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Growth Regulation of a *Salmonella* Plasmid Gene Essential for Virulence

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The *Salmonella dublin* plasmid gene *vsdC* is essential for virulence. We have constructed a *vsdC-lacZ* translational fusion to demonstrate that *vsdC* is selectively expressed during the stationary phase of bacterial cell growth. This pattern of expression has been confirmed by mRNA hybridization studies. Carbon starvation is able to induce *vsdC* expression by limiting bacterial growth. The expression of *vsdC* is dependent upon an upstream gene, *vsdA*, whose gene product possesses significant amino-terminus homology with the LysR family of transcriptional activator proteins. We have further demonstrated that *vsdC* expression is not dependent upon the known *Salmonella* chromosomal virulence regulatory loci *ompR*, *phoP*, and *cya-crp* and that *vsdC* can be expressed in a range of nontyphoidal *Salmonella* serovars, including some serovars in which introduction of the virulence plasmid does not confer mouse virulence. The *vsd* system provides a model for the study of transcriptional activation, a basis for the development of new expression vectors, and a novel mechanism of virulence gene regulation. Bacterial growth limitation within the phagosomes of host phagocytic cells may be the environmental signal inducing plasmid-mediated virulence gene expression in salmonellae.

Salmonella dublin (*Salmonella enteritidis* subsp. *dublin*) is an unusually invasive nontyphoidal *Salmonella* serovar which is host adapted for cattle. Along with *Salmonella* serovars such as *S. typhimurium*, *S. choleraesuis*, *S. enteritidis*, *S. gallinarum*, and *S. pullorum*, *S. dublin* possesses a large plasmid essential for virulence (34, 54). Although these plasmids are not identical, the sequences required for virulence are highly conserved (2, 5, 44, 48). Cured derivatives of *S. dublin*, *S. typhimurium*, *S. choleraesuis*, *S. enteritidis*, *S. gallinarum*, and *S. pullorum* have been obtained, resulting in up to a 10^6-fold increase in 50% lethal dose in animal models (4, 14, 28, 31–33, 54); reintroduction of the plasmid results in the restoration of virulence.

The plasmid pSDL2 is an 80-kb virulence plasmid isolated from *S. dublin* Lane, a strain originally obtained from a patient with bacteremia (14). Recent work in our laboratory has identified the sequence of an 8.2-kb *Sal*I-*Xho*I pSDL2 fragment which represents the region sufficient to express virulence in BALB/c mice when cloned onto a heterologous low-copy-number replicon (35). Six sequential open reading frames, designated *vsdA* to *vsdF*, have been identified in this fragment.

The *vsdA* gene is nearly identical to the gene previously named *vagA* or ORF1 or *mkaC* in *S. typhimurium* (41, 45, 53) and encodes a 34-kDa protein possessing significant amino-terminus homology with the LysR family of transcriptional activator proteins (29, 35). The *vsdC* gene, analogous to *mkaA* and *mkfB* in *S. typhimurium*, is the largest gene in the pSDL2 virulence region, encoding a 66-kDa protein product with the distinctive structural feature of nine consecutive proline residues in the central portion of the peptide (35). We have constructed translation termination linker insertions in *vsdA* and *vsdC* that abolish virulence, confirming that these loci are required for pathogenicity (48a).

Previous work in *S. typhimurium* has identified several regulatory genes involved in *Salmonella* virulence. The pleiotropic chromosomal regulatory loci *ompR* (20), *phoP* (23), and *cya-crp* (18) have been shown to be essential for virulence expression. Furthermore, although the specific regulatory mechanisms are not known, the ability of salmonellae to invade epithelial cells has been shown to be induced by oxygen limitation and logarithmic growth (22, 38). However, regulatory mechanisms of the *Salmonella* plasmid-encoded virulence genes have not been identified previously.

In order to examine environmental signals affecting *vsdC* expression, we constructed a translational fusion combining the amino terminus of *vsdC* with *lacZ*, the structural gene encoding the β-galactosidase enzyme of *Escherichia coli*. *lacZ* was selected as an easily assayable reporter gene, with no background activity in salmonellae.

In the present study, analyses of reporter gene expression, mRNA synthesis, deletion mutagenesis, and gene complementation have demonstrated a novel regulatory system resulting in the selective transcription of *vsdC* during the stationary phase of bacterial growth. *vsdC* activation by the *vsdA* gene product can be triggered specifically by carbon source deprivation. We propose that nutrient deprivation within the phagosomes of host phagocytic cells may be the environmental signal activating *Salmonella* plasmid virulence gene expression.

MATERIALS AND METHODS

Bacterial strains and media. The bacteria used in this study are shown in Table 1. Luria-Bertani broth (10 g of tryptone, 5 g of yeast extract, 10 g of NaCl [all in 1 liter]) or M9 minimal medium (7 g of Na_2HPO_4, 3 g of KH_2PO_4, 0.5 g of NaCl, 1 g of NH_4Cl, 5 mg of thiamine, 0.1 mg of NAD, 120

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TABLE 1. Bacterial strains and serovars

	Relevant feature(s)	Reference
Escherichia coli TB1	*hsdR hsdM*$^+$ *lacZ*ΔM15	3
Salmonella typhimurium LB5000	$r_{LT}^- r_{SA}^- r_{SB}^-$	10
Salmonella dublin Lane	Contains pSDL2 virulence plasmid	14
Salmonella dublin LD842	Plasmid-cured derivative of Lane	14
Salmonella typhimurium BRD578	*ompR* mutation	20
Salmonella typhimurium 7953	*phoP* mutation	23
Salmonella typhimurium 4064	Δ*cya*-Δ*crp*	17
Salmonella heidelburg	See text	48
Salmonella newport	See text	48
Salmonella derby	See text	48
Salmonella saintpaul	See text	48

mg of $MgSO_4$, 0.1 to 4.0 g of glucose [all in 1 liter]) at 37°C were used for all experiments. Agar (1.5%) was added to solid medium. Antibiotics used for selection were penicillin (400 μg/ml) and chloramphenicol (20 μg/ml), both from Sigma (St. Louis, Mo.). X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) was added to solid medium (30 μg/ml) as indicated.

