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Genes Essential for Amber Disease in Grass Grubs Are Located on the Large Plasmid Found in *Serratia entomophila* and *Serratia proteamaculans*

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The bacteria *Serratia entomophila* and *S. proteamaculans* cause amber disease in the grass grub, *Costelytra zealandica* (Coleoptera: Scarabaeidae), an important pasture pest in New Zealand. Disease symptoms include rapid cessation of feeding and amber coloration of larvae. A 105-kb plasmid (designated pADAP) has consistently been found only in pathogenic isolates of both species. Investigations into the involvement of pADAP in amber disease have been hindered by the lack of both a selectable marker on the plasmid and a reliable transposon delivery system. Kanamycin-resistant transposon insertions into three cloned *Hin*dIII fragments (9.5, 9.6, and 10.6 kb) were isolated and introduced into pADAP by shuttle mutagenesis. Inserts into the 9.5- and 9.6-kb *Hin*dIII fragments on pADAP did not alter disease-causing ability. When plasmids with inserts into the 9.6-kb region were conjugated into plasmid-minus, nonpathogenic isolates of *S. entomophila* and *S. proteamaculans*, all of them became pathogenic. Transposon insertions into two regions of the 10.6-kb *Hin*dIII fragment continued to cause cessation of feeding but failed to produce amber coloration. Further analysis of a mutant from each amber-minus region (pADK-10 and pADK-13) demonstrated that the antifeeding effect was produced only at dosages higher than that of the wild-type strain. Complementation with the wild-type *Hin*dIII fragment restored full-blown disease properties for pADK-13, but not for pADK-10.

Amber disease is a chronic disease of larvae of the New Zealand grass grub, *Costelytra zealandica* (Coleoptera: Scarabaeidae), which is caused by strains of both *Serratia entomophila* and *S. proteamaculans* (family *Enterobacteriaceae*). It was first described by Trought et al. (32) and subsequently developed into a commercially available biological control agent for *C. zealandica* in New Zealand (18). This insect disease has many unique features. These species are known to cause amber disease only in a single type of indigenous New Zealand scarab larva. The infection cycle is also unusual, with bacterial cells adhering preferentially to the crop lining of larvae. An infected host ceases feeding and clears its gut within days of ingesting pathogenic cells (17). Clearing the gut results in the characteristic amber color of infected hosts, hence the name of the disease. Although larvae cease feeding within days, death does not occur for weeks or months. Bacteria are restricted to the gut, mainly in the foregut (crop) region around the cardiac valve, until late in the disease, when bacteria break into the hemocoel and death occurs.

It is unusual to have a novel insect disease that is caused by two bacterial species, and the involvement of extrachromosomal elements has been hypothesized (16), especially as *S. entomophila* and *S. proteamaculans* are not the most closely related *Serratia* spp. (9). DNA-DNA reassociation values between *S. entomophila* and *S. proteamaculans* were only 32 to 40% (9). Approximately 50% of *S. entomophila* and *S. proteamaculans* field isolates are able to cause disease in *C. zealandica* larvae. Subsequent research confirmed that a 105-kb plasmid (designated pADAP) was present only in pathogenic isolates of both species (7). Heat curing pathogenic isolates of the plasmid also resulted in the loss of virulence (7). However, further studies to elucidate the role of plasmid-based genes in disease have been hampered by the lack of a selectable marker and a reliable transposon delivery system. To date, molecular analysis of amber disease has concentrated solely on *S. entomophila* (8). The only transposon delivery system that has been used successfully with this species (33) is a broad-host-range delivery system for Tn*phoA* (31). Unfortunately, application of this system to the study of plasmid-encoded determinants has proven to be unsuitable, as selection of nonpathogenic strains largely resulted in mutants that had lost pADAP (6).

In this paper, we report on successful marking of pADAP and subsequent manipulations which have increased our knowledge of the genetic elements involved in amber disease development.

MATERIALS AND METHODS

Bacterial isolates and methods of culture. Table 1 lists the bacterial isolates and plasmids used in this study; all were held in the AgResearch (Lincoln, New Zealand) insect pathogen culture collection. Classifications were determined primarily by reactions on three test media (DNase agar, adonitol agar, and itaconate agar [ITA]), according to the system previously described (22), with the identities of isolates confirmed by using API20E and API50CH test strips (Analytab Products, Marcy-l'Etoile, France). Bacteria were grown in Luria-Bertani (LB) broth or on LB agar (LA) (25) at 37°C for *Escherichia coli* and 30°C for *Serratia* spp., unless otherwise stated. The antibiotic concentrations (in micrograms per milliliter) used for *Serratia* spp. were as follows: kanamycin, 100; chloramphenicol, 90; tetracycline, 30; streptomycin, 100. For *E. coli* strains, the concentrations used were as follows: kanamycin, 50; chloramphenicol, 30; tetracycline, 15; streptomycin, 100.

