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Plasmid Virulence Gene Expression Induced by Short-Chain Fatty Acids in *Salmonella dublin*: Identification of *rpoS*-Dependent and *rpoS*-Independent Mechanisms

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The *Salmonella* plasmid virulence *spvABCD* genes are growth phase regulated and require RpoS for maximal expression in stationary phase. We identified a growth phase-independent expression of *spv* which is mediated by short-chain fatty acids. During this fatty acid-mediated expression of *spv*, RpoS is required for induction only during exponential phase. In stationary phase, an *rpoS*-independent mechanism is responsible for expression of *spv*.

The large virulence plasmids of nontyphoid *Salmonella* share a highly conserved region containing the *spvABCD* operon, which is responsible for expression of the plasmid-mediated virulence function (12, 13). In nutrient-rich cultures, the *spvABCD* genes are not induced in exponential growth but are turned on when cells enter stationary phase (6, 8). In stationary growth, two regulatory factors are involved in *spvABCD* expression. One is RpoS, a σ subunit of RNA polymerase that governs expression of various stationary phase-induced genes. In *Salmonella rpoS* mutants, expression of the *spv* operon is substantially reduced (9, 14, 15, 20). RpoS is responsible for induction of the second regulator, SpvR, which is encoded by the virulence plasmid (4, 15). SpvR induces *spvABCD* expression and also positively regulates its own expression (1, 3, 4, 15, 26). It belongs to the LysR-MetR-type family of transcriptional activator proteins and binds specifically to the promoter regions of *spvA* and *spvR* (8, 11, 16–18).

Although the intracellular host cell environment has been shown to induce *spv* gene expression (10, 23), the specific molecular signals that are responsible for this induction are still unknown. Glucose starvation has been found to serve as a signal for *spv* induction in *Salmonella dublin* (8); however, other factors, including pH and iron limitation, may also play roles (27). We have recently observed that acetic acid and other short-chain fatty acids (SCFA) induce substantial *spv* expression, suggesting that accumulation of metabolic products might be involved as well (7). In this study, we analyzed SCFA-mediated *spv* expression in *S. dublin* and investigated the roles of RpoS and SpvR in this expression.

Effect of SCFA on *spvABCD* expression. The effects of various concentrations of a series of SCFA on the expression of the *spvABCD* operon were determined. We monitored this expression by measuring β-galactosidase activities of pFF14, a pACYC184 derivative containing a *spvRAB*::*lacZ* fusion which was transformed into *S. dublin* LD842, a plasmid-cured derivative of wild-type *S. dublin* Lane (5, 8, 19). The sodium salts of the fatty acids C_1 to C_6, C_8, and C_{10} were added to early-log-phase cultures of LD842(pFF14) growing in M9 medium (8), and β-galactosidase activities were determined after 1 h. The pHs did not differ significantly among the media (Table 1). Significant dose-dependent *spvB* expression was induced by C_3, C_4, C_5, and C_6, with highest levels being induced by C_5 (valerate), and a striking cutoff between C_6 and C_8, implying a structure-function relationship with an optimum at C_5 (Fig. 1). To investigate the SCFA effect during the growth cycle, *spvB* expression of LD842(pFF14) was monitored in valerate (C_5)-rich Luria-Bertani broth (LB) cultures until cells reached stationary growth (Fig. 2). The same strain growing in LB served as a control (Fig. 2). After an approximately 40-fold induction within 1 h of exposure to valerate, *spvB* expression continued to rise parallel to the stationary phase-induced rise in expression in the control. Throughout the growth cycle, *spvB* expression of LD842(pFF14) remained higher than in the control; however, in stationary cultures, expression was at the most only twofold as high, suggesting that the most prominent effect of SCFA on *spv* expression occurs during log-phase growth. We noted that the addition of SCFA to the culture media slowed growth, raising the question of whether growth inhibition itself was responsible for induction of *spvB* (Fig. 2). However, we did not find an association between the increase in generation time and the induction of *spvB* (Table 1). C_8 and C_{10} definitely lacked activity at 10 mM, even though they produced significant growth inhibition, and shorter-chain compounds barely slowed growth at 5 and 10 mM, although they led to significant *spvB* induction.