Plasmids. Maps of the plasmids constructed for this study, and sequences shared with the *S. dublin* virulence plasmid pSDL2 (5, 14), are shown in Fig. 1. The *Sal*I B fragment of pSDL2 cloned into the *Sal*I site of pBR322 (9), with the *vsd* genes in a counterclockwise orientation (pSD15.2), was obtained from G. Chikami; pFF12 was constructed by ligating the 8.3-kb *Bam*HI-*Nhe*I fragment of pSD15.2 to the 3.1-kb *Bam*HI-*Xba*I fragment of pPA52 (from P. Agron), which contains the *lacZ* gene missing the first 21 bp. The resulting construct contains *vsdA*, *vsdB*, and a translational fusion containing the first 900 bp of *vsdC* and the last 3,051 bp of *lacZ*, in a pBR322 replicon. pFF14 was constructed by ligating the 3.6-kb *Sal*I-*Hin*dIII fragment of pACYC184 (12) to the 7.7-kb *Sal*I-*Hin*dIII fragment of pFF12, thereby replacing the pBR322 replicon of pFF12 with a p15A replicon containing the *cat* gene. pFF16 was created by deleting the 3.2-kb *Eag*I fragment from pFF14, leaving a *vsdB* carboxy-terminus remnant and the *vsdC-lacZ* fusion. pFF18 was constructed to provide a high-copy-number source of *vsdA* in *trans*, by ligating the 2.3-kb *Sal*I-*Eco*RI fragment of the pSDL2 *Sal*I B fragment into pUC19 (58). pFF24 consists of the 3.5-kb *Xho*I-*Kpn*I fragment of pUC:MK-20 ligated to the 7.0-kb *Sal*I-*Kpn*I fragment of pFF14; pUC:MK-20 consists of the 13-kb *Sal*I fragment from pSDL2::Tn*5*-*oriT*15-2 (5) cloned into pUC18 (58). Tn*5*-*oriT*15-2 is located at bp 1247 of the *Sal*I B fragment (35); pFF24 therefore contains a 365-bp Tn*5* remnant, followed by a *vsdA* carboxy-terminus remnant, *vsdB*, and *vsdC* fused to *lacZ* as in pFF14. pFF26 is identical to pFF14, except for the presence of a suppressible multiple reading frame termination linker containing an *Xba*I site at the *Hpa*I site in *vsdA*, thereby creating a nonsense mutation in *vsdA*. pFF26 was constructed by ligating the 7.0-kb *Sal*I-*Kpn*I fragment of pFF14 to the 4.3-kb *Sal*I-*Kpn*I fragment of pCR5-A1.

DNA isolation and manipulation. Plasmids were constructed in *E. coli* TB1 (3). Transformation of *Salmonella* strains was facilitated by the use of DNA isolated from restriction-deficient *S. typhimurium* LB5000 (10); subsequent steps were done by the method of Dagert and Ehrlich (18). Rapid clonal analysis of plasmid DNA (6) and agarose gel electrophoresis (49) were performed according to the conventional published methods.

Determination of β-galactosidase activity. Quantitative determination of β-galactosidase activity was performed as previously described by Miller (39). Optical densities were measured with an LKB Ultrospec II spectrophotometer. Each experiment was repeated at least once.

RNA isolation and Northern blot (RNA blot) analysis. RNA was isolated by the hot phenol method as described previously (37). Culture samples (10 ml each) were grown to the desired optical density of culture at 600 nm [OD_{600}]) and

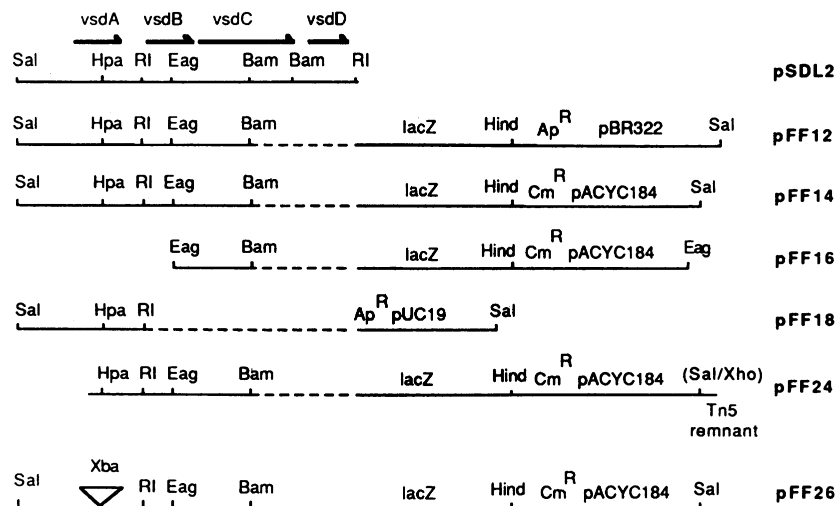

FIG. 1. Maps of plasmids. Relevant sequences of the *Sal*I B fragment of pSDL2 (5), the *S. dublin* virulence plasmid, are shown in the top line. *lacZ* was obtained from pPA52 (P. Agron). pFF26 contains a translational termination linker with an *Xba*I site, inserted into the *Hpa*I site of the *vsdA* gene. Abbreviations: Sal, *Sal*I; Hpa, *Hpa*I; RI, *Eco*RI; Eag, *Eag*I; Bam, *Bam*HI; Hind, *Hin*dIII; Xho, *Xho*I; Xba, *Xba*I; Ap^R, β-lactamase; Cm^R, chloramphenicol acetyltransferase.

