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A Strain of *Serratia marcescens* Pathogenic for Larvae of *Lymantria dispar*: Infectivity and Mechanisms of Pathogenicity

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The ED_{50} of a strain of *Serratia marcescens* for microinjected instar III and IV gypsy moth larvae was 7.5 and 14.5 viable cells, respectively. Percentage and rate of mortality were found to be highly variable among replicates of the same instar and between instars in free-feeding bioassays. Mortality in second instar larvae occurred before ecdysis, whereas practically no mortality occurred in third and fourth instars until the molting period. Neither Boivin endotoxin preparations nor culture filtrates were toxic to instar III larvae when administered per os or by microinjection. Histological evidence indicated that the microorganism invaded the hemocoel of healthy or predisposed insects through the gut wall. The rapid multiplication of the bacterium in the hemocoel of infected insects, followed by death in the absence of extensive tissue damage, indicated mortality was due to a septicemia. The histological and biological evidence presented indicated that the microorganism would be less than effective if utilized as a conventional microbial insecticide.

INTRODUCTION

In a previous communication, Podgwaite and Campbell (1972) described a number of aerobic bacterial pathogens isolated from larvae of the gypsy moth, *Lymantria dispar*. In preliminary bioassays, one of these microorganisms was found to be highly pathogenic for second-instar larvae. This bacterium was subsequently characterized in greater detail and identified as a nonpigmented strain of *Serratia marcescens* (Podgwaite and Cosenza, 1976). It was distinguished from closely related members of the Enterbacteriaceae by its inability to produce gas from glucose, insitol, glycerol, and cellobiose; its rapid liquefaction of gelatin; and its failure to ferment raffinose or arabinose. The percentage of G + C in DNA from this bacterium was within the range reported for known strains of *S. marcescens*. Although there have been numerous reports of *S. marcescens* infecting insects (Bucher, 1959; Stephens, 1959; Stevenson, 1959), there is a paucity of information regarding the invasive mechanism of this bacterium. Because of this and the potential of *S. marcescens* as a microbial control agent, the nonpigmented strain was studied further to (1) determine its infectivity for larval stages of the gypsy moth and (2) gain some insight into its mechanisms of pathogenicity. The following is a report on these studies.

MATERIALS AND METHODS

Maintenance of bacterial cultures. The strain of *S. marcescens*, herein referred to as 922A, used in these studies was transferred monthly on Trypticase soy agar slants (BBL) and maintained at 4° C.

General insect-rearing procedures. Gypsy moth larvae used in infectivity tests were reared from egg masses that were collected from areas of sparse populations where natural disease incidence was low. Eggs were kept at 4°C before being dehaired and surface-treated with 0.5% sodium hypochlorite for 10 min. Larvae were reared on artificial diet (Odell and Rollinson, 1966) in sterile plastic Petri dishes (13 cm) at room temperature, 24° ± 2°C, with 16 hr of light. Whenever possible, test and control larvae for a given set of trials were hatched from the same egg mass. Generally, the test and control groups consisted of five replicates of 10 larvae each. Foliage used for free-feeding trials was tender red or white oak leaves of a uniform size (14–16 cm^2).

Dose-feeding experiments. Test insects were fed tender oak leaves dipped in serial log dilutions of a 16-hr Trypticase soy broth (TSB) (BBL) culture of 922A. The most concentrated (high dose) contained 1.95×10^9 viable cells/ml; the least concentrated (low dose) contained 19.5 viable cells/ml. Control insects were fed on oak leaves dipped in sterile TSB. Larvae were allowed to feed undisturbed for 48 hr on the test material before being transferred to an artificial diet for the remainder of the trial. Differences in mortality occurring between doses were assessed by using the χ^2 test.

Pathogenicity for larval stages. The relative pathogenicity of 922A for different larval instars was tested by allowing instar II, III, and IV larvae to feed on oak leaves dipped in concentrated, washed cellular suspensions of the microorganism in sterile distilled water. Larvae used for these tests were pooled from several egg masses, but, for a given instar, were the same age. Each group of larvae was challenged 2 days after the larvae had molted to the appropriate instar, and the larvae were allowed to feed on the test material for 48 hr before being transferred to the artificial diet for the duration of the trial. Each test was concluded 2 days after the last larva of a given instar group had molted to the succeeding instar. We assessed their mortality and analyzed the data by using the χ^2 test.

