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Plasmid-Located Pathogenicity Determinants of *Serratia entomophila*, the Causal Agent of Amber Disease of Grass Grub, Show Similarity to the Insecticidal Toxins of *Photorhabdus luminescens*

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***Serratia entomophila* and *Serratia proteamaculans* cause amber disease in the grass grub *Costelytra zealandica* (Coleoptera: Scarabaeidae), an important pasture pest in New Zealand. Larval disease symptoms include cessation of feeding, clearance of the gut, amber coloration, and eventual death. A 115-kb plasmid, pADAP, identified in *S. entomophila* is required for disease causation and, when introduced into *Escherichia coli*, enables that organism to cause amber disease. A 23-kb fragment of pADAP that conferred disease-causing ability on *E. coli* and a pADAP-cured strain of *S. entomophila* was isolated. Using insertion mutagenesis, the pathogenicity determinants were mapped to a 17-kb region of the clone. Sequence analysis of the 17-kb region showed that the predicted products of three of the open reading frames (*sepA*, *sepB*, and *sepC*) showed significant sequence similarity to components of the insecticidal toxin produced by the bacterium *Photorhabdus luminescens*. Transposon insertions in *sepA*, *sepB*, or *sepC* completely abolished both gut clearance and cessation of feeding on the 23-kb clone; when recombined back into pADAP, they abolished gut clearance but not cessation of feeding. These results suggest that SepA, SepB, and SepC together are sufficient for amber disease causation by *S. entomophila* and that another locus also able to exert a cessation-of-feeding effect is encoded elsewhere on pADAP.**

Amber disease of the New Zealand grass grub *Costelytra zealandica* (Coleoptera: Scarabaeidae) is caused by some strains of *Serratia entomophila* and *Serratia proteamaculans* (*Enterobacteriaceae*). The disease was first described by Trought et al. (40), and an isolate of *S. entomophila* was subsequently developed into a commercially available biological control agent for *C. zealandica* in New Zealand (26). The disease is highly host specific, affecting only a single species of scarab that is indigenous to New Zealand (24). The disease is chronic, with a prolonged infection phase before bacteria invade the hemocoelic cavity, causing death (25). Amber disease has a distinct phenotypic progression, with infected hosts ceasing feeding within 2 to 4 days of ingesting pathogenic bacteria. At this time, levels of the major gut digestive enzymes decrease sharply (23) and the normally black-gray gut clears (25), resulting in a characteristic amber color of the infected insects. The infected larva may remain in this state for a prolonged period (1 to 3 months) before the infecting bacteria eventually invade the hemocoel, causing death.

The disease determinants of *S. entomophila* are encoded on a 115-kb plasmid designated pADAP, for amber disease-associated plasmid (17). pADAP has been transferred by conjugation to *Enterobacter agglomerans*, *Escherichia coli*, a *Klebsiella* sp., and the *Serratia* species *S. proteamaculans*, *S. marcescens*, and *S. liquefaciens*. Acquisition of pADAP by these species confers pathogenicity towards grass grub larvae (18).

To identify pathogenicity determinants on pADAP, Grkovic et al. (20) mutated a number of cloned *Hin*dIII fragments from pADAP with the mini-Tn*10* derivative 103 and recombined the insertion mutations back into pADAP to form pADK derivatives. Bioassays of *S. entomophila* strains containing the various pADK derivatives against grass grub larvae showed that 21 of the mutations had no detectable effect on pathogenicity toward the grass grub. However, in contrast to larvae infected with wild-type *S. entomophila*(pADAP), where there is a cessation of feeding and clearance of the larval gut, seven mutants induced a phenotype of nonfeeding with no gut clearance. The mutations in these strains were all located throughout a single 11-kb *Hin*dIII fragment, but one insertion (pADK-35) in the central region of the fragment had no effect on the disease process. Complementation analysis of the pADAP recombinants that contained insertions on either side of the pADK-35 insertion (pADK-10 and pADK-13) showed that only the pADK-13 region was complemented by the subcloned 11-kb fragment. The subclone itself did not enable a pADAP-cured strain of *S. entomophila* to induce any disease symptoms, indicating that it did not contain all of the essential pathogenicity determinants of pADAP.

In this report, we describe the identification, cloning, mutagenesis, and nucleotide sequence analysis of a region of pADAP that is sufficient to confer pathogenicity toward grass grub on both *E. coli* and pADAP-cured *S. entomophila* bacteria.

MATERIALS AND METHODS

Bacterial strains and methods of culture. Table 1 lists bacterial strains and plasmids used in this study. Bacteria were grown in Luria-Bertani (LB) broth or on LB agar (37) at 37°C for *E. coli* and 30°C for *S. entomophila*. For *Serratia*, the antibiotics kanamycin, chloramphenicol, and tetracycline were used at 100, 90, and 30 μg/ml, respectively; for *E. coli*, kanamycin, chloramphenicol, tetracycline, and ampicillin were used at 50, 30, 15, and 100 μg/ml, respectively.

DNA isolation and manipulation. pADAP DNA was isolated from a 50-ml overnight culture of bacteria using a Qiagen (Hilden, Germany) plasmid maxi kit according to the manufacturer's instructions. Standard DNA techniques were carried out as described by Sambrook et al. (37). Radioactive probes were made using the Amersham (Buckinghamshire, United Kingdom) Megaprime DNA

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TABLE 1. Bacterial strains, plasmids, and bacteriophage used in this study

Strain, plasmid, or phage	Description	Reference or source
Bacterial strains		
E. coli		
DH5α	F$^-$ ϕ80d *lacZ*ΔM15 Δ*lacZYA-argFU169 recA1 endA1 supE44*	22
DH10B	F$^-$ *mcrA* Δ*mrr-hsdRMS-mcrBC*ϕ80d *lacZ*ΔM15 Δ*lacX74 endA1 recA1 deoR*Δ*ara leu 7697 araD139 galU galK nupG rpsL* λ$^-$	31
DF1	γδ transposase *tnpA*	Gibco BRL
MC1061	*sup*^{0} *hsdR mcrB araD139* Δ*araABC-leu7679* Δ*lacX74 galU galK rpsL thi*	7
MC4100	*araD139* Δ*lacZYA-argFU169 rpsL150* St^r *relA1 flbB5301 deoC1 ptsF25 rbsR*	38
XL1-BlueMRA	Δ*mcrA183* Δ*mcrCB-hsdSMR-mrr-173 endA1 supE44 thi-1 reA1 gyrA96 relA1*	Stratagene
S. entomophila		
A1MO2	Ap^r, pADAP, pathogenic	19
5.6	Heat-cured pADAP-minus derivative of A1MO2	17
5.6RC	Cm^r *recA* pADAP-minus strain	20
5.6RK	Kn^r *recA* pADAP-minus strain	This study
Plasmids		
pACYC184	Cm^r Tc^r	8
pADAP	Amber disease-associated plasmid	17
pBR322	Ap^r Tc^r	3
pBM32	23-kb *Bam*HI fragment from pMH32 cloned in pBR322	This study
pBM32-1-40	pBM32 containing mini-Tn*10* insertions	This study
pDELTA1	Ap^r Sm^r Kn^r, sucrose^r	Gibco BRL
pLAFR3	Tc^r pRK290 with λ*cos*, *lacZ*α, and multicloning site from pUC8	39
pGLA20	11-kb *Hin*dIII pADAP fragment cloned in pLAFR3	20
pADK-13	pADAP::mini-Tn*10* insertion in 10.6-kb *Hin*dIII fragment, Kn^r, nonpathogenic	20
pADK-35	pADAP::mini-Tn*10* insertion in 10.6-kb *Hin*dIII fragment, Kn^r, pathogenic	20
pMH32	23-kb *Bam*HI fragment of pADAP cloned into pLAFR3	This study
pMH41	33-kb *Bam*HI fragment of pADAP cloned into pLAFR3	This study
pBM32	23-kb *Bam*HI fragment of pMH32 cloned into pBR322	This study
pUC19	Ap^r, *lacZ*α, multicloning site	42
Bacteriophage		
λNK1316	Mini-Tn*10* derivative 103 donor λ*b*522 *c*I857 *P*am80 *nin*5	27

labeling system. Southern blot and colony hybridizations were performed as described by Sambrook et al. (37).