The role of RpoS in SCFA-mediated *spv* expression depends on the growth phase. The simplest way to explain the SCFA effect is through activation of RpoS. In *Escherichia coli*, SCFA have been found to induce *rpoS*, although the effect is weak (25). To investigate this possibility, we determined levels of *spvB* expression in an *rpoS* mutant of LD842, *S. dublin* CC1003(pFF14) (4), during growth in valerate-rich LB cultures (Fig. 2). No induction was detectable during exponential growth, indicating that, in this phase, SCFA-induced *spvABCD* expression is mediated by RpoS. When CC1003(pFF14) entered stationary growth, however, a strong induction was observed, resulting in *spvB* expression that was almost as high as that seen in LD842(pFF14) growing in LB (Fig. 2). These findings suggested that SCFA-induced *spv* transcription requires RpoS only during early exponential growth, whereas in the postexponential phase, another sigma factor is responsible. To corroborate this conclusion, we complemented *rpoS* in

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TABLE 1. Generation time determined by CFU after 1 h of exposure to SCFA

SCFA	Concn(s) of SCFA (mM)	pH of medium	No. of partitions/h	Level(s) of SpvB induction[a]
NaCl	50	7.58	2.4	68
NaCl-Brij 35	50-0.4[c]	7.48	2.3	53
C_1	50	7.62	2.2	70
C_2	50	7.60	2.2	934
C_3	50	7.58	2.0	2,740
C_4	50	7.60	2.2	2,624
C_5	50	7.59	1.8	3,234
C_6	5		2.1	730
C_6	10		1.9	915
C_6	50	7.65	1.6	2,305
C_8	5		2.0	47
C_8	10		1.8	79
C_8	50	7.61	—[b]	80
C_{10}	5		2.1	50
C_{10}	10		1.0	24
C_{10}	50	7.57	—[b]	62

[a] Values are expressed in Miller units (taken from Fig. 1).
[b] —, at 50 mM, C_8 and C_{10} were lethal to the cells.
[c] 0.4% Brij 35.

CC1003(pFF14) at different time points during growth in valerate-rich LB. We hypothesized that complementation of RpoS in exponential phase would increase SpvB expression, whereas complementation in later phases would not. To obtain an inducible *rpoS* expression vector, we cloned the amplified *rpoS* gene from *S. dublin* Lane into pTrc99A behind the *trc* promoter, resulting in pAM2 (Fig. 3). In this vector, the *trc* promoter is inactivated due to the presence of *lacI*^{q} and derepressed by IPTG (isopropyl-β-D-thiogalactopyranoside) (2). pAM2 was introduced into CC1003(pFF14), and CC1003(pFF14+pAM2) was grown in valerate-enriched medium. When *rpoS* was induced by IPTG in early logarithmic growth (Fig. 4), *spvB* expression increased 10- to 20-fold over the next 1 to 2 h and remained stable thereafter. In contrast, when *rpoS* was induced when the cells entered stationary growth (optical density at 600 nm [OD_{600}], 1.0) (Fig. 4), no

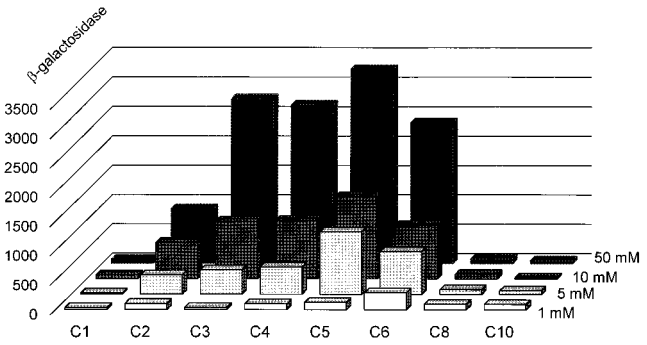

FIG. 1. Expression of *spvB*::*lacZ* in response to various fatty acids at different concentrations. The cells were grown to an OD_{600} of approximately 0.1 in M9 medium, at which point the cultures were supplemented with fatty acid sodium salts (C_1 [formate], C_2 [acetate], C_3 [propionate], C_4 [butyrate], C_5 [valerate], C_6 [caproate], C_8 [caprylate], and C_{10} [caprate]). The cultures supplemented with C_6, C_8, and C_{10} contained 0.4% Brij 35 for solubilization (24). The final concentrations tested were 1 mM (open bars), 5 mM (lightly shaded bars), 10 mM (darkly shaded bars), and 50 mM (filled bars). The cells were harvested after 1 h for measurement of β-galactosidase. M9 medium with 50 mM NaCl and M9 medium with 0.4% Brij 35 served as controls, giving β-galactosidase levels of 82 and 94 Miller units, respectively.

