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The *Salmonella typhimurium katF* (*rpoS*) Gene: Cloning, Nucleotide Sequence, and Regulation of *spvR* and *spvABCD* Virulence Plasmid Genes

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The *spv* region of *Salmonella* virulence plasmids is essential for the development of a systemic infection in mice. Transcriptional activation of the *spvABCD* operon occurs during stationary growth phase and is mediated by the regulatory gene product SpvR. We have previously shown that expression of a *spvRAB'-cat* fusion in *Escherichia coli* was dependent on the *katF* (*rpoS*) locus which encodes an alternative sigma factor (σ^S). The *katF* gene from *Salmonella typhimurium* has been cloned, sequenced, and used to construct *Salmonella katF* mutants by allelic replacement. Using these mutants, we demonstrated by mRNA and gene fusion analyses that σ^S, in conjunction with SpvR, controls the transcription of the regulatory gene *spvR*. In a second series of experiments, we sought to clarify the relationship between σ^S and SpvR in the control of *spvABCD* transcription. It was shown that expression of a transcriptional *spvAB'-lacZ* fusion could be restored in *E. coli* and *Salmonella katF* mutants when *spvR* was expressed in *trans* from an exogenous promoter. Moreover, identical *spvA* mRNA startpoints were detected in *katF*$^+$ and *katF* strains. These results indicate that the reduction of *spvABCD* transcription in *katF* mutants is mainly due to decreased expression of *spvR*. Finally, mouse inoculation studies with *S. typhimurium katF* mutants of both wild-type and virulence plasmid-cured strains suggest that *katF* contributes to *Salmonella* virulence via the regulation of chromosomal genes in addition to that of *spv* genes.

The *spvRABCD* genes are carried by the virulence plasmids of the most virulent nontyphoidal serovars of *Salmonella* strains and are required for the induction of a systemic disease in mice (11). The precise function of the *spvABCD* genes is still unknown, but these genes may increase the growth rate of *Salmonella* strains in host cells and affect the interaction of *Salmonella* strains with the host immune system (6, 11, 12).

Analysis of the regulation of *spv* gene expression was initiated with the finding that the *spvR*-encoded product was homologous to MetR, a member of the LysR family of positive regulatory proteins (30, 34). Various studies have shown that the *spvABCD* genes form an operon regulated by the SpvR protein (11). Gel mobility shift assays indicated that SpvR specifically bound to a fragment containing the *spvA* promoter(s) (21). In vitro, the expression of the *spvABCD* operon was specifically induced when the bacteria were in stationary growth phase or were cultivated under a number of stress conditions (5, 7, 43). Interestingly, it was recently shown that the expression of the *spv* genes is rapidly induced inside macrophages, epithelial cells, and hepatocytes (10, 31). It remains to be determined whether this induction involves a specific signal that is common to the intracellular compartments of cells that are invaded by *Salmonella* strains or whether this activation is due to the numerous stresses that an intracellular environment is likely to impose on the bacteria. The induction of *spv* gene expression during stationary growth phase led us to investigate the role of *katF* in *spv* gene regulation.

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In *Escherichia coli*, *katF* is involved in the selective expression of a large number of genes in stationary phase and in the resistance of the bacteria to many stress conditions (13, 18, 38). From the nucleotide sequence of the *katF* gene, the gene product is believed to be an RNA polymerase σ factor (24). However, σ activity was only recently detected for the KatF (RpoS, σ^{38}, σ^S) product (26, 42). σ^S is a member of the RpoD-related protein family, and the structural similarity in the DNA binding regions of σ^S may indicate that this protein is functionally similar to σ^{70} (20). Consistent with that notion, in vitro studies revealed that σ^S and σ^{70} proteins have some cross-specificity for promoter recognition (26, 42). However, though the levels of σ^{70} protein were shown to be almost constant throughout the growth phase of *E. coli*, the levels of the σ^S protein were found to increase during the stationary phase of growth (42). This growth phase regulation of σ^S involves both transcriptional and posttranscriptional controls and is consistent with the fact that various σ^S-dependent genes are induced during the stationary phase (18, 19, 22, 25, 35).

We have previously shown that *spvRAB'-cat* and *spvRA'-lacZ* transcriptional fusions are not fully expressed in *E. coli katF* mutants unless the *E. coli katF* gene is provided in *trans* (28). Using an insertional *katF* mutant of *Salmonella typhimurium*, Fang et al. (8) determined that *katF* is required for maximal expression of an *spvRAB'-lacZ* translational fusion and for *Salmonella* virulence. In the present study, we have tried to elucidate the relationship between *katF* and *spvR* in growth phase-dependent regulation of the *spvABCD* genes. Firstly, we cloned and sequenced the *katF* gene of *S. typhimurium*. Secondly, *Salmonella katF* mutants were constructed, and these were used to demonstrate that both σ^S and SpvR are required for the transcription of the regulatory gene *spvR*. Finally, complementation experiments using the *katF* mutants

