The identification of a spore-forming bacterium isolated from diseased honeybee brood

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(Received 12/ 5 / 2009 , Accepted 12 / 10 / 2009)

Abstract
A sporogenic bacterium isolated from diseased honeybee broods was obtained in a pure culture and standard bacteriological procedures were employed to determine its characteristics. Interpretation of the results with literatures provided a taxonomic designation of the isolate as a member of the *Bacillus cereus* group. The prime interesting point was that the isolate must be either a strain of the insect pathogen *Bacillus thuringiensis*, or a new species. However, the scrutinized experiments ascertained that the isolate to be a strain of *B. cereus.*

Introduction
Insect diseases were recorded as early as 335- 322 B.C. by Aristotle, describing diseases of the honeybee. In the 18th century, Louis Pasteur made a significant contribution to insect pathology, when he described a bacterial infection of the silkworm (*Bacillus bombycia*) [1].
An important turning point in the history of insect diseases was achieved by the German biologist Berliner [2]
who isolated a spore-forming bacterium from diseased larvae of the Mediterranean flour moth. The bacterium was named *Bacillus thuringiensis* everywhere known today for its extensive application in agriculture as a biological insecticide [3, 4].
Bacillus larvae, *B. alvei* and *B. laterosporus* are the most intensively investigated bacteria that cause honeybee diseases [5].
The present study investigated the identification of a spore-forming bacillus, isolated from honeybee brood. The work involved in the identification of the isolate was considered worthwhile based on three premises:
(a) Most members of the family Bacillaceae are saprophytes commonly found in soil, and hence any isolation from adifferent source is worthy of characterization.
(b) Interest in bacterial honeybee pathogens other than *B.*
larvae, B. alvei, and *B. laterosporus.*
(c) This bacterium may be a secondary invader encountered in the disease .

Materials and methods
New isolate . The isolate has been given a strain designation A / 91.
Control cultures. Bacteria used as positive and / or negative controls throughout the present study are those given in the Tables 2 and 3. All purchased from ATCC.
Staining methods: Bacterial morphology examined by employing the following staining techniques: Gram stain; Schaeffer-Fulton method (Endospore stain), capsule stain: using a wet India ink film, volutin or metachromatic granules, intracellular lipid stain, cell wall stain: Hale's method [6, 7], and flagella stain: using modified Fontana's method [8].
Observation of parasporal bodies:
Slopes of nutrient agar were inoculated with isolate A/91, and incubated at 35°C. The cultures were examined (18-28 hrs) using Smirnoff [9] staining technique, to observe the possible production of parasporal crystals during the speculating sequence.

Physiological and biochemical tests:[10, 11]
1. Growth temperatures.
2. Anaerobic growth.
3. Production of catalase.
4. Voges – Proskauer test.
5. Egg – yolk reaction.
6. Resistance to lysozyme.
7. Growth in sodium chloride.
8. Growth at pH 5.7.
9. Acid from carbohydrates.
10. Hydrolysis of starch.
11. Utilization of citrate.
12. Reduction of nitrate to nitrite.
13. Production of indole.
14. Deamination of phenylalanine.
15. Decomposition of casein.
16. Decomposition of tyrosine.
17. Gelatin liquefaction.
18. Urease production.
19. Growth on Sabouraud´s dextrose agar.

Results and Discussion
Colony characteristics. All plates examined revealed profuse growth after only 18 hrs. Other colony properties are listed in Table 1.
Cell morphology. Gram positive bacilli, with a tendency to form long chains. Individual rods measure 1.0-1.26x2.4-2.8 um. The cells have rounded ends, and are not encapsulated.
Endospores. The bacterium sporulates aerobically, and the endospores are ellipsoidal, one per sporangial cell in a dominant paracentral position. Each endospore measure 1.0x1.5-1.72 um. They do not distend the sporangia.
Motility. This property can provide a useful preliminary guide to identifying an unknown culture, but it is not definitive. A hanging drop preparation viewed in a light microscope. Revealed rods with rapid progressive movements.
Flagellation . Is a fairly good taxonomic criterion. Cells with peritrichous flagella being observed.
Inclusions . The rods are vacuolated and contain large fat globules, the largest measuring 0.5-0.72 um . volutin or metachromatic granules were observed .