Introduction of plasmid DNA into *E. coli* and *S. entomophila*. pLAFR3- and pBR322-based plasmids were electroporated into *E. coli* and *S. entomophila* strains using a Bio-Rad Gene Pulser (25 μF, 2.5 kV, and 200 ohms) (10).

Mutagenesis. Transposon insertions were generated in recombinant plasmids using the mini-Tn*10* derivative 103 (kanamycin resistant) carried on λNK1316, as described by Kleckner et al. (27). Insertions were recombined into pADAP by transforming strain A1MO2 (Table 1) with the desired pLAFR3-based construct. After 5 days of growth in nonselective medium, bacteria were selected for resistance to kanamycin and screened for loss of the pLAFR3 tetracycline resistance marker (approximately 34% of the kanamycin-resistant colonies were tetracycline sensitive).

Bioassay against *C. zealandica* larvae. Infection of *C. zealandica* larvae was determined by a standard bioassay (20) where healthy larvae, collected from the field, were individually fed cubes of carrot (3 mm^3) which had been rolled in colonies of bacteria grown overnight on solid medium, resulting in approximately 10^7 cells per carrot cube. Twelve second- or third-instar larvae were used for each treatment. Inoculated larvae were maintained at 15°C in ice cube trays. Larvae were fed treated carrot at day 1; at days 3 and 6, they were transferred to fresh trays containing untreated carrot (3 mm^3). The occurrence of gut clearance and cessation of feeding were monitored at days 3, 6, and 12. Strains were tested for loss of disease-causing ability by comparing numbers of diseased larvae in treated with known pathogenic and nonpathogenic bacterial controls after 12 days using a one-tailed paired *t* test.

Recovery of bacteria from larvae. To isolate bacteria from inoculated grubs, larvae were first surface sterilized by submersion in 70% methanol for 30 s. The larvae were then shaken in sterile distilled water, removed, and individually macerated in 1.5-ml microcentrifuge tubes. Each macerate was serially diluted and plated on LB medium containing antibiotics selective for the host *S. entomophila* strain. To assess the maintenance of the plasmid in the bioassayed strain, colonies were patched onto a plate containing antibiotics selective for the recombinant plasmid. Identity of plasmids in the recovered strains was checked by restriction enzyme profiling.

Nucleotide sequencing. A 9-kb *Bam*HI-*Eco*RI fragment derived from pBM32-8 (Fig. 1C) and the 8-kb *Hin*dIII fragment of pBM32 were separately cloned into the appropriate sites of the deletion factory plasmid pDELTA1 (Gibco BRL, Rockville, Md.). Deletions were generated using the deletion factory system as outlined in the manufacturer's instructions.

To identify the precise locations of mini-Tn*10* insertions, the peripheral *Bam*HI sites located within the ends of mini-Tn*10* were used in conjunction with the *Bam*HI sites of the cloned region to subclone the regions flanking the mini-Tn*10* insertion into either pACYC184 or pUC19. Sequences were generated using the mini-Tn*10*-specific primer 5′ ATGACAAGATGTGTATCCACC 3′ (27).

Plasmid templates for sequencing were prepared using Wizard (Promega, Madison, Wis.) or Quantum-Prep (Bio-Rad) miniprep kits. Sequences were determined on both strands using combinations of subcloned fragments, custom primers, and deletion products derived from the deletion factory system. The DNA was sequenced either by using [^{33}P]dCTP and the Thermosequenase cycle sequencing kit (Amersham) or by automated sequencing using an Applied Biosystems 373A or 377 autosequencer. Sequence data were assembled using SEQMAN (DNASTAR Inc., Madison, Wis.). Databases at the National Center for Biotechnology Information were searched using BLASTN and BLASTX (1). Searches for open reading frames (ORFs), DNA repeats, and inverted repeats were undertaken using DNAMAN (Lynnon BioSoft, Quebec, Quebec, Canada). Searches for protein motifs were carried out using Blocks (http://www.blocks.fhcrc.org/), ExPASy (http://www.expasy.ch/), and Gene Quiz (http://columba.ebi.ac.uk:8765/gqsrv/submit).

Nucleotide sequence accession number. The sequence determined in this study has been deposited in GenBank under accession number AF135182.

RESULTS

Cloning the major virulence determinants from pADAP. Complementation analysis of the pADK-10 and -13 mutants with the 11-kb *Hin*dIII fragment cloned in pLAFR3 to give pGLA20 showed that only the pADK-13 insertion was complemented, suggesting that the locus inactivated by the pADK-10 insertion was not fully contained within the fragment (20). To define the region required to complement the pADK-10 mutation, a 13-kb *Bgl*II fragment from pADK-35 that included the