Estimation of ED_{50} by intracoelomic injection. Trials were conducted using third- and fourth-instar, diet-reared larvae. For a given bioassay, larvae from the same egg mass, of the same instar, same age, and approximately the same weight were used. Dosages were prepared from a concentrated cellular suspension of 922A in sterile distilled water. Viable cell counts per dose were determined either from dilutions by using the drop plate method (Reed and Reed, 1948) or by plating the actual amount injected. All larvae were anesthetized with CO_2 before treatment.

An ISCO Model M microapplicator (Instrumentation Specialties Co., Inc.) equipped with either a 27- or 30-gauge needle was used for injections. Test insects were intracoelomically injected laterally, in the area above and between the first and second thoracic legs, with 1 μl of cellular suspension. Control insects were injected similarly with 1 μl of sterile distilled water. Wounds caused by the injection procedure were immediately covered with hot, sterile Vaspar (Vaseline–paraffin, 1:1, v/v). Larvae were reared on artificial diet for 48 hr after injection. Larval deaths in that period were recorded, and ED_{50}'s were determined from dose–percent effect curves by the method of Litchfield and Wilcoxon (1949).

Endotoxin bioassays. Endotoxin from 922A was prepared by a modification of the method of Boivin, Mesrobeanu, and Mesrobeanu (1933) and bioassayed per os and by intracoelomic injection against instar III larvae.

For free-feeding tests, groups of 10 larvae were allowed to feed on two uniformly cut (1 cm^2) diet blocks, each containing 0.5 mg of endotoxin preparation. Control insects were fed normal diet. Larvae were reared for 15 days and were constantly observed for gross changes in behavior or other symptoms of disease.

Dosage levels of 2 and 1 μg of endotoxin per animal were used for microinjection assays. Twenty instar III larvae weighing, on the average, 70 mg received the appropriate dosage in 1 μl of sterile distilled water. Con-

trol animals received 1 μl of sterile distilled water. On a w/w basis, the dosages used corresponded to 100 and 50 times the LD_{50} value of *S. marcescens* endotoxin for mice (Cundy and Nowotny, 1968). Larvae were reared on artificial diet and observed for symptoms of disease.

Toxicity of culture filtrates. Filtered (0.22-μm filter) supernatant fluid from a TSB culture of 922A in the logarithmic phase of growth was bioassayed per os and by intracoelomic injection against instar III larvae. Twenty larvae were fed oak leaves dipped in the filtrate. Twenty larvae were fed untreated foliage. Additionally, 20 larvae were injected with 1 μl of filtrate. Controls received an equal amount of sterile TSB. All larvae were reared for 10 days and were observed for symptoms of disease.

Histology. Instar II larvae were fed oak foliage dipped in an aqueous suspension of 922A. Control insects were fed untreated oak foliage. Twenty-four hours after feeding and at subsequent 24-hr periods, living and dead larvae from test and control groups were sampled and examined by the following procedures.

Living and dead insects were fixed in Bouin's solution for 24–72 hr. After fixation, larvae were dehydrated in three to five changes of 90% isopropyl alcohol (13 hr) followed by three changes in 100% isopropyl alcohol (17 hr). Larvae were infiltrated under vacuum (8 hr) with Paraplast (Sherwood Medical Industries, Inc.) and embedded in the same medium. Longitudinal and cross sections (midgut region) were prepared at a thickness of 6 μm.

The following staining procedures outlined by Luna (1968) were used. Harris' hematoxylin and eosin stain was used to detect general histological changes occurring in larval tissues, particularly those of the midgut. To detect bacterial cells in tissues, the staining procedure of MacCallum and Goodpasture was used.

RESULTS

Results of a dose-feeding experiment with the microorganism are shown in Table 1. No mortality occurred in any of the treated groups until the third day of the trial. In the two lowest doses tested, mortality was first evident on the fourth day. Peak mortality in all groups occurred between the third and fifth day after feeding. There were no significant differences in the percentage of mortality caused by the three highest dose levels, nor were the two lowest doses significantly different in this regard. However, each high dose was significantly different from each low dose in percentage of mortality.

TABLE 1
Free-Feeding Bioassay of 922A against Instar II Gypsy Moth Larvae

Dose (cells/ml of suspension)	Larvae tested (No.)	Mortality (%)
0 (Control)	50	0
1.95×10^9	50	28
1.95×10^7	50	30
1.95×10^5	50	22
1.95×10^3	50	8
1.95×10^1	50	8

The relative pathogenicity of 922A for instar II–IV larvae is shown in Table 2. The microorganism killed 32, 95, and 42% of the instar II, III, and IV larvae, respectively. Almost all mortality in the second instars occurred before ecdysis, whereas very little mortality occurred in the third- and fourth-instar group until the molting period. The peak mortality in these two groups occurred while the animals were molting to the next instar.

