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Restricted Ingestion of Bacteria by Fire Ants

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Fire ant queens and workers from colonies fed to repletion on *Serratia marcescens, Bacillus thuringiensis,* or *Bacillus sphaericus* were aseptically dissected, and homogenates of their thoracic and gastric guts were plated on appropriate media to determine whether bacteria were ingested. All three species of bacteria were effectively excluded from the gut of both queens and workers. A small, slow-growing, Gram-negative bacterium, noted in some of the test queens, was subsequently isolated from the gut of 8 (13.8%) of 58 queens from field colonies. This bacterium was partially characterized, but was not identified. Implications for microbial formicide research are discussed. © 1996 Academic Press, Inc.

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INTRODUCTION

The imported fire ants, *Solenopsis invicta* Buren and *Solenopsis richteri* Forel, are medical and agricultural pests from South America that infest over 10^8 ha in 11 southeastern states and Puerto Rico (Lofgren, 1986). Isolated infestations have also been detected and destroyed in Arizona and California. If they become established in the more humid or irrigated areas of the West, their range will increase substantially. In addition, a polygynous form having denser populations is spreading within the infestation (Glancey *et al.,* 1987).

The development of a biological formicide for fire ants is a daunting task. Formicides are effective only if they destroy the reproductive capacity (kill or sterilize the queen or queens) of ant colonies. Agents which act rapidly kill only worker ants, reducing the size of colonies temporarily, but do not effectively eliminate them. Therefore, delayed action (defined by Banks *et al.* (1977) as less than 15% mortality after 14 hr and more than 89% mortality within 14 days) is essential to ensure distribution of a toxicant to the queen(s) before large numbers of workers die.

Since serial dilution of ingested toxicants occurs through trophallaxis, effective toxicants exhibit delayed action over a concentration range of at least 100-fold (Banks, 1990). The probability of finding a bacterial exotoxin with these properties seems remote. Thus, unless cells or spores are actually ingested by queens, elimination of fire ant colonies by bacteria is improbable.

Miller and Brown (1983) reported that fire ant workers ingest several species of bacteria. Their methods did not, however, differentiate between bacteria from the gut or from the infrabuccal cavity. Particles filtered from food in the pharynx are diverted to the infrabuccal cavity, molded into pellets, expelled, and fed to fourth-instar larvae only (Petralia and Vinson, 1978). Glancey *et al.* (1981) determined that the pharyngeal filters of fire ant workers remove latex microspheres greater than 0.88 ± 0.02 μm in diameter from honey-water. We, therefore, tested whether selected bacteria are indeed ingested before initiating a program of screening entomopathogenic bacteria to identify strains virulent for fire ants. We also isolated and partially characterized cultures of a small, slow-growing bacterium from the gut of field-collected fire ant queens, for biotechnology offers possibilities for transforming weak or opportunistic pathogens, and even commensals, into virulent pathogens.

MATERIALS AND METHODS

Three species of bacteria were chosen because of their availability and ease of identification and because some strains are pathogenic to other insects, not because of any expected pathogenicity toward the fire ants. Cells of *Serratia marcescens* Bizio (18-hr cultures), spores of *Bacillus thuringiensis* Berliner var. *israelensis* de Barjac (3.2×10^6/ml) or *Bacillus sphaericus* Meyer and Neide (strain 2362) (3.1×10^6/ml) were mixed (fresh daily) with boiled egg yolk to a paste consistency. These pastes were fed daily for 14 days to small polygene colonies of fire ants (two colonies per

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bacterium; total of six colonies). Each day for the last 10 days of the test, each colony was also fed one living corn earworm larva immediately after the larva had been injected with 0.25 ml of bacterial cell or spore suspension. The ants readily consumed the diet as we observed daily and confirmed by isolating *S. marcescens* from the midgut of fourth-instar larvae. *S. marcescens* was subcultured every afternoon to provide 18-hr cultures the following morning. Spore suspensions were prepared once and stored in a refrigerator; viability was confirmed by streaking diluted aliquots on nutrient yeast salt agar (Meyer and Yousten, 1978).

Each ant colony consisted of 12 queens, approximately 5000 workers, and 1.5 g of brood. They were maintained in soil-free nests (Banks *et al.,* 1981) at 28°C in a walk-in rearing chamber.

Ten queens from each colony were surface-sterilized (Poinar and Thomas, 1978) and dissected aseptically in Grace's insect cell culture medium (GCCM). Each specimen was first decapitated to eliminate the bacteria-laden infrabuccal cavity. The petiole was then severed and the thorax and gaster were dissected separately, extracted in 0.5 ml GCCM in sterile 3-ml glass tissue grinders and plated on the appropriate agar medium. The thoracic (or pharyngeal) crop was usually removed intact from the thorax, but in a few difficult cases the entire thorax was extracted. Because the venom is antibacterial (Jouvenaz *et al.,* 1972), the venom gland was removed intact before further dissection of the gaster. The gastric gut (gastric crop, midgut, hindgut) was then excised and extracted as a unit.

In addition, 10 workers were captured as they left each egg yolk–bacteria paste, having fed to repletion. These 30 workers were surface sterilized and decapitated, and their venom glands were removed; however, because of their small size, the thoraces and gasters were not separated, but extracted as a unit.

The homogenates of ants from colonies fed *S. marcescens* were plated on mannitol–salt agar (Harned, 1954), on which this bacterium produces the red pigment prodigiosin in abundance, permitting instant species identification. The extracts of ants from colonies fed spores were plated on NYSA, which stimulates sporulation and thereby aids identification. *B. sphaericus* was tentatively identified by the characteristic swollen, terminal spore; identity was confirmed by bioassay against highly susceptible, laboratory reared *Culex quinquefasciatus* mosquitoes. Bacteria having parasporal bodies were considered to be *B. thuringiensis.* All plates were incubated at 28°C and examined at 24 and 48 hr.

