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Serratia marcescens as a bacterial pathogen of *Rhagoletis pomonella* flies (Diptera: Tephritidae)

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Abstract. A nonpigmenting strain of *Serratia marcescens* Bizio isolated from dead and apparently diseased wild apple maggot flies, *Rhagoletis pomonella* (Walsh), was shown to be pathogenic to healthy apple maggot flies upon ingestion. The microorganism was detected in live adult alimentary canal organs four days post ingestion but produced death in some flies within 24 h when flies fed on a cell concentration of 4.7×10^4 cfu/ml and within 8 h when flies fed on filter-sterilized culture medium that previously contained a 21 h culture of *S. marcescens*. Increasing the cell concentration 10,000 fold did not lead to an increased rate of kill. Young flies (7–10 days old) were more susceptible to infection leading to death than were older flies (21–28 days old). The potential use of *S. marcescens* cells as control agents against apple maggot flies is negated by their pathogenicity to vertebrates; however, the potential use of toxic compounds produced by this strain of *S. marcescens* is discussed.

INTRODUCTION

Control of *Rhagoletis pomonella* (Walsh), a major apple pest in the United States, depends almost exclusively on the application of organophosphate insecticides azinphosmethyl or phosmet. Growing concerns about public health, environmental pollution, insecticide resistance, and rising costs associated with pest control press growers to consider alternative methods of pest control that are less invasive or more cost effective. The use of natural pathogens to cause epizootics is an alluring possibility. Specificity, persistence, and transovarial infection are a few characteristics of insect pathogens that make them attractive for insect pest management programs. Ample information exists regarding the success of certain natural pathogens for insect control, notably *Bacillus thuringiensis* used to control several genera of numerous insect orders, and *Bacillus popilliae* used to control Japanese Beetle, *Popillia japonica* Newman. Little is known about diseases in the apple maggot fly, and the information that does exist (e.g. Jacques et al., 1969; Fay, 1989) does not report specific causative agents and associated pathogenicity.

To begin to understand and evaluate any potential use of a pathogen for control of apple maggot fly, i.e. use of a toxic metabolite from a pathogen, we chose to examine the pathogenicity of a nonpigmenting strain of *Serratia marcescens* Bizio that was isolated earlier from dead and apparently diseased wild apple maggot flies and identified using standard methods (Brenner, 1992). Strains of *S. marcescens* have been reported to be recovered from other tephritids such as diseased *Ceratitis capitata* Weidemann and *Dacus* (*Bactrocera*) *dorsalis* Hendel flies (Grimont & Grimont, 1978) and these bacteria may possess some utility as insect control agents. With this idea in mind, we examined this microorganism for its ability to produce disease; that is, to become established within the fly, to resist the host's immune defenses, and to exploit the host to the point of disability and/or death. We sought to identify conditions for pathogenicity of *S. marcescens* to *R. pomonella* by feeding *S. marcescens* to adult *R. pomonella* and examining (1) the effects of two different ingested doses, (2) age-mortality relationships, (3) persistence and/or establishment of *S. marcescens* in alimentary canal organs of *R. pomonella*, and (4) the effect of the presence of *S. marcescens* on other bacterial populations present within alimentary canal organs of *R. pomonella*. We also demonstrated Koch's postulates and sought to determine if this strain produced any toxic metabolite(s) against *R. pomonella*.

MATERIALS AND METHODS

Bacterial cell preparation and fly feeding procedure. An 18 h culture of *S. marcescens*, obtained from dead *R. pomonella* eclosed from pupae collected in the field, was grown in 7 ml trypticase soy broth (Difco Laboratories, Detroit, MI) (TSB) at 28°C, not shaken, was placed in a Dynac™ centrifuge (Clay-Adams Inc., Parsinnany, NJ) and spun at 5000 rpm for 20 min at room temperature. The supernatant fluid was discarded, and the remaining pellet of bacterial cells was resuspended in 500 µl of sterile distilled water (pH 7), which was just enough liquid to assist in the removal of the cells by micropipette. The cell-water suspension was enumerated by serial dilution using the spread-plate technique on prepoured trypticase soy agar (TSA) plates in triplicate. Colony-forming units were enumerated after 18 h of incubation at 30°C. We determined that after 18 h of incubation in TSB, this strain of *Serratia* in culture had reached a density

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TABLE 1. Percent mortality of apple maggot flies (10–28 days old) caused by ingestion of *S. marcescens* recovered from dead *S. marcescens*-fed apple maggot flies.