Tables 3 and 4 show results of tests to determine the ED_{50} of the microorganism for

TABLE 2
Relative Pathogenicity of 922A for Instar II–IV Larvae by Free Feeding

Instar	Dose (cells/ml of suspension)	Larvae tested (No.)	Mortality (%)
II	1.95×10^9	50	32
II	0 (Control)	50	0
III	9.3×10^8	50	95
III	0 (Control)	50	4
IV	6.7×10^9	45	42
IV	0 (Control)	45	0

TABLE 3
Bioassay of 922A against Instar III Larvae (Microinjection)

Dose (viable cells injected)[a]	Larvae treated (No.)	Mortality at 48 hr (%)
0	19	0
4670	20	95
467	20	80
46	19	100
13.5	20	40
7.5	19	53
4.86	19	47
2.6	20	35

[a] ED_{50} = 7.5; for 95% probability, 14.03 = upper limit and 4.01 = lower limit.

instar III and instar IV larvae. The ED_{50} for microinjected instar III larvae was determined to be 7.5 viable cells with the upper and lower 95% confidence limits being 14.03 and 4.01, respectively. The ED_{50} for instar IV larvae was somewhat higher, 14.5 cells, with 52.2 and 4.03 being the upper and lower confidence limits. All dead animals from these tests were found to contain high intracoelomic populations of the microorganism at the time of death.

Animals fed Boivin endotoxin extracted from 922A displayed no abnormal behavior or other signs of disease 15 days after feeding. The test specimens that were sacrificed and dissected at this time and compared to dissected control specimens showed no gross pathologies of internal organs or tissues.

Animals injected with endotoxin preparations also did not show any observable pathological changes. There was one death in the 2-μg test group, which could not be attributed to endotoxin affects. Hemolymph samples taken from test and control animals 1, 6, and 24 hr after injection revealed no significant differences in number and types of hemocytes present nor any obvious pathologies thereof.

TABLE 4
Bioassay of 922A against Instar IV Larvae (Microinjection)

Dose (viable cells injected)[a]	Larvae treated (No.)	Mortality at 48 hr (%)
0	20	0
4,000,000	20	100
400,000	20	100
40,000	20	100
4,000	20	100
400	20	95
40	19	68

[a] ED_{50} = 14.5; for 95% probability, 52.2 = upper limit and 4.03 = lower limit.

Culture filtrates from 922A did not induce any disease in larvae when the treatment was either per os or by microinjection. Treated animals could not be shown to differ from controls when they were dissected and examined for gross pathological changes.

Larvae that ingested a lethal dose of 922A whole cells succumbed in 1–15 days. Sixteen to twenty-four hours before death, infected animals became sluggish and ceased to feed. Some displayed a diarrhetic discharge. Integumental coloration differences between healthy and diseased larvae were not evident at death, but the coloration of infected animals darkened slightly after death.

Gross changes in internal organs and tissues of diseased animals were not evident at death; however the hemolymph of diseased animals appeared milky white or tan, which was distinct from the clear or blue hemolymph of healthy animals. Post mortem histolytic activity was rapid. Internal organs and tissues rapidly lost their integrity and eventually decomposed within 24–48 hr.

Those animals killed by 922A all harbored an actively multiplying population of the microorganism in the hemocoel (Fig. 1). Longitudinal sections through the midgut of healthy and moribund larvae are compared in Figures 2–5. Sections of moribund larvae revealed the microorganism to be widespread throughout the hemocoel but not associated with any tissue or organ system other than the hemolymph.

Figure 3 shows the midgut of a typical moribund larva infected with 922A. Although there was separation of the basement membrane from the epithelial cells, there was little cellular damage at this time. This specimen shows disintegration of the peri-