Plasmid visualization and DNA isolation and manipulation. Large plasmids, such as pADAP, were visualized by the method of Kado and Liu (19). For restriction digestion or other manipulations, pADAP DNA was isolated from up to 250 ml of bacteria by using CsCl density gradients, as previously described (25). Other plasmid DNAs were prepared by standard alkaline lysis methods (20). Restriction digestions, agarose gel electrophoresis, ligations, and transformations of competent *E. coli* cells were performed by standard methods (14, 25). Southern hybridizations were performed with a nonradioactive ECL kit (Amersham) (30).

Mobilization of pLAFR3-based plasmids into *S. entomophila*. pGLA# deriv-

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TABLE 1. Bacterial isolates, bacteriophages, and plasmids used in this study

Strain, bacteriophage, or plasmid	Phenotype and/or comment	Source or reference
Strains		
S. entomophila		
A1MO2	Pathogenic wild type; pADAP	Fairton, New Zealand
154	Pathogenic wild type; pADAP	Canterbury, New Zealand
562RC	Wild type; *recA* Cm^r; pathogenic	10
A1MRC	A1MO2, *recA* Cm^r; pathogenic	This work
5.6	A1MO2 derivative; $plasmid^-$ (heat cured); nonpathogenic	7
5.6RC	5.6, *recA* Cm^r	This work
UC16	Str^r spontaneous $plasmid^-$; A1MO2 derivative	H. K. Mahanty
220	Str^r ITA^-; nonpathogenic	France
314	Str^r; nonpathogenic	Taupo, New Zealand
440	Str^r; nonpathogenic; 70-kb plasmid[a]	Chatham Islands, New Zealand
328	Str^r; nonpathogenic	Moteuka, New Zealand
S. proteamaculans		
374	Str^r; nonpathogenic; 90-kb plasmid[a]	Tihoi, New Zealand
377	Str^r; nonpathogenic; 90-kb plasmid[a]	Taupo, New Zealand
491	Str^r; nonpathogenic	Balcarce, Argentina
197	Str^r; nonpathogenic	Paewhenua, New Zealand
216	Str^r; nonpathogenic	Templeton, New Zealand
461	Str^r; nonpathogenic	Czechoslovakia
193	Str^r; nonpathogenic	Canterbury, New Zealand
S. plymuthica 385	Str^r; nonpathogenic; isolated from *C. zealandica*	Rakaia, New Zealand
E. coli		
DH5α	*endA1 hsdR17 supE44* Δ*lacU169* (ϕ80*lacZ*ΔM15) *thi-1 recA1 gyrA96 relA1*	13
MC1061	*rpsL*(Str^r) *araD139* Δ(*ara-leu*)*7696 galE15 galK16* Δ(*lac*)*X74 hsdR2* ($r_K^-m_K^+$) *mcrA mcrB1*	3
MC4100	*rpsL150* (Str^r) *araD139* Δ(*argF-lac*)*U169 relA1 flbB5301 deoC1 ptsF25 rbsR*	28
ZK353	W3110; Rif^r	R. Kolter
Bacteriophages		
ϕCW1	*S. entomophila*-specific generalized transducing phage	11
λNK1316	Mini-Tn*10* derivative 103 donor, λ*b*522 *c*I857 *P*am80 *nin5*	21
Plasmids		
pADAP	Wild-type amber disease-associated plasmid	7
pADK-6	pADAP::mini-Tn*10* insert in 9.6-kb *Hin*dIII region; Kan^r; pathogenic	This study
pADK-7	pADAP::mini-Tn*10* insert in 9.6-kb *Hin*dIII region; Kan^r; pathogenic	This study
pADK-13	pADAP::mini-Tn*10* insert in 10.6-kb *Hin*dIII region; Kan^r	This study
pADK-10	pADAP::mini-Tn*10* insert in 10.6-kb *Hin*dIII region; Kan^r	This study
pGLA#	*Hin*dIII clones of pADAP in pLAFR3	This study
pGLA20	10.6-kb *Hin*dIII clone of pADAP in pLAFR3	This study
pGRK23	2.7-kb *Kpn*I fragment from pGLA20 cloned in pLAFR3	This study
pLR14	pLAFR3-based plasmid for construction of *recA S. entomophila* strains; Cm^r Tet^r	10
pNK2859	Plasmid vehicle for mini-Tn*10* derivative 103	21
pLAFR3	Tet^r *lacZ*α^+; 22-kb *tra*	29
pRK2013	Kan^r *tra*$^+$	5