Treatment	No. tested	Average percent mortality 24 h
High dose[a]	58	50a
Low dose[b]	60	40a
Control	60	5b

[a]High dose = 4.7×10^8 cfu/ml
[b]Low dose = 4.7×10^4 cfu/ml
Values followed by the same lower case letter are not significantly different at p = 0.05 level according to least significant difference tests.

of 4.7×10^4 colony-forming units (cfu)/ml. Fifty µl of the cell suspension was then dispensed aseptically and equally onto 8 sterile paper disks (7 mm dia) made from Whatman No. 1 filter paper (Whatman Internat'l. Ltd, Maidstone, England) contained in a sterile Petri dish. Ten µl of a 10% yeast hydrolysate/sugar solution (fly food) was also added to each disk and mixed well with the bacterial cells. Apple maggot flies, eclosed from wild-origin pupae, were separated into groups based on age and were starved of protein and sugar for 18 h. Flies were then placed individually into Petri dishes containing the bacterial cells on paper disks and were allowed to feed. Flies that fed only on the yeast hydrolysate/sugar solution (plus 50 µl sterile dH_20) on paper disks served as controls. Flies were watched carefully and, after all flies had fed and displayed distended abdomens, the flies were removed and placed in plexiglass-wire cages containing fresh water, sugar, and protein. Percent mortality was recorded after 24 and 120 h .

High and low dose tests. A concentration of 4.7×10^8 cfu/ml, obtained by dilution technique, was chosen arbitrarily and considered to be a "high" dose of *S. marcescens*, while a concentration of 4.7×10^4 cfu/ml was considered to be a "low" dose of the bacterial cells. Recall that this low dose did cause mortality within 24 h (Table 1.). From this point onward, high and low doses refer to these cell concentrations respectively. Four groups of 15 adult *R. pomonella* were fed the high dose and 4 groups of 15 adult *R. pomonella* were fed the low dose of *S. marcescens* following the disk method described above. Four groups of 15 adult *R. pomonella* were fed yeast hydrolysate/sugar and served as control groups. Percent mortality was recorded at 24 and 120 h.

Age-mortality relationship tests. High and low doses of *S. marcescens* were fed to *R. pomonella* considered to be young (7–10 days old) and old (21–28 days old). Six cages for each age and dose (15 flies per cage) were tested using the paper disk method described above. "Young" and "old" fly groups serving as control groups (N = 90) were given only yeast hydrolysate/sugar. Percent mortality was recorded at 24 and 120 h.

Detection, establishment and persistence of *Serratia marcescens* within the host. *R. pomonella* (7–10 days old) were fed a dose of 4.7×10^4 cfu/ml and were sacrificed 1–2 min, 15 min, 30 min, 1 h, 24 h, and 4 d post-feeding while at the same time parts of their alimentary canal tract (esophageal bulb, crop, and a section of midgut) were removed aseptically from each fly. The organs were placed individually into 7 ml of TSB and incubated for 18–24 h at 28°C. The cultures were streaked individually onto TSA to obtain pure colonies, incubated at 28°C, and bacterial colonies were subcultured onto biochemical media for identification. The API 20E (Analytab Products, Inc., Plainview, NY) microbiological identification system was also used as part of the identification process. *R. pomonella* that fed on yeast hydrolysate/sugar alone were likewise dissected at the same time intervals and served as control groups. The presence, not quantity, of *S. marcescens* in the removed parts of the alimentary canal was recorded. Recovered *S. marcescens* were fed to small groups (10) of flies (6) ranging in age between 10 to 28 days old to confirm Koch's postulates.

Toxicity tests using *S. marcescens* supernatant. A 21 h culture of *S. marcescens* grown in TSB and centrifuged at 5000 rpm for 15 min at 4°C. The supernatant was removed and filter-sterilized using a 0.22 µm filter (Acrodisc, Gelman Sciences, Ann Arbor, MI). A sample of the supernatant was placed into TSB which was incubated overnight and observed for any bacterial growth. Lack of growth confirmed successful filter sterilization. Fifty µl of sterile supernatant was applied to sterile paper disks as described earlier. Adult *R. pomonella* "young" and "old" flies, as described earlier, were allowed to feed on the soaked disks contained in a sterile Petri dish. Once the flies were observed to have fed and their abdomens were distended, the flies were individually removed and placed by age into separate cages containing fresh food and water. Preliminary feeding tests revealed that some flies that fed on the supernatant died just hours after feeding. Therefore, percent mortality was recorded at 8 and 24 h.

Statistical analysis. Data acquired from all studies were analyzed using the Statistix© Analytical Software for analysis of variance tests (SAS Institute, 1985).

