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Expression of the Antifeeding Gene *anfA1* in *Serratia entomophila* Requires RpoS

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The *rpoS* gene of *Serratia entomophila* BC4B was cloned and used to create *rpoS*-mutant strain BC4BRS. Larvae of the New Zealand grass grub *Costelytra zealandica* infected with BC4BRS became amber colored but continued to feed, albeit to a lesser extent than infected larvae. Subsequently, we found that expression of the antifeeding gene *anfA1* in *trans* was substantially reduced in BC4BRS relative to that in the parental strain BC4B. Our data show that a functional *rpoS* gene is vital for full expression of *anfA1* and for development of the antifeeding component of amber disease.

The soil-borne bacterium *Serratia entomophila* causes amber disease in a major New Zealand pasture pest, the grass grub *Costelytra zealandica* (White) [Coleoptera: Scarabideae]. Understanding the molecular mechanism of the disease process is an important objective of our research into the biological control of grass grub infection in New Zealand. The disease process, as described by Jackson et al. (6), is multifactorial. As *S. entomophila* cells adhere to the grass grub crop and propagate around the cardiac valve, larvae cease feeding, clear their gut, and become amber-colored. Eventually the bacteria cross the crop wall and infect the hemolymph, causing general septicemia and death. Recent research has shown that factors responsible for the gut clearance and amber coloration of larvae are encoded by a 105-kb cryptic plasmid named pADAP (5). Additionally, a locus designated *amb1* has been implicated in pili-mediated adherence (24) and evidence suggests that a second locus named *amb2* encodes an antifeeding toxin (17).

RpoS (σ^S) is a class 2 sigma factor that regulates many of the changes necessary for bacterial survival under stress conditions by altering the promoter specificity of RNA polymerase (E) (12). The ability of RpoS to influence disease processes caused by *Salmonella* (2, 10) and *Pseudomonas fluorescens* Pf-5 (21) led us to investigate whether RpoS has a role in the regulation of one or more components of the *S. entomophila* amber disease system.

Identification and characterization of the *rpoS* gene of *S. entomophila*. The *rpoS* gene of *S. entomophila* BC4B was cloned by digesting genomic DNA of this strain with restriction endonucleases *Sal*I and *Eco*RI and ligating all fragments to similarly digested vector pBR322. *rpoS*-containing clones were isolated by electroporating ligated DNA into *Escherichia coli rpoS* mutant ZK918 and selecting for restoration of RpoS-regulated low pH survival as described by Zambrano et al. (25). A clone containing a 4.2-kb *Sal*I-*Eco*RI BC4B genomic DNA fragment containing *rpoS* was named pSERS1 (Table 1; Fig. 1). ZK918 (Table 1) contains a λ lysogen in which *lacZ* expression is under the control of the RpoS-dependent *E. coli bolA* promoter. *bolA* is responsible for cell morphology changes during entry to stationary phase by *E. coli* cells (11). Complementation of ZK918 by pSERS1 led to the expression of *lacZ* and restoration of hydroperoxidase HPII activity due to the *rpoS*-dependent expression of *katE* (15), further verifying that pSERS1 contains an *rpoS* analogue.

To determine the DNA sequence of the *S. entomophila rpoS* gene, *Bam*HI and *Bam*HI-*Pst*I fragments of pSERS1 were subcloned into vector pBluescript KS(+) and sequenced by using the dideoxynucleotide chain termination method (20). Sequencing of double-stranded DNA was performed with a T7 sequencing Kit (Pharmacia) using T3, T7, SK, or specific primers. This procedure yielded 1,176 bp of DNA sequence, including the entire predicted *S. entomophila rpoS* gene. Analysis of the *S. entomophila rpoS* nucleotide sequence with the computer program DNASIS (Hitachi Software Engineering Co.) predicted an open reading frame of 999 bp encoding a putative protein with a molecular mass of 38.3 kDa. Nucleotide and predicted protein sequences were aligned with known sequences in the GenBank sequence database by using BLAST (1). The predicted RpoS protein of *S. entomophila* showed overall identities to the RpoS proteins of *Salmonella enterica* serovar Typhimurium (94%), *E. coli* (93.7%), *Shigella flexneri* (90.6%), and *P. fluorescens* (75%) (respective GenBank EMBL database no. X77752, U29579, P35540, and U34203).