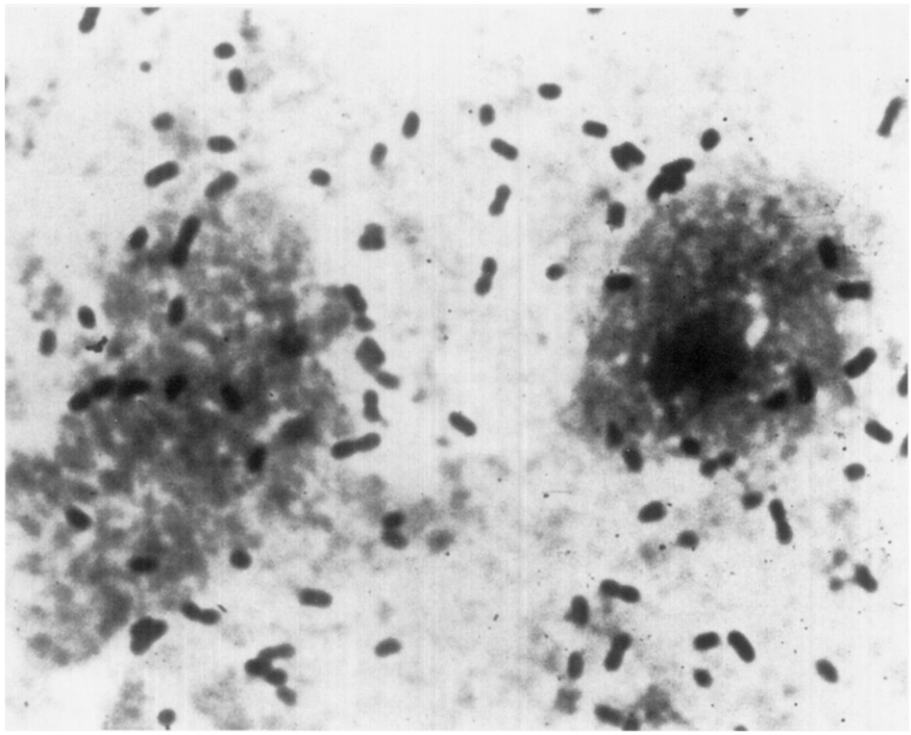

FIG. 1. Hemolymph from a moribund larva infected with 922A. Giemsa; ×4250.

trophic membrane; however, this was not a consistent finding with other diseased specimens. There appeared to be no paralysis of the alimentary tract as evidenced by the relaxed nature of the gut musculature.

Moribund animals frequently displayed a separation of the midgut epithelium from the basement membrane (Fig. 4) and occasionally a loss of cellular integrity in areas of the midgut epithelium. These appeared as "holes" generally two or three cell-widths thick in the midgut wall (Fig. 5). Other than this infrequent cellular damage, midgut epithelium of moribund animals displayed little difference from that of healthy uninfected controls.

DISCUSSION

The dose-feeding experiments revealed the ever-present variation encountered in free-feeding bioassays and are in general agreement with the work of Steinhaus (1959). He tested a number of pigmented strains of *S. marcescens* against several lepidopterous species and observed considerable variation in pathogenicity, not only according to the particular strain used but also when any one strain was tested at different times under standardized experimental conditions.

Steinhaus (1959) also indicated that older insects of a particular species were more susceptible than younger ones to *S. marcescens* infection. Results of the present study support this view, but the data (Table 2) suggest a different mode of action in older insects. Newly molted instar II larvae begin to succumb to infection 1–3 days after feeding on contaminated foliage. Instar III and IV larvae, however, generally do not begin to die until the succeeding period of ecdysis, but during this period mortality is often dramatically high. This might be explained by the greater voracity of later-stage larvae. An alternative explanation relates to the histolytic activity that occurs in gut and other tissues of many insects during the molting