RESULTS

Preliminary tests showed that a cell concentration of 4.7×10^4 cfu/ml produced death in approximately 48% of flies that were approximately 14 days old. Tests were then designed to determine a concentration of this *S. marcescens* needed to produce approximately 50% death of flies within a predetermined time period for a broad age range of flies. These tests showed that a cell concentration of 4.7×10^4 cfu/ml produced death in 43% of the flies after 24 h with no appreciable additional death after 120 h (data not shown). All control flies (not exposed to the pathogen) were alive at 120 h.

Flies that fed on the two different concentrations of *S. marcescens*, 4.7×10^4 cfu/ml and 4.7×10^8 cfu/ml respectively, produced nearly identical results (Table 1). There was no significant difference in fly mortality between concentrations. However, significant differences in mortality did occur between fly ages; younger flies were significantly more susceptible to infection and death than older flies at each concentration (Table 2).

To determine the presence and persistence of *S. marcescens* within infected *R. pomonella,* flies fed on bacterial suspensions and alimentary canal organs were sampled for S. *marcescens* starting at 1–2 min and ending at 4 days post-ingestion. *S. marcescens* were recovered 1–2 min after feeding and until the end of the study (Table 3). By day's end of the fourth day (post-ingestion), only 30 percent of the infected flies remained alive and of these 30%, 66% harbored *S. marcescens.* The control flies did not harbor any *Serratia* sp. in samples of their alimentary canals (data not shown).

Microorganisms present within the alimentary canal of *R. pomonella* maintained in the laboratory before experi-

TABLE 2. Percent mortality of young and old apple maggot flies caused by ingestion of two different concentrations of *Serratia marcescens*.

Treatment	No. tested	Average percent mortality			
		Young (7–10 days old)		Old (21–28 days old)	
		24 h	120 h	24 h	120 h
High dose[a]	90	30a	33a	12b	20b
Low dose[b]	90	27a	31a	18b	20b
Control (uninfected)	90	6c	6c	10bc	15b

[a]High dose = 4.7×10^8 cfu/ml
[b]Low dose = 4.7×10^4 cfu/ml
Values followed by a different lower case letter are significantly different at p = 0.05 level according to least significant difference tests.

mentation were primarily *Pseudomonas* sp., *Alcaligenes* sp., and *Acinetobacter* sp. The control flies were found to contain large numbers of *Pseudomonas* sp., and lesser numbers of *Alcaligenes* sp. and *Acinetobacter* sp. (nonfermenters), with very few *Enterobacter* spp. and *Klebsiella* spp. (butylene glycol fermenters) throughout the study. After feeding on *S. marcescens*, however, infected flies showed an increase in isolation of the number of bacterial species belonging to the family Enterobacteriaceae (Table 4). In these test flies, *Klebsiella* sp. dominated, followed closely by *Enterobacter* sp., with only 1 *Pseudomonas* sp. and no *Alcaligenes* or *Acinetobacter* sp. recovered. *S. marcescens* re-isolated from test flies was subsequently used to test Koch's Postulates. We determined that, indeed, this strain of *S. marcescens* was responsible for apple maggot fly death (data not shown) in mortality studies.

The supernatant from a 21 h old culture of *S. marcescens* was highly toxic to flies, killing on average 59% of the young flies within 8 h and 72% within 24 h. Eighty percent of old flies were dead within 24 h (Table 5). There was no difference in mortality in relation to age.

DISCUSSION

Our findings indicate that a nonpigmenting strain of *S. marcescens* isolated from diseased and/or dead apple maggot flies kills, on average, 40% of test flies (10–28 days old) within 24 h post-ingestion at a concentration of 4.7×10^4 cfu/ml. Increasing the number of cells ingested by the flies 10,000 fold did not lead to a substantial increase in the rate or amount of kill. There may be at least five possible explanations for this occurrence: (1) the rapidity of kill eliminates or disguises any obvious dosage effect, (2) the doses tested are actually two closely-aligned points on a dose-response curve, (3) virulence is related to an exotoxin produced by the bacteria, whose lethal concentration is not based solely on cellular number, (4) enough *S. marcescens* were digested by some flies to release toxic or lethal amounts of Lipid A (endotoxin) (Atlas, 1988) or (5) cells entered the insects' hemolymph via small wounds or other entrance sites during feeding. Further investigation is needed to evaluate these possibilities and/or any other subtle effects of dosage.

TABLE 3. Establishment and persistence of *Serratia marcescens* in laboratory-tested *Rhagoletis pomonella*.

