol precipitated, washed with 70% ethanol, vacuum dried, and redissolved in loading buffer (deionized formamide containing 10 mM EDTA, 0.3% bromphenol blue, and 0.3% xylene cyanol). The samples were incubated for 2 min at 90°C and analyzed on an 8% sequencing gel. After electrophoresis, the gels were dried and subjected to autoradiography. To locate the DNase I footprint, C+T-specific degradation of the DNA fragments was performed (29).

RESULTS

The initial aim of this study was to purify SpvR and examine the interaction of this transcriptional regulatory protein with DNA. Overexpression of *spvR* from the inducible *tac* promoter in *E. coli* DH5α or *S. dublin* resulted in protein which was predominantly aggregated in inclusion bodies. Using several other *E. coli* strains or providing *groESL* of *E. coli* on a plasmid did not increase the solubility of SpvR (data not shown) (8, 34). Hence, we decided to construct SpvR fusion proteins.

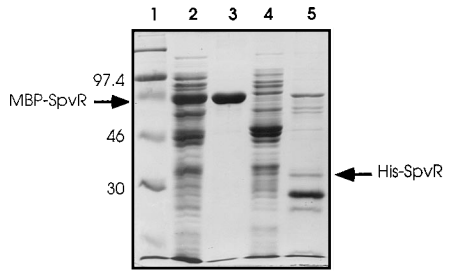

FIG. 1. Coomassie blue-stained SDS-polyacrylamide gel of the purified fusion proteins. Samples include soluble cell fractions of *E. coli* DH5α(pPG6) and DH5α(pPG13) (lanes 2 and 4, respectively), MBP-SpvR fusion protein as eluted from the amylose affinity column (lane 3), protein fraction containing His-SpvR as eluted from the Ni^{2+}-NTA resin column (lane 5), and molecular weight standards (lane 1). The sizes of the molecular mass standards are indicated in kilodaltons.

Purification of SpvR fusion proteins. The vector pMal-c2 was used to fuse *spvR* to the *E. coli malE* gene, which encodes the maltose-binding protein (MBP), resulting in plasmid pPG6. The MBP protein part allows purification of the fusion protein by affinity chromatography. To release a native-size SpvR protein from this fusion, a cleavage site for factor Xa protease is positioned adjacent to the N-terminal end of SpvR. Analysis of extracts of IPTG-induced *E. coli* DH5α(pPG6) cells by SDS-PAGE showed that MBP-SpvR was highly produced and present in the soluble cell fraction. Subsequently, the MBP-SpvR fusion protein was purified by amylose affinity chromatography, yielding about 10 mg of at least 95% pure protein (Fig. 1). After treatment with factor Xa, only a minority fraction of the fusion protein was cut, and even prolonged cleavage with 10 times more factor Xa did not significantly increase the yield of cleaved SpvR protein. Thus, the uncleaved MBP-SpvR fusion protein was used for in vitro studies.

Since the MBP-SpvR fusion protein is not efficiently cleaved, we also constructed a smaller fusion protein by using the vector pTrcHisA (Invitrogen). The resulting plasmid, pPG13, encodes the His-SpvR fusion protein which carries 14 extra amino acids at the N-terminal end of SpvR. This additional peptide sequence contains six consecutive histidine residues and has high affinity for Ni^{2+}-charged resin (20). *E. coli* DH5α(pPG13) and DH5α(pTrcHisA) were used to analyze the overexpression of His-SpvR by SDS-PAGE. It was impossible to identify a fusion protein band in crude extract of DH5α(pPG13) compared with the control strain (data not shown). Thus, the His-SpvR fusion protein, like wild-type SpvR, seems to aggregate mainly in inclusion bodies. Nevertheless, the soluble cell fraction of a 4-liter DH5α(pPG13) culture was loaded onto a Ni^{2+}-NTA column to purify or at least enrich the His-SpvR protein. We used gel mobility shift assays to identify fractions containing DNA binding activity (see below). The active fractions were pooled and concentrated, and analysis by SDS-PAGE showed that a protein band of the predicted size was present (Fig. 1). Western blot (immunoblot) analysis with a polyclonal SpvR antibody confirmed that the pooled fractions contained His-SpvR (data not shown). Several other proteins, some of them at higher concentration than His-SpvR, could be seen. However, the affinity chromatography resulted in an enrichment of the fusion protein (compare lanes 4 and 5 in Fig. 1).