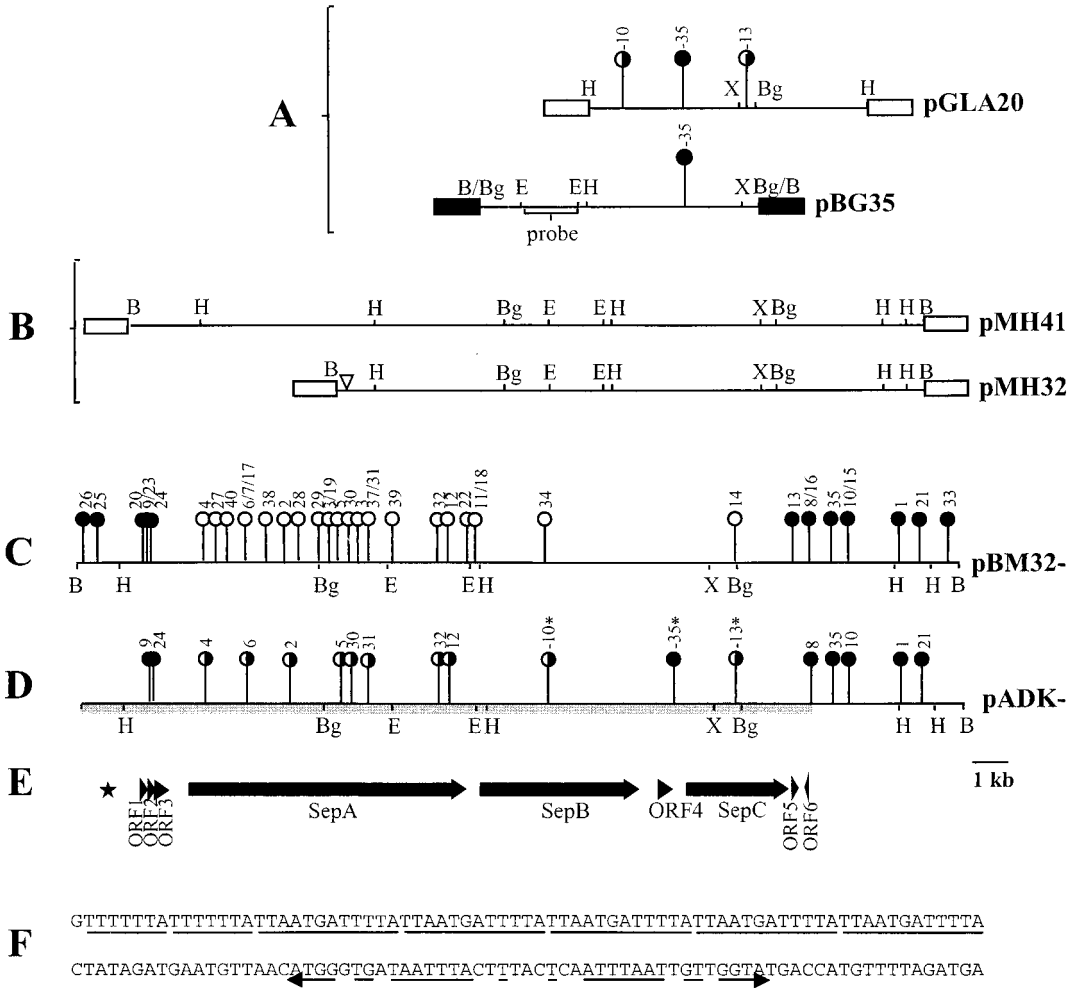

FIG. 1. (A) The *Hin*dIII fragment from pADAP cloned into pLAFR3 to form pGLA20, showing locations of the mini-Tn*10* 103 insertion mutations at positions -10, -13, and -35 (18). Results of bioassay of mutants against the grass grub are shown. The map of pBG35 shows the relative position of pGLA20-35 mutation and the location of the 2.2-kb *Eco*RI fragment used as a probe to screen the pADAP *Bam*HI library. (B) Restriction enzyme maps of the pathogenic clones pMH32 and pMH41. (C) Locations and phenotypes of mini-Tn*10* insertions in pBM32. (D) Bioassay results of the pADK recombinants. (E) Schematic diagram of the sequenced region. (F) Nucleotide sequence of the 7-bp repeat, five-copy 12-bp repeat, and the downstream degenerate 34-bp inverted repeat. *, pADK mutations isolated by Grkovic et al. (20); filled circles, mutations that resulted in an unaltered pathogenic phenotype (clear gut, nonfeeding); open circles, mutations that resulted in the abolition of pathogenicity; half-filled circles, mutations that induced a nonfeeding pathotype without clearance of the gut; ▽, site of internal deletion; ■, pBR322 vector DNA; □, pLAFR3; ★, location of nucleotide repeats. Arrows indicate ORFs and their orientation. Abbreviations for restriction enzymes: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; X, *Xba*I.

mini-Tn*10* insertion and encompassed the sites of both the pADK-10 and pADK-35 mutations was cloned into the *Bam*HI site of pBR322 to form pBG35. pBG35 was placed separately in *trans* with pADK-10 and pGLA20 in the pADAP-cured *S. entomophila* strain 5.6RC, and the resultant strains were bioassayed against grass grub larvae. Results showed that pBG35 complemented strain 5.6RC(pADK-10) but did not confer the ability to induce any of the disease symptoms on strain 5.6RC (pGLA20), suggesting that there must be a region of pADAP in addition to that encoded by the pGLA20 and pBG35 fragments needed to induce amber disease.

Restriction enzyme mapping of pGLA20 and pBG35 showed that neither contained a *Bam*HI site, indicating that the cloned DNA from both plasmids was contained within a large (>15-kb) *Bam*HI fragment of pADAP. A *Bam*HI library of pADAP was made and screened using a 2.2-kb *Eco*RI fragment derived from pBG35 (Fig. 1A) as the probe. Several probe-positive clones were isolated, and all had similar restriction enzyme profiles. However, one (designated pMH32) was smaller, with an inserted *Bam*HI fragment of only 23 kb, compared with the 33-kb insert of the other clones (e.g., pMH41 [Fig. 1B]). The difference between pMH32 and pMH41 was found to be a 10-kb truncation at one end of pMH32 that included one *Hin*dIII site (Fig. 1B). Recent sequence data have shown that the site of truncation is at bp 170 of the sequence generated in this study (M. R. H. Hurst, unpublished data).

When bioassayed against grass grub larvae, *E. coli* strains containing pMH32 or pMH41 induced the full symptoms of amber disease (i.e., gut clearance and cessation of feeding activity). However, about 10 days after infection, approximately 25% of the grass grub larvae fed the *E. coli* strains recovered from a diseased to a healthy phenotype. This may reflect either poor persistence of the *E. coli* strains in the grass grub or poor expression of the cloned genes. Therefore, all further studies of the cloned loci were done in *S. entomophila* backgrounds.

TABLE 2. Disease-causing ability of the cloned virulence-encoding region, its mutated derivatives, and pADAP recombined mutations[a]

Positive (A1MO2), 91–100%[b]				Negative (untreated carrot), 5.3–22.8%[b]			
Construct	Mean % showing disease symptoms	One-tailed *t* test[c]		Construct	Mean % showing disease symptoms	One-tailed *t* test	
		df	*P*			df	*P*
Virulence-encoding clones							
pBM32	97.8	3	NV	pMH32	97.0	2	0.211
pMH41	95.5	1	0.250				
pBM32 derivatives[d]							
pBM32-1	97.0	2	0.500	pBM32-21	88.7	2	0.211
pBM32-2	16.5	1	0.052	pBM32-22	8.3	2	0.005*
pBM32-3	4.0	1	0.015*	pBM32-23	91.5	3	0.196
pBM32-4	12.50	1	0.050	pBM32-24	94.0	2	0.500
pBM32-5	25.0	—		pBM32-25	94.0	2	0.211
pBM32-6	4.0	1	0.029*	pBM32-26	93.3	3	NV
pBM32-7	8.0	—		pBM32-27	10.5	1	0.041*
pBM32-8	94.0	2	0.211	pBM32-28	94	2	0.001*
pBM32-9	93.3	3	0.091	pBM32-29	8.0	1	NV
pBM32-10	95.8	5	0.298	pBM32-30	8.0	1	NV
pBM32-11	2.9	3	0.029*	pBM32-31	10.5	1	0.056
pBM32-12	18.5	1	0.011*	pBM32-32	10.7	2	0.002*
pBM32-13	94.0	2	0.500	pBM32-33	91.0	4	0.035*
pBM32-14	8.0	1	0.031*	pBM32-34	0.0	2	0.000*
pBM32-15	95.5	1	NV	pBM32-35	87.5	3	0.187
pBM32-16	100.0	—		pBM32-36	97.0	2	0.211
pBM32-17	21.0	—		pBM32-37	2.7	2	0.000*
pBM32-18	91.0	1	0.250	pBM32-38	4.0	1	0.015*
pBM32-19	8.0	—		pBM32-39	4.0	1	0.029*
pBM32-20	93.0	4	0.102	pBM32-40	0.0	1	NV
pADAP pADK derivatives[e]							
pADK-1	97.0	2	0.211	pADK-21	94.0	2	0.092
pADK-4	8.3 (NF)	2	0.004*	pADK-22	12.0 (NF)	1	0.002*
pADK-5	12.0 (NF)	1	0.014*	pADK-23	93.3	3	0.091
pADK-6	4.0 (NF)	1	0.013*	pADK-24	91.0	—	
pADK-8	8.0 (NF)	—		pADK-30	13.7 (NF)	2	0.006*
pADK-9	97.0	2	0.211	pADK-31	4.0 (NF)	1	0.013*
pADK-10	88.7	2	0.131	pADK-32	19.0 (NF)	2	0.004*
pADK-11	12.0 (NF)	1	0.014*	pADK-34	13.3 (NF)	2	0.000*
pADK-12	95.5	1	0.250	pADK-35	100.0	3	NV
pADK-13	97.8	3	0.196	pADK-39	8.0 (NF)	1	0.028*

[a] NF, larvae were healthy in appearance but unable to feed.
[b] Range of controls over 80 batches.
[c] One-tailed paired *t* test over batches of the percentage of *C. zealandica* larvae showing full disease symptoms (amber coloration and inhibition of feeding) after ingestion of *Serratia entomophila* at day 12. NV, no variation; *, significant difference ($P < 0.05$); —, only one batch.
[d] See Fig. 1C for locations of pBM32 mutations.
[e] See Fig. 1D for locations of pADK mutations.

Plasmids pMH32 and pMH41 were subsequently introduced into an *S. entomophila* strain cured of pADAP (5.6RC), and the strains were bioassayed against grass grub larvae. The strains gave the same disease progression as the wild type, and no larvae were recovered (Table 2).

Effects of mini-Tn*10* insertions in pBM32 on disease-causing ability. To facilitate mutagenesis, the 23-kb *Bam*HI fragment from pMH32 was cloned into the medium-copy-number plasmid pBR322 to give pBM32. Bioassays of the strains containing pBM32 showed that it conferred the ability to induce amber disease in an *S. entomophila* (5.6RC) background (Table 2). Plasmid pBM32 was mutated with the mini-Tn*10* transposon derivative 103, and the sites of the insertions were mapped (Fig. 1C). Bioassays of *S. entomophila* strain 5.6RC derivatives containing the resultant mutated plasmids showed that the disease determinants were confined to a central 17-kb region of the *Bam*HI fragment. Each strain either had no effect or caused full disease symptoms (cessation of feeding and gut clearance) (Table 2).