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype or phenotype[a]	Source or reference
Strains		
S. typhimurium		
C52 and C53	Isogenic derivatives of C5 (only C52 carries the 90-kb virulence plasmid pIP1350 of strain C5)	29
C52K	Derived from C52, *katF*::*kan*	This study
C53K	Derived from C53, *katF*::*kan*	This study
E. coli		
MC1061	*araD139* Δ(*ara-leu*)*-7679 rpsL galU galK* Δ(*lacIPOZY*)*X74*	3
QC1673	*E. coli* K10, HfrC, *relA pit-10 tonA21 thi* Δ*lacIZ*	P. L. Boquet
QC1672	Derived from QC1673, *katF* (*appR190*)	P. L. Boquet
ZK916	W3110 Δ*lacU169* tna-2 λMAV10	2
ZK918	ZK916, *katF*::*kan*	2
MC1061K	MC1061, *katF*::*kan* (P1 transduction of *katF*::*kan* mutation from ZK918)	This study
Plasmids		
pACYC184	Cloning vector, Cm^r Tc^r	4
pACYC177	Cloning vector, Cb^r Km^r	4
pUC19	Cloning vector, Cb^r	44
pVK100	Cloning vector, Tc^r Km^r	16
pUC4K	Source of the *kan* cartridge	Pharmacia
pQF50	Promoter-probe vector (polylinker inserted between 2 *trpA* terminators and a promoterless *lacZ* gene, Cb^r)	9
pSTK1/pSTK2	6-kb *Bgl*II fragment carrying the *katF* gene of *S. typhimurium* C52 in pACYC184	This study
pSTK3	*Bam*HI-*Hin*dIII deletion derivative of pSTK2	This study
pUCK2	2.3-kb *Bam*HI-*Sca*I fragment from pSTK2 in pUC19	This study
pUCK3	3-kb *Sca*I-*Hin*dIII fragment from pSTK2 in pUC19	This study
pUCK3Km	pUCK3, but carries a 1.3-kb *Hin*cII fragment (from pUC4K) containing the *kan* gene, substituted for 0.8-kb *Hpa*I-*Pst*I fragment containing *katF*	This study
pSTK4	2.3-kb *Bam*HI-*Hin*dIII fragment from pUCK2 in pACYC184	This study
pSTK5	2.3-kb *Bam*HI-*Sph*I fragment from pUCK2 in pACYC184	This study
pSTK6	*Hpa*I-*Nru*I deletion derivative of pSTK3	This study
pSTK7	2.3-kb *Bam*HI fragment from pUCK3 in pACYC177 (*Nru*I)	This study
pSTC2/pSTC3	*spvR* in pACYC184	5
pSTC10	*Sca*I deletion derivative of pSTC3	5
pSTC17	1.1-kb *Eco*RI fragment containing *spvR* gene from pSTC19Δ162 in pACYC184 (orientation of *spvR* is identical to that of *cat* gene in which insert was cloned)	This study
pSTC19	1.9-kb *Pst*I fragment carrying *spvR* (from pIP1367) in pUC19 (*Bam*HI and *Kpn*I sites of pUC19 are located upstream of *spvR* promoter region)	This study
pSTC19Δ162	Same as pSTC19, but contains deletion of *spvR* promoter region except 12 bp upstream of ATG start codon (*spvR* upstream region removed by exonuclease III digestion after restriction by *Bam*HI and *Kpn*I)	This study
pSTF4	Transcriptional fusion *spvRAB'-lacZ* in pQF50	5
pSTF5	Transcriptional fusion *spvAB'-lacZ* in pQF50	5
pSTF9	Transcriptional fusion *spvRAB'-cat* in pVK100	28
pSTF12	Transcriptional fusion *spvR'-lacZ* in pQF50 (0.6-kb *Sph*I-*Sca*I fragment of pIP1367)	This study
pSTF13	Transcriptional fusion *spvR-lacZ* in pQF50 (1.4-kb *Cla*I-*spvR* stop codon amplified by PCR from pIP1367)	This study
pIP1367	*spvRABC* genes in pUC19	27
pIP1350	Wild-type 90-kb virulence plasmid of strain C52	29
pDEB2	*katF* gene of *E. coli* in pUC19	2

[a] Cb^r, carbenicillin resistant; Cm^r, chloramphenicol resistant; Km^r, kanamycin resistant; Tc^r, tetracycline resistant.

indicated that σ^S is not indispensable for *spvABCD* transcription when *spvR* is expressed from an exogenous promoter. Murine infection studies suggested that *katF* may contribute to *Salmonella* virulence via the regulation of both chromosomal and *spv* genes.

MATERIALS AND METHODS

Strains, plasmids, and growth conditions. Strains and plasmids used in the study are listed in Table 1. Strains were routinely grown at 37°C in Luria broth medium (LB) (32) supplemented with the appropriate antibiotics. These were carbenicillin at 100 μg/ml, chloramphenicol at 30 μg/ml, tetracycline at 20 μg/ml, and kanamycin at 100 μg/ml.

DNA manipulations. Recombinant DNA techniques and methods for genetic exchange were as previously described (5, 32, 40). PCR amplification with the GeneAmp kit (Perkin-Elmer Cetus) was used to construct the *spvR-lacZ* fusion in pSTF13 (Table 1) and to obtain fragments internal to the *spvA*, *spvR*, and *katF* genes. The amplified fragments were recovered by electroelution and purified with Elutip columns (Schleicher and Schuell). Oligonucleotides were synthesized with an Applied Biosystems model 391 DNA synthesizer. The double-

stranded Nested Deletion kit (Pharmacia) was used to construct exonuclease III deletions in the *spvR* promoter region of pSTC19 (Table 1). The positions of the deletions were determined by DNA sequencing.

DNA sequencing. The nucleotide sequence was determined on both strands by the dideoxynucleotide chain termination method (33). Sequencing of double-stranded DNA was performed with the Sequenase sequencing kit (U.S. Biochemical Corp.) and with universal or specific synthetic primers. Nucleotide sequence data were analyzed with the Genetics Computer Group (University of Wisconsin, Madison) sequence analysis software package (Data General UNIX computer at the Service d'Informatique Scientifique, Institut Pasteur).

RNA manipulations. Total cellular RNA from stationary-phase cultures (optical density at 600 nm of 2 to 3) of *S. typhimurium* and *E. coli* grown at 37°C in LB was extracted by the hot acid-phenol method (1). RNA was incubated at 37°C for 1 h in the presence of DNase I (Pharmacia; 0.3 U/μg) to further eliminate contamination by DNA. For RNA slot blots, samples (1 and 7 μg of RNA) were heated for 5 min at 65°C and spotted onto Immobilon-N membrane (Millipore) using a Bio-Dot microfiltration apparatus (Bio-Rad). Prehybridization and hybridization were performed at 65°C in the hybridization buffer RPN 131 (Amersham).

Determination of the 5′ ends of *spvA* mRNA was performed by primer extension as described below. An oligonucleotide complementary to the *spvA* start codon region (5′-GGTG GTCTGATTCATATTCATAAATAATGATGACTC-3′) was labeled at the 5′ end with [γ-^{32}P]ATP (110 TBq/mmol; Amersham) and T4 polynucleotide kinase (Pharmacia). The ^{32}P-labeled primer (10^6 cpm) was hybridized with 100 μg of total RNA. The cDNA was extended at 42°C for 1 h with avian myeloblastosis virus reverse transcriptase (Boehringer). The length of the cDNA was measured with a DNA sequencing gel (33).

Enzyme assays. Bacterial extracts for chloramphenicol acetyltransferase assays were prepared as previously described (28). The amount of protein was measured by the Coomassie brilliant blue assay (Pierce Chemical Co.). The chloramphenicol acetyltransferase activity was determined by the spectrophotometric method of Shaw (36) and was expressed as the change in A_{412} per minute per milligram of protein. The β-galactosidase activity was measured as described by Miller (23) and was expressed in Miller units (23).

Mouse infection. Female C57BL/6 mice 5 to 6 weeks old were obtained from the Centre d'Elevage R. Janvier (Le Genest Saint Isle, France) and maintained in our animal facilities on a diet of mouse chow and water ad libitum. Techniques for infecting mice by the oral route and methods for the evaluation of bacterial growth in the spleen have been reported previously (29). Prior to infection of the mice, the antigenic formulae of *S. typhimurium* strains were confirmed by slide agglutination using rabbit antisera specific for O- and H-antigen factors (Diagnostics Pasteur) and the plasmids harbored by the strains were verified.