Creation and description of an *S. entomophila rpoS* mutant. An *rpoS*-negative mutant strain of BC4B was created to assess whether RpoS is involved in the amber disease process. A 680-bp *Bam*HI fragment of pSERS1 was replaced with a 1.4-kb *Bam*HI kanamycin marker cassette from pNK2859 (9) to create clone pSERS3 (Fig. 1). Homologous recombination between the interrupted *rpoS* allele of pSERS3 and the functional *rpoS* allele of BC4B created strain BC4BRS. Recombination was confirmed by Southern hybridization (data not shown). The presence of pADAP in BC4B and BC4BRS was confirmed by the preparation of plasmid DNA from these strains by the method of Kado and Liu (8).

RpoS is responsible for the expression of numerous proteins in *E. coli* during the stationary phase (13, 22). Similarly, two-dimensional gel electrophoresis of total cell proteins expressed during stationary phase by BC4B and BC4BRS showed many significant differences (data not shown) indicating the pleiotrophic effect of the *rpoS* mutation in *S. entomophila*.

Two differences were noted between the *S. entomophila rpoS* mutant created during this study and *rpoS* mutants of *E. coli*. Firstly, *E. coli* possesses two distinct catalases-hydroperoxidases, HPI(*katG*) and HPII(*katE*), of which HPII expression is regulated by *rpoS* (15, 16). In comparison, *S. entomophila* ap-

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TABLE 1. Bacterial strains and plasmids used during this investigation

Strain or plasmid	Genotype or description	Reference or source
Strains		
E. coli ZK918	ZK126 *rpoS*(IS insert) λ(*bolA*::*lacZ*) lysogen[a]	R. Kolter
S. entomophila		
BC4B	EMS-induced phage^R derivative of A1MO2; Path$^+$	19
BC4BRS	Marker-interrupted *rpoS*-minus mutant of BC4B	This study
Plasmids		
pADAP	Cryptic plasmid found in pathogenic strains of *S. entomophila* and *S. proteamaculans* encoding amber disease determinants	4
pALC	pLacZ3 containing a chloramphenicol cassette and an *anfA1*::*lacZ* fusion	This study
pLacZ3	*lacZ* translational-fusion vector	7
pLC	pLacZ3 containing a chloramphenicol cassette	This study
pSERS1	pBR322 with a 4.2-kb *Eco*RI-*Sal*I genomic DNA fragment with the *rpoS* gene of BC4B	This study
pSERS2	pACYC184 with a 4.0-kb *Sal*I-*Ava*I genomic DNA fragment with the *rpoS* gene of BC4B	This study
pSERS3	Deletion of a 0.6-kb *Bam*HI fragment of pSERS1 and insertion of a *Bam*HI fragment from pNK2859 (10) encoding kanamycin resistance	This study
pSERS4	*Eco*RI-*Sal*I fragment from pSERS3 cloned into pLAFR3	This study

[a] IS, insertion sequence.

pears to possess a single, highly active catalase that is produced in similar amounts by BC4B and BC4BRS. Secondly, the ability of *S. flexneri* and *E. coli* to withstand several hours below pH 3 during stationary phase is regulated by *rpoS* (23). In contrast, viable cell counts of BC4B and BC4BRS dropped away rapidly below pH 4.5, the point at which even log phase *E. coli* and *S. flexneri* cells can survive. Therefore, it appears that BC4B does not possess at least two of the known *rpoS*-regulated stationary phase survival mechanisms of *E. coli* and *S. flexneri*.

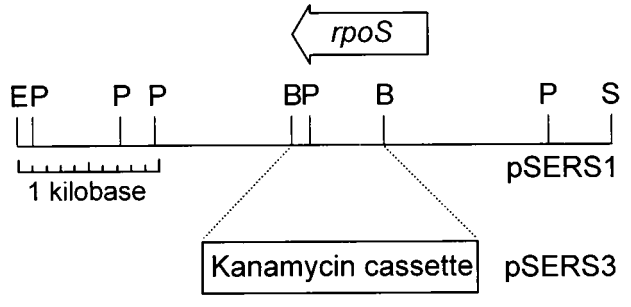

FIG. 1. Construction of strain BC4BRS, a kanamycin-marked *rpoS* mutant of *S. entomophila* strain BC4B. Restriction enzyme cleavage sites are as follows: B, *Bam*HI; E, *Eco*RI; P, *Pst*I; S, *Sal*I. A 0.6-kb *Bam*HI fragment containing part of the *S. entomophila rpoS* gene was deleted from pSERS1 and replaced with a 1.4-kb *Bam*HI fragment cloned from pNK2859 that encodes kanamycin resistance. The 4.9-kb *Sal*I-*Eco*RI insert fragment of pSERS3 was cloned into pLAFR3 and used to replace the wild-type *rpoS* gene in *S. entomophila* BC4B by allelic exchange. The resulting *rpoS* mutant strain was named BC4BRS.