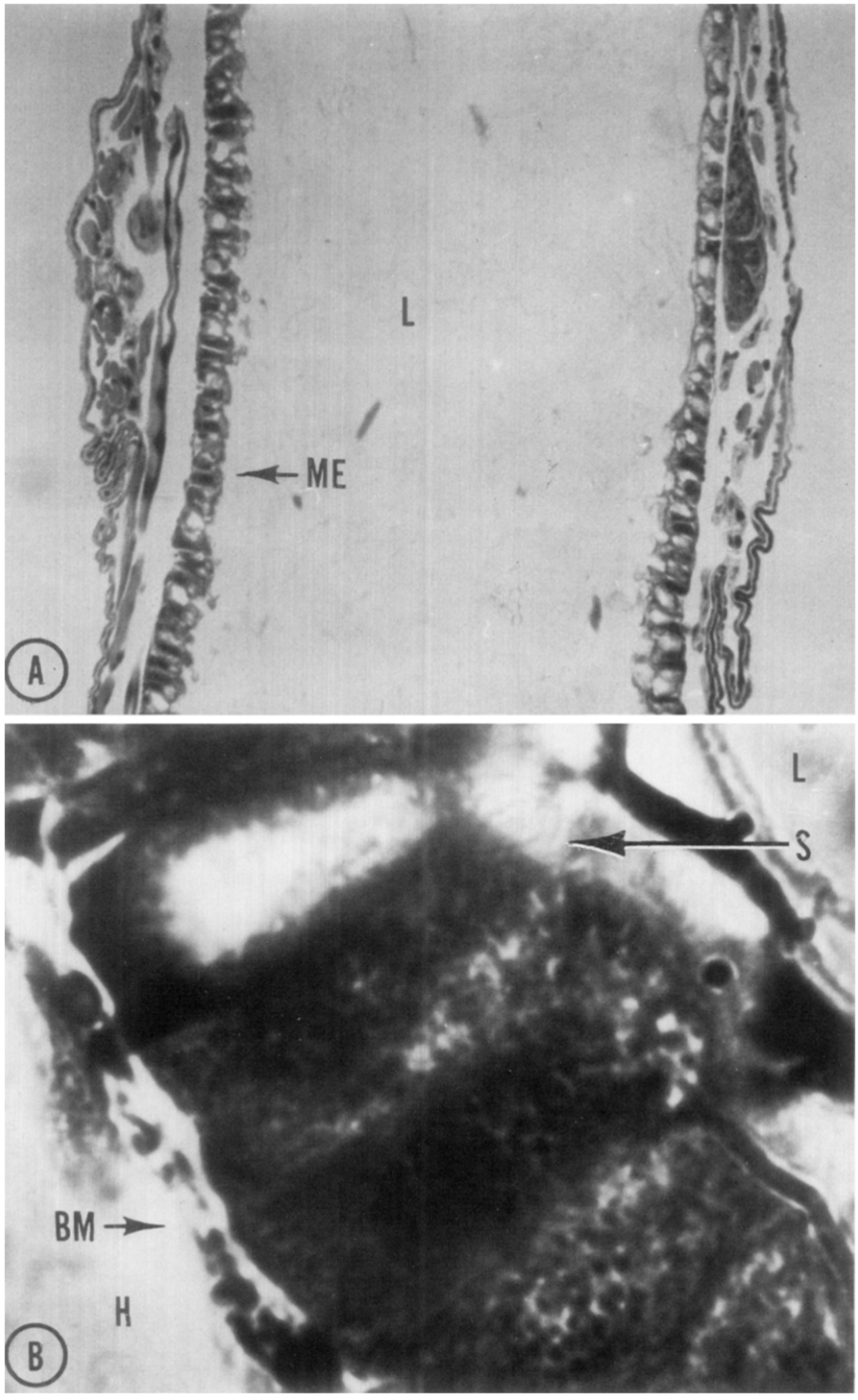

FIG. 2. Longitudinal section through the midgut region of a healthy instar II gypsy moth larva. L, lumen; ME, midgut epithelium; H, hemocoel; BM, basement membrane; S, striated border. Hematoxylin and eosin; (A) ×340; (B) ×4250.

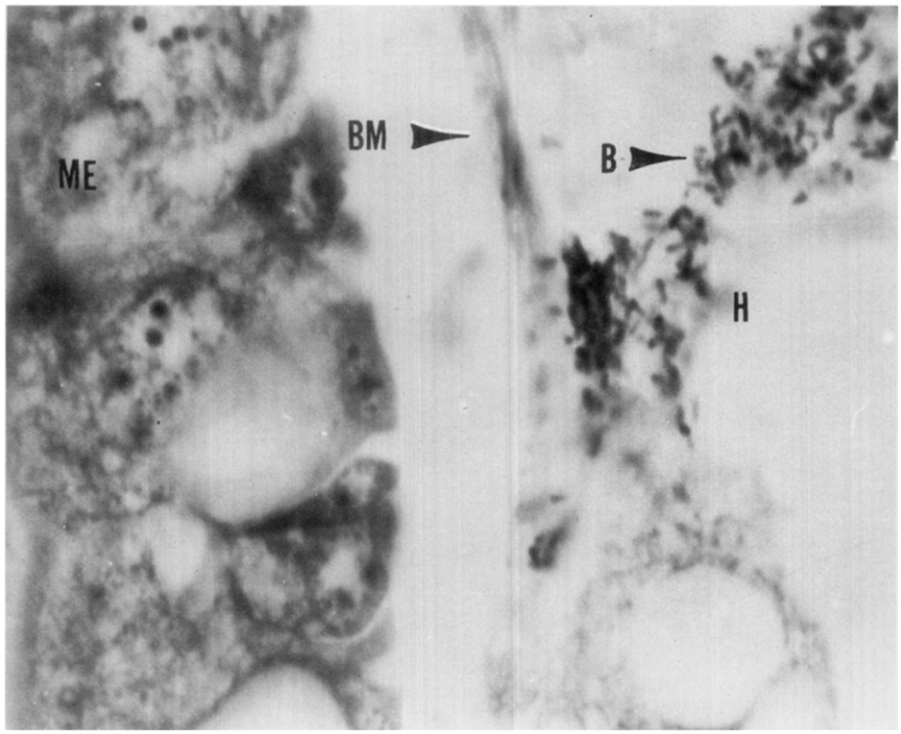

FIG. 3. Longitudinal section through the midgut of a typical larva infected with 922A. ME, midgut epithelium; BM, basement membrane; B, bacterial cells; H, hemocoel. MacCallum–Goodpasture; ×3825.

period. This would clearly predispose the individual insect to a greater chance of bacterial invasion through the gut wall. This view is supported by Bucher (1959), who noted that bacteria penetrate more easily into the hemocoel of crickets during the molting period.