In vivo activity of the SpvR fusion proteins. To determine whether the fusion proteins were able to induce *spvA* expression in vivo, pPG6 and pPG13 were introduced into *S. dublin* LD842(pSpvA-Z_{mutR}) by transformation. Plasmid pSpvA-

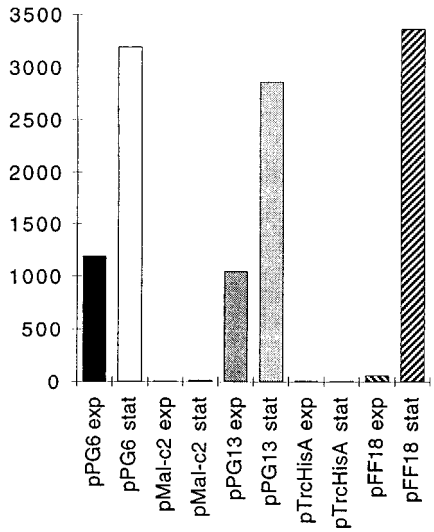

FIG. 2. In vivo activities of MBP-SpvR and His-SpvR. *S. dublin* LD842 (pSpvA-Z_{mutR}) containing either pPG6, pMal-c2, pPG13, pTrcHisA, or pFF18 was used. β-Galactosidase activity was measured from cultures in exponential phase (exp) and in stationary phase (stat). Each experiment was repeated at least three times. The data presented are from a representative experiment.

Z_{mutR} carries a *spvA-lacZ* fusion and a translation termination linker in the *spvR* gene (24). As control, the vectors pMal-c2 and pTrcHisA were also introduced into the same strain. Subsequently, overnight cultures were assayed for β-galactosidase activity. Even in the absence of IPTG induction, pPG6 and pPG13 were able to induce *spvA* expression, whereas the control strains showed only basal activity (Fig. 2). The level of induction was the same as with pFF18, which contains the *spvR* gene with its native upstream region, and addition of IPTG did not further increase the induction (Fig. 2). This result suggested that *spvA* expression was already maximally activated by the low level of fusion proteins in uninduced cells. Furthermore, the induction of *spvA* in *S. dublin* LD842(pSpvA-Z_{mutR}, pPG6) and LD842(pSpvA-Z_{mutR}, pPG13) was still growth phase dependent, although a significant level of expression was observed during exponential phase compared with the strain containing pFF18 (Fig. 2). Hence, both fusion proteins, MBP-SpvR and His-SpvR, are able to induce *spvA* expression in a growth phase-dependent manner and can be used to examine the function of SpvR in vitro.

Binding of the fusion proteins to the *spvA* and *spvR* upstream regions. Earlier gel mobility shift studies showed that the upstream region of *spvA* is retarded by adding crude extract containing overexpressed SpvR protein (23, 28). The gel mobility shift assays to determine the location of the SpvR binding sites were performed with both the MBP-SpvR and His-SpvR proteins, with identical results (Fig. 3).

The 356-bp *Eco*RI-*Xho*I DNA fragment of pPG14-1 (pPG14-1EX) containing the *spvA* promoter region was used with the purified fusion proteins in gel mobility shift assays (Fig. 3A). Both proteins were able to bind to this fragment, confirming the results with crude extract. To further define the essential region for SpvR binding, the labeled pPG14-1XE fragment was digested with different restriction enzymes and used in gel mobility shift assays (Fig. 3A). With *Dde*I-cleaved DNA, only the 266-bp fragment, not the 90-bp fragment, was shifted. Addition of fusion protein to *Pst*I-digested pPG14-1XE resulted in the shift of the 215-bp fragment. After cleavage with *Bst*UI, both fragments were retarded; however, the fusion proteins showed lower affinity to the larger fragment, suggesting that this restriction site lies within the binding site. These results show that the SpvR binding site is located within the 125-bp *Dde*I-*Pst*I region upstream of *spvA*.