[a] Indigenous plasmid in wild-type strain.

atives were introduced into *S. entomophila* by tripartite matings with the pRK2013 helper plasmid (24). Alternatively, electroporation (Bio-Rad Gene Pulser) (4) was used to transform *S. entomophila* strains with plasmids for the selection of recombinants or complementation experiments.

Shuttle mutagenesis. Transposon insertions were generated in recombinant plasmids by using the mini-Tn*10* derivative 103 (kanamycin resistant) as previously described (21). Insertions were recombined into pADAP by transforming A1MO2 with the construct and selecting for the loss of the pLAFR3 tetracycline resistance marker after growth in nonselective media.

Conjugation of pADAP derivatives. Streptomycin-resistant recipient strains were generated by stepwise selection on streptomycin plates that contained increasing concentrations of this antibiotic. Donor and recipient strains for conjugations were grown in LB broth for 12 h at 20°C; the use of older cultures was unsuccessful. Five hundred microliters each of donor and recipient cultures were mixed in a test tube and placed in a horizontal position for 1.5 h at room temperature. Then a 300-μl aliquot was concentrated by centrifugation and plated on an LA plate that contained the appropriate antibiotics. Three hundred microliters each of recipient and donor cultures were plated as controls on LA plates that contained the same antibiotics.

Construction of *S. entomophila recA* strains. Strain 5.6RC was generated by recombination of the chloramphenicol-resistant *recA* marker from pLR14 into strain 5.6 by the method of Grkovic and Mahanty (10). A1MRC strains (chloramphenicol-resistant, *recA* A1MO2 isolates containing various pADAP derivatives) were constructed by transduction of the same *recA* marker described immediately above from strain 562RC with the generalized transducing phage ϕCW1 (11).

Bioassay against *C. zealandica* larvae. Infection of *C. zealandica* larvae was determined by a standard bioassay (7), in which healthy, feeding larvae, collected from the field, were individually fed carrot squares which had been rolled in colonies of bacteria grown overnight on solid media. At least 12 second- or third-instar larvae were used for each treatment. Larvae were left to feed on treated carrots for 3 to 4 days, transferred to fresh trays, and refed untreated carrots for up to 10 to 14 days. The occurrence of amber coloration and loss of feeding were recorded every 3 to 4 days. Strains were designated as being pathogenic when amber appearance and the cessation of feeding occurred in >70% of treated larvae after ingestion of high-dosage carrots.

The strength of the antifeeding and disease effects was determined by dose-response assays. Bacteria to be tested were grown in LB shake culture overnight at 30°C, with the resultant densities estimated by plate count. Bacterial suspensions of known densities were prepared by dilutions of stock suspensions in phosphate buffer and applied in 5-μl doses to carrots. Two dose-response assays were carried out. The effects of inserts pADK-6, pADK-10, and pADK-13 were determined by dilutions of approximately 10^7, 10^5, and 10^3 CFU per dose. The effects of pADK-13 and the complemented strain pADK-13/pGLA20 were eval-