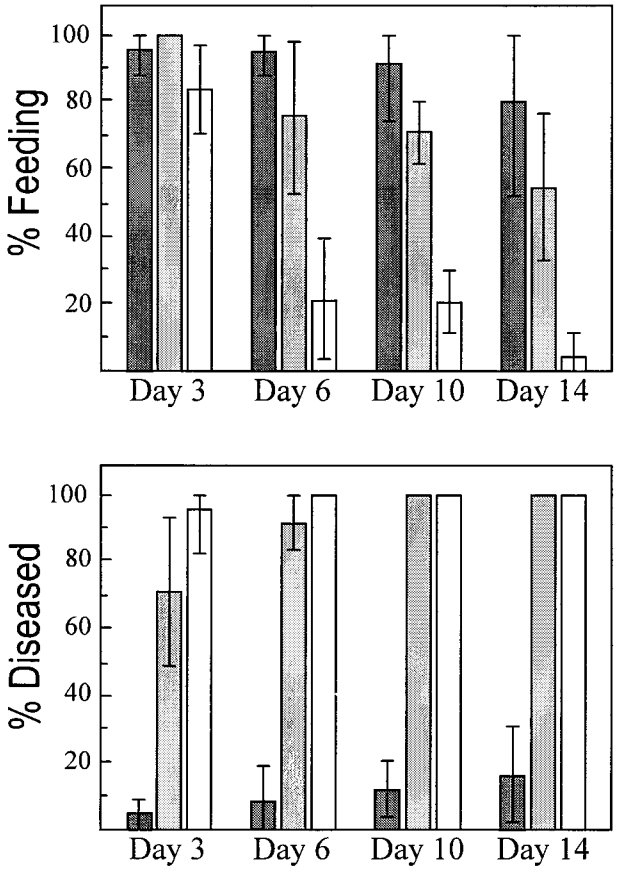

FIG. 2. Feeding and amber coloration assessment of *C. zealandica* larvae treated with water (■), BC4BRS (▩), or BC4B (□). Each result is the average of four replicates of six larvae per strain. Error bars represent 99% confidence intervals for those replicates in which variation was observed. Larvae were inoculated on day 1 and assessed for feeding and amber coloration on days 3, 6, 10, and 14. Percentage feeding and diseased represent, respectively, the number of grubs still feeding and the number of grubs that had become amber colored within each replicate.

The role of RpoS in the amber disease process. Bioassays were carried out as described previously (4) to compare the ability of BC4B and BC4BRS to cause amber disease in grass grub larvae (Fig. 2). Larvae infected with BC4BRS became amber colored, suggesting that development of this symptom is not affected by the mutation of *rpoS* in *S. entomophila*. However, most BC4BRS-infected larvae continued feeding and excreting waste, although not to the same extent as uninfected larvae. In contrast, after day 3 of the bioassay, nearly all larvae infected with BC4B had stopped feeding.

The *S. entomophila* nonpathogenic mutant UC24 possesses a Tn*phoA* insertion within a 5.3-kb region of DNA containing the locus *amb2* (17). Two genes within this locus, *anfA1* and *anfB*, are thought to be essential for the antifeeding effect (18). Because the Tn*phoA* insertion of UC24 is located within or near to the *anfA1* gene, we decided to investigate whether the expression of *anfA1* is affected by mutation to *rpoS* in *S. entomophila*.

A 1,050-bp *Hin*dIII fragment from the *amb2* region, including the region upstream of the *anfA1* translation start site and the first 20 predicted codons of *anfA1*, was cloned with fusion vectors pLacZ1, pLacZ2, and pLacZ3 (7, 17). Plasmid DNA from blue colonies on media containing 5-bromo-4-chloro-3-

TABLE 2. *lacZ*::*anfA* fusion expression in BC4B and BC4BRS[a]

Growth phase	Strain	β-Galactosidase activity	
		pLC	pALC
Exponential	BC4B	1	36
	BC4BRS	1	44
Stationary	BC4B	10	5,032
	BC4BRS	9	1,223

[a] β-galactosidase activity was measured as nanomoles of *O*-nitrophenol/min/10^6 plasmid-containing cells by the method of Miller (14). Individual assays were measured in triplicate, and the activity units of three separate assays were averaged for each data point.

indolyl-β-D-galactoside (Xg) was assessed by using *Kpn*I digestion to ensure correct orientation of the insert DNA within the vector. The correct reading frame for *anfA1*::*lacZ* fusion was provided by pLacZ3. Because *S. entomophila* has inherent resistance to ampicillin, an omega cassette encoding chloramphenicol resistance (3) was ligated to the *Eco*RI site of the pLacZ3 multicloning site upstream of the *anfA1* fusion fragment to create plasmid pALC. The cassette also provided additional translational and transcriptional stop signals to ensure that expression of *lacZ* is dependent only on information provided within the *anfA1* fusion fragment. An omega cassette encoding chloramphenicol resistance was also introduced to pLacZ3 to create plasmid pLC, which is devoid of the *anfA1* fusion fragment and thus served as a control to determine background β-galactosidase activity.