Effects of mini-Tn*10* insertions in pADAP on disease-causing ability. Grkovic et al. (20) recombined the pGLA20-based mutations −10 and −13 into pADAP by homologous recombination (Fig. 1A and D). When bioassayed, *S. entomophila* strains containing either of these mutant pADAP plasmids caused a partial disease condition, inducing cessation of feeding but not gut clearance and amber coloration. This was in contrast to the complete abolition of disease observed with pADAP-cured *S. entomophila* strains containing mutant pBM32 plasmids with similar insertions (Table 2). To determine the phenotype of the pBM32-based insertions in a pADAP background, DNA fragments containing the pBM32 insertions at positions −1, −2, −4, −5, −6, −8, −9, −10, −12, −21, −24, −30, −31, −32, and −35 and flanking DNA were cloned separately into pLAFR3, and the inserted transposon was introduced into pADAP by homologous recombination (Fig. 1D). The resultant recombinant *S. entomophila* strains were checked by Southern analysis to confirm that recombination had occurred as expected and that no pLAFR3 vector

sequences were present (data not shown). The strains were then assayed against grass grub larvae (Table 2). Mutations that did not affect the disease process in pBM32 also had no effect on disease when recombined into pADAP. However, strains with the pADAP mutations that totally abolished the disease process when in pBM32 caused cessation of feeding but not gut clearance of the grubs (Fig. 1C and D).

Assessment of the stability of pBM32 and its mutated derivatives during the course of the bioassays showed that greater than 90% of the recovered *Serratia* strains contained the plasmid of interest.

Sequence analysis of the disease-encoding region. The *Bam*HI fragment (18,937 bp) derived from pBM32-8 was sequenced on both strands using a combination of constructed deletions, plasmid subclones, and custom-made primers. Structural analysis of the DNA sequence using DNAMAN showed that there was a 7-bp direct repeat at bp 671 to 684, followed by a 12-bp sequence repeated five times between positions 685 and 744. Downstream of the repeat region was a degenerate 39-bp inverted repeat (bp 763 to 801) (Fig. 1E and F). These repeat motifs are in a region of DNA that is AT rich and lacks any potential ORFs.

Translation of the nucleotide sequence revealed the presence of nine ORFs of more than 90 codons. Eight of the ORFs were oriented in the same direction, and the other was oriented in the opposite direction (Fig. 1E). Sequence similarity searches showed that the deduced products of seven of these ORFs showed similarity with known proteins (Table 3). ORF1 (100 amino acid residues) and ORF2 (91 amino acid residues) had no similarity to any proteins in the current databases. Products of three of the ORFs showed similarity to different protein components of insecticidal toxins of *Photorhabdus luminescens* (5). These ORFs were designated *sep* (*sepA*, *sepB*, and *sepC*), for *Serratia entomophila* pathogenicity.

The predicted protein product of *sepA* had high similarity throughout its entire length to the *P. luminescens* insecticidal toxin complex proteins TcbA, TcdA, TcaB, and TccB, with greatest similarity at the carboxyl terminus (Table 3; Fig. 2). Analysis for protein motifs showed that the tripeptide cell-binding motif Arg-Gly-Asp (RGD), implicated in the binding of various adhesion proteins produced by parasites and viruses to eukaryotic cells (29), is present in SepA and the *P. luminescens* TcdA, TcbA, and TcaB proteins (Fig. 2).

SepB and the *P. luminescens* insecticidal toxin complex protein TcaC showed similarity throughout their length, and both SepB and TcaC showed high amino-terminal similarity to the *Salmonella enterica* serovar Typhimurium virulence protein SpvB (21) (Fig. 3). The similarity of SepB and TcaC to SpvB diminished after SpvB amino acid residue 356.

SepC showed strong similarity to the amino-terminal region of the insecticidal toxin complex protein TccC, up to amino acid residue 732 of SepC (Fig. 4A). A number of putative bacterial cell wall-associated proteins also showed similarity to SepC, including the wall-associated precursor protein of *Bacillus subtilis* (WapA) (15), members of the *E. coli rhs* (recombination hot spot) elements (41), and hypothetical wall-associated proteins from *Streptomyces coelicolor* A3(2) and *Coxiella burnetii* (Table 3; Fig. 4B). Comparison of SepC to its homologues showed that all showed amino acid similarity to the carboxyl end of a so-called Rhs core region (41) (Fig. 4B) and diverged from each other in amino acid composition at a conserved glycine residue (SepC amino acid residue 666 [Fig. 4B and 5]). Further comparison of SepC with members of the Rhs family showed that it contained nine partial or complete matches to the Rhs core protein peptide motif GxxRYxY DxxGRL(I/T) (12, 41) (Fig. 4A).

TABLE 3. Similarities of products of putative ORFs to protein sequences in the database detected using BlastP

ORF, size (amino acids)	Protein homologue, size (amino acids)	Degree of similarity[a]	Function of similar protein	Organism	Accession no.
SepA, 2,376	TcbA, 2,504	34/50, 41–1628; 57/72, 1630–2374	Insecticidal toxin complex protein	*P. luminescens*	AF047457
	TcdA, 2,516	38/55, 33–1289; 40/54, 1499–1625; 58/71, 1630–2374	Insecticidal toxin complex protein	*P. luminescens*	AF188483
	TcaB, 1,189	29/50, 936–1198; 38/54, 1625–2374	Insecticidal toxin complex protein	*P. luminescens*	AF046867
	TccB, 1,565	31/51, 930–1204; 36/51, 1575–2373	Insecticidal toxin complex protein	*P. luminescens*	AF047028
	TcaA, 1,095	36/56, 94–183; 18/39, 435–928	Insecticidal toxin complex protein	*P. luminescens*	AF046867
	TccA, 965	27/45, 115–280	Insecticidal toxin complex protein	*P. luminescens*	AF047028
SepB, 1,428	TcaC, 1,485	49/63, 1–1263; 64/78, 1270–1421	Insecticidal toxin complex protein	*P. luminescens*	AF046867
	SpvB, 591	40/52, 9–365	*Salmonella* virulence protein	*Salmonella* serovar Typhimurium	S22664
SepC, 938	TccC, 1,043	53/66, 3–782	Insecticidal toxin complex protein	*P. luminescens*	AF047028
	Gene sc2h4.02, 2,183	23/34, 68–677	Hypothetical wall-associated protein	*S. coelicolor* A3(2)	CAA20596.1
	WapA, 2,334	20/36, 48–625; 22/34, 255–677	Wall-associated protein precursor	*B. subtilis*	Q07833
	Orf 774, 334	21/34, 181–684	Hypothetical wall-associated protein	*C. burnetii*	CAA75841
	Rhs core, 1,420	21/36, 35–300; 21/35, 237–677	Rhs core protein	*E. coli*	ACC32471
ORF3, 144	Gene 15, 263	45/62, 1–139	Morphogenesis protein of bacteriophage B103	*B. subtilis*	CAA67646.1
	Gene 19, 146	46/61, 1–143	Phage P22 lysozyme EC3.2.1.17	*Salmonella*	Q37896
ORF4, 191	Gp55, 181	28/42, 1–184	Bacteriophage N15 protein	*E. coli*	AAC19092.1
ORF5, 236	Tnp1294, 312	50/69, 18651–18391; 55/74, 18934–18752	Transposase	*E. coli*	S49612
ORF6, 310	IS*9J*	39/58, 18675–18394; 39/56, 18934–18725; 30/48, 18391–18164	IS*9J* transposase	*E. coli*	S23782

[a] Amino acid similarity (percent identity/percent similarity over the indicated range of amino acid residues) in relation to sequence generated in this study. Percent identities and similarities were calculated in relation to the deduced gene products of the sequenced ORF. Similarities were considered potentially significant if the BlastP score exceeded e^{-5}.

