Properties of *Serratia marcescens* Isolated from Diseased Honeybee (*Apis mellifera*) Larvae

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Twenty-three strains of *Serratia marcescens* were isolated in pure culture from diseased honeybee (*Apis mellifera*) larvae in the Sudan. All the strains belonged to biotype A4(b). DNAase and lipase production, pectolytic activity, and citrate utilization were useful criteria for distinguishing *S. marcescens* from closely related bacteria. A challenge experiment using pure cultures of *S. marcescens* biotype A4(b) proved this organism to be pathogenic for honeybee larvae.

INTRODUCTION

Many bacteria occur in insects, but most are commensals or secondary invaders of diseased individuals. Sometimes they are uncommon isolates or of uncertain taxonomic affiliation (Bailey, 1981). However, there are certain bacteria which can cause specific diseases in adults and/or larvae of honeybees (*Apis mellifera*). These include *Bacillus larvae*, the cause of the American foul brood, and '*Melissococcus pluton*', the cause of the European foul brood. There are, in addition, many bacteria which have been isolated from the intestinal tract of the honeybee, but little is known of their natural history. This paper describes the isolation and partial characterization of *Serratia marcescens* from diseased honeybee larvae.

METHODS

History of the disease outbreak. Thirty colonies of Carniolian honeybees were brought from the Egyptian Ministry of Agriculture to be stationed in El Damar (Northern Sudan) in May, 1984. Before being transferred to a permanent site, the colonies were placed in the apiary of the Faculty of Agriculture, University of Khartoum. The colonies were re-hived in Langstroth hives, supplied with sucrose syrup (1:1, v/v). Colony manipulation, including feeding, was done at 12 d intervals for two months. Almost half the colonies had died by the end of July of the same year; this coincided with the drought season when only a few acacia trees were in bloom. The following observations were recorded. An increasing number of creeping worker bees was observed on the ground of the apiary. An unpleasant odour was noticed associated with two colonies: examination revealed dead larvae inside capped cells that were yellowish to light brown in colour. They were soft in consistency and mucoid when punctured. Most of the affected larvae were in the prepupal stage. The cappings of the cells containing dead broods were punctured and sunken in appearance. Dead and living brood were observed in the same comb. Only two combs had dead brood in these two infected colonies. The apiary was closely observed for six months with continuous examination of dead larvae for pathogenic bacteria.

Investigations into the disease outbreak. Cappings of cells containing dead brood were removed. Both dead and apparently healthy larvae were aseptically removed, placed in sterile containers and immediately transferred to the laboratory, where they were surface sterilized by dipping them in 70% (v/v) ethanol, allowed to dry and crushed between sterile slides. Larval contents were streaked on several freshly prepared 10% (v/v) sheep blood agar (2.5%, w/v, nutrient agar; Difco) plates which were incubated at 35 °C aerobically and examined at 24 h intervals for 7 d before being discarded as negatives. Direct smears prepared from dead larvae and from the growth obtained on blood agar were fixed and stained with Gram's stain. Isolated organisms were subjected to biochemical tests according to Davis & Ewing (1964), Ewing *et al.* (1969) and Cowan (1957). They were biotyped according to Grimont *et al.* (1977) and Grimont & Grimont (1978). Other confirmatory tests, including DNAase

and lipase production, pectolytic activity and alginate utilization were included. Isolates were compared with a reference strain of *S. marcescens* obtained from the National Medical Laboratory, Khartoum, and designated strain 24.

DNAase production. Three methods were compared. (1) The standard Oxoid test using Oxoid DNA agar and the method of Jeffries *et al.* (1957). (2) The modification of Schreier (1969), using toluidine blue. Toluidine blue was added to Oxoid DNA agar to a final concentration of 0.01%. The resulting dye-DNA complex produces a clear blue medium. DNAase breaks down this complex, changing the blue to rose-pink. (3) The modification of Smith *et al.* (1969). Methyl green was added to Oxoid DNA agar to give a final concentration of 0.02% (Black *et al.*, 1971). The dye combines with DNA in the agar medium at pH 7.5. Hydrolysis of the complex by DNAase results in the production of a clear zone around the colonies. A negative control organism (*Escherichia coli*) was included for each of these tests.