Nucleotide sequence accession number. The sequence data reported in this communication will appear in the EMBL/GenBank/DDBJ nucleotide sequence databases under the accession number X77752.

RESULTS

The *katF* gene of *S. typhimurium*: cloning, nucleotide sequence, and complementation of an *E. coli katF* mutant. Plasmid pDEB2 containing the cloned *E. coli katF* gene (Table 1) was used as a probe against *S. typhimurium* C52 total DNA digested with various restriction endonucleases (data not shown). A 6-kb *Bgl*II fragment from C52 DNA hybridized with the *E. coli katF* gene. To clone the *Bgl*II fragment containing the C52 *katF* sequence, size-fractionated *Bgl*II fragments (between 5 and 10 kb) of C52 total DNA were ligated in the *Bam*HI site of pACYC184. Recombinant plasmids were transformed into the *E. coli* MC1061K strain, which contains a chromosomal deletion removing the 3′ end of *katF* from codon 71 (Table 1). A 0.75-kb fragment containing the 3′ end of the *E. coli katF* gene (from codon 84) was obtained by PCR amplification from plasmid pDEB2 and used as a probe to screen transformants by colony hybridization. Two clones containing the recombinant plasmids pSTK1 and pSTK2, respectively (Fig. 1A), were selected. Both plasmids contained a common 6-kb *Bgl*II fragment cloned in both orientations in pACYC184. Hybridization of the DNA plasmid digests with the probe indicated that the *katF* sequence was localized to a 2.3-kb *Bam*HI-*Sca*I fragment (Fig. 1A).

Complementation experiments using isogenic *E. coli katF* and *katF*$^+$ strains (ZK918 and ZK916, respectively) containing chromosomal *lacZ* transcriptional fusions in the *katF*-regulated *bolA* gene (2, 13) were performed. As shown in Fig. 1A, plasmids pSTK1 and pSTK2 restored wild-type levels of *lacZ* expression in the *katF* strain ZK918. The size of the chromosomal insert containing the *Salmonella katF* gene was further reduced by subcloning the 2.3-kb *Bam*HI-*Sca*I fragment in pACYC184 to generate plasmids pSTK4 and pSTK5, which differed only in the orientation of the insert DNA but which both complemented the *katF* mutation of strain ZK918 (Fig. 1A). The lack of complementation observed with plasmid pSTK6 suggested that the *Hpa*I restriction site was located within the *S. typhimurium katF* gene (Fig. 1A).

Sequence analysis of the *katF* region using plasmid pUCK2 was undertaken (Table 1). Figure 1B indicates the location of a 1,574-bp sequence on pUCK2. Examination of the sequence showed one open reading frame of 990 bp located 7 bp downstream of a potential ribosome-binding site sequence AGGAG (37) (Fig. 1B). This open reading frame encoded a protein of 330 amino acids with a predicted molecular weight of 38 kDa which showed levels of identity of 99 and 95% with the KatF (RpoS and σ^S) proteins of *E. coli* and *Shigella flexneri*, respectively (15, 24, 39, 42). Thus, this protein was henceforth referred to as *S. typhimurium* σ^S. In *E. coli*, a gene encoding a lipoprotein precursor (*nlpD*) was recently identified upstream of *katF* (14). In the *S. typhimurium katF* upstream region, the 3′ end of an open reading frame encoding a product 100% identical to the *E. coli* NlpD carboxy-terminal part was detected (Fig. 1B). DNA sequencing downstream of the *Sca*I site on pUCK2 (Fig. 1B) further confirmed the presence of the *nlpD* gene and indicated that the *Sca*I site was located within the *nlpD* sequence.

Construction of *Salmonella katF* mutants. Plasmid pUCK3Km (Fig. 2A) was used to construct *katF* mutants from both wild-type and virulence plasmid-cured strains of *Salmonella* (C52 and C53, respectively). This plasmid contained a deletion of the 0.8-kb *Hpa*I-*Pst*I fragment within the *katF* gene of *S. typhimurium* replaced by the 1.3-kb *Hin*cII fragment encoding the kanamycin resistance (*kan*) gene from plasmid pUC4K (Table 1). After electroporation in *S. typhimurium*, plasmid pUCK3Km appeared to be unstable. Recombination of the *kan* cartridge into the host genome, with simultaneous loss of plasmid pUCK3Km, resulted in the isolation of clones that were resistant to kanamycin and sensitive to carbenicillin. The Km^r Cb^s clones obtained from the *Salmonella* strains C52(pUCK3Km) and C53(pUCK3Km) were designated C52K and C53K, respectively. To verify that the *kan* cartridge was