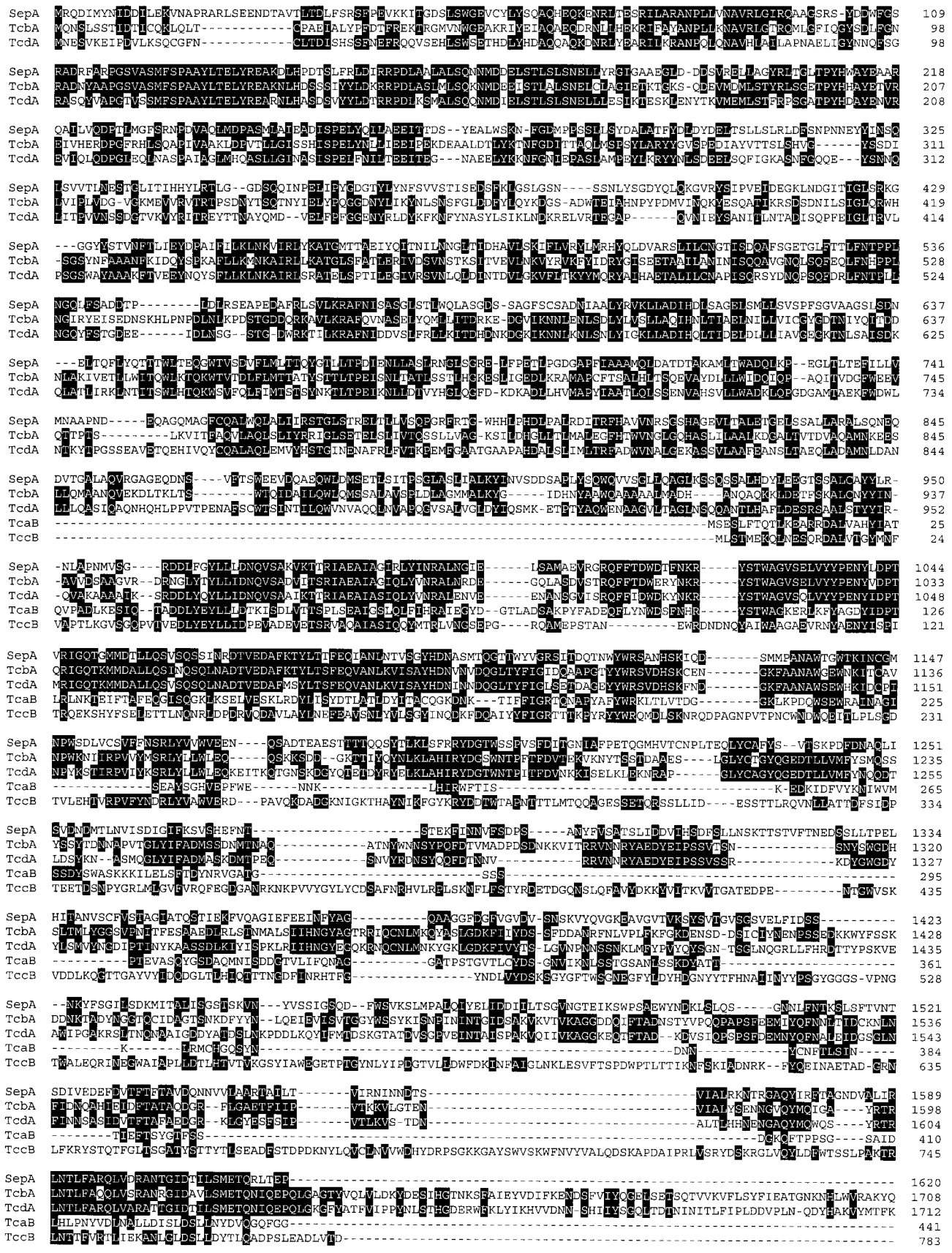

FIG. 2. Alignment of amino acid sequences of the SepA and *P. luminescens* toxin components TcbA, TcdA, TcaB, and TccB. ■, RGD motif.

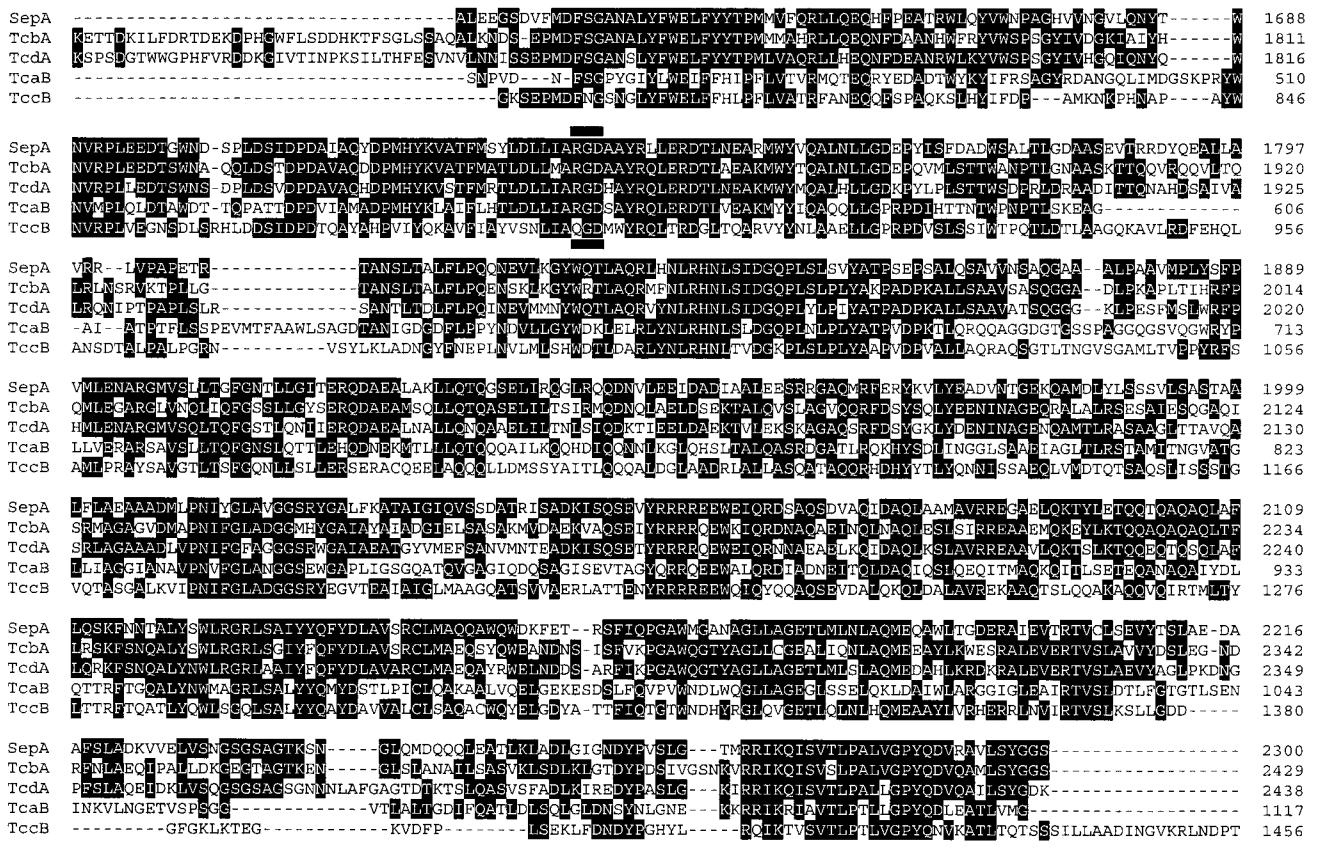

FIG. 2—*Continued.*

We found that the *sepA* and *sepB* genes were more GC rich (54 and 58% G+C) than their *P. luminescens* counterparts (43 and 44% [*tcbA* and *tcdA*] and 51% [*tcaC*] G+C), while *sepC* and *tccC* had similar GC contents (55 and 54% G+C). Similar to *rhs* elements, *sepC* is relatively GC rich (*sepC* bp 1 to 2031, 60% G+C) preceding the juxtaposition of the core and variable region but decreases in GC content thereafter (*sepC* bp 2032 to 2922, 44% G+C) (Fig. 5). The GC content of the *sep* genes was similar to that of the *S. entomophila* genome, which is 58% G+C (19). *tccC* also shows a strong reduction in GC content at the junction of the core and variable regions, but thereafter its 3′ region is GC rich. This may reflect the highly hydrophobic nature of the carboxyl terminus of TccC together with its high content of glycine and alanine residues, which together comprise 40% of the amino acids of the region and are encoded by GC-rich codons.