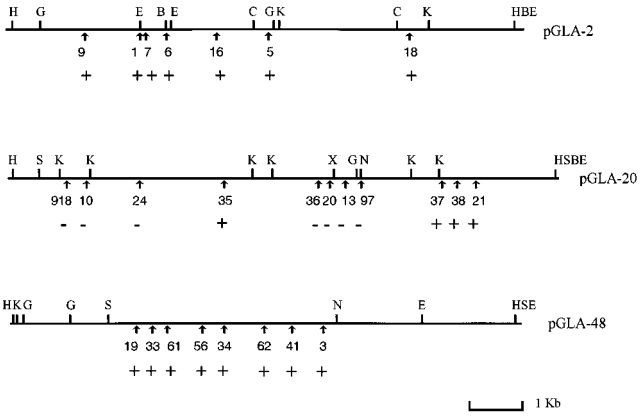

FIG. 1. Restriction maps and transposon insertion points in *Hin*dIII pADAP subclones pGLA2 (9.6 kb), pGLA20 (10.6 kb), and pGLA48 (9.5 kb). Transposon insertion points are represented by arrows. The phenotype of each mutant after recombination into pADAP is indicated by + (full-blown disease symptoms) or − (amber minus; failure to clear gut). Restriction enzymes are abbreviated as follows: B, *Bam*HI; C, *Cla*I; E, *Eco*RI; G, *Bgl*II; H, *Hin*dIII; K, *Kpn*I; N, *Nhe*I; S, *Sal*I; X, *Xba*I.

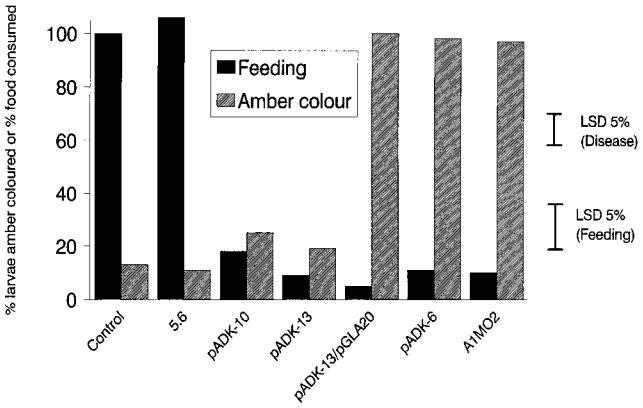

FIG. 2. Effects of mini-Tn*10* insertion mutants (pADK#) and a complemented *S. entomophila* strain (pADK-13/pGLA20) in high-dosage assays on amber coloration and feeding between days 4 to 7 after inoculation of third-instar *C. zealandica* larvae. Wild-type pathogenic (A1MO2) and nonpathogenic (5.6) standards were included for comparison. Data are means of at least three tests, with 12 larvae per treatment at 15°C. LSD, least significant difference.

uated in a further dose-response assay, with five dosage rates (log dilutions ranging from 10^8 to 10^3 CFU per dose). In each assay, 12 to 24 larvae were treated at each dosage rate and doses were randomized within three experimental blocks. As described above, larvae were examined and fresh carrots were provided. Appropriate pathogenic and nonpathogenic standards were used in all assays. The strength of the antifeeding response (amount of carrot consumed) or disease response (onset of amber coloration) to each strain was determined by regression analysis and comparison of the slopes of fitted lines.

Histopathology. To examine the adhesion and colonization of bacteria in the foreguts of larvae, individual third-instar larvae were inoculated as described above and left for 20 days at 15°C. Then foreguts were removed intact, fixed in 2.5% glutaraldehyde in 0.025 M phosphate buffer (pH 7.2) for 2 h, postfixed in 1% OsO_4 for 1 h, dehydrated by an ethanol and acetone series, and embedded in Spurr's resin. Sections (1 to 2 μm) were cut for light microscopy with a Pyramatome (LKG) and briefly stained (30 s) with 1% Toluylene blue–1% sodium tetraborate.

RESULTS

Subclones. Restriction digestion of pADAP with *Hin*dIII produced 16 fragments, 13 of which were successfully shotgun cloned into pLAFR3. These clones ranged in size from 10.6 to 0.8 kb and were designated pGLA# (Table 1). The two largest fragments, approximately 22 and 23 kb, were not cloned. Tripartite mating was used to mobilize pGLA clones into strain 5.6, a nonpathogenic, plasmid-minus *S. entomophila* derivative. None of the resulting transconjugants expressed any disease symptoms in *C. zealandica* larvae (data not shown).

Introduction of a kanamycin-resistant transposon into pADAP by shuttle mutagenesis. The overall approach of this study was to insert mini-Tn*10* transposons into subclones of pADAP and then introduce mutated subclones into pADAP-containing cells by tripartite mating. Through homologous recombination, a subclone with a transposon was recombined into pADAP, resulting in a marked and mutated pADAP.