centrifuged. Each pellet was resuspended in 2 ml of 0.02 M sodium acetate–1 mM EDTA (pH 5.3). Two milliliters of 1% sodium dodecyl sulfate (SDS)–0.02 M sodium acetate was added, and the suspension was immediately extracted with 5 ml of phenol saturated with water at 65°C. After reextraction with phenol, the aqueous phase was layered onto a 3-ml 5.7 M CsCl cushion and centrifuged 40,000 rpm at 20°C for 12 h in a horizontal rotor in a Beckman L5-50 ultracentrifuge. The RNA pellet was then washed with 75% ethanol, air dried, and resuspended in DEPC (diethyl pyrocarbonate)-treated water. RNA concentrations were determined by measuring A_{260}. For Northern blot analysis, 50-μg total RNA samples were electrophoresed on a horizontal denaturing formaldehyde-agarose gel (49) and transferred to a nylon membrane (Oncor, Gaithersburg, Md.). Hybridization using a double-stranded *vsdC*-specific probe randomly primed with [^{32}P] dCTP was performed overnight at 45°C in the presence of 50% formamide. The membrane was washed three times for 1 h at room temperature and once at 50°C with 0.1% SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–1% SDS and then was exposed to Kodak XAR5 film for 8 to 18 h with a Quantum III intensifying screen.

RESULTS

***vsdC* is selectively expressed during the stationary phase of bacterial cell growth.** *S. dublin* LD842 carrying pFF14, containing the *vsdC-lacZ* fusion, was used in initial screening studies to examine the effect of various environmental manipulations on *vsdC* expression. No significant changes in LacZ production, as measured by color differences on medium containing X-Gal were seen with alterations in medium, temperature, osmolarity, potassium concentration, calcium concentration, phosphate concentration, iron availability, oxygen concentration, pH, or redox state (data not shown). *vsdC* expression in *S. dublin* LD842 cells containing pFF14 was then determined at different phases of bacterial cell growth. An overnight culture was diluted 1:100 into prewarmed medium at 37°C and placed on a rotary shaker. Aliquots were removed at various time intervals, and β-galactosidase activity was assayed. Growth phase was determined by measurement of culture OD_{600}. A plot of β-galactosidase activity versus optical density from a representative experiment is shown in Fig. 2. Expression of β-galactosidase activity from the *vsdC-lacZ* fusion on pFF14 is seen to rise in late logarithmic phase, reaching maximal values in the stationary phase of cell growth. A nearly 40-fold difference in *vsdC* expression, as measured by the β-galactosidase activity of the VsdC-LacZ fusion protein, is seen between the mid-logarithmic and stationary phases of growth.

Growth-phase-dependent regulation at the transcriptional level was confirmed by Northern blot hybridization analysis of mRNA. Total cellular RNA was isolated from *S. dublin* Lane (containing pSDL2) at different phases of cell growth. RNA concentration was determined spectrophotometrically, and equivalent amounts of RNA were analyzed by Northern blotting as described above. A ^{32}P-labelled 400-bp *Bam*HI-*Dra*I fragment of the *vsdC* gene was used as a probe. As shown in Fig. 3a, the amount of RNA transcript containing *vsdC* is increased dramatically during the stationary phase of growth. This confirms that *vsdC* is selectively expressed during the stationary phase of growth and that this regulation occurs in the native pSDL2 plasmid as well as in the pFF14 construct. A ^{32}P-labelled 441-bp *Nsi*I-*Xho*I fragment of the constitutively expressed *vsdF* gene (35) provided a control probe. As seen in Fig. 3b, no significant change in *vsdF*

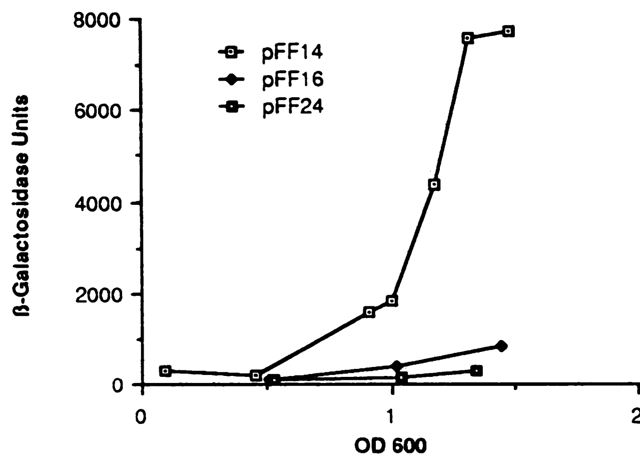

FIG. 2. *vsdC* Expression relative to bacterial cell growth. β-Galactosidase activity expressed by cured strain *S. dublin* LD842 (14) carrying pFF14, pFF16, and pFF24 at different phases of cell growth was measured and corrected for cell density, by the method of Miller (39). Data from a representative experiment are shown; an identical pattern of expression was observed in all experiments. The culture transition to stationary phase for LD842 in LB medium begins at an OD_{600} value of approximately 1.0. The selective expression of *vsdC* during the stationary phase of growth and the requirement of *vsdA* for *vsdC* expression are demonstrated.

transcription from pSDL2 occurs during different phases of growth; this excludes the possibility that the growth-related change in *vsdC* expression is the result of a change in plasmid copy number.

***vsdC* expression is induced by carbon starvation.** β-Galactosidase expression by *S. dublin* LD842 containing pFF14 was measured in M9 minimal medium containing variable amounts of glucose as the carbon source, to determine whether carbon deprivation could induce *vsdC* expression in stationary cells at different culture densities. Cultures grown overnight were diluted 10^5-fold into prewarmed M9 medium supplemented with 0.4, 0.1, 0.025, or 0.01% glucose and placed on a rotary shaker at 37°C. β-Galactosidase activity was measured at 24 and 30 h; an additional measurement was made at 48 h for the culture containing 0.4% glucose because these cells required more generations to attain stationary phase. As shown in Fig. 4, *vsdC* is activated as cessation of bacterial growth from carbon starvation occurs, independent of culture density. As cells remain at stationary phase for longer periods of time, production of the VsdC-LacZ fusion protein increases even as total cell mass (OD_{600}) remains constant.