The hydropathicity profile of each of the Sep proteins was examined using the Kyte and Doolittle algorithm (28) and compared to the profiles of relevant *P. luminescens* homologues. None of the Sep or Tc proteins contained a characteristic signal sequence (16) or regions capable of spanning the cell membrane, except for the *P. luminescens* protein TccC, which has a highly hydrophobic carboxyl terminus from amino acid residue 719 onward (Fig. 5).

ORF3 precedes *sepA* (Fig. 1E) and has high similarity to the morphogenesis protein encoded by gene 15 of the *B. subtilis* bacteriophage B103 (33) and the product of gene 19 from the *Salmonella* bacteriophage P2, a protein essential for the lysis of the bacterial cell wall (35). Hydropathicity analysis showed that the translated product of ORF3 contains a hydrophobic amino terminus capable of spanning the lipid bilayer.

Located between *sepB* and *sepC* is ORF4, the translated product of which has similarity to the *E. coli* bacteriophage N15 gp55 protein, a protein of unknown function (Table 3), and contains an amino terminus capable of spanning the lipid bilayer.

Identification of mini-Tn*10* location by sequence analysis. Analysis of the insertion points of the mini-Tn*10* insertions (Fig. 1C) within the putative ORFs (Table 4) revealed that ORF3 and ORF4 were interrupted by the insertions that had no effect on the disease process. However, the pADAP-35 mutation was at the 3′ end of ORF4, resulting in a truncation of the final 11 amino acid residues of ORF4, which may not have affected protein function. Further mutagenesis of ORF4 is therefore required to confirm that it has no role in disease. The mutations that caused loss of disease-causing ability all resided within *sepA*, *sepB*, or *sepC*. No mutation mapped to ORF1, ORF2, or ORF5.

Complementation analysis. The sequence data indicated that pBG35 and pGLA20 contain *sepB* and *sepC*, respectively.

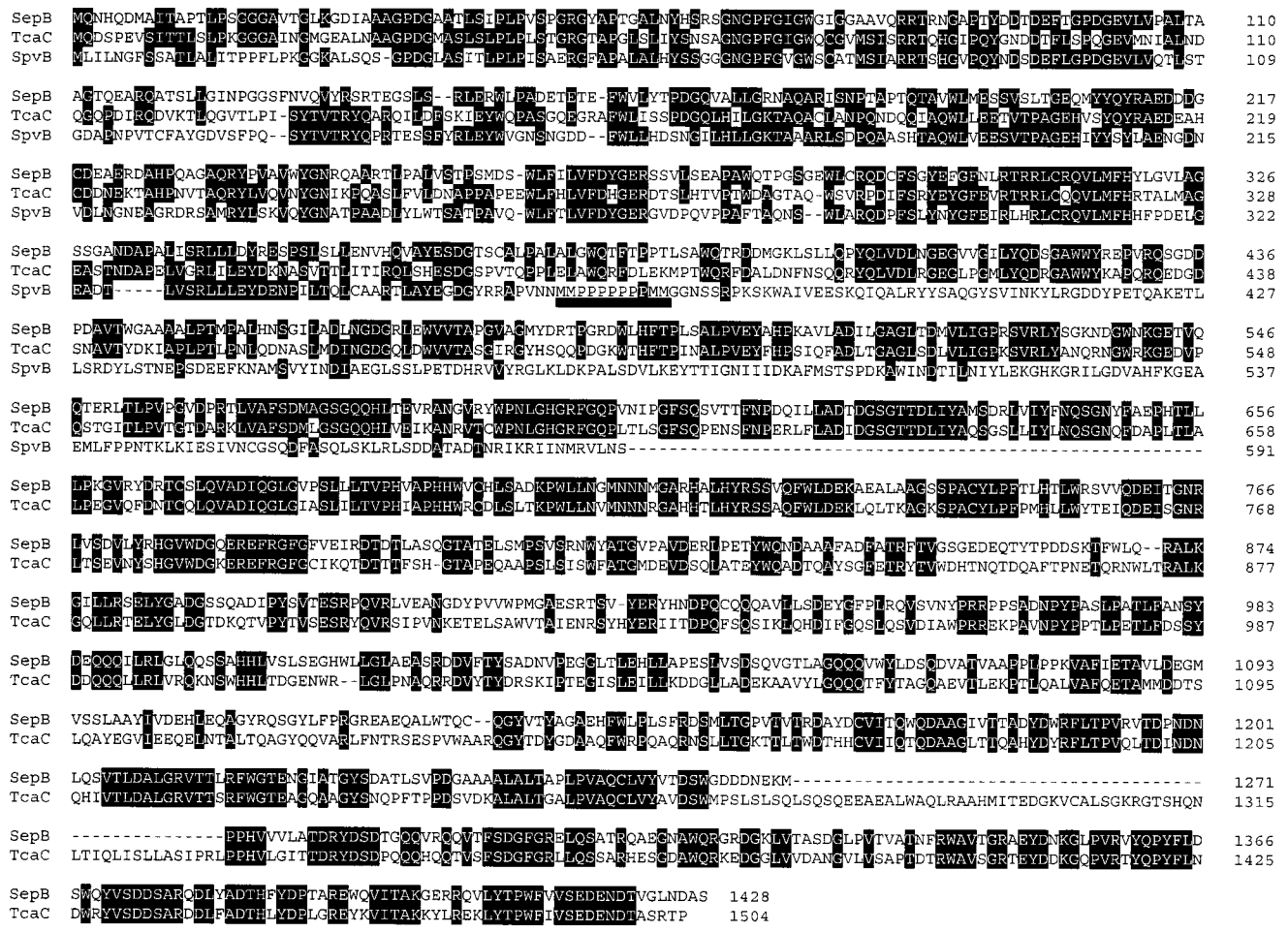

FIG. 3. Alignment of amino acid sequences of the SepB, *P. luminescens* toxin component TcaC, and SpvB.

The complementation data obtained with these plasmids indicate that both genes are essential for virulence. Attempts to complement *sepA* mutants with the 8.45-kb *Hin*dIII fragment encompassing the *sepA* gene cloned into pLAFR3 were unsuccessful. In these bioassays, 80% of the grubs remained healthy but nonfeeding. However, over 90% of *S. entomophila* bacteria isolated from macerates of healthy nonfeeding grubs had lost the complementing plasmid, whereas 80% isolated from diseased grubs retained the plasmid.

DISCUSSION

The large conjugative plasmid pADAP is present in all *S. entomophila* and *S. proteamaculans* strains capable of causing amber disease of the New Zealand grass grub *C. zealandica*; it encodes the genes responsible for the symptoms of amber disease, including cessation of feeding and the gut clearance that results in amber coloration of the grub (17). We have defined a 16.9-kb region of pADAP that is sufficient to confer disease-causing ability to *C. zealandica* on pADAP-cured strains of *S. entomophila* and on strains of *E. coli*. Mutagenesis and sequence analyses of the region indicated that it encodes three genes, *sepA*, *sepB*, and *sepC*, that are required for pathogenicity. The products of these genes show similarity to components of the insecticidal toxin complexes of the entomopathogen *P. luminescens*.