Time elapsed from inoculation until dissection	Group dissected	Identified bacterial isolates recovered by dissection			
		Sm[a]	Other Enterobacteriaceae	PS[b]	Other aerobes
1–2 min	Treatment[c]	T[d]	F[e]	D[f]	F
	Control[g]	Nr[h]	F	D	F
15 min	Treatment	T	F	D	F
	Control	Nr	F	D	F
30 min	Treatment	T	D	F	T
	Control	Nr	F	D	f
1 h	Treatment	F	D	F	Nr
	Control	Nr	F	D	F
24 h	Treatment	F	D	T	F
	Control	Nr	F	D	F
4 d	Treatment	T	D	F	Nr
	Control	Nr	F	D	F

[a]Sm = *Serratia marcescens*
[b]PS = *Pseudomonas* sp.
[c]Treatment = *S.marcescens*-fed *R. pomonella*
[d]T = trace bacterial species recovered
[e]F = few bacterial species recovered
[f]D = dominant bacterial species recovered
[g]Control = normal , protein - fed *R. pomonella*
[h]Nr = not recovered

TABLE 4. Demonstrated shift in bacterial species in the alimentary canals of *Rhagoletis pomonella* after being infected with *Serratia marcescens*.

	Number of bacterial species recovered from alimentary canal organs		
	Classification	Before infection	After infection
Pseudomonas spp.	NF	3	1
Acinetobacter sp.	NF	1	0
Alcaligenes sp.	NF	1	0
Klebsiella sp.*	F	1	3
Serratia sp.*	F	0	2
Enterobacter sp.*	F	0	2

*Members of the family Enterobacteriaceae
NF = Nonfermenter
F = Fermenter

TABLE 5. Average percent mortality of young and old *R. pomonella* flies after ingestion of supernatant from a 21 h culture of *Serratia marcescens*.

Treatment	No. tested	Average percent mortality			
		Young (7–10 days old)		Old (21–28 days old)	
		8 h	24 h	8 h	24 h
Supernatant	90	59a	72a	60a	80a
Control	90	0b	0b	0b	0.11b

Values followed by a different lower case letter are significantly different at p = 0.05 level according to least significant difference tests.

Young *R. pomonella* appeared to be more sensitive to the presence of this bacterial pathogen than older more mature flies (Table 2). This is contrary to studies that suggest decreased enzyme action and immune response are characteristic of older mature flies (Christensen et al., 1986, Jianyong et al., 1992), making them more sensitive to the effects of bacterial pathogens. In addition, during the course of our study a few of the test flies (young and old) did not appear to be adversely affected upon exposure to the pathogen. This suggests that those flies were insensitive to the pathogen's presence or that some protective mechanism was present in such flies (i.e. induction of bactericidal peptides) but not in the majority of the fly population.

Several studies exist that describe acquired humoral immunity against bacteria and toxins in insects (e.g. Krieg, 1987), although Chadwick (1971) found that *S. marcescens* survived treatment with immunized hemolymph. In our experiments, such a protective mechanism is not known but merits further investigation. In addition, tighter age-dependency experiments should be conducted with companion microbiological and immunological analyses. It is also possible that these flies did not ingest or feed as much as the other flies, however, this is unlikely as each fly was observed to feed and only flies with extended abdomens post-feeding were used for the study.

We have also shown that *S. marcescens* was responsible for the death of *R. pomonella* flies by fulfilling the requirements of Koch's postulates. We isolated continuously this strain of *S. marcescens* from dead flies and used these cultures to reinfect additional flies whom also died. The gut environment of the fly appears to favor growth of *S. marcescens* and possibly other bacteria once introduced. Several different tephritid pest species possess few bacteria on eclosion (Lauzon et al., 2000, unpublished). It is likely that the first bacteria that arrive to or are already present in the gut that can tolerate or thrive under gut conditions are those that become established. Enteric bacteria, *Enterobacter* spp. and *Klebsiella* spp. are isolated repeatedly from the alimentary canal organs of *R. pomonella* despite the numerous different types of bacteria and fungi that these flies consume while feeding on natural food sources, such as fecal material (e.g. Lauzon et al., 1998, 2000). *Serratia* spp., *Enterobacter* and *Klebsiella* spp. are all members of the family Enterobacteriaceae. Perhaps the gut environment is exceptionally supportive of these types of bacteria. In addition, a dramatic shift in the kinds and numbers of bacteria within the alimentary canal of flies infected with *S. marcescens* suggests that fundamental changes are occurring within the flies when they are exposed to this pathogen. The exact mechanism for this shift is not known but could be related to pH effects on bacterial growth. In preliminary experiments, we demonstrated that *S. marcescens* grows well in an acidic (pH 5.0) environment (data not shown). *Pseudomonas* spp. and related bacteria, which were found to be dominant inhabitants of alimentary canals of laboratory-maintained apple maggot flies before testing, favor utilization of amino acids as an energy source and are sensitive to acidic pH. Conversely, *Serratia* spp. are rapid fermenters of carbohydrates. The rapid utilization of carbohydrates results in the release of small amounts of acids from the breakdown of glucose, and appear to tolerate an acidic environment very well. A pH shift downward would tend to favor bacteria tolerant of an acidic environment; however, further work is necessary before a more definitive statement can be made as to why a population shift occurred. Kodama & Nakasuji (1971) described a situation where the presence of *Streptococcus faecalis* and *S. faecium* in the midgut of silkworms enhances the pathogenicity of *Serratia piscatorum* by lowering the midgut pH to one that favors the growth of the *Serratia* sp. Several investigators have reported numerous factors that affect susceptibility of insects to bacterial infection, including age (Beegle et al., 1981), plant allelochemical-induced stress (Felton & Dahlman, 1984), diet (James & Lighthart, 1992), and general changes of microbiotia within insects housed in laboratories (Lighthart, 1988). Whatever the explanation, the strain of *S. marcescens* tested here does kill *R. pomonella,* which indicates that further study is merited to understand the nature of pathogenicity associated with this strain of *S. marcescens*.

