BACILLUS PULVIFACIENS (N. SP.), AN ORGANISM ASSOCIATED WITH POWDERY SCALE OF HONEYBEE LARVAE[1]

H. KATZNELSON[2]

Division of Bacteriology and Dairy Research, Department of Agriculture, Ottawa, Canada

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In a recent investigation on the cause of death of honeybee larvae it was observed (le Maistre, 1949) that cells of the honeycomb contained scale (thin, dried remnants of dead larvae) that was dry, powdery, and light brown in color, and therefore quite unlike scale resulting from European and American foulbrood diseases. Comb with this unusual scale was sent to Ottawa for further study. Microscopic examination of the powdery material revealed many large spores with thick walls and fairly numerous short rods. Samples of this powdery material were removed aseptically into sterile water and loopfuls streaked or plated in appropriate dilutions with yeast beef agar; portions were also heated for 10 minutes at 80 C prior to plating. After two days' incubation at 33 C every sample yielded a pure culture of an organism that produced colonies varying from Brazil red (Ridgway, 1912), to brownish-orange, to light brown in color; all colonies examined showed a rather short, well-stained, gram-positive rod. After 5 to 8 days' incubation these colonies yielded large oval spores identical with those originally found in the powdery scale. Another phenomenon was observed at this time; many of the pigmented colonies and of the cultures isolated from these showed at their periphery much lighter and even creamy whitish growth. When cultures were transferred to fresh medium or streaked on fresh plates both deeply pigmented and whitish colonies appeared. After suitable incubation all cultures showed short gram-positive rods and after further incubation typical and similar spores. It was also observed that after several transfers on yeast beef agar the cultures began to lose their pigment and spore-producing capacity. However, by culturing on the pollen extract medium of Smith *et al.* (1949) and on glucose agar these characteristics were retained.

The procedure outlined in the key for aerobic mesophilic sporeforming bacteria by Smith *et al.* (1946) was used for identification, after repurification of the cultures. The organism forms spores in definitely swollen sporangia; produces acid but no gas from glucose; does not hydrolyze starch, does not produce acetylmethylcarbinol, nor utilize citrates; and lowers the pH of glucose proteose peptone broth to 6.0 to 6.4. According to these characteristics, the organism corresponds with *Bacillus laterosporus*; however, other characteristics are quite different as the following detailed description will show:

The vegetative rods are 0.3 μ to 0.6 μ by 1.5 μ to 3.0 μ, with rounded, not pointed ends; on nutrient agar motility is restricted to a small proportion of cells

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[2] Bacteriologist.

but is more extensive on pollen extract medium; the rods are distinctly gram-positive. The sporangia are definitely bulged and spindle-shaped. Spores are 0.8 μ to 1.2 μ by 1.5 μ to 1.8 μ, oval, terminal, not lateral, with remnants of the sporangium often adhering to give a thick wall that takes stain and that is at times almost pointed at the ends. Sporulation is variable, poor on nutrient agar and good on glucose nutrient agar and pollen extract agar; spores are produced only after 5 to 7 days' incubation.

Colonies on yeast beef agar are thin, opaque, round, entire, slightly irregular, not spreading, and, especially when first obtained from powdery scale in the comb, Brazil red or brownish in color; older colonies show a lighter colored periphery; variations are whitish, small, round, entire, glistening, and translucent to opaque. The pigmented colonies often show sectors of light whitish growth at their edges after 6 to 8 days' incubation. Nutrient agar slants show fair, flat, smooth, nonspreading growth that is deeply pigmented, cream-colored or light brown, and translucent to opaque. Glucose nutrient agar shows heavier growth, becoming somewhat wrinkled in older cultures. Nutrient broth shows poor though uniform turbidity, which is heavier in glucose nutrient broth.

All cultures give a negative Voges-Proskauer reaction and do not utilize citrates. Fermentation tests were made on nutrient agar as the organism does not use inorganic nitrogen. Of the 16 substances tested, acid is produced from glucose, glycerol, and mannitol. Good to excellent growth is obtained with glucose, maltose, galactose, and mannose; fair to moderate growth with fructose, glycerol, mannitol, and salicin; and slight growth with 6 other carbohydrates. The pigmented strains show strong color with glucose, fructose, maltose, galactose, mannose, and salicin.

Starch hydrolysis is negative, cellulose is not fermented, nitrates are reduced to nitrites, gelatin and casein are hydrolyzed. Methylene blue reduction varies considerably with the strains, most of which reduce it completely within 4 to 5 days, begin to reoxidize it within 8 days, and complete this reaction within 15 days.

Maximum temperature for growth is 45 C; growth is poor at 20 C and moderate at 25 C after 3 days. Growth at 35 and 45 C is moderate to good within 2 days. Pigmentation is extensive at both 35 and 45 C and brilliant at the latter temperature. Spores are produced in abundance at 35 and 45 C but very poorly if at all at 20 or 25 C.

It is apparent that the newly isolated bacillus differs from *B. laterosporus* in the size and shape of vegetative cells; in its distinctly gram-positive staining and its poor motility; in the size, shape, position, staining, and appearance of the spore; in its poor nonspreading growth on nutrient agar; in the production of the Brazil red to brownish pigment; in its fermentation reactions and its inability to utilize inorganic nitrogen; and in its strongly dissociative tendency. This newly isolated organism also differs markedly from *Bacillus larvae*, which produces longer gram-variable vegetative cells showing a tendency for filament formation, forms smaller spores (usually under special conditions of culturing), and does not grow on ordinary laboratory media (Breed *et al.*, 1948; Lochhead, 1928). On the basis of these characteristic differences there appears to be justification for

considering the newly isolated organism a different species, for which the name *Bacillus pulvifaciens* is suggested.

Since the organism was isolated from larval scale, attempts were also made to determine its ability to cause disease in honeybee larvae. Accordingly, three colonies of bees were fed 50 per cent sugar syrup containing the following suspensions: colony 7—unheated suspension of spores from powdery scale; colony 107—as above but heated 10 minutes at 80 C; colony 115—spore suspension from agar cultures.

Weekly examination of the larvae disclosed no infection; however, on the fourth week one cell from colony 7 showed a decomposing larva from which was obtained a pure culture of the pigmented bacillus. The experiment was repeated and again one comb from colony 7 showed several larval scales that disintegrated when touched and that on microscopic examination showed the typical spores of this bacillus. Culturing of this material yielded the pigmented organism.

Since this work was done late in the season (August and September), it is possible that conditions did not favor extensive development of this organism. It is also possible that conditions in Ottawa were not so conducive to infection as those in Alberta, where the powdery scale was first observed. Further work is required to establish whether this organism is a pathogen or merely a contaminant brought in by bees which develops in larvae dead from chilling, poison, or other causes.

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SUMMARY

An aerobic sporeforming bacillus producing a reddish brown pigment and possessing strong dissociative tendencies was isolated from powdery scale of honeybee larvae. The name *Bacillus pulvifaciens* (n. sp.) is suggested for this organism.

It has not yet been definitely established whether this organism is a contaminant of dead larvae or the cause of their death.

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