Third and fourth instar larvae were found to be highly susceptible to the bacterium by intracoelomic injection (Tables 3 and 4). The ED_{50} for instar III larvae was between 4 and 14 viable cells, whereas for instar IV larvae it was higher, between 4 and 52 viable cells. Bucher (1959) found that the ED_{50} of *S. marcescens* by injection was 10–50 cells for adult grasshoppers. The ED_{50} for *Schistocerca gregaria* (Forskal) was determined to be 10 cells (Stevenson, 1959); and for *Galleria mellonella* (L.), it was determined to be 40 cells per animal (Stephens, 1959). Slatten and Larson (1967) reported an intracoelomic ED_{50} value of 5.1 ± 1 cells for the adult boll weevil *Anthonomus grandis* Bohenan. The roach *Blatella germanica* was found to be susceptible to only massive intracoelomic doses, the ED_{50} being 4×10^4 cells (Heimpel and West, 1959). Thus, isolate 922A seems to compare in degree of virulence to reported strains of *S. marcescens*.

Although the toxic effects of endotoxins on insects have received little study, the results obtained by administering endotoxin by two routes to gypsy moth larvae are in agreement with those few observations by other researchers using different insect species.

Chadwick (1968) reported the Boivin antigen of *Pseudomonas aeruginosa* to be nontoxic to larvae of *G. mellonella* in large doses. Supporting this, Schwalbe and Boush (1971) found that Cr^{51} labeled endotoxin is treated as a foreign material by *G. mellonella* and is effectively removed from circulation by cyst formation in the hemocoel with ultimate deposition in the cuticle. These researchers also suggested that there was a hemocyte proliferation after endotoxin injec-