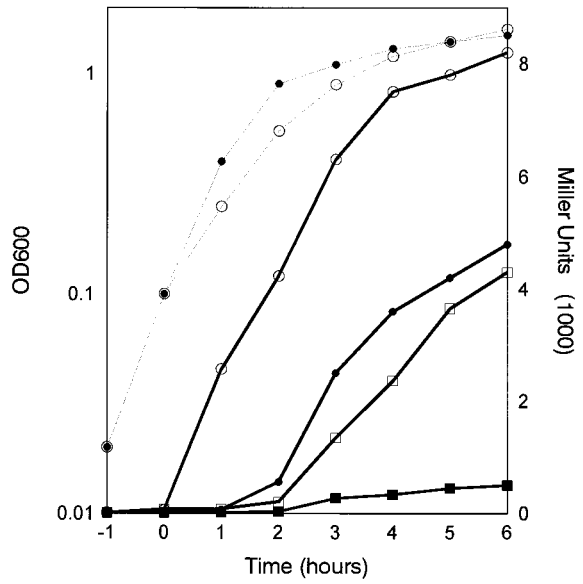

FIG. 2. Effect of valerate on *spvB* expression during growth in *S. dublin* LD842 and in *rpoS* mutant *S. dublin* CC1003. Symbols: filled circles, LD842(pFF14) in LB supplemented with 50 mM sodium chloride; open circles, LD842(pFF14) in LB supplemented with 50 mM sodium valerate; filled squares, CC1003(pFF14) in LB supplemented with 50 mM sodium chloride; open squares, CC1003(pFF14) in LB supplemented with 50 mM sodium valerate; solid lines, β-galactosidase levels; dotted lines, growth curves. The cultures were supplemented with sodium valerate or sodium chloride at time zero. The growth curves of the two strains growing in LB and of the two strains growing in LB-valerate were similar; thus, only two OD_{600} curves are shown.

effect was observed, suggesting that once *rpoS*-independent *spv* transcription was initiated by valerate, RpoS no longer interfered with this expression. In LB cultures, IPTG induction of *rpoS* in CC1003(pFF14+pAM2) at an OD_{600} of 1.0 resulted in *spvB* expression levels that were comparable to those in LD842(pFF14), ensuring that functional RpoS was produced (data not shown). Furthermore, we constructed an *rpoS*::*lacZ* fusion plasmid, pAM2-Z, and introduced it into CC1003 (Fig. 3). During growth in valerate-supplemented cultures, this plasmid provided high β-galactosidase levels (>10,000 Miller units) after IPTG induction, confirming that *rpoS* expression from the *trc* promoter was not abolished by valerate in the culture media.

It was remarkable that *spvB* expression remained constant after RpoS was induced during early growth (Fig. 4). A similar effect was also observed in the LB control medium (data not shown), indicating that this effect was not restricted to valerate-rich media but was rather a consequence of unphysiological RpoS overproduction during early growth.

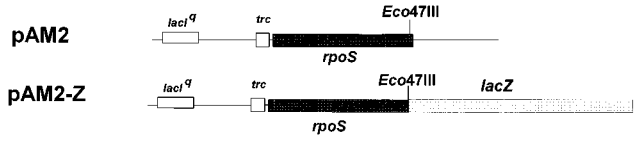

FIG. 3. Construction of an *rpoS* overexpression vector and an *rpoS*::*lacZ* fusion. The *rpoS* gene was amplified from genomic *S. dublin* Lane DNA by PCR with the oligomers 5′AAGGGATCCCGGGTAGGAGC3′ and 5′ATCCCGCC GCTGCTGGCAGC3′ as primers. The resulting 990-bp *rpoS* gene was sequenced and cloned behind the *trc* promoter of pTrc99A, resulting in pAM2. Only 21 bp lie between the *trc* promoter and *rpoS*. pAM2-Z, a *lacZ* fusion at the *Eco*47III site (nucleotide +974 of the *rpoS* gene) was used to confirm IPTG-induced overexpression of *rpoS*.