Expression of *vsdC* requires *vsdA*. On the basis of the DNA sequence of the *vsdA* gene, we have proposed that this protein may activate expression of the downstream virulence loci shown in Fig. 1 (35). Therefore, we investigated the role of the *vsdA* gene in the growth-regulated expression of *vsdC*. Since pFF14 contains *vsdA* and *vsdB* sequences in addition to the *vsdC-lacZ* fusion, pFF16 (containing *vsdC-lacZ* only) and pFF24 (containing *vsdB* and *vsdC-lacZ*) were constructed to determine whether *vsdA* is required for *vsdC* expression. The β-galactosidase activity of *S. dublin* LD842 carrying either pFF16 or pFF24 is shown in Fig. 2. In the absence of intact *vsdA* sequences, the expression of *vsdC* is profoundly reduced. Comparably reduced levels of β-galactosidase activity expressed by *S. dublin* LD842 carrying pFF26 (*vsdA* nonsense mutation, *vsdB*, *vsdC-lacZ*) con-

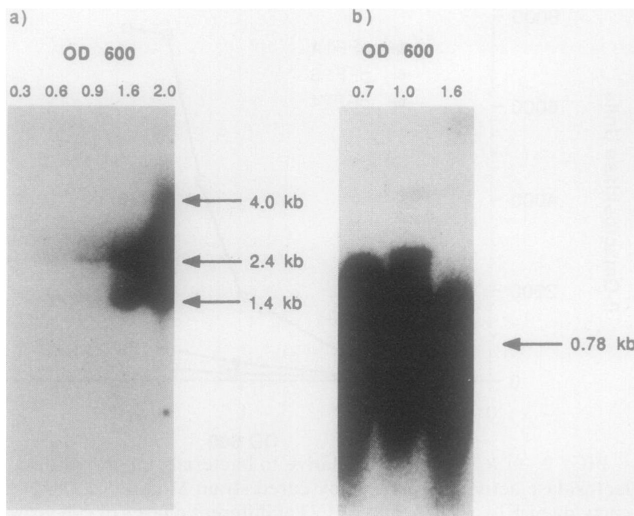

FIG. 3. Northern blot analysis of *vsdC* mRNA. *S. dublin* Lane was grown to OD_{600} values of 0.3, 0.6, 0.7, 0.9, 1.0, 1.6, and 2.0. Portions (50 μg each) of total RNA from each sample were electrophoresed, blotted onto a nylon membrane, and hybridized either to a ^{32}P-labelled 400-bp *Bam*HI-*Dra*I fragment of the *vsdC* gene (a) or to a ^{32}P-labelled 441-bp *Nsi*I-*Xho*I fragment of the *vsdF* gene (b), which provided a constitutively expressed control. RNA obtained from *S. dublin* LD842 at different phases of growth served as a negative control and showed no hybridization. The presence of multiple RNA species in panel a suggest that *vsdC* transcription may arise or terminate at multiple sites. The approximate sizes of the *vsdC* RNA bands were determined by the use of radiolabelled RNA standards to be 4.0, 2.4, and 1.4 kb. Selective expression of *vsdC* during the stationary phase of growth is confirmed in the native pSDL2 plasmid. In contrast, no relationship between growth phase and *vsdF* expression is observed.

firmed that *vsdC* expression requires the *vsdA* gene product (data not shown).

***vsdA* can activate *vsdC* in *trans*.** The β-galactosidase activity of *S. dublin* LD842 containing pFF24 (*vsdB* and

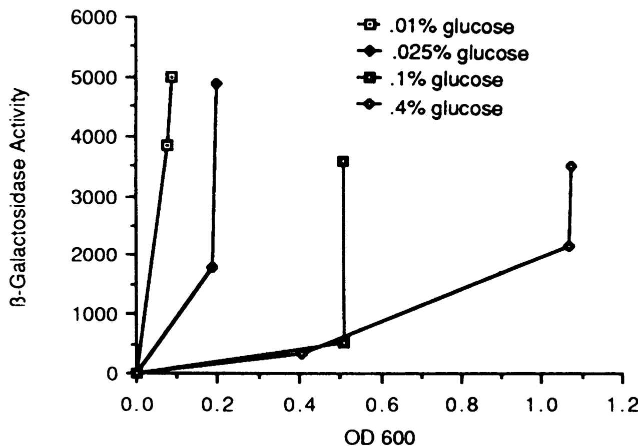

FIG. 4. *vsdC* expression is induced by carbon starvation. An overnight culture of *S. dublin* LD842 with pFF14 was diluted 10^5-fold into M9 medium with 0.4, 0.1, 0.025, or 0.01% glucose. *vsdC* is activated as the carbon source is depleted, arresting bacterial growth at different culture densities. β-Galactosidase activity (39) was measured at 24 and 30 h; an additional measurement was made at 48 h for the culture containing 0.4% glucose.

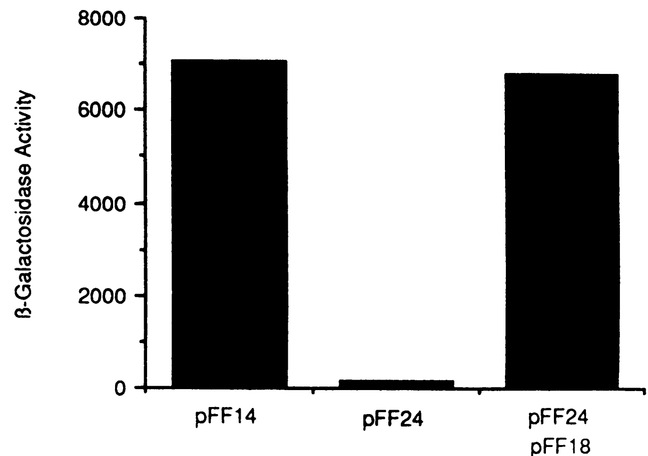

FIG. 5. Complementation by *vsdA* in *trans*. The β-galactosidase activity expressed during the stationary phase of growth by *S. dublin* LD842 containing pFF14 or pFF24, with and without pFF18 (*vsdA* in *trans*), is shown. The presence of *vsdA* in *trans* is able to fully complement the deletion of *vsdA* on pFF24.

vsdC-lacZ) was measured with *vsdA* supplied on a heterologous replicon to determine whether the loss of *vsdA* could be complemented in *trans* with respect to *vsdC* expression. The plasmid pFF18 (pUC19 derivative) was used as the source of *vsdA*. Measurements were performed on stationary cultures grown synchronously. As shown in Fig. 5, *vsdA* in *trans* (pFF18) fully complements the deletion of *vsdA* on pFF24.