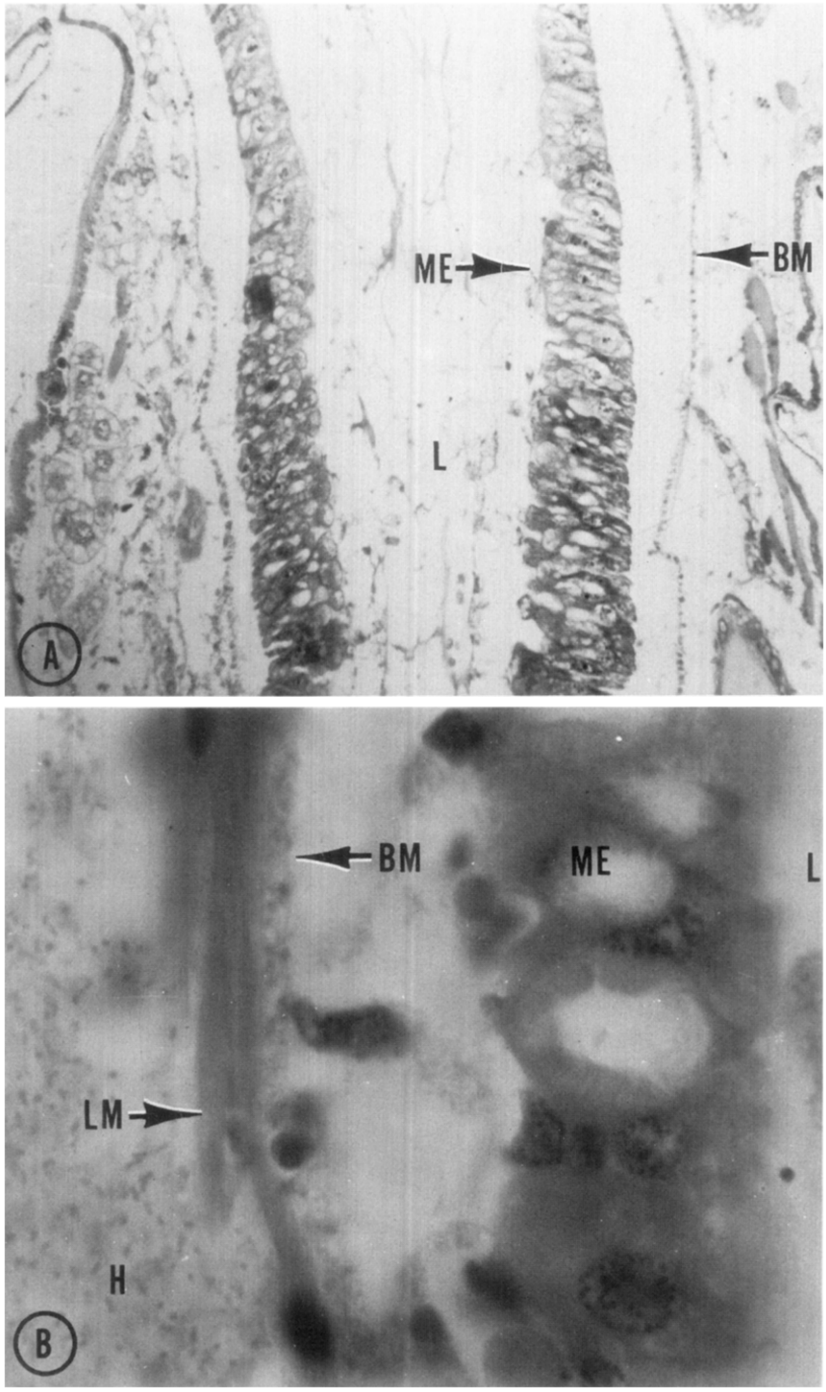

FIG. 4. Longitudinal section through the midgut of a moribund larva infected with 922A, L, lumen; H, hemocoel, ME, midgut epithelium; BM, basement membrane; LM, longitudinal muscle. Hemotoxylin and eosin; (A) ×340, (B) ×3825.

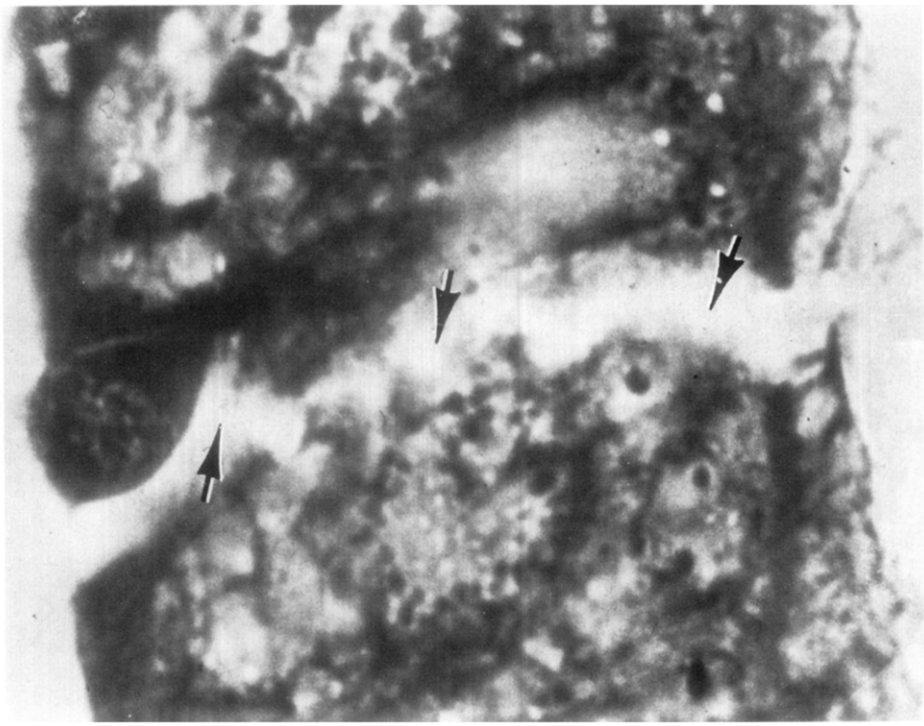

FIG. 5. Longitudinal section through the midgut of a moribund larva infected with 922A. Arrows indicate area of cellular disruption. Hematoxylin and eosin; ×4250.

tion but that there were no abnormalities in cellular appearance. Hemocyte proliferation could not be demonstrated in this study, and cellular abnormalities were not in evidence.

The histological evidence presented suggests that invasion is through the midgut epithelium, but bacterial cells were never found in epithelial cells or interstitial spaces.

The rapid multiplication of the microorganism in the hemocoel followed by death of the animal without any extensive physical damage to the internal cellular systems suggests that animals infected with the microorganism die of a septicemia. Although bioassay results indicated that larvae are predisposed to passive invasion just before or during ecdysis, the microorganism does possess enzyme systems that, in theory, could be responsible for its invasive nature.