Since the *lacZ* fusion experiments indicated that SpvR regulates its own transcription, the 479-bp *Xba*I-*Xho*I fragment of pPG9 (pPG9XX) containing the upstream region of *spvR* was also used in binding studies (Fig. 3B). MBP-SpvR and His-SpvR were able to bind to this DNA region. Digestion with *Dde*I and *Ase*I showed that the binding region was located between the cleavage sites of these restriction enzymes. When pPG10-1XX was cut with *Bst*EII, both fragments were bound by the fusion proteins but with much lower affinity, suggesting that this restriction enzyme site lies within the SpvR binding site. The essential DNA region for SpvR binding was located on the 147-bp DNA fragment (Fig. 3B).

Gel mobility shift assays were also performed to determine the binding affinities of MBP-SpvR to the *spvA* and *spvR* promoter regions. The labeled pPG10-1XX and pPG14-1EX fragments (0.1 nM) were tested over a range of MBP-SpvR concentrations (0.3 nM to 1.5 μM; Fig. 4). The complexes were separated and subjected to autoradiography (Fig. 4A and C), and the fraction of DNA bound or unbound at each MBP-SpvR concentration used was quantitated by densitometric scanning of the autoradiograph. Subsequently, the data were converted to Bjerrum plots in which the fraction of free DNA was plotted versus the log MBP-SpvR concentration (Fig. 4B and D). The apparent dissociation constant (K_d) for MBP-SpvR binding, i.e., the concentration at which 50% saturation of the sites occurs, can be estimated from these plots. Because the concentration of DNA was much lower than the protein concentration in the reaction mixture, the total concentration of MBP-SpvR could be considered as equal to the concentration of free protein. The measured values of K_d for the *spvA* and *spvR* promoter sites were 25 and 55 nM, respectively (Fig. 4B and D). Hence, MBP-SpvR has an approximately twofold-higher affinity for the *spvA* promoter than for the *spvR* promoter. Competition gel mobility shift assays using labeled pPG10-1XX fragment (*spvR* promoter) and increasing amounts of unlabeled competitor DNA comprising either the *spvA* or *spvR* promoter region confirmed the lower K_d for the *spvA* promoter (data not shown). These gel mobility shift assays revealed the presence of two different retarded complexes, showing another characteristic of the fusion protein binding. Hence, it is likely that the more slowly moving complex has an increased number of fusion proteins bound to the DNA fragment, although this has not been determined empirically.

DNase I protection assays. DNase I footprint experiments were performed to establish the precise location of the SpvR binding site within the promoter regions of *spvA* and *spvR*. The *Sac*II-*Hin*dIII fragments of plasmids pPG14-1 and pPG14-2 were used to examine the top and bottom strands of the *spvA* promoter, respectively. Both fusion proteins were used at saturating concentrations (5 μM) to determine the protected DNA region. Comparison of the sequence patterns produced either in the absence or in the presence of MBP-SpvR or His-SpvR demonstrated protected regions of 43 and 54 nucleotides on the top and bottom strands, respectively (Fig. 5A and data not shown). The binding sites extended from positions −71 to −29 on the top strand and from positions −79 to −26 on the bottom strand relative to the first transcriptional start site of *spvA* (Fig. 6A). With each strand, three DNase I-hypersensitive sites were present: at positions −87, −64, and −42 on the top strand and at positions −101, −78, and −55 on the bottom strand. The first hypersensitive sites on each strand were not as obvious when MBP-SpvR was used, revealing the