The β-galactosidase activity expressed by *S. dublin* LD842 carrying pFF14 and pFF18 was determined at different phases of growth, to determine whether the high-level expression of *vsdA* in *trans* regulated by a heterologous promoter (P*lac*) would abolish the growth-dependent regulation of *vsdC* expression. Northern blot analysis using a *vsdA* probe confirmed a high degree of *vsdA* expression from pFF18 (data not shown). Although the addition of pFF18 to pFF14 resulted in a modest increase in *vsdC* expression during logarithmic growth, growth-dependent regulation with maximal expression occurring during stationary phase was still evident.

***vsdC* expression is not dependent upon *ompR*, *phoP*, or *cya-crp*.** *ompR* (20), *phoP* (23), and *cya-crp* (17) are pleiotropic chromosomal regulatory loci which have been demonstrated to be essential for full *Salmonella* virulence. pFF14 was introduced into *S. typhimurium* BRD578 (*ompR* mutation), *S. typhimurium* 7953 (*phoP* mutation) and *S. typhimurium* 4064 (Δ*cya* Δ*crp*), and β-galactosidase activity was measured at the stationary phase of growth. High levels of *vsdC* expression were detected in these different genetic backgrounds (data not shown), demonstrating that *vsdC* is not dependent upon these known *Salmonella* virulence regulatory loci.

Comparison of *vsdC* expression in different *Salmonella* serovars. pFF14 was introduced into *Salmonella heidelburg*, *S. newport*, *S. derby*, and *S. saintpaul*, and the β-galactosidase activity of these strains was measured. While none of these *Salmonella* serovars normally harbors a virulence plasmid, the introduction of the *S. dublin* virulence plasmid has been found to confer mouse virulence in *S. heidelburg* and *S. newport*, but not in *S. derby* or *S. saintpaul* (48). The results of this experiment in comparison with the β-galac-

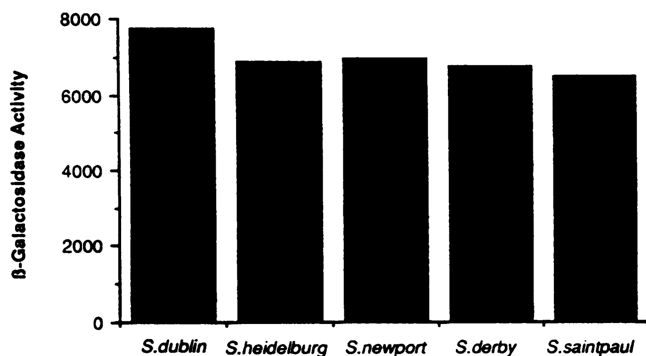

FIG. 6. *vsdC* expression in different *Salmonella* serovars. The β-galactosidase activity expressed by various *Salmonella* serovars carrying pFF14 at the stationary phase of growth is virtually identical.

tosidase activity expressed from *S. dublin* LD842 containing pFF14 are shown in Fig. 6. No significant differences in *vsdC* expression between these strains and *S. dublin* was detected.

DISCUSSION

We have identified a novel system of gene regulation in the *Salmonella* virulence plasmid. This study has demonstrated that *vsdC* is selectively expressed during the stationary phase of bacterial cell growth and can be induced specifically by carbon starvation. A fundamental pathogenic property of salmonellae is believed to be its ability to survive within phagocytic cells (24, 25), and *vsdC* is one of a group of plasmid genes which are believed to permit salmonellae to escape confinement by phagocytic cells of the reticuloendothelial system (27). There is some evidence that macrophages act to limit the growth of phagocytosed organisms by limiting essential nutrients within the phagosome (40). The resulting cessation of bacterial growth in this microenvironment may be the environmental signal which activates the expression of the *Salmonella* plasmid-encoded virulence genes at the appropriate time.

An inverse relationship between growth rate and the expression of a virulence-associated gene was reported in *Shigella flexneri* (19), but in this instance the variation in gene expression was attributed to a global alteration in DNA superhelicity, rather than to a specific transcriptional regulatory mechanism. The *Salmonella* genes expressing adherence to epithelial cells have been demonstrated recently to be regulated by growth, as well as by oxygen deprivation (22, 38). However, this relationship between growth and the expression of invasion genes is in fact precisely the opposite of *vsdC* regulation; cells lose their invasive potential in the stationary phase of growth.

In order to cause systemic infection, free-living salmonellae are required to adapt to radically differing environmental conditions; ingested organisms must survive exposure to gastric acid, adhere to and invade intestinal epithelia, multiply within the mononuclear phagocytic cells of the reticuloendothelial system, and eventually cause bacteremia and microabscess formation, resulting in their exposure to serum bactericidal factors and neutrophils (28). The plasmid virulence genes are required only after invasion from the intestine to the reticuloendothelial system has occurred (28). The completely different growth conditions required for expression of the *Salmonella* invasion loci and the *vsdC* gene illustrates the specificity of virulence gene regulatory mechanisms for particular microenvironments found at different stages of bacterial infection.