Upon further incubation, innumerable minute colonies of bacteria developed on several plates (gasters) which had initially appeared to be sterile. Therefore, an additional 58 queens were collected from field colonies and examined within 4 hr. After surface sterilization, the venom glands were carefully removed free of adhering tissues. To avoid the possible loss of any section of hindgut, the gasters of these queens were not separated from the thoraces. The gasters were homogenized in BHI broth, plated on BHI agar, inoculated into tubes of fluid thioglycollate medium (Difco), and incubated for 2 weeks. Because of the high density of the slow-growing, minute colonies, cells from multiple colonies were transferred into BHI broth, serially diluted, and streaked on BHI agar. The morphology of colonies on the dilution plates was uniform; therefore one colony from each queen was subcultured and maintained for characterization.

The isolates were first tested for Gram reaction and fermentation of glucose and lactose. Isolates of the bacterium were analyzed with a computerized bacterial identification system (BioLog Inc., Hayward, CA) to determine whether it matched species included in its software library and to assess whether all the isolates were of the same species. This system determines 95 physiological characters and statistically correlates the similarity of the unidentified bacterium to species in either a Gram-positive or a Gram-negative library. Each test was conducted in triplicate and, due to the slow growth of this organism, read on 3 additional, successive days. Motility was tested in BHI medium containing 0.5% agar; anaerobic growth was tested in fluid thioglycollate medium. Gram-stained cells ($N = 50$) were measured with a Vickers-A.E.I. splitting image micrometer.

RESULTS AND DISCUSSION

Only 1 of the 72 queens in the six test colonies (12 per colony, of which 10 were dissected) died during the test period. Worker and brood mortality was comparable to that of our other insectary colonies, which served as controls.

S. marcescens was not recovered from any of the 20 queens and 10 workers fed this bacterium. Cells of *S. marcescens* measure about 0.5 × 0.5–1.0 μm and have four peritrichous flagella (Breed *et al.,* 1957). Since the smaller cells are below the reported filtration threshold of fire ants, we assume the flagella become tangled in the filter. In contrast, the midgut of fourth-instar larvae were red from prodigiosin, and *S. marcescens* was isolated from all 10 specimens plated. It is possible, but seems improbable, that adult fire ants also ingested *S. marcescens,* but unlike larvae, immediately digested them. Rapid digestive inactivation of bacterial spores rather than exclusion from the gut seems even less likely.

Bacillus sphaericus was recovered from the gaster of only 1 of 20 queens. The thoracic crop of this specimen was sterile, but the gaster yielded 104 colonies of bacteria, of which 10 were *B. sphaericus.* The gasters of 3 other queens yielded several colonies each of bacteria,

but none were *B. sphaericus*. Whether these bacteria were from the gut or survived surface sterilization is, of course, unknown. Spores of *B. sphaericus* measure 0.7–1.2 μm in diameter (Breed *et al.,* 1957). Thus, the smaller spores, if naked, should pass the pharyngeal filter. However, the sporangium adhered strongly to the spores in this species, thus increasing their size substantially. All of the 10 workers fed *B. sphaericus* spores yielded sterile plates.

B. thuringiensis was not recovered from any of the 20 queens from colonies fed this bacterium. Seven of the 10 workers yielded sterile plates; the remaining 3 yielded 4, 6, and 14 colonies of bacteria, all of which were *B. thuringiensis*. The spores of this species measure 1.0–1.5 μm in diameter and are thus large enough to be filtered by worker fire ants. Through trophallaxis, food may be filtered repeatedly before reaching the queen.

The slow-growing bacterium was isolated from only 9 (15%) of the 60 queens from colonies fed bacteria and, later on, also from 8 (13.8%) of the 58 queens from field colonies. Since it is somewhat fastidious and occurred in significant numbers, we initially thought it might be a gut symbiont. However, its presence in only a small proportion of the population of fire ants argues against this interpretation. Intracellular symbiotic bacteria occur in the ovariols and midgut epithelium of the wood ant, *Formica fusca,* certain carpenter ants, *Camponotus* spp., and possibly species of the tribe Cephalotini (Holldobler and Wilson, 1990). However, intracellular symbionts are extremely fastidious; they have not been cultured even in insect tissue culture medium (Douglas, 1989). The ecological relationships of fire ants and this bacterium are unknown and we observed nothing to suggest that it is pathogenic to fire ants.

This bacterium is a Gram-negative, nonmotile, facultative anaerobe measuring about 0.5 × 0.5–1.8 μm. It does not grow at 37°C and grows very slowly at 28°C (faint turbidity appears in BHI broth tubes after 2–3 days; in nutrient broth, after 10–12 days). After 8 days at 28°C, colonies on BHI agar were 0.4–1.5 mm in diameter, hyaline, butyrous in texture, with margins entire. Glucose and lactose are not fermented. Within the BioLog Gram-negative library, all isolates of the fire ant bacterium formed a similarity grouping, suggesting that it is one species. It correlated poorly with various species of *Neisseria* and *Pseudomonas,* from which it was easily differentiated morphologically.

It has been suggested to us that extensive screening of entomopathogenic bacteria, most of which are gut toxins, would probably identify a strain pathogenic for fire ants. This study demonstrates that such a screening program must be carefully planned with due consideration given to the filtration capabilities of fire ants. Candidates should be sought among the nonflagellated species small enough to pass repeated pharyngeal filtration. Viruses (Avery *et al.,* 1977) and mollicutes (Tully, 1989) are even smaller than bacteria and, for this reason, may be better candidates for development as formicides.

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