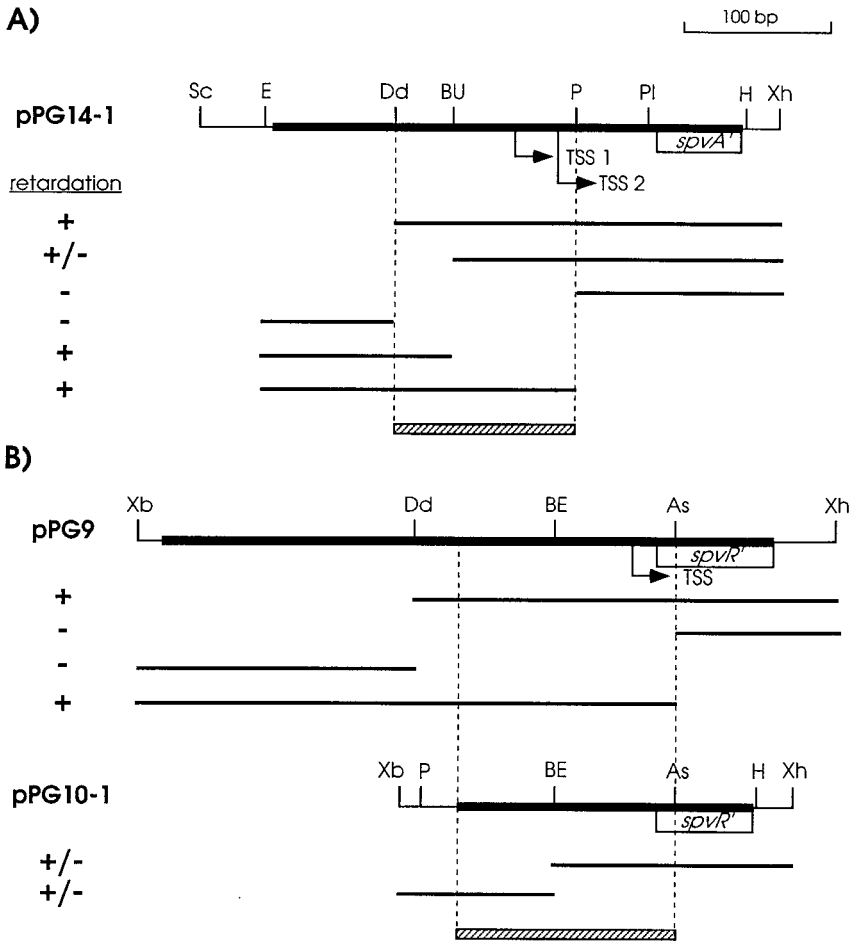

FIG. 3. Summary of gel mobility shift assays with the SpvR fusion proteins. (A) *Eco*RI-*Xho*I DNA fragment of pPG14-1 containing the *spvA* promoter region. (B) *Xba*I-*Xho*I DNA fragment of pPG9 and pPG10-1, both containing the *spvR* promoter region. The DNA was labeled and then digested with the enzyme indicated. The fragments that were produced are indicated by lines. +, +/−, and − indicate strong, weak, and no binding of the fusion protein, respectively. Vector DNA is indicated by thin lines; *spv* DNA is shown as thick lines; open boxes indicate genes; hatched bars show the essential DNA regions for binding of the fusion proteins. Abbreviations: As, *Ase*I; BE, *Bst*EII; BU, *Bst*UI; Dd, *Dde*I; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; Pl, *Ple*I; Sc, *Sac*II; Xb, *Xba*I; Xh, *Xho*I; TSS, transcriptional start site.

only difference between the use of MBP-SpvR and His-SpvR in the footprint assays.