Our study has begun to clarify the molecular components of the *Salmonella* virulence plasmid regulatory system. The significant loss of β-galactosidase activity expressed by *S. dublin* LD842 containing pFF24 (*vsdB vsdC-lacZ*) in comparison to pFF14 (*vsdA vsdB vsdC-lacZ*) demonstrates that *vsdA* is necessary for growth-regulated *vsdC* expression. The ability of *vsdA* to restore full *vsdC* expression in *trans* is consistent with a role of the VsdA protein as a transcriptional activator. The sharp fall in *vsdC* expression induced by a nonsense mutation in *vsdA* (pFF26) provides further confirmation of this model. Hybridization analysis of mRNA using a *vsdC* probe establishes that growth-dependent regulation of *vsdC* occurs at a transcriptional level, demonstrable in the native pSDL2 virulence plasmid as well as in the recombinant pFF14 construct. Northern blot analysis demonstrates the presence of multiple bands which hybridize to *vsdC*, suggesting that *vsdC* transcription may arise at multiple separate sites, that transcription may terminate at different sites, or that specific processing of a large transcript which encompasses *vsdC* is taking place.

DNA sequence analysis reveals that the VsdA protein has significant amino-terminus homology with a large family of bacterial transcriptional activator proteins (29) which includes the following: MetR (43) and OxyR (15) of *S. typhimurium*; LysR (52), IlvY (57), CysB (42), and Mor (56) of *E. coli*; NodD (21) of *Rhizobium meliloti*; AmpR (30) of *Enterobacter cloacae*; CatR (47) and NahR (50) of *Pseudomonas putida*; TrpI (13) of *Pseudomonas aeruginosa*; IrgB (26) of *Vibrio cholerae*; MleR (46) of *Lactococcus lactis*; and GltC (8) of *Bacillus subtilis*. The region of homology encompasses a proposed helix-turn-helix DNA binding motif (35). Interestingly, at least some of these proteins (MetR, LysR, and IlvY) are known to be involved in pathways which are induced during the stringent response to amino acid starvation. Thus, these regulatory loci, like *vsdA*, participate in pathways which are activated at a time when most cellular genes are repressed (11).

Small inducer molecules (e.g., diaminopimelic acid for LysR, acetohydroxybutyrate or acetolactate for IlvY, flavenoids for NodD, homocysteine for MetR, and *O*-acetylserine for CysB) are believed to act as coactivators in these systems (29, 55), possibly altering the conformation of the regulatory protein once it has bound its operator site (55). Overexpression of a transcriptional activator protein fails to result in maximal gene expression in the absence of the corresponding inducer molecule. Although *vsdA* is required for maximal activation of *vsdC* in the stationary phase of growth, overexpression of *vsdA* in *trans* did not result in the maximal expression of *vsdC* during logarithmic growth. This is consistent with the hypothesis that an as yet unidentified coactivator molecule which rises in concentration during stationary phase is required for maximal *vsdC* activation.

Several *Salmonella* chromosomal regulatory loci have been demonstrated to be associated with virulence, including *ompR* (20), *phoP* (23), and *cya-crp* (17). These loci are of particular interest in view of the recent identification of growth-regulated loci which are *ompR* (16) or *cya-crp* (51) dependent in *E. coli*. However, our data indicate that *vsdC* expression is not dependent upon these chromosomal regulatory loci in salmonellae. Additional transcriptional regulatory systems have been identified recently in *E. coli* which allow changes in gene expression during stationary phase (1,

7, 36); the relationship between these systems and *vsdC* expression is not known.

All *Salmonella* serovars virulent in mice (e.g., *S. dublin*, *S. typhimurium*, *S. choleraesuis*) possess a virulence plasmid. Introduction of the *S. dublin* virulence plasmid (48) confers the ability to cause virulence in mice to some plasmid-free serovars (*S. heidelburg*, *S. newport*), but not to others (*S. derby*, *S. saintpaul*). The intact expression of *vsdC* in *Salmonella* serovars (*S. derby*, *S. saintpaul*), in which virulence plasmid introduction is not sufficient to confer virulence in mice indicates that a failure to properly express plasmid virulence genes may not be the reason for the avirulence of these strains. Taken together with the slightly higher intrinsic virulence of *S. heidelburg* and *S. newport* even prior to the addition of the virulence plasmid (48), this result suggests that the presence of an additional unrelated chromosomal virulence factor in *S. heidelburg*, *S. newport*, and the mouse-virulent *Salmonella* serovars, but not present in *S. derby* and *S. saintpaul*, may be a more likely explanation for the observed results.

In summary, we have described a new system which regulates the expression of *Salmonella* plasmid virulence genes in response to bacterial cell growth and nutrient availability. The *vsd* genes provide a convenient model system for the study of the LysR family of bacterial activator proteins. This system will also provide the basis for the development of a vector system to allow the selective expression of a gene product during the stationary phase of growth, facilitating the production of proteins with deleterious effects on cell growth. Perhaps most significantly, these observations provide novel insights into the environmental signals triggering the expression of microbial virulence mechanisms.