Transposon insertions were attempted with the largest subclones. Mini-Tn*10* (kanamycin-resistant) transposon insertions into the 9.5- (pGLA48), 9.6- (pGLA2), and 10.6-kb (pGLA20) *Hin*dIII subclones were isolated. Restriction maps were generated, and the points of transposon insertions were determined (Fig. 1). Then all insertions were recombined into pADAP in the wild-type A1MO2 background, and the resulting kanamycin-resistant, tetracycline-sensitive derivatives were bioassayed after plasmid visualization to confirm the loss of the pLAFR3-based plasmid.

Effects of mini-Tn*10* insertions into pADAP on disease-causing ability. Insertions into the pGLA2 and pGLA48 regions of the plasmid (Fig. 1) had no effect on the disease-causing ability of *S. entomophila*, as larvae ceased feeding and cleared their guts in the same manner as when they had been infected with the pathogenic standard (A1M02). However, insertions into the pGLA20 region did alter pathogenicity, with larvae failing to produce all of the symptoms of the full-blown disease. Larvae treated with mutants containing 7 of the 11 inserts into the pGLA20 region ceased feeding but did not clear their guts or take on the typical amber appearance of diseased larvae (Fig. 1 and 2). These amber-minus mutants fell into two groups that were separated by an insertion with unaltered pathogenicity (pADK-35) (Fig. 1). One amber-minus mutant from each group, pADK-10 and pADK-13, was chosen for further analysis. Multiple tests of these strains in high-dosage assays with carrots rolled in bacteria confirmed that these mutants produced significant reductions in feeding but no expression of amber coloration (Fig. 2). Mutant pADK-6, with an insertion in the pGLA2 region, produced antifeeding and gut clearance to the same extent as wild-type standards in high-dosage assays and was used for comparison.

Dose-response assays allowed the strength of these effects to be evaluated. Generally, pathogenic strain pADK-6 produced rates of disease and antifeeding responses that were similar to those of wild-type pathogenic isolates (Fig. 3 and 4). In contrast, the antifeeding effects produced in larvae that had been treated with strains pADK-10 and pADK-13 were significantly reduced ($P < 0.05$), compared with those of the wild-type pathogenic strain or pADK-6 (Fig. 3). A similar reduced level of antifeeding was produced by pADK-13 in the second assay (Fig. 4b).

Examinations of thin sections of fixed foreguts demonstrated that bacteria adhered to this crucial region in similar numbers upon inoculation with pADK-13 and pADK-10, compared with that of a standard pathogenic wild-type strain, despite not displaying the phenology of the full-blown disease (data not shown). For pADK-6, pADK-10, pADK-13, pADK-35, and pADK-34, the recombination of the mini-Tn*10* insertion into the correct *Hin*dIII fragment was confirmed by Southern hybridization, with the *Bam*HI kanamycin-resistant fragment from pNK2859 as the probe (data not shown).

Complementation. As the pADK-10 and pADK-13 inser-

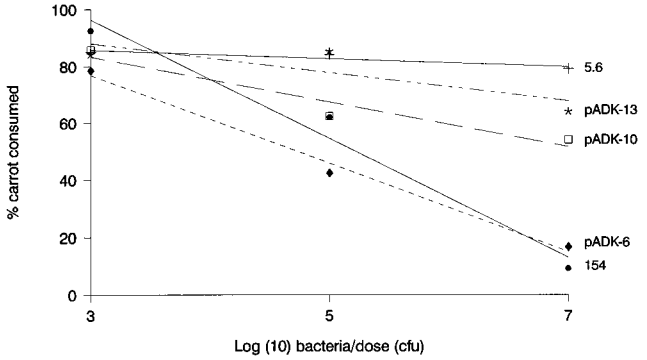

FIG. 3. Effects of mini-Tn*10* insertion mutants pADK-6, pADK-10, and pADK-13 on feeding between days 4 to 7 after inoculation by third-instar *C. zealandica* larvae treated with three dosages of bacteria. Wild-type pathogenic (154) and nonpathogenic standards (5.6) were included for comparison (12 to 24 larvae per treatment at 15°C). The slopes of lines were as follows: pADK-6, −15.4; pADK-10, −6.5; pADK-13, −2.9; 154, −20.8; 5.6, −1.5. The least significant difference (5%) was 10.7.

tions altered disease phenology, the ability of the wild-type pGLA20 fragment to complement the pADK-10 and pADK-13 mutations was determined in a recombination-deficient background by constructing *recA* A1MO2/pADK# derivatives (A1MRC strains). The pADK-13 mutation was successfully complemented by the wild-type *Hin*dIII fragment, resulting in the restoration of amber coloration (Fig. 2) and gut clearance. However, pGLA20 failed to restore to pADK-10 the ability to cause amber coloration and gut clearance. Although complementation of pADK-13 by pGLA20 resulted in full-blown-disease symptoms, the results of the dose-response assay for this mutant suggest that it produces disease and antifeeding effects at a rate intermediate between that of the wild-type strain and that of antifeeding mutant pADK-13 (Fig. 4).