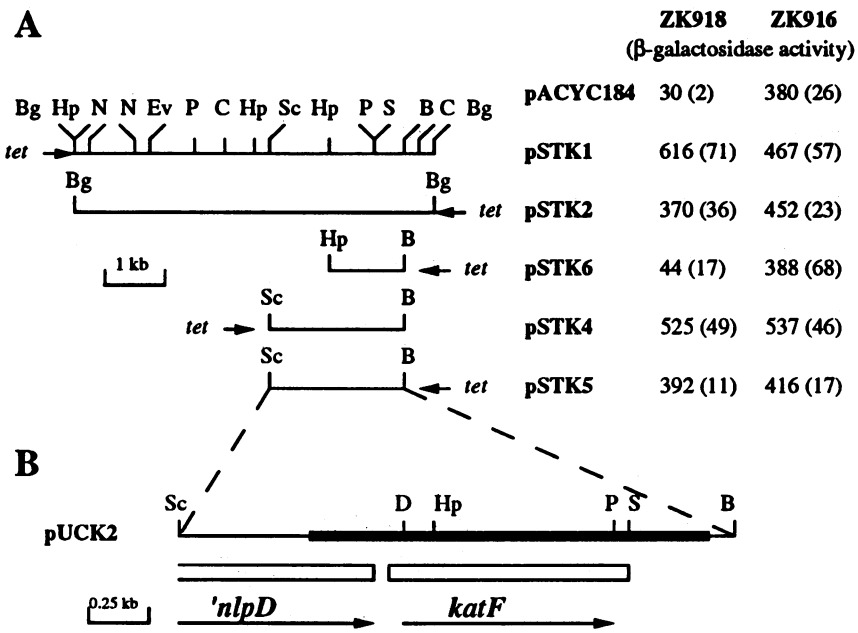


FIG. 1. Cloning and identification of the *katF* gene from *S. typhimurium* C52. (A) Physical map of pSTK1 and derivative plasmids and complementation analysis in *E. coli* ZK918 (*katF*). The plasmids shown are all pACYC184 derivatives. Only insert sequences are shown, and the direction of transcription is indicated for the *tet* gene into which sequences are cloned. β-galactosidase activities expressed by the chromosomal *bolA-lacZ* fusion of the *katF* mutant ZK918 and the parental strain ZK916 harboring the different plasmids and the vector pACYC184 are reported on the righthand side of the figure. β-Galactosidase activities were measured after overnight growth in LB and are expressed in Miller units (23). Values are the averages of at least three independent experiments. The standard error of the mean is indicated in parentheses. (B) Sequence analysis of the *katF* region. The thick line on the restriction map of pUCK2 shows the pUCK2 DNA region that was sequenced on both strands (accession number X77752). The position and orientation of the relevant genes are indicated by open boxes and arrows, respectively, beneath the map. Only part of the *nlpD* gene has been sequenced. The nucleotide sequence of the *katF* gene from *S. typhimurium* 14028s was recently deposited in the GenBank database (Z14965). The deduced amino acid sequence of σ^S from strain 14028s is identical to that of strain C52 except for codon 53. The restriction sites shown are *Bam*HI (B), *Bgl*II (Bg), *Cla*I (C), *Dra*I (D), *Eco*RV (Ev), *Hpa*I (Hp), *Nru*I (N), *Pst*I (P), *Sal*I (S), and *Sca*I (Sc).

inserted at the correct location in *Salmonella* strains C53K and C52K, Southern hybridization experiments in which plasmids pSTK1 and pUC4K were used as probes against total DNA from wild-type and derivative mutant *Salmonella* strains were undertaken. Plasmid pSTK1 revealed a 6-kb *Bgl*II fragment and two *Cla*I fragments of 3.5 and 3.3 kb in C52 DNA (Fig. 2B), whereas no hybridization was detected when pUC4K was used as a probe (data not shown). In the *Salmonella* mutant strain C52K, the size of the fragment carrying the *katF* gene was modified. Since the *kan* cartridge contains a *Cla*I site but no *Bgl*II site (Fig. 2A), the 6-kb *Bgl*II fragment and the 3.3-kb *Cla*I fragment, which contained the *katF* sequence and hybridized with the pSTK1 probe in the wild-type strain DNA, were converted respectively into a 6.5-kb *Bgl*II fragment and two 2-kb *Cla*I fragments in the mutant strain (Fig. 2B). Identical results were obtained for C53 and C53K DNA, respectively (data not shown). As expected, only the 6.5-kb *Bgl*II fragment and the 2-kb *Cla*I fragments were detected in C52K and C53K DNA when pUC4K was used as a probe (data not shown).

Transcription of the *spv* genes in *katF* mutants of *E. coli* and *S. typhimurium*. We have previously shown that the level of expression of an *spvRAB'-cat* transcriptional fusion was much lower in the *E. coli katF* strain ZK918 than in the isogenic *katF*$^+$ strain ZK916 (28). In addition, complementation was observed with the cloned *katF* gene of *E. coli* (28). Fang et al. (8) also observed that a *katF* mutation in *Salmonella* sp. resulted in decreased expression of a cloned translational *spvRAB'-lacZ* fusion. Since the transcription of the *spvABCD* operon is controlled by the SpvR regulatory protein (5, 11), it was of interest to determine whether a *katF* mutation would affect the expression of the *spvR* gene itself.

To compare the transcription levels of *spvR* in *katF* mutants and wild-type strains of *E. coli* and *S. typhimurium*, we used plasmid pSTF9 which carries a transcriptional fusion, *spvRAB'-cat*, cloned into the low-copy-number vector pVK100 (Fig. 3). Total RNAs were isolated from stationary-phase cultures of *Salmonella* and *E. coli* strains containing pSTF9 and hybridized by dot blot with an *spvR* intragenic fragment used as a probe. Plasmid pVK100 and fragments specific to the *spvA*, *katF*, and *tet* genes were used as probes in control experiments. The chloramphenicol acetyltransferase activity expressed from the *spvRAB'-cat* fusion on pSTF9 was also determined. The results are presented in Fig. 4. As expected, transcription of the *katF* gene was detected only in the *katF*$^+$ strains. A decreased transcription of *spvA*, *spvB* (*spvRAB'-cat*), and *spvR* was observed in the *katF* strains of *E. coli* and *Salmonella* compared with that of wild-type strains. This phenomenon appeared to be specific to *spv* genes and did not result from a variation in the copy number of pSTF9, since the mRNAs expressed from pVK100-borne genes (e.g., the *tet* gene) were detected at similar levels in the *katF* mutants and in the wild-type strains. In contrast to the vector pACYC177 (data not shown), plasmid pSTK7 which carries the cloned *S. typhimurium katF* gene was able to restore wild-type transcription levels of *spvR*, *spvA*, and *spvB* in the *katF* mutant C53K (Fig. 4). This confirmed that *katF* was required for maximal *spvR* and *spvABCD* transcription levels. Interestingly, a significant basal level of *spvR* transcription was detected in the *katF Salmonella* mutant.

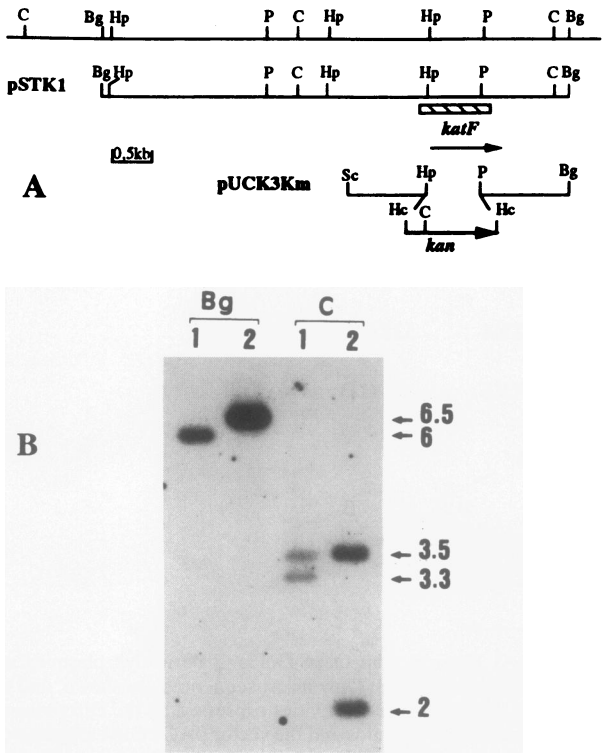


FIG. 2. Construction of *S. typhimurium katF* mutants by gene disruption. (A) Restriction map of the *katF* chromosomal DNA region from *S. typhimurium* C52 and plasmid pSTK1 in which the 6-kb *Bgl*II fragment encompassing *katF* was cloned. The *katF*::*kan* mutation on pUCK3Km was integrated into the chromosome of strains C52 and C53 by homologous recombination to give strains C52K and C53K, respectively (for details, see Results). Restriction sites are indicated as in Fig. 1, with the addition of *Hin*cII (Hc). (B) Southern analysis of *Salmonella katF* strain DNAs with plasmid pSTK1 as a probe. Lanes: 1, C52 DNA; 2, C52K DNA. The chromosomal DNA was digested with *Bgl*II (Bg) and *Cla*I (C). Identical results were obtained with C53 and C53K DNAs. The sizes in kilobases of hybridization bands (arrows) are shown on the right-hand side of the autoradiograph.