Lipase production. The method was essentially that of Nemato & Nakazawa (1978). Tween 80 was incorporated at a final concentration of 1% (v/v) in Heart Infusion Agar (Difco). Lipase production was detected by formation of a cloudy precipitate around the colonies.

Alginate utilization and pectolytic activity tests. These were done according to Black *et al.* (1971).

Biotyping. The M70 inorganic mixture of Veron (1975) was used for carbon source utilization studies. The biotyping was done according to Grimont & Grimont (1978).

Tetrathionate reductase test. This was done according to Grimont & Grimont (1978). The medium contained $K_2S_2O_8$, 5 g; bromothymol blue, 0.2% aqueous solution, 25 ml; peptone water powder (Difco) 10 g; NaCl, 5 g, and distilled water up to 1 l (pH 7.4); it was filter-sterilized. Negative and positive controls were included in each batch of tests. Tubes were incubated at 37 °C for 7 d with frequent examination before they were discarded.

Sensitivity to various antibiotics and sulphonamides. Sensitivity to the following antibiotics was tested using the disk method according to Bauer *et al.* (1966): carbenicillin (100 µg), kanamycin (10 µg), streptomycin (10 µg), ampicillin (25 µg), gentamicin (10 µg), nalidixic acid (30 µg), chloramphenicol (25 µg), polymyxin B (300 µg), penicillin (1 unit), tetracycline (25 µg), erythromycin (5 µg) and novobiocin (25 µg) (all from Oxoid); oxacillin (1 µg) and clindamycin (2 µg) (both from Difco); and sulphamethizole (200 µg) (from Mastring-S).

Mycology. Infected larvae were crushed in a sterile pestle and mortar with 0.5% lactalbumin hydrolysate containing 200 IU penicillin and 200 µg streptomycin ml⁻¹ to make a final 10% (w/v) homogenate. This was inoculated on Sabouraud's agar, and incubated at 28 °C for 2 weeks with frequent examination for fungal growth.

Attempts to reproduce the disease artificially. An apparently healthy brood comb was selected by careful inspection of colonies in an apiary at Gezera (central Sudan), where the disease we describe is unknown. The brood comb frame (40 × 23 cm) contained sealed and unsealed broods and about 2.5 kg sealed honey on either side. It consisted of 950 unsealed cells with only a few sealed ones. Samples of larvae, collected at random, were tested for the absence of *S. marcescens*. The frame was suspended in a rectangular wooden box 53 × 11 cm, 25 cm tall, with glass on either side and a 5 cm diameter observation hive hole covered with wire mesh. This hole was also used for injection of food (concentrated sucrose syrup, used to soak a piece of cotton wool placed in a McCartney bottle). The top of the box was closed with a sliding piece of wood. Four hundred and fifty adult honeybees were admitted to the box.

Growth of *S. marcescens* biotype A4(b) was scraped and washed off a blood agar plate and diluted with sterile phosphate-buffered saline (0.145 M-sodium chloride, 0.15 M-sodium phosphate, pH 7) to contain 10⁶ c.f.u. ml⁻¹; 3 ml of this bacterial suspension was sprayed over a 10 cm² area at one corner of the frame. A similar box was used as a control. Instead of bacterial suspension, it was sprayed with sterile phosphate-buffered saline. Both boxes were incubated at 35 ± 0.5 °C and carefully inspected twice a day for 10 d, for any deaths or changes in odour. In a trial to study the survival of *S. marcescens* on the sprayed area, the area was swabbed with a sterile cotton swab every day and the swabs were cultured on blood agar.

On the 10th day the experiment was stopped and the adult bees were killed with an insecticide spray.