The 2.7-kb *Kpn*I fragment in which the pADK-13 insertion is located (Fig. 1) was cloned into pUC19 and then into pLAFR3 via flanking sites in the pUC19 polylinker. The resulting plasmid, pGRK23, failed to complement the pADK-13 mutation.

Conjugation. The presence of a kanamycin resistance marker in a non-disease-encoding region of pADAP allowed conjugation experiments to be conducted with wild-type, nonpathogenic field isolates and laboratory strains that had either spontaneously lost or been heat cured of pADAP. Both A1MO2/pADK-6 and A1MO2/pADK-7 were successfully used as donor strains. In every case, the introduction of plasmids from either strain into a nonpathogenic background resulted in pathogenic transconjugants (Table 2). In total, six isolates of *S. proteamaculans* and five isolates of *S. entomophila* were successfully conjugated with pADAP derivatives. The acquisition of pADK-6 or pADK-7 was confirmed by plasmid visualization (Fig. 5), and the backgrounds of transconjugants were confirmed, when possible, by using differentiating media as described previously (22). *S. entomophila* recipient strains 5.6RC (*recA* DNase$^-$) and 440 (possession of a 70-kb plasmid) were also identifiable by their respective characteristics. For both *S. entomophila* and *S. proteamaculans*, only one plasmid-minus, streptomycin-resistant strain failed to be a successful recipient for conjugation (Table 2). In addition to New Zealand strains, two exotic *S. proteamaculans* isolates (from Argentina [491] and Europe [461]) and the only exotic *S. entomophila* isolate (strain 220 from France) became pathogenic after the introduction of pADK-6 by conjugation (Table 2).

In the case of two New Zealand nonpathogenic *S. proteamaculans* isolates with resident ~90-kb plasmids (374 and

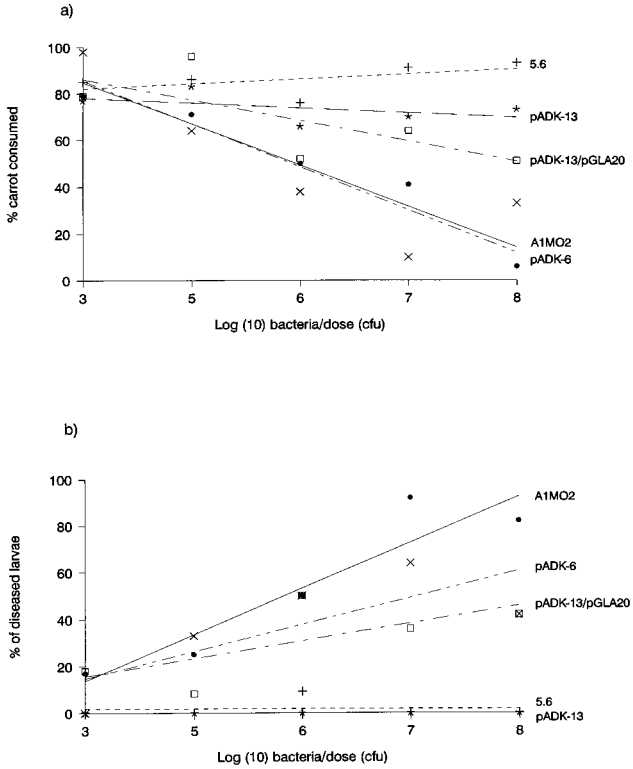

FIG. 4. Effects of mini-Tn*10* insertion mutants, pADK-13, and complemented strain pADK-13/pGLA20 on expression of feeding (a) and amber disease (b) after being applied to carrots at various bacterial doses. The unaltered pathogenic mutant (pADK-6) strain and wild-type pathogenic (A1MO2) and nonpathogenic (5.6) strain were included for comparison (12 larvae per treatment at 15°C). The slopes of lines in panel a were as follows: pADK-6, −15.8; pADK-13/pGLA20, −6.7; pADK-13, −1.7; A1MO2, −13.2; 5.6, 1.5. The least significant difference (5%) in panel a was 10.7. The slopes of lines in panel b were as follows: pADK-6, 9.8; pADK-13/pGLA20, 6.0; pADK-13, 0; A1MO2, 16.3; 5.6, 0.1. The least significant difference (5%) in panel b was 12.4.