The cloned pathogenicity region conferred the ability to initiate all symptoms of amber disease on pADAP-cured *S. entomophila* strains, and insertion mutations in any of *sepA*, *sepB*, or *sepC* abolished disease. Complementation studies confirmed that *sepB* and *sepC* were both required for disease, but attempts to complement *sepA* mutants were unsuccessful. This was attributed to instability of the plasmid clone encoding SepA, suggesting that overexpression of the SepA protein may be detrimental to the growth of the host bacterium. Nevertheless, the fact that *sepB* mutants were complemented by a clone lacking *sepA* strongly suggests that the *sepA* mutant phenotype was not due to a downstream effect of the transposon insertion on *sepB* or *sepC* expression. Hence, it is likely that *sepA*, *sepB*, and *sepC* together comprise the entire complement of essential virulence genes on pADAP. However, when the *sep* insertion mutations were transferred to pADAP, the resultant strains showed a partial disease phenotype, inducing cessation of feeding but not gut clearance. This result suggests that another locus able to exert a cessation-of-feeding effect may be present elsewhere on pADAP. The findings that pADAP-cured strains of *S. entomophila* containing the cloned *sep* genes cause cessation of feeding and that higher doses of *sepB*(pADK-10) or *sepC*(pADK-13) mutants are required to induce cessation of feeding compared to the wild-type strain, as shown in dose-response assays (20), indicate that the *sep* gene products are

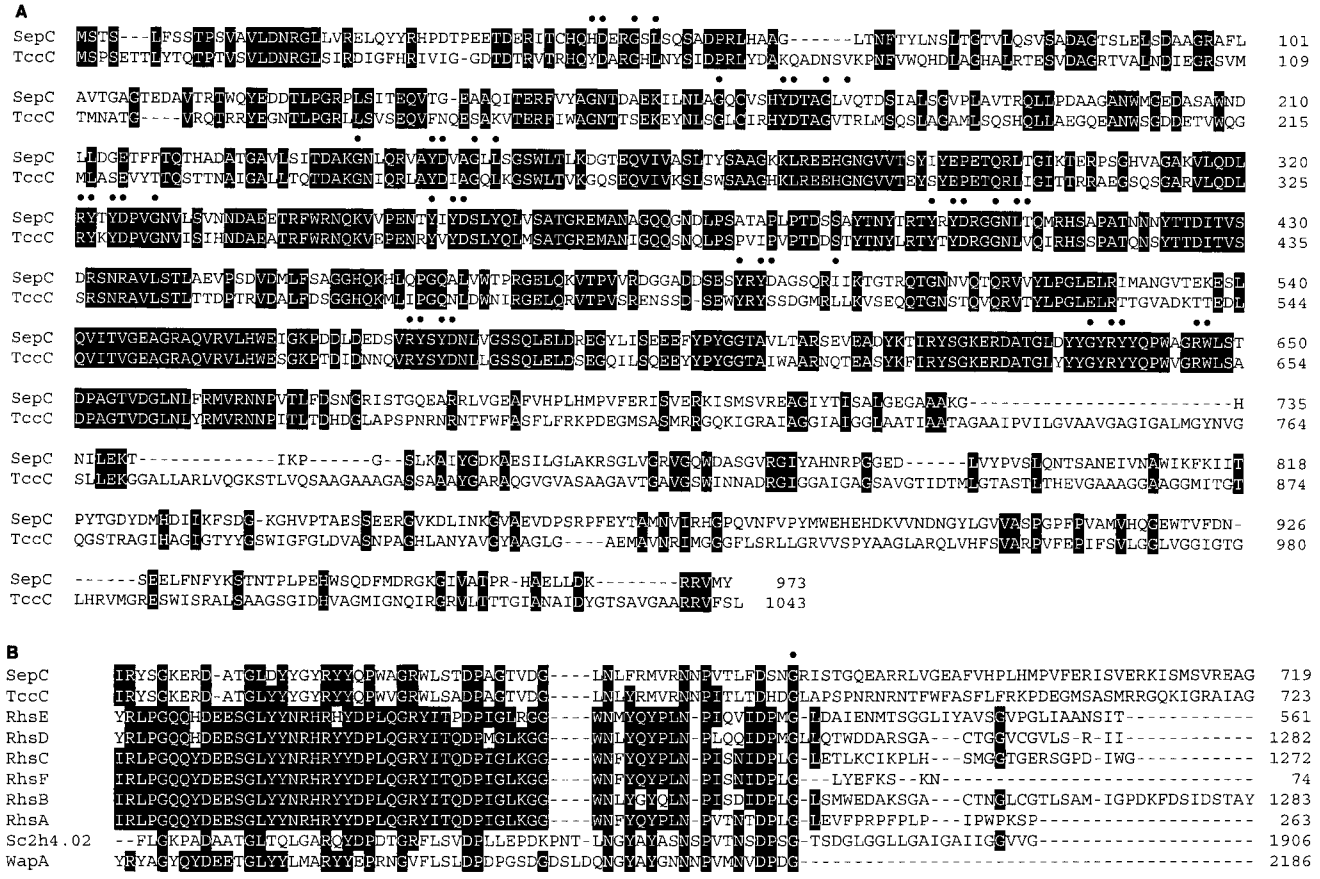

FIG. 4. (A) Alignment of amino acid sequences of the SepC and *P. luminescens* toxin component TccC. Conserved positions of the repeat motif GxxRYxY DxxGRL(I/T) (●) are marked. (B) Alignment of amino acid sequences of SepC to the *P. luminescens* toxin component TccC, the Rhs elements (RshE, P24211; RshD, P16919; RshC, P16918; RshF, I69801; RshB, P16917; RshA, P16916), the hypothetical protein SC2H4.02 from *S. coelicolor* A3(2), and the wall-associated protein of *B. subtilus* (WapA). ●, position of the conserved glycine residue which characterizes the junction between the conserved carboxyl end of the Rhs core and the variable carboxyl terminus.

likely to play a key role in induction of the cessation-of-feeding response.

Another locus, *amb2*, that is required for induction of both symptoms of amber disease has already been described for *S. entomophila* (32). The cloned *amb2* locus confers a cessation-of-feeding effect on *E. coli* strains harboring it, and *amb2* mutants of *S. entomophila* are nonpathogenic. However, *amb2* is different from the loci described in this work, as it maps to the chromosome of *S. entomophila* and does not show sequence similarity to the *sep* genes. Further work is required to determine the relationships or interactions, if any, between the *amb2* and *sep* loci.

Comparison of the predicted translated products of the *sep* genes showed they have similarity to the proteins that are components of the insecticidal toxin complexes of the enterobacterium *P. luminescens* (a symbiont of entomopathogenic nematodes of the family Heterorhabditidae). Bowen et al. (5) found that several *P. luminescens* strains secrete high-molecular-weight toxins that have strong insecticidal activity toward a large number of insects, including species of Coleoptera, Dictyoptera, Hymenoptera, and Lepidoptera. The physiological effects exerted by these toxins on susceptible insects are very similar to the effects exerted by δ-endotoxins of *Bacillus thuringiensis* and include cessation of feeding, loss of gut peristalsis, paralysis of the insect, and death (2, 5). Four *P. luminescens* toxin complexes were resolved on a native gel and termed toxin complexes Tca, Tcb, Tcc, and Tcd (5, 11). The Tcb and Tcd complexes are encoded by single-gene loci, but Tca and Tcc could be further resolved into a number of different polypeptides by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The loci encoding Tca and Tcc comprise four genes, three of which (*tcaABC* or *tccABC*) are oriented in the same direction, while the fourth, *tcaZ* or *tccZ*, is located downstream and oriented in the opposing direction.

The *sep* gene products show similarity to members of each of the *P. luminescens* loci, *tca* (SepA to TcaB; SepB to TcaC), *tcb* (SepA to TcbA), *tcc* (SepA to TccB; SepC to TccC), and *tcd* (SepA to TcdA). SepA and its *P. luminescens* homologues share an RGD motif. The RGD motif is present in cell surface adhesins produced by the human pathogen *Bordetella pertussis*, namely, the filamentous hemagglutinin (34) and the outer membrane protein pertactin (29), and has been implicated in enhancing the binding of *B. pertussis* to eukaryotic cells. The RGD motif found in SepA falls in a region of high similarity between SepA and its *P. luminescens* counterparts, and it seems possible that it may play a role in mediating the attachment of the proteins and/or the bacteria to the insect cell membrane.