RESULTS

The disease outbreak

The infected brood revealed dead larvae inside capped cells. The cell cappings were brown, punctured and sunken in appearance. There were dead and living larvae in the same comb. The dead larvae were whitish, sticky and had a foul odour. Direct smears from dead larvae revealed minute Gram-negative rods arranged singly and in groups. They were isolated in pure culture. Growth on blood agar consisted solely of small white colonies 1–2 mm in diameter, slightly raised with convex surfaces. They were non-haemolytic, Gram's stain revealed minute Gram-negative rods indistinguishable morphologically from those seen in the smears taken from dead larvae. Twenty-three strains were isolated, and designated 1–23.

No fungi were isolated from the infected larvae by the techniques described in Methods.

Table 1. *Properties of 23 S. marcescens* strains isolated from honeybee larvae, and one reference strain

Test	No of strains*			
	Positive	Late positive	Weak†	Negative
KCN	21	2	1	0
Citrate	7	0	1	16
Glucose (gas)	0	0	18	6
Arabinose	1	0	0	23
Inositol	3	6	(9)	6
Rhamnose	0	0	(4)	20
Trehalose	5	19	0	0
Indole	0	0	6	18
Urease	0	20	2	2
Methyl red	20	—	1	3
Voges-Proskauer	16	—	8	0
Loeffler's	20	0	4	0
Growth on erythritol	22	1	0	1
Pigment	1‡	0	0	23

* All strains were positive for motility (six strains were motile at 25 °C but not at 37 °C), produced acid from glucose, utilized glycerol, maltose, mannitol, salicin, sorbitol, sucrose, gluconate and aesculin as carbon sources, reduced nitrate, and produced lipase, DNAase and gelatinase. None of the strains produced oxidase, or utilized malonate, dulcitol, lactose, xylose or raffinose as sole carbon source. None produced H₂S or reduced tetrathionate, and none grew on trigonelline, 3-hydroxybenzoic acid, 4-hydroxybenzoic acid, benzoate or DL-carnitine.

† Parentheses indicate a late and weak reaction.

‡ Strain no. 5 produced a pink pigment on lactose-peptone water, but not on milk agar or potato agar.

Properties and biotyping of the strains

The biochemical properties of the isolates, and of a reference strain of *S. marcescens*, are shown in Table 1. All the strains were shown to produce DNAase by all three methods. They also produced lipase and utilized alginate as a sole source of carbon (the latter demonstrated after 14 d incubation). Pectolytic activity was demonstrated by a reduction of the pH of the medium to less than 6.2. The organisms were thus confirmed to be non-pigmented isolates of *S. marcescens*. No such organisms were isolated from apparently healthy larvae.

All the strains grew on adonitol, and all except strain 2 grew on erythritol. None of the strains tested grew on trigonelline, 3-hydroxybenzoic acid, 4-hydroxybenzoic acid, benzoate or DL-carnitine. None reduced tetrathionate. The strains were thus diagnosed as biotype A4(b) (Grimont *et al.*, 1977, 1979; Grimont & Grimont, 1978).

In the disk assays (see Methods), all the strains were susceptible to carbenicillin, kanamycin, streptomycin, ampicillin, gentamicin, nalidixic acid and chloramphenicol, but resistant to oxacillin, clindamycin, polymyxin B, penicillin, erythromycin, novobiocin and sulphamethizole. In tests in which antibiotics were added to the medium (blood agar), all the strains were resistant to cephalixin (100 µg ml⁻¹), 22 were resistant to polymyxin B (38 µg ml⁻¹) and 21 were resistant to a mixture of the two (at the stated concentrations).