To analyze the *spvR* promoter region, the *Pst*I-*Hin*dIII fragments of plasmids pPG10-1 and pPG10-2 were used. As shown in Fig. 5B for the top strand and summarized in Fig. 6B, distinct regions were protected on both the top and bottom strands. The sizes of these footprints (46 nucleotides on the top strand and 48 nucleotides on the bottom strand) were similar to those obtained with the *spvA* promoter region. The binding site extended from positions −23 to −68 and −25 to −72 on the top and bottom strands, respectively. In contrast to the *spvA* footprint, only two DNase I-hypersensitive sites were present on the top (−42 and −63) or bottom strand (−31 and −51) of the *spvR* upstream region, suggesting that the DNA-protein complexes of the fusion proteins with *spvA* and *spvR* do not have identical structures.

DISCUSSION

SpvR has been shown by *lacZ* fusion studies to be required for the regulation of *spvA* and *spvR*. Furthermore, the homology to the LysR-type transcriptional regulators suggested that SpvR was able to bind directly to the promoter regions of its target genes to induce expression. The experiments described in this report were designed to show the DNA binding ability of SpvR and to identify the exact SpvR binding sites.

Overexpression of native *spvR* resulted in protein aggregated in inclusion bodies. Therefore, we decided to construct SpvR fusion proteins to overcome the insolubility of the native protein and to facilitate the purification. MBP-SpvR was produced in large quantities and as soluble protein, whereas His-SpvR was only marginally present in the soluble cell fraction. Amylose affinity chromatography resulted in at least 95% pure MBP-SpvR protein (Fig. 1). But even after extensive cleavage of the fusion protein with factor Xa, only a portion of MBP-SpvR was cut, suggesting the presence of two different populations of protein. Although the His-SpvR protein was less abundant, the Ni^{2+}-NTA affinity resin was used to purify the fusion protein. The chromatography did not lead to pure His-SpvR but it did enable us to significantly enrich the protein (Fig. 1).

Despite differences in size and in the nature of the fusions, both MBP-SpvR and His-SpvR were indistinguishable from the wild-type protein in the growth phase-dependent induction of *spvA* expression in vivo. Thus, both fusions showed intact biological activity and were suitable to study the SpvR DNA binding characteristics in vitro. MBP-SpvR and His-SpvR be-

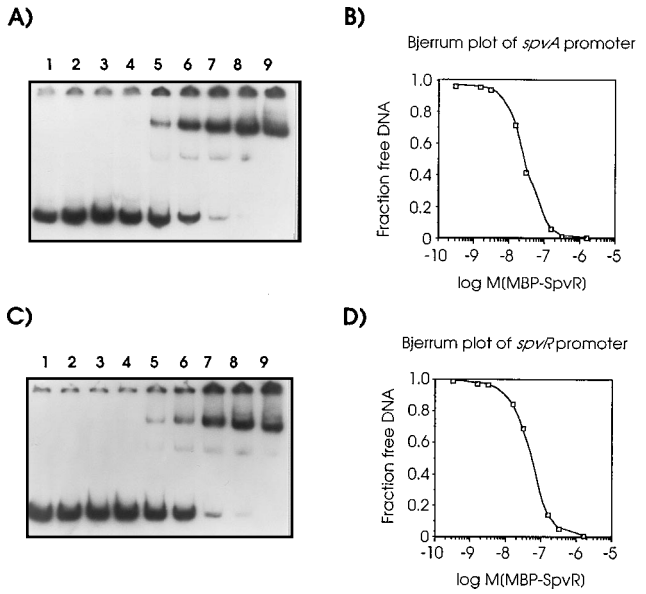

FIG. 4. Comparison of the affinities of MBP-SpvR for the *spvA* and *spvR* promoter regions. Approximately 2 fmol of labeled pPG14-1EX (A) or pPG10-1XX (C) was incubated with increasing amounts of MBP-SpvR as follows: lane 1, no protein; lane 2, 0.3 nM; lane 3, 1.5 nM; lane 4, 3 nM; lane 5, 15 nM; lane 6, 30 nM; lane 7, 150 nM; lane 8, 300 nM; lane 9, 1.5 μM. (B and D) Bjerrum plots (fraction of free DNA versus log MBP-SpvR concentration) of data obtained with pPG14-1EX (B) and pPG10-1XX (D).

haved similarly in gel mobility shift and DNase I protection assays, indicating that the MBP part does not interfere with the DNA binding activity of SpvR (see below).