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REFERENCES

1. **Aldea, M., T. Garrido, C. Hernández-Chico, M. Vicente, and S. R. Kushner.** 1989. Induction of growth-phase-dependent promoter triggers transcription of *bolA*, an *Escherichia coli* morphogene. EMBO J. **8:**3923–3931.
2. **Baird, G. D., E. J. Manning, and P. W. Jones.** 1985. Evidence for related virulence sequences in plasmids of *Salmonella dublin* and *Salmonella typhimurium*. J. Gen. Microbiol. **131:**1815–1823.
3. **Baldwin, T. O., T. Berends, T. A. Brunch, T. F. Holzman, S. K. Rausch, L. Shamansky, M. L. Treat, and M. M. Ziegler.** 1984. Cloning of the luciferase structural genes from *Vibrio harveyi* and expression of bioluminescence in *Escherichia coli*. Biochemistry **23:**3663–3667.
4. **Barrow, P. A., and M. A. Lovell.** 1989. Functional homology of virulence plasmids in *Salmonella gallinarum*, *Salmonella pullorum*, and *Salmonella typhimurium*. Infect. Immun. **57:**3136–3141.
5. **Beninger, P. R., G. Chikami, K. Tanabe, C. Roudier, J. Fierer, and D. G. Guiney.** 1988. Physical and genetic mapping of the *Salmonella dublin* virulence plasmid pSDL2. J. Clin. Invest. **81:**1341–1347.
6. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7:**1513–1523.
7. **Bohannon, D. E., N. Connell, J. Keener, A. Tormo, M. Espinosa-Urgel, M. M. Zambrano, and R. Kolter.** 1991. Stationary-phase-inducible "gearbox" promoters: differential effects of *katF* mutations and role of σ^{70}. J. Bacteriol. **173:**4482–4492.
8. **Bohannon, D. E., and A. L. Sonenshein.** 1989. Positive regulation of glutamate biosynthesis in *Bacillus subtilis*. J. Bacteriol. **171:**4718–4727.
9. **Bolivar, F., R. L. Rodriguez, P. J. Greene, M. C. Betlach, H. L. Heyneker, H. W. Boyer, J. H. Crosa, and S. Falkow.** 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene **2:**95–113.
10. **Bullas, L. R., and J.-I. Ryu.** 1983. *Salmonella typhimurium* LT2 strains which are $r^- m^+$ for all three chromosomally located systems of DNA restriction and modification. J. Bacteriol. **156:**471–474.
11. **Cashel, M., and K. E. Rudd.** 1987. The stringent response, p. 1410–1438. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
12. **Chang, A. C., and S. N. Cohen.** 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the p15A cryptic miniplasmid. J. Bacteriol. **134:**1141–1156.
13. **Chang, M., A. Hadero, and I. P. Crawford.** 1989. Sequence of the *Pseudomonas aeruginosa trpI* activator gene and relatedness of *trpI* to other procaryotic regulatory genes. J. Bacteriol. **171:**172–183.
14. **Chikami, G. K., J. Fierer, and D. G. Guiney.** 1985. Plasmid-mediated virulence in *Salmonella dublin* demonstrated by use of a Tn*5*-*oriT* construct. Infect. Immun. **50:**420–424.
15. **Christman, M. F., G. Storz, and B. N. Ames.** 1989. OxyR, a positive regulator of hydrogen peroxide-inducible genes in *Escherichia coli* and *Salmonella typhimurium*, is homologous to a family of bacterial regulatory proteins. Proc. Natl. Acad. Sci. USA **86:**3484–3488.
16. **Connell, N., Z. Han, F. Moreno, and R. Kolter.** 1987. An *E. coli* promoter induced by the cessation of growth. Mol. Microbiol. **1:**195–201.
17. **Curtiss, R., III, and S. M. Kelly.** 1987. *Salmonella typhimurium* deletion mutants lacking adenylate cyclase and cyclic AMP receptor protein are avirulent and immunogenic. Infect. Immun. **55:**3035–3043.
18. **Dagert, M., and S. D. Ehrlich.** 1979. Prolonged incubation in calcium chloride improves the competence of *Escherichia coli* cells. Gene **6:**23–28.
19. **Dorman, C. J., N. N. Bhriain, and C. F. Higgins.** 1990. DNA supercoiling and environmental regulation of virulence gene expression in *Shigella flexneri*. Nature (London) **344:**789–792.
20. **Dorman, C. J., S. Chatfield, C. F. Higgins, C. Hayward, and G. Dougan.** 1989. Characterization of porin and *ompR* mutants of a virulent strain of *Salmonella typhimurium*: *ompR* mutants are attenuated in vivo. Infect. Immun. **57:**2136–2140.
21. **Eglehoff, T. T., R. F. Fisher, T. W. Jacobs, J. T. Mulligan, and S. R. Long.** 1985. Nucleotide sequence of *Rhizobium meliloti* 1021 nodulation genes: *nodD* is read divergently from *nodABC*. DNA **4:**241–248.
22. **Ernst, R. K., D. M. Dombrowski, and J. M. Merrick.** 1990. Anaerobiosis, type 1 fimbriae, and growth phase are factors that affect invasion of HEp-2 cells by *Salmonella typhimurium*. Infect. Immun. **58:**2014–2016.
23. **Fields, P. I., E. A. Groisman, and F. Heffron.** 1989. A *Salmonella* locus that controls resistance to microbicidal proteins from phagocytic cells. Science **243:**1059–1062.
24. **Fields, P. I., R. V. Swanson, C. G. Haidaris, and F. Heffron.** 1986. Mutants of *Salmonella typhimurium* that cannot survive within the macrophage are avirulent. Proc. Natl. Acad. Sci. USA **83:**5189–5193.
25. **Finlay, B. B., and S. Falkow.** 1989. *Salmonella* as an intracellular parasite. Mol. Microbiol. **3:**1833–1841.
26. **Goldberg, M. B., S. A. Boyko, and S. B. Calderwood.** 1991. Positive transcriptional regulation of an iron-regulated virulence

gene in *Vibrio cholerae*. Proc. Natl. Acad. Sci. USA **88**:1125–1129.