Table 1. characteristics of isolated Colonies on streaks incubated at 35 °C for 18 hrs

Shape	Irregular
Margin	Undulate
Elevation	Flat
Surface	Smooth
Colour	Whitish
Size	6-8 mm
Translucency	Opaque
Any change in the medium	None

The visuial examiination of A/ 91 isolate , i.e. , cultural characteristics , light microscopy and phase contrast microscopy, showed observations that correlated precisely with generic description for the genus *Bacillus* [10, 12].

Members of the genus *Bacillus* have been subdivided into three groups on the basis of spore and sporangial swelling. These groups have been used by various workers, and of notable value when one seeks to rapidly reduce the taxonomy of a *Bacillus* isolate to just a group of species. The A / 91 isolate can be allocated to group 1A [13], which has properties and species given in key 1.

The common characteristics of strains of *B. cereus* are well documented by [14, 15]. Table 4 summarizes these characters, together with the corresponding properties of the A/91 isolate which are obtained in the present study.

B. cereus variety *B. anthracis* (designated *B. anthracis*, [10] it was found to be nonmotile, encapsulated *in vivo*, and does not decarboxylate tyrosine. *B. cereus* variety *mycoides* (designated *B. mycoides*, [10] is reported to be nonmotile and forms distinctive rhizoid colonies on agar. Its maximum growth temperature was between 35-40°C.

Table 2. fermentatıon of carbohydrates

Carbohydrate	(+) control [1]	(-) control [2]	Isolate A/ 91
Glucose	+	-	+
Galactose	-	-	-
Fructose	+	-	+
Mannose	+	-	-
Arabinose	+	-	-
Xylose	_+٣	-	-
Cellobiose	+	-	+
Lactose	+	-	-
Maltose	+	-	+
Sucrose	+	-	+
Raffinose	+	-	-
Rhamnose	-	-	-
Mannitol	+	-	-
Glycerol	+	-	+
Inulin	+	-	-
Salicin	+	-	+

1: Bacteria that produce acid :
B. cereus : sucrose, fructose, maltose, and glycerol.
B. megaterium: inulin and salicin .
B. subtilis: galactose, mannose, and xylose.
B. polymyxa : glucose, arabinose, cellobiose, lactose, raffinose, rhamnose, and mannitol.
2: Bacteria that do not produce acid:
B. cereus: inulin, xylose, raffinose, and rhamnose.
B. sphaericus: rhamnose and xylose.
The remainder of the carbohydrates used *B. sphaericus* as negative control.
3: Positive for *B. subtilis*. Negative for *B. megaterium*.

Table 3. Positive and negative biochemical properties of isolate A/ 91 .

Tests	Positive * control	Neg ative * control
Catalase	*B. subtilis*	*Streptococcus faecalis*
Urease	*Proteus vulgaris*	*B. polymyxa*
Lecithinase	*B. cereus*	*B. brevis*
Facultative anaerobe	*B. cereus*	*B. megaterium*
Growth in : pH 5.7	*B. polymyxa*	*B. firmus*
7% Nacl	*B. firmus*	*B. polymyxa*
0.001 % Lysozyme	*B. cereus*	*B. megaterium*
Citrate utilization	*B. subtilis*	*B. firmus*
Nitrate reduction	*B. subtilis*	*B. sphaericus*
Production of 1. Acetoin 2. Indole	 *B. subtilis* *B. coli*	 *B. megaterium* *B. cereus*
Hydrolysis of Starch	*B. subtilis*	*E. coli*
Casein	*B. cereus*	*E. coli*
Gelatin	*B. subtilis*	*E. coli*
Deamination of Phenylalanine	*B. sphaericus*	*B. polymyxa*
Decarboxylation of Tyrosine	*B. brevis*	*B. polymyxa*

* Controls indicated, behaved as expected.