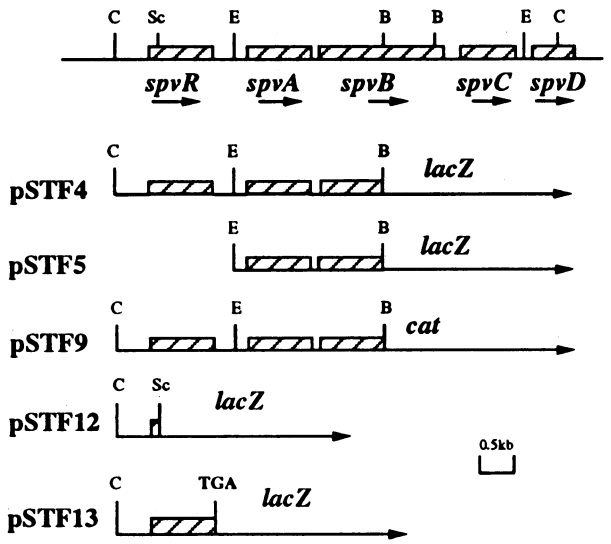


FIG. 3. Transcriptional fusions in the *spv* genes. The top diagram shows the genetic organization of the *spv* virulence gene cluster of plasmid pIP1350 (the presence of an *spvD* gene on pIP1350 is putative). The gene fusions shown below are cloned into the promoter probe vector pQF50 (35 copies per cell; Table 1) except for plasmid pSTF9 (low-copy-number vector pVK100; Table 1). The *spv* genes are represented by boxes, with arrows indicating the direction of transcription. The *lacZ* and *cat* genes are represented by arrows and are not drawn to scale. The restriction sites from the *spv* region presented are *Bam*HI (B), *Cla*I (C), *Eco*RI (E), *Sac*I (S), and *Sca*I (Sc).

Expression of a cloned *spvAB'-lacZ* fusion in *E. coli* and *S. typhimurium katF* mutants when *spvR* is expressed from an exogenous promoter. We have previously shown that the presence of *spvR* induced the transcription of an *spvAB'-lacZ* fusion in *Salmonella* and *E. coli* strains (5). To further investigate the role of *katF* in the transcription of the *spv* genes, the same experiment was performed with *katF* mutants. Whilst plasmids pSTC2 and pSTC3 both encode the *spvR* gene, only the latter induced expression of the *spvAB'-lacZ* fusion in *katF* mutants of *E. coli* and *Salmonella* (Table 2; Fig. 5). As the *spvR* gene in pSTC3 is oriented in such a way that its transcription could be initiated from the *cat* promoter, it is likely that the expression of *spvR* from this promoter was responsible for the enhanced transcription of the *spvAB'-lacZ* fusion in *katF* mutants. In agreement with that conclusion, deletion of the *spvR* promoter region on pSTC3 (plasmid pSTC17; Fig. 5) did not affect the ability of the plasmid to induce high levels of expression of the *spvAB'-lacZ* fusion in the *katF* mutants (Table 2). Moreover, identical *spvA* transcriptional starts sites were detected in the *Salmonella katF*$^+$ and *katF* strains when *spvR* was expressed from the *cat* promoter of pSTC17 (Fig. 6). These results indicated that the decrease of *spvABCD* transcription observed in the *katF* mutants compared with transcription in wild-type strains was mainly due to decreased expression of *spvR*.

SpvR is required for *katF*-dependent transcription of *spvR* in stationary phase. To further confirm the role of *katF* in the

strain	specific mRNAs					CAT activity
	spvR	*spvA*	*katF*	*tet*	pVK100	
C53 (pSTF9)						10.3
C53K (pSTF9)						0.5
C53K (pSTF9, pSTK7)						23.5
ZK916 (pSTF9)						16.9
ZK918 (pSTF9)						< 0.5

FIG. 4. Transcription of *spvA*, *spvB*, and *spvR* in isogenic *katF*$^+$ and *katF* strains of *E. coli* and *Salmonella*. The *E. coli* and *Salmonella* strains were transformed with the plasmids indicated. Determination of chloramphenicol acetyltransferase (CAT) activities (change in A_{412} per minute per milligram) and analysis of specific mRNAs were performed in stationary-phase LB cultures. For mRNA analysis, total RNAs (1 and 7 μg) were blotted onto filters and probed with PCR products specific for the *spvA* (codons 7 to 238), *spvR* (codons 13 to 250), and *katF* (codons 80 to 310) genes from *S. typhimurium*. The *tet* gene (1-kb *Hin*dIII-*Nru*I fragment from pACYC184; Table 1) and the vector pVK100 (Table 1) were used as controls. The autoradiographs were developed after 24 (*spvA* and pVK100) or 48 (*spvR*, *katF*, and *tet*) h of exposure. A representative experiment is shown. Similar results were observed in each of the three experiments performed.

TABLE 2. β-Galactosidase activity of isogenic *katF*$^+$ and *katF* strains of *E. coli* and *S. typhimurium* carrying an *spvAB'-lacZ* fusion in complementation experiments[a]

Plasmid (relevant genes)	β-Galactosidase activity (SEM)[b]			
	E. coli		*S. typhimurium*	
	QC1672 *katF*	QC1673 *katF*$^+$	C53K *katF*	C53 *katF*$^+$
pSTF5 (*spvAB'-lacZ*)	2.6 (0.2)	2.2 (0.4)	7.4 (2.4)	3.2 (1.8)
pSTF5 + pSTC10 (*spvAB'-lacZ* + truncated *spvR*)	2.7 (0.5)	1.7 (0.4)	6.3 (1.5)	7 (1.5)
pSTF5 + pSTC2 (*spvAB'-lacZ* + *spvR*)	12.5 (1.2)	288 (39)	7.9 (2.5)	191 (42)
pSTF5 + pSTC3 (*spvAB'-lacZ* + *spvR*)[c]	328 (61)	374 (29)	310 (60)	708 (83)
pSTF5 + pSTC17 (*spvAB'-lacZ* + *spvR*)[c]	500 (68)	323 (50)	345 (31)	859 (180)
pSTF4 (*spvRAB'-lacZ*)	32 (4)	601 (95)	9.1 (3)	407 (35)
pQF50	<1	<1	<1	<1

[a] β-Galactosidase activities of isogenic *katF*$^+$ and *katF* strains of *E. coli* and *S. typhimurium* containing the indicated plasmids were estimated by the method of Miller (23) after overnight growth in LB. The *S. typhimurium* strains C53 and C53K were cured for the virulence plasmid pIP1350 (Table 1). Genes carried by the recombinant plasmids are indicated. Plasmids pSTF4, pSTC10, and pQF50 were used as controls.