Reproduction of the disease artificially

On the 5th day of incubation an offensive smell was detected in the experimental box. Adult bees were distracted by an electric lamp placed at one corner of the box and the frame was carefully removed. The cell cappings were removed. One hundred and thirty five larvae were found dead, each with its head pointing outwards from its cell. This represented a mortality rate of 16%. The larvae were difficult to remove completely and resembled those observed in the natural infection. The rest of the larvae proceeded normally to the pupal stage. Cultures made of surface-sterilized dead larvae yielded pure colonies of *S. marcescens*; this organism was not

isolated from any of the living larvae, whether removed from the infected box or from the control. Swabs taken from the sprayed areas 24 h after spraying were negative for *S. marcescens*.

In a separate experiment to study the bactericidal effect of honey on *S. marcescens*, diffusion tests in Noble agar (Difco) plates inoculated with *S. marcescens* showed that concentrated honey was completely bactericidal after 24 h at 35 °C. Honey diluted 1 in 2 was slightly bactericidal and honey diluted 1 in 3 had no effect.

DISCUSSION

The genus *Serratia* consists of small Gram-negative rods, which usually produce a characteristic red or pink pigment, prodigiosin, although white to rose-red strains frequently occur. Ordinarily they are common saprophytes found in water, milk and other foods. Three species of *Serratia* have been recorded as being associated with insects, but only *S. marcescens* has been considered as potentially pathogenic for insects (Steinhaus, 1967). The association of *S. marcescens* with larvae of the silkworm (*Bombyx mori*) is well established. Studies by Masera in the 1930s (see Steinhaus, 1967) on the pathogenicity of *S. marcescens* towards a number of insects, including silkworm larvae, showed that the organism was lethal only when inoculated into the body of the larvae, not when introduced with food, and that the percentage of deaths was higher the nearer the insect was to the prepupal stages (a result which was also noticed in the present study). The pathogenicity of *S. marcescens* for the larvae of honeybees is less well established, although Wille & Pinter (1961) confirmed the association of *S. marcescens* with septicaemia in adult honeybees in Switzerland. One reason for the paucity of reports concerning *S. marcescens* may be the incorrect identification of this organism, especially in the case of non-pigmented strains. It appears that some of the cultures isolated from honeybees suffering from septicaemia and investigated by Landerkin & Katznelson (1959) probably belonged to the *Serratia* group.

In the present study, the mortality among experimentally infected larvae was 16%, a percentage similar to that found in the natural infection. Koch's postulates were thus verified since the strain was capable of inducing infection in healthy larvae and the same organism was re-isolated from experimentally infected larvae. The organism was never isolated from healthy larvae, suggesting that a 'silent-carrier' state was not occurring. Moreover, some of the healthy larvae were intentionally starved to death. In none of them was *S. marcescens* isolated. Ninety percent of the affected larvae were in the prepupal stage.

It seems that adult bees are resistant to such infection. Apart from six adult bees which were accidentally destroyed during the introduction of the brood comb into the experimental box, none died. The question arises as to how the infection took place. The following hypothesis is suggested. The bacteria may have been picked up by the adult bees when passing over the infected area and then introduced by them to the larvae in food. Infection must have taken place within the first 24 h after spraying, since swabs of the sprayed area 24 h after inoculation were negative for *S. marcescens*.

The bactericidal effect of honey, confirmed in our experiments, may be attributed to various factors, among which are the high concentration of sugars, low pH and/or the presence of 10-hydroxydecenoic acid (Bailey, 1981). In our experiment honey did not protect larvae against infection, probably because of dilution by the digestive secretions in the larval gut or by a change in the pH.

For many years, *S. marcescens* was thought to be non-pathogenic for vertebrates, but it is now known to be pathogenic for man under appropriate conditions (Woodward & Clark, 1913; Robinson & Woolley, 1957). The true medical importance of this organism will be known only if clinical laboratories are capable of identifying non-pigmented strains correctly (Wilfert *et al.*, 1970).

The isolation of *S. marcescens* in pure culture from diseased larvae and the reproduction of the disease experimentally is evidence of the pathogenic nature of a particular biotype for larvae of the honeybee. The role of other biotypes warrants further investigation.

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