To determine the effect of a *rpoS* mutation in *S. entomophila* on the expression of *anfA1*, both pLC and pALC were introduced to BC4B and BC4BRS by electroporation. Cultures of these strains were grown in Luria-Bertani (LB) broth, supplemented with ampicillin (100 μg ml^{-1}) and chloramphenicol (30 μg ml^{-1}). Samples were taken when cultures had reached mid-exponential phase (optical density at 600 nm [OD_{600}], 0.3 to 0.6) and after 24 h (OD_{600}, 3.4 to 3.6). Cells were rinsed three times with minimal A media, and β-galactosidase expression was measured by the method of Miller (14). Viable cell counts on LB agar plates and LB agar plates supplemented with ampicillin and chloramphenicol indicated that plasmid maintenance was approximately 100% for exponential phase cells but dropped after 24 h of culture growth, to 50 to 80% for pLC and to 12 to 20% for pALC. Therefore, β-galactosidase activity was determined as nanomoles of *O*-nitrophenol/min/10^6 plasmid-containing cells.

The expression of *anfA1* was found to be low during the exponential phase and was induced to a high level after 24 h of growth (Table 2). *anfA1* expression in an *rpoS* mutant, measured as β-galactosidase activity per 10^6 cells, is approximately 25 times greater in stationary-phase cells than exponential-phase cells. In BC4B, *anfA1* expression is approximately four times greater in the stationary phase than in BC4BRS. The data presented in Table 2 indicate that stationary-phase-specific expression of *anfA1* is predominantly *rpoS* dependent, but a residual level of expression is *rpoS* independent, and this may be due to regulation by other sigma factors. It is therefore proposed that *rpoS* mediates its influence on the antifeeding potential of *S. entomophila*, at least in part, via its effect on the expression of *anfA1*. A residual level of *rpoS*-independent *anfA1* expression would result in low-level production of the antifeeding toxin during stationary phase. A reduced dose of antifeeding toxin may be responsible for the observation that *C. zealandica* larvae infected with BC4BRS continue to feed, but less actively than untreated larvae (data not shown). Indeed, Nunez-Valdez and Mahanty (17) found that repeated feeding of *C. zealandica* larvae with *E. coli* cells containing the *amb2* locus on a multicopy plasmid was necessary for the expression of the antifeeding phenotype, indicating that the toxin may act in a dose-dependent manner.

RpoS has been shown to have a role in the transcription of the plasmid-carried virulence operon *spvRABCD*, which is common to many *Salmonella* serovars (2, 10). In addition, the production of antifungal compounds and the suppression of fungal pests are influenced by RpoS in the potential biological control bacterium *P. fluorescens* Pf-5 (21). The present study has determined that RpoS influences the antifeeding component of amber disease and the expression of the antifeeding gene *anfA1*, which is required by *S. entomophila* BC4B for full biological control of *C. zealandica*.

Nucleotide sequence accession number. The DNA sequence data for the *S. entomophila rpoS* gene described in this paper have been deposited in the GenBank database under accession no. U35777.