[b] Values in Miller units are the averages of at least three independent experiments.

[c] *spvR* was transcribed from the *cat* promoter of the vector pACYC184; in the case of pSTC17, the *spvR* promoter region was also deleted.

transcriptional control of *spvR*, we examined the expression of an *spvR'-lacZ* transcriptional fusion in the *Salmonella* virulence plasmid-cured strains C53 (*katF*$^+$) and C53K (*katF*). Surprisingly, the *spvR'-lacZ* fusion on pSTF12 (Fig. 3) was poorly transcribed whatever the host strain and even displayed a slightly higher level of expression in the *katF* mutant than in the wild-type strain (Table 3). Taira et al. (41) initially demonstrated that a translational *spvR'-lacZ* fusion was positively regulated by the SpvR product itself. Therefore, we hypothesized that autoregulation might play a role in the *katF*-mediated regulation of *spvR*. Consistent with that notion, plasmids pSTC2, pSTC3, and pSTC17 (Fig. 5) induced a *katF*-dependent expression of the *spvR'-lacZ* fusion in pSTF12 (Table 3). In addition, the *spvR-lacZ* fusion on plasmid pSTF13 (in which the promoterless *lacZ* gene is located immediately downstream of the TGA stop codon of *spvR*; Fig. 3) displayed *katF*-dependent and growth phase-dependent transcription (Table 3; Fig. 7). These results confirmed that *spvR* is transcribed in the stationary phase of growth in a *katF*-dependent manner and further indicated that the *katF*-dependent transcription of *spvR* required the SpvR protein. In the *katF* mutant C53K, a low but significant level of transcription of the *spvR'-lacZ* fusion on pSTF12 was detected (Table 3). This *katF*-independent transcription of *spvR* did not appear to be significantly regulated by SpvR, since expression of *spvR* from the *cat* promoter of pSTC17 enhanced by only twofold the transcription of the *spvR'-lacZ* fusion in the *katF* strain C53K (Table 3). Interestingly, the constitutive (i.e., σ^S- and SpvR-independent) transcription of *spvR* was lower in the

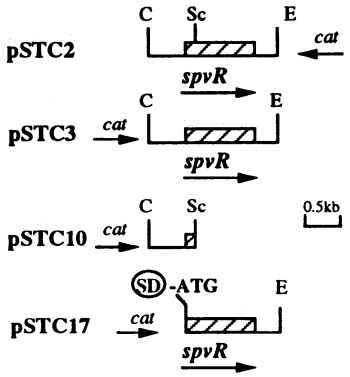
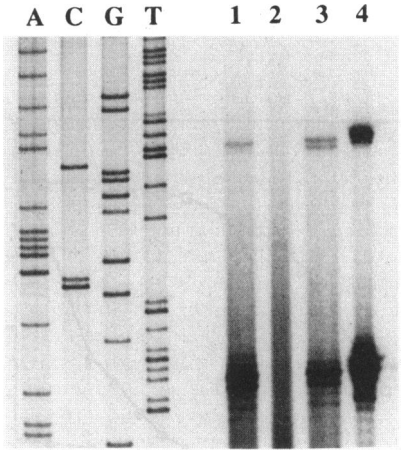


FIG. 5. Physical maps of the recombinant plasmids used for *spvR* complementation in *trans*. Only insert sequences of the pACYC184 derivatives are shown, and the direction of transcription is indicated for the *cat* gene, in which sequences are cloned. The *spvR* gene is represented by a box, with the arrow indicating the direction of transcription. In pSTC17 (Table 1), only 12 bp containing the Shine-Dalgarno sequence (SD) have been conserved upstream of the start codon of *spvR* (ATG). Restriction sites are indicated as in Fig. 3.



-10 * *
5' TAAATAAACTCAATATAAGCCACTCATTTT

-10 * *
CTGGCAATACAAAATA-N_{67}-ATG *spvA* 3'

FIG. 6. Mapping of the 5' end of *spvA* mRNA. A 5'-^{32}P-labeled primer complementary to the *spvA* coding strand was annealed to total RNA isolated from LB stationary-phase cultures of C53(pSTF4) (lane 1), C53K(pSTF4) (lane 2), C53K(pSTF5, pSTC17) (lane 3), and C53(pSTF5, pSTC17) (lane 4). The primer was extended with reverse transcriptase, and the products were submitted to electrophoresis on a sequencing gel. The DNA sequencing ladder (lanes A, C, G, and T) was prepared by using the same primer to sequence pSTC2 template (Table 1). The start sites are indicated by asterisks on the depicted sequence. Putative −10 boxes are underlined.

TABLE 3. Expression of *spvR'-lacZ* and *spvR-lacZ* transcriptional fusions in *Salmonella katF* isogenic strains[a]

Plasmid (relevant genes)	β-Galactosidase activity (SEM)[b]	
	C53K *katF*	C53 *katF*$^+$
pSTF12 (*spvR'-lacZ*)	28.5 (9)	10 (3)
pSTF12 + pSTC10 (*spvR'-lacZ* + truncated *spvR*)	44.5 (13)	9 (4)
pSTF12 + pSTC2 (*spvR'-lacZ* + *spvR*)	49 (14)	136 (40)
pSTF12 + pSTC3 (*spvR'-lacZ* + *spvR*)[c]	56 (8)	228 (32)
pSTF12 + pSTC17 (*spvR'-lacZ* + *spvR*)[c]	71 (16)	313 (91)
pSTF13 (*spvR-lacZ*)[d]	16.1 (6)	134 (54)

[a] β-Galactosidase activities of the *S. typhimurium* virulence plasmid-cured strains C53 (*katF*$^+$) and C53K (*katF*) containing the indicated plasmids were estimated by the method of Miller (23) after overnight growth in LB. Genes carried by the recombinant plasmids are indicated. Plasmid pSTC10 was used as a negative control for complementation experiments.

[b] Values in Miller units are the averages of at least three independent experiments.