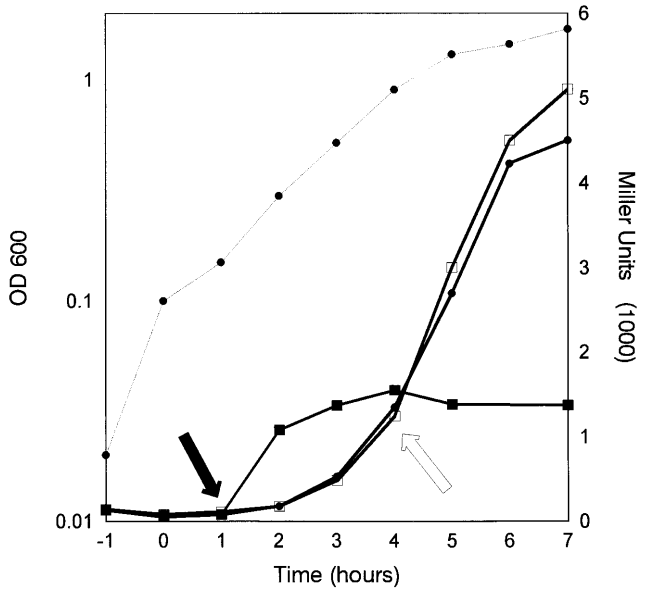

FIG. 4. Influence of RpoS overproduction on *spvB*::*lacZ* expression during growth in valerate-supplemented medium. Symbols: filled circles, CC1003(pFF14+ pAM2) in LB-valerate medium; filled squares, CC1003(pFF14+pAM2) in LB-valerate medium after addition of 0.5 mM IPTG during exponential growth (the filled arrow indicates the time point where IPTG was given); open squares, CC1003(pFF14+pAM2) in LB-valerate medium after addition of IPTG in stationary growth (the open arrow indicates the time point where IPTG was given). The LB medium was supplemented with 50 mM sodium valerate at time zero. The growth curves of the three strains were similar; thus, only one OD_{600} curve is shown (dotted line).

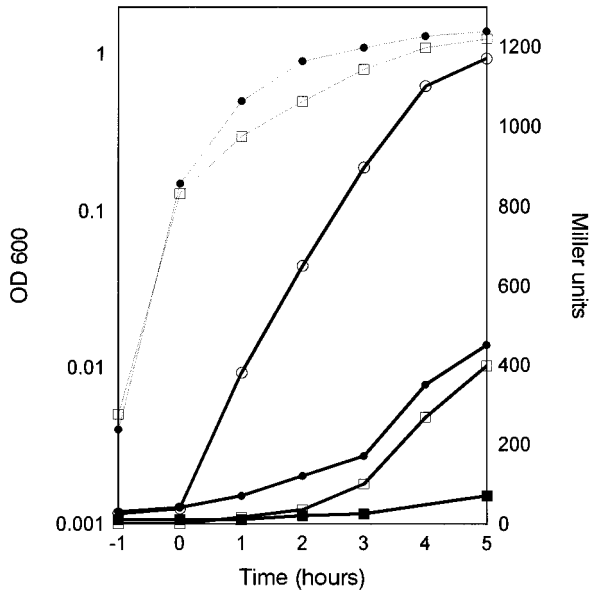

FIG. 5. Effect of valerate on *spvR* during growth in wild-type *S. dublin* Lane and in *rpoS* mutant *S. dublin* CC1002. Symbols: filled circles, *S. dublin* Lane (pSpvR-Z) in LB; open circles, *S. dublin* Lane(pSpvR-Z) in LB-valerate medium; filled squares, CC1002(pSpvR-Z) in LB; open squares, CC1002(pSpvR-Z) in LB-valerate medium; solid lines, β-galactosidase levels; dotted lines, growth curves. The cultures were supplemented with sodium valerate (50 mM) or sodium chloride (50 mM) at time zero. The growth curves of the two strains growing in LB and of the two strains growing in LB-valerate were similar; thus, only two OD_{600} curves are shown.