Table 4 Common properties of *B. cereus* and isolate A / 91

Properties	*B. cereus*	Isolate A / 91
Gram reactıon	+	+
Diameter over 1.0 µm	+	+
Sporangia not appreciably Swollen by ellipsoidal endospores	+	+
Cells rich in lipids	+	+
Catalase	+	+
Egg- yolk lecithinase	+	+
Anaerobic growth	+	+
Survive 50°C	-	-
V – P test	+	+
Growth with lysozyme	+	+
Acid from :		
Glucose	+	+
Mannitol	-	-
Xylose	-	-
Hydrolysis of :		
Casein	+	+
Starch	+	+
Nitrite from nitrate	+	+
Decarboxylate tyrosine	+	+
Deamination of phenylalanine	-	-

B. cereus variety *thuringiensis* (designated *B. thuringiensis*, [10] is distinguished by the production in the cell of parasporal crystals .

The characteristics of *B. cereus* are different from those given for its varieties above [10]. The properties of A/91 isolate are also different from those given for the varieties of *B. cereus* given above. Now, since the properties of A/91 isolate are in line with those of *B. cereus* Table 4, and with descriptions for *B. cereus* in the literatures cited above. Consequently, the A/91 isolate may be assigned to *B. cereus.*

The majority of the results proved entirely, *viz*: physiological and biochemical reactions for the A/91 isolate correlated with properties of *B. cereus* summarized in Tables 3 and 4; cell morphology, cell motility, the ability to deaminate phenylalanine, to decarboxylate tyrosine, starch hydrolysis, and many other properties. These results have been interpreted in conjunction with descriptions of *Bacillus spp*. given in the *Bergey's manual of systematic bacteriology* (vol. 2) [10, 14] and collated with identification keys for the genus [12].

The results for the fermentation of carbohydrates (Table 2), are in line with findings of [11]. Cultural characteristics (Table 1) also agree with descriptions reported by [11, 12]. A study has been made to reinforce the evidence pertaining to the identification of the A/91 isolate

as *B. cereus*, is the distribution of intracellular stainable lipid inclusion granules. Microscopic examinations of fixed smears of *B. cereus* stained with Sudan Black B [16] revealed blue–black or sometimes blue–grey granules match those of the A/91 isolate. This study also demonstrated that the amount of lipid material remained constant for a given species, and hence the positive correlation between the microscopic observations may be regarded as further corroborative evidence for the identity of the A/91 isolate.

The only significant distinctions between *B. cereus*, and the closely related insect pathogen *B. thuringiensis*, are the production of parasporal crystals and pathogenicity for larvae of the Lepidoptera in the latter organism [17]. One was led to the conclusion therefore, that the isolate must be *B. thuringiensis*.

Strains of *B. thuringiensis* sometimes lose their ability to form crystals having been maintained on laboratory media, and no parasporal inclusions were observed during microscopic examination of the A/91 isolate.

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تشخيص البكتريا المولدة للابواغ في حضنة مصابة لنحل العسل

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(تاريخ الاستلام: ١٢ / ٥ /٢٠٠٩ ، تاريخ القبول: ١٢ / ١٠ / ٢٠٠٩)

الملخص

تم عزل البكتريا المولدة للابواغ من حضنة مصابة لنحل العسل في مزرعة نقية. وقد استخدمت الطرق البكتريولوجية القياسية لإيجاد صفات هذه المزرعة اثبت تفسير النتائج المتحصل عليها بالمقارنة مع المراجع العلمية أن البكتريا المعزولة تنتمي إلى مجموعة البكتريا *Bacillus cereus* . وكان الاهتمام الرئيسي في هذه الدراسة قد انصب على إن المزرعة النقية قد تكون للبكتريا *B. thuringiensis* أو أنها لنوع جديد . ولكن التجارب الدقيقة قد أكدت أن العزلة هي سلالة للبكتريا *B. cereus*.