[c] *spvR* was transcribed from the *cat* promoter of pACYC184; in the case of pSTC17, the *spvR* promoter region was also deleted.

[d] The promoterless *lacZ* gene was located immediately downstream of the TGA stop codon of *spvR*.

katF$^+$ strain C53 than in the *katF* strain C53K (pSTF12; Table 3).

Virulence of *S. typhimurium katF* mutants in mice. To evaluate the role of *katF* in *Salmonella* virulence, we compared the effects of a *katF* mutation in the wild-type strain C52 (which contains the virulence plasmid pIP1350) and in the isogenic plasmid-cured strain C53. For that purpose, groups of five C57BL/6 mice were infected by the oral route with each of the *katF*$^+$ strains and *katF* mutants. Five days after infection, mice were sacrificed to determine the number of viable bacteria per spleen. All mice infected with strain C52 died between 6 and 8 days postinfection (27). The *katF* mutation considerably affected the virulence of the pIP1350-proficient strain, whereas the mutation had no detectable effect on the virulence of the attenuated plasmid-cured strain (Table 4). In addition, the virulence level of the *katF* mutant C52K was identical to that of the plasmid-cured *katF*$^+$ and *katF* strains (C53 and C53K, respectively). In contrast to the vector pACYC184, plasmids pSTK4 and pSTK5, which carry the *Salmonella katF* gene (Fig. 1), were both able to restore virulence to C52K, confirming that *katF* was indeed required for virulence (Table 4). To further investigate a putative role of *katF* in the control of chromosomal virulence genes, besides the regulation of *spv* genes, we compared the kinetics of infection of strains C53 and C53K. Although both strains displayed similar apparent growth in vivo 5 days postinfection, the *katF* mutant appeared less virulent than the wild-type strain at day 11 and was more rapidly eliminated in the following days (Table 5). As a result, the *katF*$^+$ strain was able to persist in the spleen 3 weeks postinfection, whereas the persistence of the *katF* mutant was strongly affected (Table 5).

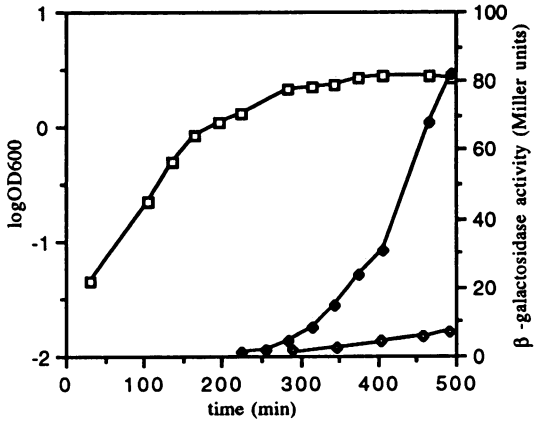


FIG. 7. *spvR-lacZ* expression relative to bacterial cell growth. Exponential-phase cultures (optical density at 600 nm of 0.5) of strains C53(pSTF13) and C53K(pSTF13) were diluted 1:100 into prewarmed LB at 37°C in order to prolong the exponential phase. Aliquots were removed at various time intervals (between 1 and 8 h of growth) and used to measure β-galactosidase activity according to the method of Miller (23). The growth phase was determined by measurement of culture turbidity at an optical density of 600 nm. The measurements were repeated twice, and curves from a representative experiment are shown. The growth curves were similar for both strains. Symbols: open squares, growth curve; closed diamonds, β-galactosidase activity of C53(pSTF13); open diamonds, β-galactosidase activity of C53K(pSTF13).

TABLE 4. Virulence of the *Salmonella* parental strains (C52 and C53) and *katF* mutants (C52K and C53K) and complementation experiments[a]

Strain	Genotype	pIP1350	Plasmid in *trans*	CFU/spleen (SEM)
C52	*katF*$^+$	+	None	6.8 (0.65)
C52K	*katF*	+	None	3.76 (0.23)
C52K	*katF*	+	pACYC184	3.87 (0.3)
C52K	*katF*	+	pSTK4	6.14 (0.13)
C52K	*katF*	+	pSTK5	6.1 (0.42)
C53	*katF*$^+$	−	None	3.9 (0.8)
C53K	*katF*	−	None	3.55 (0.38)

[a] The presence (+) or absence (−) of the 90-kb virulence plasmid pIP1350 is indicated. Groups consisting of five female C57BL/6 mice were orally infected with 10^8 bacteria. Five days postinfection, mice were sacrificed and the number of CFU per spleen was determined. Experiments were repeated at least three times. Results are expressed as means of $\log_{10}$ values. In all cases, the stability of the recombinant plasmid in vivo was checked and the restriction map of plasmid DNA extracted from spleen homogenate cultures was controlled.

DISCUSSION

Recent studies have shown that an *spvRAB'-lacZ* fusion was not fully expressed in *S. typhimurium* and in *E. coli* strains mutated within the *katF* gene, which encodes a sigma factor involved in the selective expression of a large number of genes in stationary phase (8, 28). In this study, we elucidated the relationships between *katF* (*rpoS*) and the regulatory gene *spvR* in the growth phase-dependent regulation of the *spvABCD* operon.

TABLE 5. Kinetics of murine infection with the virulence plasmid-cured strains C53 (*katF*$^+$) and C53K (*katF*)[a]

Strain	Genotype	CFU/spleen (SEM) on postinfection day:			
		5	11	15	21
C53	*katF*$^+$	3.5 (0.23)	4.42 (0.1)	4.2 (0.25)	3.68 (0.27)
C53K	*katF*	3.57 (0.44)	3.27 (0.45)	2.67 (0.67)	1.83 (0.33)

[a] Groups consisting of 20 female C57BL/6 mice were orally infected with 10^8 bacteria. At various times postinfection (5, 11, 15, and 21 days), mice were sacrificed and the number of CFU per spleen was determined. Experiments were repeated at least three times. Results are expressed as means of $\log_{10}$ values.

We have cloned the *katF* gene from *S. typhimurium* C52. The *Salmonella katF* gene was able to complement an *E. coli katF* mutant (Fig. 1). A high degree of conservation of the *katF* gene was then confirmed at the nucleotide sequence level between the coding regions of *katF* in *S. typhimurium*, *E. coli*, and *S. flexneri*. In addition, we have shown that *katF* is located downstream of the *nlpD* gene in *S. typhimurium*, as is the case in *E. coli* (14).