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REFERENCES

1. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
2. **Fang, F. C., S. J. Libby, N. A. Buchmeier, P. C. Loewen, J. Switala, J. Harwood, and D. G. Guiney.** 1992. The alternative σ factor KatF (RpoS) regulates *Salmonella* virulence. Proc. Natl. Acad. Sci. USA **89:**11978–11982.
3. **Fellay, R., J. Frey, and H. Krisch.** 1987. Interposon mutagenesis of soil and water bacteria: a family of DNA fragments designed for in vitro insertional mutagenesis of Gram-negative bacteria. Gene **52:**147–154.
4. **Glare, T. R., G. E. Corbett, and T. J. Sadler.** 1993. Association of a large plasmid with amber disease of the New Zealand grass grub, *Costelytra zealandica*, caused by *Serratia entomophila* and *S. proteamaculans*. J. Invertebr. Pathol. **62:**165–170.
5. **Grkovic, S., T. R. Glare, T. A. Jackson, and G. E. Corbett.** 1995. Genes essential for amber disease in grass grubs are located on the large plasmid found in *Serratia entomophila* and *Serratia proteamaculans*. Appl. Environ. Microbiol. **61:**2218–2223.
6. **Jackson, T. A., A. M. Huger, and T. R. Glare.** 1993. Pathology of amber disease in the New Zealand grass grub, *Costelytra zealandica* (Coleoptera: Scarabaeidae). J. Invertebr. Pathol. **61:**123–130.
7. **Jain, C.** 1993. New improved *lacZ* gene fusion vectors. Gene **133:**99–102.
8. **Kado, C. I., and S. T. Liu.** 1981. Rapid procedure for the detection and isolation of large and small plasmids. J. Bacteriol. **145:**1365–1373.
9. **Kleckner, N., J. Bender, and S. Gottesman.** 1991. Uses of transposons with emphasis on Tn*10*. Methods Enzymol. **204:**139–179.
10. **Kowarz, L., C. Coynault, V. Robbe-Saule, and F. Norel.** 1994. The *Salmonella typhimurium katF* (*rpoS*) gene: cloning, nucleotide sequence, and regulation of *spvV* and *spvABCD* virulence plasmid genes. J. Bacteriol. **176:**6852–6860.
11. **Lange, R., and R. Hengge-Aronis.** 1991. Growth phase-regulated expression of *bolA* and morphology of stationary-phase *Escherichia coli* cells are controlled by the novel sigma factor σ^S. J. Bacteriol. **173:**4474–4481.
12. **Loewen, P. C., and R. Hengge-Aronis.** 1994. The role of the sigma factor σ^S (KatF) in bacterial global regulation. Annu. Rev. Microbiol. **48:**53–80.
13. **McCann, M. P., J. P. Kidwell, and A. Matin.** 1991. The putative σ^S factor KatF has a central role in development of starvation-mediated general resistance in *Escherichia coli*. J. Bacteriol. **173:**4188–4194.
14. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
15. **Mulvey, M. R., P. A. Sorby, B. L. Triggs-Raine, and P. C. Loewen.** 1988. Cloning and physical characterisation of *katE* and *katF* required for catalase HPII expression in *Escherichia coli*. Gene **73:**337–345.
16. **Mulvey, M. R., and P. C. Loewen.** 1989. Nucleotide sequence of *katF* of *Escherichia coli* suggests KatF protein is a novel σ transcription factor. Nucleic Acids Res. **17:**9979–9991.
17. **Nunez-Valdez, M. E., and H. K. Mahanty.** 1996. The *amb2* locus from *Serratia entomophila* confers anti-feeding effect on larvae of *Costelytra zealandica* (Coleoptera: Scarabaeidae). Gene **172:**75–79.
18. **Nunez-Valdez, M. E.** 1994. Identification and analysis of the virulence factors

in *Serratia entomophila* causing amber disease to the grass grub *Costelytra zealandica*. A molecular genetics approach. Ph.D. thesis. University of Canterbury, Christchurch, New Zealand.
19. **O'Callaghan, M., T. A. Jackson, and H. K. Mahanty.** 1992. Selection, development and testing of phage-resistant strains of *Serratia entomophila* for grass grub control. Biocontrol Sci. Technol. **2:**297–305.
20. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
21. **Sarniguet, A., J. Kraus, M. D. Henkels, A. M. Muehlchen, and J. E. Loper.** 1995. The sigma factor σ^S affects antibiotic production and biological control activity of *Pseudomonas fluorescens* PF-5. Proc. Natl. Acad. Sci. USA **92:**12255–12259.
22. **Schellhorn, H. E., J. P. Audia, L. I. C. Wei, and L. Chang.** 1998. Identification of conserved, RpoS-dependent stationary-phase genes of *Escherichia coli*. J. Bacteriol. **180:**6283–6291.
23. **Small, P., D. Blankenhorn, D. Welty, E. Zinser, and J. I. Slonczewski.** 1994. Acid and base resistance in *Escherichia coli* and *Shigella flexneri*: role of *rpoS* and growth pH. J. Bacteriol. **176:**1729–1737.
24. **Upadhyaya, N. M., T. R. Glare, and H. K. Mahanty.** 1992. Identification of a *Serratia entomophila* genetic locus encoding amber disease in New Zealand grass grub (*Costelytra zealandica*). J. Bacteriol. **174:**1020–1028.
25. **Zambrano, M. M., D. A. Siegele, M. Almirón, A. Tormo, and R. Kolter.** 1993. Microbial competition: *Escherichia coli* mutants that take over stationary phase cultures. Science **259:**1757–1760.