Its lecithinase activity could account for some loss of cellular activity by action on gut cell membrane. However, it seems unlikely that this effect is maximized in vivo because optimum lecithinase activity occurs at a temperature and *p*H not normal to the gypsy moth larval gut. It is more likely that chitinase is involved in the invasiveness of the microorganism.

The ability of 922A to degrade chitin has been demonstrated in culture under a temperature and *p*H regime within the range found in the alimentary tract of the insect (Podgwaite and Cosenza, 1976). Because the foregut and hindgut are lined with chitin and the peritrophic membrane also contains this macromolecule, this barrier must be breached to allow hemocoelomic invasion.

Once the gut barrier is breached, the ultimate damage to the insect in all probability is due to the integral metabolism of the bacterium rather than to the effects of a single toxic entity such as the crystal or exotoxins of *Bacillus thuringiensis* or the proteinases of *Pseudomonas aeruginosa*.

The evidence presented, along with what is known of the physical factors affecting non-

spore-forming bacteria, suggests that 922A would be less than effective if utilized as a conventional microbial insecticide. Its variable pathogenicity, even when administered in massive doses under ideal conditions, indicates there would be great risk in applying the microorganism to a gypsy moth-infested forest stand and expecting a high degree of insect control. Additionally, there is little reason to expect the microorganism to be any less susceptible to sunlight and temperature-desiccation effects than other non-spore-forming bacteria.

However, assuming that the microorganism is nonpathogenic to humans and lower vertebrates and that the application problems can be overcome with the use of adjuvants and extenders, it is at least reasonable to study further the effects of utilizing 922A either in combination with other microbials or in stressed populations where its capabilities as a pathogen could be maximized.

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REFERENCES

BOIVIN, A., MESROBEANU, J., AND MESROBEANU, L. 1933. Technique pour le preparation des polyosides microbiens specifiques. *C. R. Soc. Biol.*, **113**, 490–492.

BUCHER, G. E. 1959. Bacteria of grasshoppers of western Canada. III. Frequency of occurrence, pathogenicity. *J. Insect Pathol.*, **1**, 391–405.

CHADWICK, J. S. 1968. Some aspects of immune responses in insects. *In Vitro*, **3**, 120–128.

CUNDY, K. R., AND NOWOTNY, A. 1968. Comparisons of five toxicity parameters of *Serratia marcescens* endotoxins. *Proc. Soc. Exp. Biol. Med.*, **127**, 999–1003.

HEIMPEL, A. M., AND WEST, A. S. 1959. Notes on the pathogenicity of *Serratia marcescens* Bizio for the cockroach *Blattella germanica* L. *Canad. J. Zool.*, **37**, 169–172.

LITCHFIELD, J. T., JR., AND WILCOXON, F. 1949. A simplified method of evaluating dose-effect experiments. *J. Pharmacol. Exp. Ther.*, **96**, 99–113.

LUNA, L. G. (ed.). 1968. "Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology," 258 pp., McGraw-Hill, New York.

ODELL, T. M., AND ROLLINSON, W. D. 1966. A technique for rearing the gypsy moth, *Porthetria dispar* L., on an artificial diet. *J. Econ. Entomol.*, **59**, 741–742.

REED, R. W., AND REED, G. B. 1948. "Drop plate" method of counting viable bacteria. *Canad. J. Res.*, **26**, 317–326.

PODGWAITE, J. D., AND COSENZA, B. J. (1976) A strain of *Serratia marcescens* pathogenic for larvae of *Lymantria dispar:* Characterization. *J. Invertebr. Pathol.*, in Press.

SCHWALBE, C. P., AND BOUSH, G. M. 1971. Clearance of Cr^{51}-labeled endotoxin from hemolymph of actively immunized *Galleria mellonella*. *J. Invertebr. Pathol.*, **18**, 85–88.

SLATTEN, B. H., AND LARSON, A. D. 1967. Mechanism of pathogenicity of *Serratia marcescens*. I. Virulence for the adult boll weevil. *J. Invertebr. Pathol.*, **9**, 78–81.

STEINHAUS, E. A. 1959. *Serratia marcescens* Bizio as an insect pathogen. *Hilgardia*, **28**, 351–380.

STEPHENS, J. M. 1959. Immune response of some insects to some bacterial antigens. *Canad. J. Microbiol.*, **5**, 203–228.

STEVENSON, J. P. 1959. Epizootiology of a disease of the desert locust, *Schistocerca gregaria* (Forskal), caused by nonchromogenic strains of *Serratia marcescens* Bizio. *J. Insect Pathol.*, **1**, 232–244.