Gel mobility shift assays demonstrated that the fusion proteins specifically retarded the same *spvA* promoter region that crude extract containing overexpressed native SpvR did (23, 28). Recent studies have indicated a role of SpvR in its own regulation (2, 22, 40). In *S. dublin*, SpvR is not required for its own transcription, but increased levels of SpvR stimulate *spvR* expression by a mechanism that requires RpoS (5). We were able to show that the fusion proteins bind to the upstream region of *spvR*, indicating that SpvR is directly involved in the regulation of its own expression. The essential DNA regions for SpvR binding to *spvA* and *spvR* were identified on 125- and 147-bp fragments, respectively (Fig. 3). Measurement of the apparent K_d and competition gel mobility shift assays revealed that MBP-SpvR has a twofold-higher affinity to the *spvA* promoter than to the *spvR* promoter.

To reveal the precise SpvR binding site, DNase I protection experiments were performed with the labeled top and bottom strands of the *spvA* and *spvR* promoter fragments (Fig. 5 and 6). The protected region of the *spvA* DNA comprised 54 bp and was located between bp −79 and −26. With a size of 50 bp between bp −23 and −72, the footprint of the *spvR* promoter region was similar. The locations of the binding sites are consistent with the recognition sites of other LysR-type regulators, which are usually located between bp −18 and −80 (37). Other LysR-type regulators were shown to protect DNA regions of about 26 to 54 bp and are assumed to be dimers or tetramers in vivo (37). MetR was shown to bind as a dimer, and the resulting footprint was 26 bp in length (27, 30). Therefore, the size of the SpvR footprints favors the presence of two SpvR binding sites in each region. This conclusion is supported by the presence of two retarded complexes in the gel mobility shift assays. Furthermore, the underrepresentation of the faster-moving complex suggests that cooperativity is involved.

On each DNA strand, the hypersensitive sites are separated by approximately 20 to 22 bp (two helix turns), indicating that they are on the same side of the DNA molecule. They may have become hypersensitive for DNase I digestion as a result of bending or distortion of the DNA. Although the protected regions of the *spvA* and *spvR* promoter fragments were similar in size, the patterns of the DNase I-hypersensitive sites differed. The *spvA* footprint showed three sites on each strand, whereas the *spvR* footprint had only two on each strand, suggesting that SpvR binding to the *spvA* or *spvR* DNA fragment results in a different DNA-protein complex. The finding that expression of the *spvABCD* operon in vivo is more dependent on SpvR supports this idea (5).

To identify a possible consensus motif for SpvR binding, the sequences of the two footprints were compared. Many LysR-type-regulated promoters contain the characteristic sequence $TN_{11}A$. This motif is usually part of a larger dyadic sequence (13, 37). A dyadic sequence (TG**T**GCN$_7$GC**A**CA) consistent with this consensus motif is present within the protected region of *spvA*. Its location (−69 to −53) is also similar to that of the typical LysR-type recognition site (37). The *spvR* footprint shows a similar motif (TG**T**GCN$_7$GG**T**CA), although the adenosine of the typical LysR-type consensus is not conserved. Nevertheless, studies of OxyR binding sites showed that its recognition sequences are not as conserved as those found for other LysR-type regulators (42). The binding sites of CysB do not show the typical LysR-type consensus sequence. Analysis of nine CysB binding sites revealed that the CysB tetramer binds to sequences composed of two 19-bp half-sites with dyad symmetry (21). In this respect, comparison of the SpvR protected regions reveals a palindromic sequence (TNTGCANA)