SCFA induce *spvR* in the absence of RpoS. To evaluate the role of the second positive regulator SpvR in SCFA-mediated *spvABCD* expression, we measured *spvR* expression in response to valerate. For this, we transformed pSpvR-Z, a *spvR-lacZ* fusion plasmid, into *S. dublin* Lane (5). This strain was chosen because it contains a wild-type copy of *spvR* on the virulence plasmid pSDL2 which supplies functional SpvR for positive autoregulation (4, 6, 26). One hour after we added valerate to the culture medium of exponentially growing Lane(pSpvR-Z), a level of induction 10-fold higher than that of the control growing in LB was observed (Fig. 5). In CC1002(pSpvR-Z), the *rpoS* mutant of *S. dublin* Lane, very little *spvR* expression was detectable during growth in LB (Fig. 5), confirming earlier findings that RpoS plays an important role in *spvR* transcription (4). However, in the presence of valerate, significant *spvR* was induced when cells entered stationary growth, reaching almost the levels seen in wild-type Lane(pSpvR-Z) growing in LB (Fig. 5). The mechanism by which SCFA induce *spvR* expression in the absence of *rpoS* remains unclear. Since SpvR autoregulates its own expression and σ^{70} RNA polymerase may mediate some (although limited) *spvR* transcription (4, 15), it is conceivable that SCFA somehow interact with SpvR and/or σ^{70} RNA polymerase at the *spvR* promoter, resulting in substantial enhancement of *spvR* expression.

Role of SpvR in SCFA-mediated *spvABCD* expression. It has been recently shown that SpvR overproduction from an exogenous promoter may bypass the requirement for RpoS in *spvABCD* regulation, suggesting that SpvR is able to interact with σ^{70} RNA polymerase at the *spvA* promoter under certain circumstances (4, 14, 15). Thus, our findings of significant *spvR* induction by SCFA in the *rpoS* mutant may sufficiently explain the inducing effect of SCFA on *spvABCD* transcription in the *rpoS* mutant background (Fig. 2 and 5). To establish the role of SpvR in SCFA-mediated *spvABCD* activation, we measured *spvB* expression in valerate-rich cultures, using pFF26, a pFF14 derivative carrying a *spvR'AB-lacZ* fusion defective in *spvR* (8). pFF26 was introduced into LD842 and CC1003, and β-galactosidase levels were determined during growth in valerate-rich LB medium. In both strains, no significant *spvB* expression occurred throughout the growth cycle, indicating that SpvR is indispensable for fatty acid-mediated *spvABCD* induction in the $rpoS^+$ and in the *rpoS* mutant backgrounds. Since in the *rpoS* mutant background SpvR is synthesized only during stationary phase, *spvABCD* expression is restricted to this growth phase under these conditions.

Other regulatory pathways which are unrelated to SpvR might be involved in SCFA-mediated *spvABCD* expression but were not evaluated in this study. For example, SCFA may reduce the negative control of the cyclic AMP-cyclic AMP receptor protein system or the nucleoid-associated protein H-NS. Both systems were recently reported to be involved in negative control of the *spv* operon (21, 22).

The significance of SCFA as signals for virulence expression remains unknown. Certain *E. coli* mutants are able to take up and grow on SCFA C_3 to C_6 as a sole carbon source (24); however, we were not able to demonstrate this capability in wild-type *S. dublin* (17a). The hypothesis that SCFA are endogenous metabolic products that accumulate within the bacterial cell during infection is speculative. Although SCFA may not be the physiological inducers of *spv* expression in vivo, they may be related to them. The finding that small molecules trigger expression of the plasmid virulence genes adds a new aspect to the concept of *spv* regulation. Starvation and exhaustion of energy sources are widely considered to serve as signals for *spv* expression; however, the marked induction of *spv* ex-

pression by SCFA during exponential growth indicates that the lack of nutrients is not a prerequisite for *spv* expression.

Nucleotide sequence accession number. The *rpoS* sequence is accessible under the EMBL, GenBank, and DDBJ nucleotide sequence data library accession number X82129.

The first two authors contributed equally to this work.

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