27. **Gulig, P. A.** 1990. Virulence plasmids of *Salmonella typhimurium* and other salmonellae. Microb. Pathog. **8**:3–11.
28. **Heffernan, E. J., J. Fierer, G. Chikami, and D. Guiney.** 1987. Natural history of oral *Salmonella dublin* infection in BALB/c mice: effect of an 80-kilobase-pair plasmid on virulence. J. Infect. Dis. **155**:1254–1259.
29. **Henikoff, S., G. W. Haughn, J. M. Calvo, and J. C. Wallace.** 1988. A large family of bacterial activator proteins. Proc. Natl. Acad. Sci. USA **85**:6602–6606.
30. **Honoré, N., M. H. Nicolas, and S. T. Cole.** 1986. Inducible cephalosporinase production in clinical isolates of *Enterobacter cloacae* is controlled by a regulatory gene that has been deleted from *Escherichia coli*. EMBO J. **5**:3709–3714.
31. **Hovi, M., S. Sukupolvi, M. F. Edwards, and M. Rhen.** 1988. Plasmid-associated virulence of *Salmonella enteritidis*. Microb. Pathog. **4**:385–391.
32. **Jones, G. W., D. K. Rabert, D. M. Svinarich, and H. J. Whitfield.** 1982. Association of adhesive, invasive, and virulent phenotypes of *Salmonella typhimurium* with autonomous 60-megadalton plasmids. Infect. Immun. **38**:476–486.
33. **Kawahara, K., Y. Haraguchi, M. Tsuchimoto, N. Terakado, and H. Danbara.** 1988. Evidence of correlation between 50-kilobase plasmid of *Salmonella choleraesuis* and its virulence. Microb. Pathog. **4**:155–163.
34. **Korpela, K., M. Ranki, S. Sukupolvi, P. H. Mäkelä, and M. Rhen.** 1989. Occurrence of *Salmonella typhimurium* virulence plasmid-specific sequences in different serovars of *Salmonella*. FEMS Microbiol. Lett. **58**:49–54.
35. **Krause, M., C. Roudier, J. Fierer, J. Harwood, and D. Guiney.** 1991. Molecular analysis of the virulence locus of the *Salmonella dublin* plasmid pSDL2. Mol. Microbiol. **5**:307–316.
36. **Lange, R., and R. Hengge-Aronis.** 1991. Identification of a central regulator of stationary-phase gene expression in *Escherichia coli*. Mol. Microbiol. **5**:49–59.
37. **Laoide, B. M., and A. Ullmann.** 1990. Virulence dependent and independent regulation of the *Bordetella pertussis cya* operon. EMBO J. **9**:999–1005.
38. **Lee, C. A., and S. Falkow.** 1990. The ability of *Salmonella* to enter mammalian cells is affected by bacterial growth state. Proc. Natl. Acad. Sci. USA **87**:4304–4308.
39. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
40. **Murray, H. W.** 1988. Interferon-gamma, the activated macrophage, and the host defense against microbial challenge. Ann. Intern. Med. **108**:595–608.
41. **Norel, F., M. R. Pisano, J. Nicoli, and M. Y. Popoff.** 1989. Nucleotide sequence of the plasmid-borne virulence gene *mkfA* encoding a 28 kDa polypeptide from *Salmonella typhimurium*. Res. Microbiol. **140**:263–265.
42. **Ostrowski, J., G. Jagura-Burdzy, and N. M. Kredich.** 1987. The sequences of the *cysB* regions of *Salmonella typhimurium* and *Escherichia coli*. J. Biol. Chem. **262**:5999–6005.
43. **Plamann, L. S., and G. V. Stauffer.** 1987. Nucleotide sequence of the *Salmonella typhimurium metR* gene and the *metR-metE* control region. J. Bacteriol. **169**:3932–3937.
44. **Poppe, C., R. I. Curtiss, P. A. Gulig, and C. L. Gyles.** 1989. Hybridization studies with DNA probe derived from the virulence region of the 60 Mdal plasmid of *Salmonella typhimurium*. Can. J. Vet. Res. **53**:378–384.
45. **Pullinger, G. D., G. D. Baird, C. M. Williamson, and A. J. Lax.** 1989. Nucleotide sequence of a plasmid gene involved in the virulence of salmonellas. Nucleic Acids Res. **17**:7983.
46. **Renault, P., C. Gaillardin, and H. Heslot.** 1989. Product of the *Lactococcus lactis* gene required for malolactic fermentation is homologous to a family of positive regulators. J. Bacteriol. **171**:3108–3114.
47. **Rothmel, R. K., T. L. Aldrich, J. E. Houghton, W. M. Coco, L. N. Ornston, and A. M. Chakrabarty.** 1990. Nucleotide sequencing and characterization of *Pseudomonas putida catR*: a positive regulator of the *catBC* operon is a member of the LysR family. J. Bacteriol. **172**:922–931.
48. **Roudier, C., M. Krause, J. Fierer, and D. G. Guiney.** 1990. Correlation between the presence of sequences homologous to the *vir* region of *Salmonella dublin* plasmid pSDL2 and the virulence of twenty-two *Salmonella* serotypes in mice. Infect. Immun. **58**:1180–1185.

48a. **Roudier, C.** Unpublished data.

49. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
50. **Schell, M. A., and P. Wender.** 1986. Identification of the *nahR* gene product and nucleotide sequences required for its activation of the *sal* operon. J. Bacteriol. **166**:9–14.
51. **Schultz, J. E., G. I. Latter, and A. Matin.** 1988. Differential regulation by cyclic AMP of starvation protein synthesis in *Escherichia coli*. J. Bacteriol. **170**:3903–3909.
52. **Stragier, P., and J. C. Patte.** 1983. Regulation of diaminopimelate decarboxylase synthesis in *Escherichia coli*. III. Nucleotide sequence and regulation of the *lysR* gene. J. Mol. Biol. **168**:333–350.
53. **Taira, S., and M. Rhen.** 1989. Identification and genetic analysis of *mkaA*—a gene of the *Salmonella typhimurium* virulence plasmid necessary for intracellular growth. Microb. Pathog. **7**:165–173.
54. **Terakado, N., T. Sekizaki, K. Hashimoto, and S. Naitoh.** 1983. Correlation between the presence of a fifty-megadalton plasmid in *Salmonella dublin* and virulence for mice. Infect. Immun. **41**:443–444.
55. **Urbanowski, M. L., and G. V. Stauffer.** 1989. Genetic and biochemical analysis of the MetR activator-binding site in the *metE-metR* control region of *Salmonella typhimurium*. J. Bacteriol. **171**:5620–5629.
56. **Warne, S. R., J. M. Varley, G. J. Boulnois, and M. G. Norton.** 1990. Identification and characterization of a gene that controls colony morphology and autoaggregation in *Escherichia coli* K12. J. Gen. Microbiol. **136**:455–462.
57. **Wek, R. C., and G. W. Hatfield.** 1986. Nucleotide sequence and *in vivo* expression of *ilvY* and *ilvC* genes in *Escherichia coli* K12. J. Biol. Chem. **261**:2441–2450.
58. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33**:103–119.