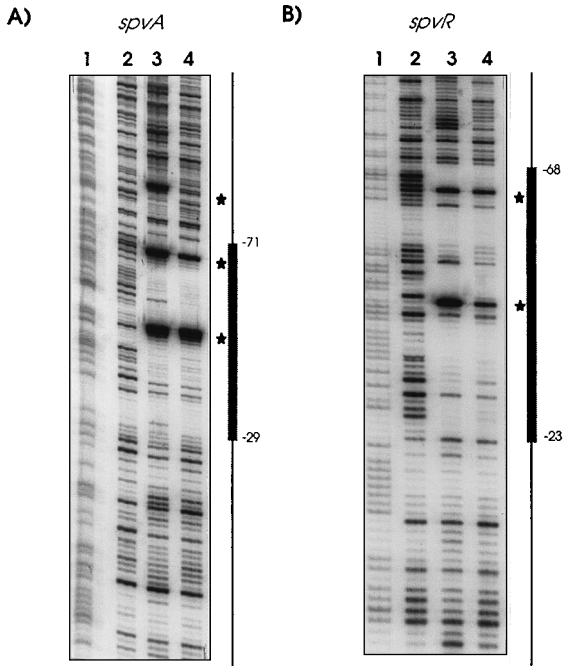

FIG. 5. DNase I footprinting analysis of SpvR fusion protein binding to the *spvA* and *spvR* promoter regions. DNase I protection assays of the top strand of *spvA* (A) and the top strand of *spvR* (B) were carried out as described in Materials and Methods. Lanes: 1, C+T-specific Maxam-Gilbert reaction; 2, DNase I plus BSA (30 μg); 3, DNase I plus His-SpvR protein fraction (52 μg of total protein); 4, DNase I plus MBP-SpvR (7.5 μg, corresponding to 5 μM). The positions of the protected regions are marked with shaded bars and numbers. DNase I-hypersensitive sites are indicated by asterisks.