377), the introduction of pADK-6 or pADK-7 resulted in the loss of resident plasmids from recipient strains (Fig. 5). Plasmid visualizations were carried out for eight independent transconjugants from two separate experiments for isolates 374 and 377, with the same results. The only other recipient with a resident plasmid, *S. entomophila* isolate 440, retained its ~70-kb plasmid after it had acquired pADK-6 (Fig. 5).

Attempted conjugations of pADK-6 into a *S. plymuthica* strain (385) isolated from *C. zealandica* and into *E. coli* MC4100, MC1061, and ZK353 were unsuccessful.

DISCUSSION

Shuttle mutagenesis was a highly successful approach in overcoming the lack of a reliable transposon delivery system in *S. entomophila*. The use of such techniques in analyses of an increasingly diverse number of species is becoming widespread (12, 24, 27). The generation of kanamycin-resistant pADAP derivatives confirmed for the first time the direct involvement of this plasmid in the development of amber disease. Conjugational transfer of a marked pADAP between pathogenic and nonpathogenic isolates of *S. entomophila* and *S. proteamaculans* resulted in disease expression by transconjugants. Even exotic (non-New Zealand) strains of both species (only one exotic isolate of *S. entomophila* exists [9]) were conjugated and became pathogenic, suggesting that any chromosomal factors

TABLE 2. Conjugation between *S. entomophila* and *S. proteamaculans* plasmid-minus recipients and an A1MO2/pADK-6 (Kan^r) donor

Isolate	Species	Source or reference	Kanamycin-resistant transconjugant colonies obtained[a]	Size (kb) of plasmid in nonpathogenic isolate	Size (kb) of plasmid(s) visualized	Disease in larvae
5.6RC	*S. entomophila*	A1MO2 derivative	+[b]		105	
UC16	*S. entomophila*	A1MO2 derivative	+		105	+
197	*S. proteamaculans*	Paewhenua, New Zealand	+		105	+
216	*S. proteamaculans*	Templeton, New Zealand	+		105	+
220	*S. entomophila*[c]	France	+		105	+ (weak)
314	*S. entomophila*	Taupo, New Zealand	+		105	+ (weak)
374	*S. proteamaculans*	Tihoi, New Zealand	+	90	105[d]	+
377	*S. proteamaculans*	Taupo, New Zealand	+	90	105[d]	+
440	*S. entomophila*	Chatham Islands, New Zealand	+	70	70, 105	+
461	*S. proteamaculans*	Czechoslovakia	+		105	+
491	*S. proteamaculans*	Argentina	+		105	+
193	*S. proteamaculans*	Canterbury, New Zealand	−			
328	*S. entomophila*	Moteuka, New Zealand	−			
385	*S. plymuthica*	Rakaia, New Zealand	−			
MC1061	*E. coli*	3	−			
MC4100	*E. coli*	28	−			
ZK353	*E. coli*	R. Kolter	−[e]			

[a] All recipients were resistant to 100 μg of streptomycin per ml, unless otherwise stated.
[b] Resistant to chloramphenicol and *recA* mutants.
[c] Possibly a confused isolate. It was negative on ITA but tested as *S. entomophila* on API strips.
[d] After conjugation, strains 374 and 377 lost resident ~90-kb plasmids (Fig. 5).
[e] Resistant to rifampin.

involved in this disease are highly conserved in *Serratia* spp. As yet, no *Serratia* species other than *S. entomophila* and *S. proteamaculans* have been conjugated. Electroporation also has failed to introduce pADAP into other species. Successful transfer of pADAP to other species would be highly significant for both understanding the disease process and possibly broadening the host range of amber disease, currently restricted to a single scarab species that is indigenous to New Zealand. The demonstration that plasmid-borne genes are involved in the amber disease-causing abilities of *Serratia* spp. is yet another example of an interesting biological feature encoded on plasmids, such as the *Rhizobium* nitrogen fixation genes (1, 23), the *Agrobacterium tumefaciens* tumor-inducing plasmid (26), and the insect toxin genes of *Bacillus thuringiensis* (15).