SepB shows strong similarity to *P. luminescens* TcaC throughout its length, and both proteins show strong amino-terminal similarity to the amino terminus of the *Salmonella* virulence gene product SpvB (21). The region of similarity in

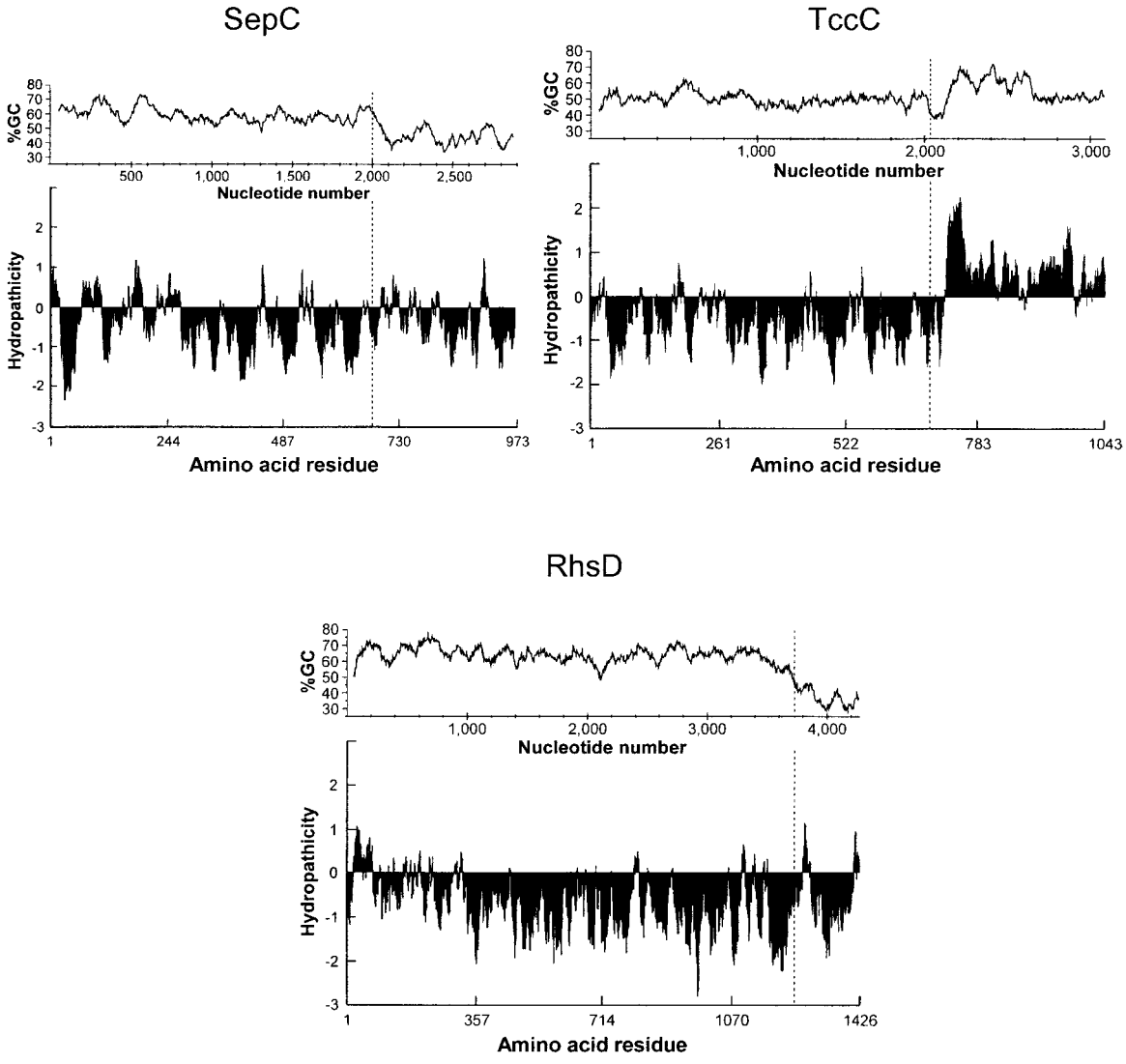

FIG. 5. GC content (window size, 100; window position shift, 3) and hydropathicity plots of SepC, TccC, and RhsD (scanning window of 17 amino acid residues). Each vertical dashed bar denotes the position of the conserved glycine residue which characterizes the junction between the conserved carboxyl end of the Rhs core and the variable carboxyl terminus.

relation to SpvB terminates 10 amino acid residues upstream of the proline-rich region postulated to divide SpvB into separate domains (36). This may indicate a vital role for the amino termini of the three proteins in interacting with an evolutionarily conserved eukaryotic protein. SpvB is believed to enhance the survival of virulent *Salmonella* in macrophages, but its mechanism of action is unknown (30). Based on its similarity to SpvB, it was suggested that TcaC may act by attacking insect hemocytes (5). However, hemocytes reside within the insect hemocoel, and *S. entomophila* does not invade the hemocoel until late in the infection process (25), suggesting that SepB may act in some other way.

The strong similarity of SepC to TccC is confined to the first 680 amino acids of the ~1,000-amino-acid proteins. The region common to SepC and TccC also shows similarity to the *B. subtilis* wall-associated protein WapA (a prototype of a family of hypothetical cell wall-associated bacterial proteins) and to members of the *E. coli rhs* element family. The Rhs family of elements has an unusual structure, with a GC-rich (62%) core of about 3.7 kb common to all members, followed by an AT-rich (60%) extension region of 400 to 600 bp that is unique to each member of the family. A single ORF runs through the GC-rich core and terminates in the extension region, which also encodes a second small ORF (12, 41). Though smaller than the typical Rhs elements, *sepC* encodes a hydrophilic protein core that contains nine variants of the Rhs peptide motif repeat GxxRYxYDxxGRL(IT) (12, 41) (Fig. 4A). There is also high similarity between SepC, TccC, WapA, and SC2H4.02 from *S. coelicolor* A3(2) to the carboxyl end of the Rhs core, which ends at a conserved glycine residue (Fig. 4B) (41). After the glycine residue the similarity between each of the proteins diminishes, resulting in different carboxy termini, as also occurs with the Rhs elements. The function of Rhs proteins is yet to be established, but they have been proposed to be cell surface-associated ligand-binding proteins on the basis of their similarity to WapA (15). The variable carboxyl termini may be the result of acquisition of new protein domains by modular evolution.

TABLE 4. Positions of mini-Tn*10* insertions

Mini-Tn*10* insertion	ORF	Position (bp) downstream of initiation codon
9/23	ORF3	120
24	ORF3	345
4	*sepA*	747
27	*sepA*	1037
40	*sepA*	1097
6	*sepA*	1727
38	*sepA*	2887
2	*sepA*	3197
5	*sepA*	3737
3	*sepA*	3697
30	*sepA*	4467
31	*sepA*	4627
12	*sepB*	182
22	*sepB*	172
11	*sepB*	362
10	*sepB*	2162
35	ORF4	557
13	*sepC*	2525
8		18937

The similarity between the *sep* and *tc* gene products suggests that they are members of a new family of insecticidal toxins. The lack of DNA similarity as opposed to protein similarity between *sep* and *P. luminescens tc* genes, together with the difference in GC content of the *sepA* and *sepB* genes compared to their *tc* homologues, suggests that these genes were present in a common enterobacterial ancestor of *P. luminescens* and *S. entomophila* and were not acquired by a more recent horizontal transfer event.

The involvement of similar disease determinants suggests that the histopathology of the diseases induced by *P. luminescens* and *S. entomophila* might be similar, despite the fact that amber disease is chronic whereas *P. luminescens* causes acute infections. Blackburn et al. (2) examined histopathology of the midgut of *Manduca sexta* larvae after treatment with purified Tca (TcaA, TcaB/SepA, TcaC/SepB, and TcaZ) through feeding on a diet cube and intracoelomic injection. Ingestion of Tca protein resulted in cessation of feeding, swelling of the midgut columnar epithelial cells, extrusion of vesicles into the gut lumen, and complete destruction of the midgut epithelium within 12 h. Injection of protein also resulted in distortion of the midgut cells. In contrast, *S. entomophila* infection has no observed histological effect on the midgut epithelial cells of *C. zealandica* but did show a reduction in the number of fat cells to almost undetectable levels and an emptying of the larval gut (25). Studies with purified Sep proteins are required to determine whether these differences reflect a different mode of action of the proteins or a toxin dose effect. Such studies will also indicate whether the remarkable host specificity of amber disease is a property of the Sep proteins or some other aspect of the disease process.

In summary, we have identified three *S. entomophila* genes, *sepA*, *sepB*, and *sepC*, that encode proteins with strong similarity to *P. luminescens* insecticidal toxins and are required for the causation of amber disease in the scarab *C. zealandica*. The similarity between *S. entomophila* and *P. luminescens* toxins suggests that they are members of a new family of insecticidal toxins. To further understand the disease process, purification of the Sep proteins and analysis of their expression and mode of action are being undertaken.

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