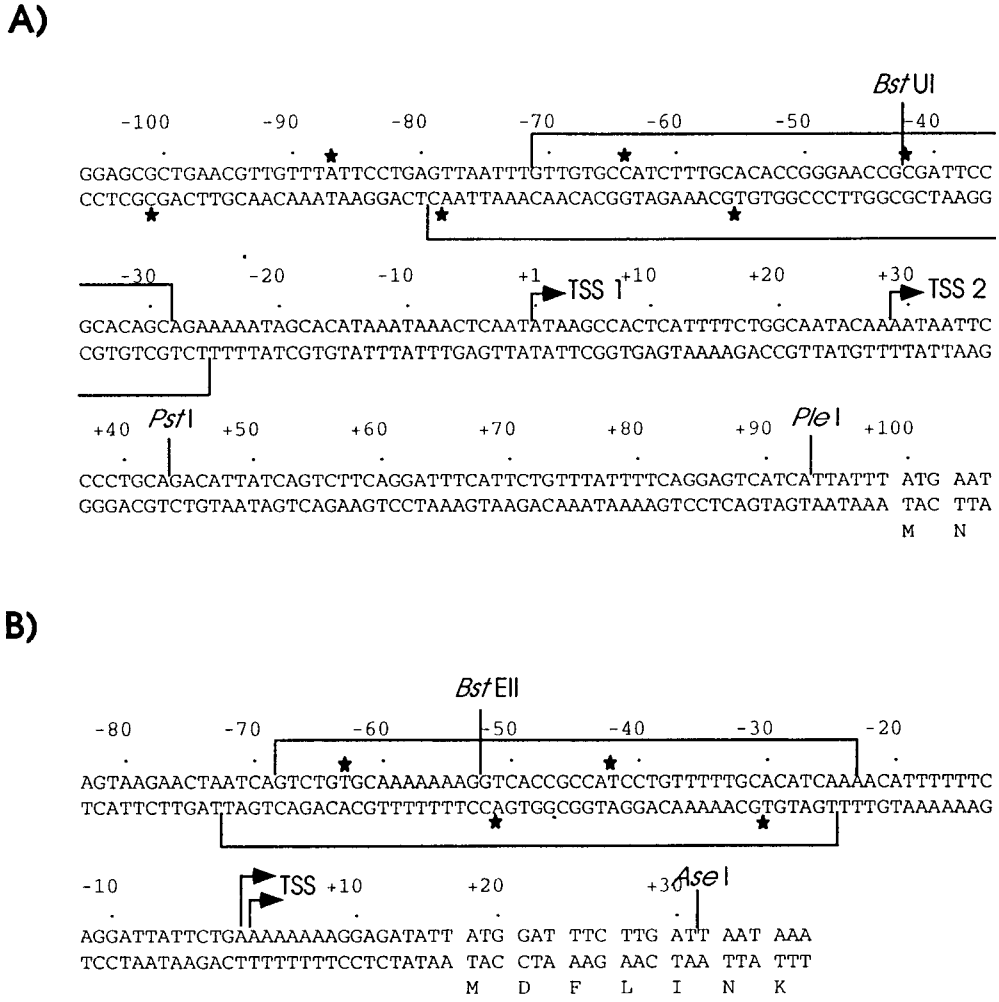

FIG. 6. Summary of DNase I protection analysis of the *spvA* (A) and *spvR* (B) promoter regions. The protected regions on each strand are indicated by brackets, and the DNase I-hypersensitive sites are shown by asterisks. The translational and transcriptional start sites (TSS 1, TSS 2, and TSS) of the *spvA* and *spvR* genes are shown. The important restriction enzyme sites are marked above the nucleotide sequence.

that is present twice on each strand (Fig. 7). In the case of the *spvR* footprint, these motifs are part of a larger point symmetry with the center between bp −45 and −46. Furthermore, the DNase I-hypersensitive sites are also located symmetrically to this mirror point. The *spvA* footprint does not show such a high symmetry. However, additional experiments are required to determine which motif is important for recognition by SpvR.

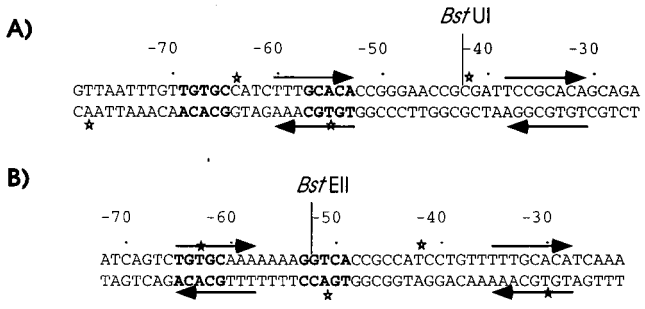

FIG. 7. Comparison of the two SpvR-binding sites of the *spvA* and *spvR* genes. The boldface nucleotides show the dyadic sequences consistent with the putative consensus motif ($TN_{11}A$) of many LysR-type transcriptional regulators. The arrows indicate the conserved palindromic sequences with the consensus motif T N TGCA NA. DNase I-hypersensitive sites are shown by asterisks. The *spvA* and *spvR* sequences are numbered relative to the first transcriptional start site.

The work presented here showed that the constructed SpvR fusion proteins can be used to examine the in vitro DNA binding characteristics of SpvR. The *spv* regulatory system is the first well-characterized instance of induction of virulence genes by the growth phase, and SpvR is the first identified coregulator acting with RpoS. Further investigation of the molecular mechanism of *spv* regulation will enhance the understanding of this type of gene regulation.

ACKNOWLEDGMENTS

We are grateful to M. Krause (University of Zürich) for the kind gift of SpvR antibodies. We thank C. Y. Chen and S. J. Libby for helpful discussion and critical review of the manuscript.

This work was supported by grants from the Swiss National Science Foundation (to P.G.), the National Institute of Health (AI-32178 and DK-35108 to D.G.G.), and the U.S. Department of Agriculture (93-37204 to D.G.G.).

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