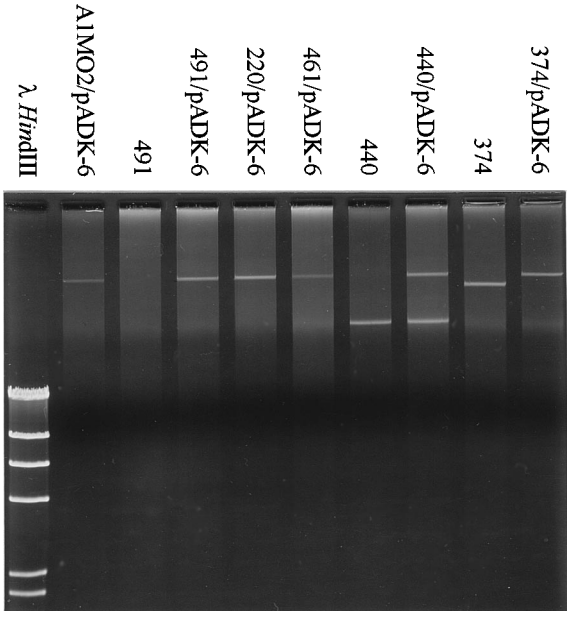

FIG. 5. Plasmid visualizations of donor (lane 2), recipient (lanes 3, 7, and 9), and transconjugant (lanes 4 through 6, 8, and 10) strains. Lane λ*Hin*dIII, molecular size markers.

The loss by both *S. proteamaculans* 374 and 377 of their 90-kb plasmids upon acquisition of pADK-6 by conjugation indicated that the 90-kb plasmids are incompatible with pADAP, a situation that arises when plasmid partition and/or replication functions are not able to distinguish between two plasmids (2).

Individual *Hin*dIII subclones of pADAP did not result in the expression of any disease symptoms when they were introduced into isolate 5.6, a plasmid-cured nonpathogenic isolate. The lack of any disease symptoms, particularly after pGLA20 had been introduced into 5.6, indicated that important amber disease determinants are located on more than just this one *Hin*dIII fragment. On the basis of the complex nature of this disease, a relatively large number of genes are likely to be involved in the disease process. The failure of most inserts to affect pathogenicity in high-dosage tests indicated that these inserts had no significant effect on the disease process. This was borne out by the results from the first quantitative assay, although the second assay suggested that the disease-causing ability of pADK-6 was reduced.

Amber-minus mini-Tn*10* insertions into the 10.6-kb pGLA20 fragment continued to adhere to the foreguts of infected larvae but failed to clear and produce the characteristic amber coloration, as well as having reduced antifeeding effects. Mutants pADK-10 and pADK-13 produced relatively weak antifeeding effects. After complementation of pADK-13 by pGLA20, both amber coloration and greater suppression of feeding occurred. Each effect was expressed at a lower level than that of the wild-type strain, suggesting some effect of genetic manipulation on pathogenesis, but this requires further study. What causes the antifeeding effect is unknown, but it is not a direct aversion to bacteria, as larvae do not resume

feeding when they are offered uninoculated carrots. The successful complementation of the pADK-13 mutation by the wild-type *Hin*dIII fragment indicated that pGLA20 encodes all of the information necessary for full restoration of disease-causing ability to this mutant. The failure of pGLA20 to restore the amber coloration effect to pADK-10 suggests that pGLA20 does not contain the entire sequence of interest. Therefore, identification and analysis of the *Hin*dIII fragment adjoining the pADK-10 region may yield further sequences involved in the development of amber disease. The involvement of at least two genes in the production of amber coloration in diseased larvae is indicated by the large span of pGLA20 DNA over which insertions with the same phenotype are spread, the separation of the pADK-10 and pADK-13 groups of mutants by the fully pathogenic pADK-35 insertion, and the ability of the wild-type pGLA20 clone to complement pADK-13, but not pADK-10. Our analysis of this region, including sequencing, continues.

The shuttle mutagenesis method employed here made it possible to directly examine the involvement of plasmid-borne genes in amber disease development. The isolation of individual genes responsible for amber disease development opens the way for future genetic manipulations to increase virulence and/or broaden the host range. Obtaining further transposon insertions into uninvestigated regions of pADAP, coupled with generating a restriction map for the entire plasmid, is required to elucidate the full extent of amber disease determinants encoded by this plasmid. Work continues on a means of isolating transposon insertions directly in pADAP.

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