S. marcescens has been reported as a pathogen of several economically important insect pests such as the boll weevil, *Anthonomus grandis* Boheman (Ourth & Smalley, 1980), the tobacco hornworm, *Manduca sexta* (Linnaeus) (Dunn & Drake, 1983), and the house fly, *Musca domestica* Linnaeus (Benoit et al., 1990), as well as beneficial arthropods such as the predatory mite *Metaseiulus occidentalis* (Nesbitt) (Lighthart et al., 1988), and the honey bee, *Apis mellifera* Linnaeus (El Sanousi et al., 1987). *S. marcescens* also has been reported as an insect pathogen of moderate virulence that causes a fatal septicemia after penetration through the insect's gut wall and subsequent invasion of the hemocoel (Lysenko, 1985; Krieg, 1987). Though pathogenicity has been reported for several insect species, most methodologies used include only injection of *Serratia* sp. into the insect (Steinhaus, 1963) rather than introduction of the bacteria into the insect through ingestion. Ingestion of *S. marcescens* is generally seen as of little consequence to insects (e.g. Podgwaite & Cosenza, 1976), however, Bracken & Buchner (1967) did show that the ichneumonid parasitoid *Exeristes comstockii* (Cresson) acquires enough *S. marcescens* from its larval form to cause a fatal septicemia in its own and other adults.

S. marcescens is ubiquitous in nature (Brenner, 1992), but its presence and persistence is not well characterized and it is not often recovered from wild insects (Steinhaus, 1959; Krieg, 1987). Studies aimed at examining further the ecology of *S. marcescens* are discouraged because the pathogenicity of *S. marcescens* to humans, other vertebrates, and beneficial insects prevents widespread use of this microorganism per se to control insect pests. Our data indicate, however, that this strain of *S. marcescens* produces a metabolite(s) toxic to apple maggot flies, sug-

gesting the possible use of the toxic metabolite(s) produced by *S. marcescens* in apple maggot fly control strategies.

Toxins or proteases secreted by *Serratia* sp. may facilitate entrance of the bacteria into the hemocoel. At present, some 11 proteases have been described as being produced by several different strains of *S. marcescens* (e.g. Brenner, 1992), including chitinases (Lysenko, 1976). Poinar et al. (1979) found that *S. marcescens* was capable of entering the hemocoel in tsetse flies, which died soon after from fatal septicemia. Also, Asano et al. (1999) determined that synergistic effects occurred when supernatant from a culture of *Serratia marcescens* was used in conjunction with *Bacillus thuringiensis* Cry1C toxin. It remains to be determined if our strain of *S. marcescens* enters the hemocoel of apple maggot flies and if this entrance is facilitated by one or more toxin(s); however, samples of several midguts of *R. pomonella* that died in our study looked partially or completely disintegrated. Perhaps the condition of the midgut was due to autolysis and/or proteinases produced by *Serratia* growing post mortem. *Serratia* spp. typically produce compounds other than proteases and possibly, these compounds were produced by our strain that feasibly poisoned the flies. These compounds would include peptides, acids and alcohols (e.g. Brenner, 1992). Current studies are underway to characterize the candidate toxin(s) produced by this strain of *S. marcescens*. Thus far, we have conducted experimentation aimed to characterize the nature of the supernatant (CRL et al., unpubl.) and isolate any toxic components. Our efforts show some promising preliminary findings including indication that flies find toxic fractions palatible.

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