Salmonella strains mutated in the *katF* gene (C52K and C53K) were used to study the role of *katF* in *Salmonella* virulence and in the regulation of *spv* genes. Analysis of expression of *spvA*-specific mRNA and of an *spvRAB'-cat* fusion in the wild-type C53 and mutant C53K strains confirmed the essential role played by *katF* in *spvABCD* transcription (8, 28). Furthermore, a decreased transcription of *spvR* was observed in *katF* mutants of *E. coli* (ZK918) and *S. typhimurium* (C53K) compared with transcription of wild-type strains (Fig. 4). Complementation with the cloned *katF* gene demonstrated that *katF* controlled *spvR* transcription. In contrast with this result, an *spvR'-lacZ* fusion (pSTF12) expressed a very low level of β-galactosidase activity in $katF^+$ and *katF* strains (Table 3). Since it was previously shown that the *spvR* gene was subjected to positive autoregulation (11, 41), we hypothesized that the SpvR protein was required for the *katF*-dependent expression of the *spvR'-lacZ* fusion. Two findings corroborated this hypothesis. (i) Expression of *spvR* in *trans* from the *cat* promoter of pACYC184 (in plasmids pSTC3 and pSTC17) restored *katF*-dependent expression of the *spvR'-lacZ* fusion (Table 3). (ii) Expression of the transcriptional *spvR-lacZ* fusion in pSTF13 (in which the *lacZ* gene is located immediately downstream of the *spvR* stop codon) was dependent on *katF* (Table 3). Therefore, it appeared that σ^S, in conjunction with SpvR, controls the transcription of the regulatory gene *spvR*. Consistent with a *katF* regulation of *spvR*, we found that *spvR* transcription is regulated by the growth phase (Fig. 7). Although we could not exclude the possibility that the control of *katF* on *spvR* transcription was indirect, it is attractive to hypothesize that SpvR could be a coregulator for an RNA polymerase containing σ^S ($E\sigma^S$). Coregulators for $E\sigma^S$ have not yet been identified to our knowledge. Both *spvR* mRNA analysis and the study of the expression of the *spvR'-lacZ* fusion allowed us to detect a low but significant level of σ^S-independent transcription of *spvR* (Fig. 4; Table 3). This basal level of *spvR* transcription (likely to be σ^{70} dependent) may be necessary to provide an amount of SpvR sufficient to initiate the σ^S-dependent transcription of *spvR*. Surprisingly, the level of σ^S-independent expression of the *spvR'-lacZ* fusion on pSTF12 was lower in the $katF^+$ strain C53 than that in the *katF* mutant C53K (Table 3). If one assumes that the control of σ^S on *spvR* is direct, one possible explanation for this is a competition between σ^S and σ^{70}. The binding of $E\sigma^S$ to the *spvR* promoter(s) would be inefficient for transcription in the absence of SpvR (i.e., in the C53 strain containing the *spvR'-lacZ* fusion) and would further prevent the efficient binding of an RNA polymerase containing σ^{70} ($E\sigma^{70}$). In that case, one would expect the transcription of the *spvR'-lacZ* fusion to be more efficient in the absence of σ^S (i.e., in strain C53K). Clearly, in vitro transcription experiments with purified products are warranted to understand the exact molecular mechanisms of *spvR* regulation. The promoter(s) and regulatory sequences involved in *spvR* transcription will need to be further characterized by deletion analysis, primer extension, and gel mobility shift assays.

Since *spvR* transcription is controlled by *katF*, would a constitutive *spvR* transcription be able to restore the expression of the *spvABCD* operon in a *katF* mutant? The induction of *spvAB'-lacZ* expression observed in *katF* mutants of *Salmonella* and *E. coli*, when *spvR* is expressed in *trans* from an exogenous promoter (pSTC3 and pSTC17; Table 2), indicated that this was indeed the case. This suggested that the reduction of *spvABCD* transcription in *katF* mutants is due mainly to decreased expression of *spvR*.

The two *spvA* mRNA startpoints detected in *S. typhimurium* $katF^+$ $spvR^+$ strains (Fig. 6) were similar to those previously identified in *Salmonella dublin* and *Salmonella choleraesuis* (17, 21). Moreover, these two mRNA start sites were also used for *spvA* transcription in the *katF* strain C53K when *spvR* was expressed in *trans* from the *cat* promoter of pSTC17 (Fig. 6). In that case, it is likely that both *spvA* promoters are recognized by $E\sigma^{70}$. The lack of typical −35 elements in the *spvA* promoter regions might explain the requirement for the SpvR protein. It remains to be determined whether the *spvA* promoters may also be recognized by $E\sigma^S$, in addition to $E\sigma^{70}$, when SpvR is provided.

We observed during mRNA analysis that the constitutive level of *spvR* transcription that was detected in the *Salmonella katF* mutant C53K did not significantly induce transcription of *spvA* and *spvB* (Fig. 4). A similar observation was made with gene fusion analysis: the constitutive *spvR* transcription that occurred on pSTC2 and pSTF4 did not induce transcription of *spvB* in the *katF* mutant C53K (Table 2). Interestingly, this phenomenon appears to be specific to *S. typhimurium*. Indeed, although the level of *spvR* mRNA detected in *katF* strains appeared lower in *E. coli* than that in *S. typhimurium* (Fig. 4), the σ^S-independent transcription of *spvR* on pSTC2 and pSTF4 was able to induce a low but significant level of transcription of *spvB* in the *katF* mutant of *E. coli* but not in that of *S. typhimurium* (Table 2). One explanation for this might be the titration of SpvR in *S. typhimurium* by SpvR-regulated promoters. On the other hand, posttranscriptional regulation of *spvR* could be involved.

Gulig and Doyle showed that the effect of *spv* in vivo was related to the promotion of growth rather than to the prevention of killing (12). The growth advantage provided by the *spv* genes to *Salmonella* strains in vivo seems to be sufficient to convert a self-limited infection into a lethal infection. Since *katF* controls the expression of the *spv* genes, it was not surprising to find that a *katF* mutation considerably affected the virulence of *S. typhimurium* (Table 4). It has been previously shown that the oral 50% lethal dose of a *Salmonella* mutant containing the insertion of a suicide plasmid in *katF* was 1,000-fold higher than for the wild-type strain (8). No complementation experiments were reported. We further confirmed the role of *katF* in *Salmonella* virulence by complementation experiments with the cloned *katF* gene (Table 4). Moreover, to explore the possibility that *katF* may regulate chromosomal virulence genes, besides the *spv* genes, we compared the kinetics of infection of the plasmid-cured and derivative *katF* mutant *Salmonella* strains (C53 and C53K, respectively). Although the *katF* mutation had no detectable effect on the growth of strain C53 5 days postinfection, it strongly affected the persistence of the virulence plasmid-cured strain in mice 3 weeks postinfection (Table 5). This suggests that chromosomal genes involved in *Salmonella* virulence may be regulated by the *katF* gene.

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