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Note

Enzymatic activity of strains of *Ascosphaera apis*, an entomopathogenic fungus of the honey bee, *Apis mellifera* *

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Summary — Unmated +, unmated -, and mated strains of *Ascosphaera apis*, the honey bee chalkbrood pathogen, were examined for production of 19 enzymes using the API ZYM system. All strains produced alkaline phosphatase, butyrate esterase, leucine aminopeptidase, acid phosphatase, and β-glucosidase. Since valine aminopeptidase was produced by unmated but not by mated strains, it may be a useful enzyme for identification of mycelial strains of the fungus. β-Galactosidase and α-mannosidase may be candidate marker enzymes for identification of both unmated and mated strains. Few other molds associated with honey bees produce these 3 enzymes. Use of enzymes as taxonomic aids could simplify tests of bees and hive products for *A apis*, particularly unmated strains.

***Ascosphaera* / enzyme / honey bee / chalkbrood**

Ascosphaera apis is a heterothallic fungus that causes chalkbrood in larvae of the honey bee, *Apis mellifera*. Diagnosis of the disease in a bee colony is generally made on the basis of white, black, or gray larval mummies at the hive entrance, on the flight board, on the bottom board, and/or in unsealed and sealed brood cells.

Bees and hive products sometimes must be certified free of *A apis* in order to be brought into areas where the pathogen has not been found. Laboratory identification of *A apis* from apparently healthy larvae, guts of adult bees, pollen, honey, and brood food is relatively easy when + and - mycelia have mated to form characteristic spore cysts. Since mycelia alone are often isolated and can only be positively identified as *A apis* by performing mating tests (Christensen and Gilliam, 1983), easier and faster methods are needed.

Results of tests for enzymes produced and carbohydrates assimilated by *A apis* strains are not in agreement (Gochnauer

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and Margetts, 1979; Chmielewski and Glinski, 1980/1981; Kowalska, 1984; Alonso *et al,* 1990). Recently, isolates of *A apis* from Spain were examined using the API ZYM system (Alonso *et al,* 1990), but it is not clear whether these were +, -, and/or mated strains.

Using the API ZYM system, we determined and compared enzymatic profiles of *A apis* unmated +, unmated -, and sporulated mated strains having spore cysts. Such profiles may be useful not only in identification of mycelial strains of *A apis* but also in detection of strain differences and determination of the role of extracellular enzymes in virulence of *A apis.*

Fifteen strains of *A apis* were tested. Two of these were +, 7 were -, and 6 were mated strains with spore cysts. Two of these, Nebr-1(+) and Nebr-31(-), were originally collected from Nebraska in 1971 (Christensen and Gilliam, 1983). The other unmated strains were fresh isolates from chalkbrood mummies from Tucson. When necessary, + and - hyphae were separated, and mating types were assigned on the basis of fruiting in crosses with the Nebraska strains and/or with ATCC 13785, LS Olive's + *A apis* strain; crosses to obtain mated strains for API ZYM tests were then made (Christensen and Gilliam, 1983).

Preliminary tests revealed that previous methods used to prepare fungal inocula for API ZYM tests of molds (Bridge and Hawksworth, 1984; Satyanarayana *et al,* 1985; Gilliam *et al,* 1989) were unsatisfactory for *A apis* even when various combinations of these methods and appropriate media for the bee pathogen were used. Difficulties were often encountered in production of sufficient fungal inocula and in variability of the level of enzyme activity.

Therefore, based on extensive preliminary work, the following procedure was devised to give reproducible results. Unmated strains of *A apis* were grown for 9 days on plates of Sabouraud dextrose agar with 0.2% yeast extract (SDA-YE) maintained at 25 °C. Two to 3 pieces of mycelium from each culture were placed in separate sterile tissue grinders with 1–2 ml of sterile water and homogenized until a uniform turbid mycelial suspension was obtained. The suspension was examined microscopically, transferred to a sterile test tube, and standardized to McFarland No 4 with sterile water. Mated strains were grown for 2 wk on SDA–YE plates at room temperature to obtain mature spore cysts. Cultures showing good sporulation were processed as described above and included mycelia, spore cysts, and spores. API ZYM test strips (Analytab Products, Plainview, NY) were then inoculated, incubated, and read according to the manufacturer's directions.

Results of final replicated tests of 15 strains of *A apis* are shown in figure 1. Data are presented using the methods of Bridge and Hawksworth (1984) which allow easy detection of differences and similarities in profiles of enzymatic activity of *A apis* strains. Since the blocks in figure 1 represent ranges of activity, there were no differences in results between replicates. This is also probably due to the fact that the inocula were produced in exactly the same way for each replicate. All strains were tested twice at minimum; some, particularly the reference strains (Nebr-1, Nebr-31, and ATCC 13785), were tested as many as 4 times. Thus, the methods developed are reproducible.

Uninoculated Sabouraud dextrose broth with 0.2% yeast extract gave negative results in API ZYM tests. Myristate lipase, cystine aminopeptidase, trypsin, α-galactosidase, β-glucuronidase, α-glucosidase, and α-fucosidase were not produced by any strains. Only one strain produced chymotrypsin. All strains produced alkaline

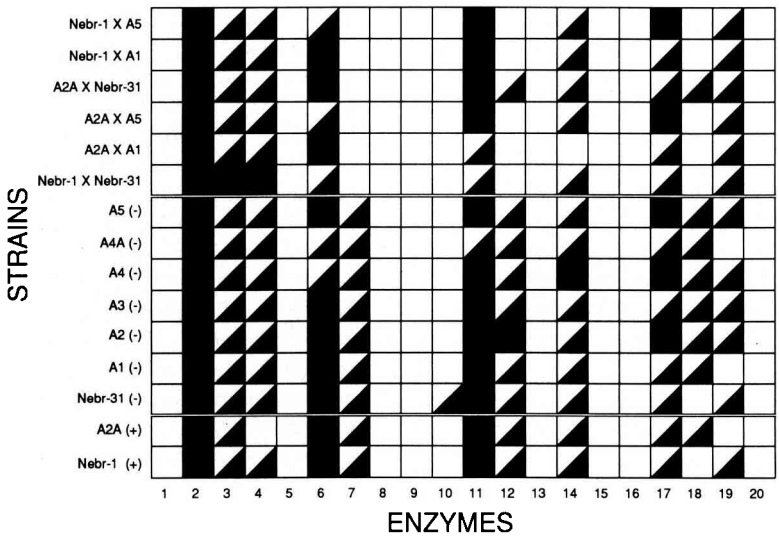

Fig 1. Results of the API ZYM enzyme test system with 15 strains of *Ascosphaera apis*. Strong positive reactions (20 to ≥ 40 nm) are indicated by a solid block, and moderate reactions (5 to 20 nm) by a half-solid block. 1 = Control; 2 = alkaline phosphatase; 3 = butyrate esterase; 4 = caprylate esterase–lipase; 5 = myristate lipase; 6 = leucine aminopeptidase; 7 = valine aminopeptidase; 8 = cystine aminopeptidase; 9 = trypsin; 10 = chymotrypsin; 11 = acid phosphatase; 12 = phosphoamidase; 13 = α-galactosidase; 14 = β-galactosidase; 15 = β-glucuronidase; 16 = α-glucosidase; 17 = β-glucosidase; 18 = *N*–acetyl-β-glucosaminidase; 19 = α-mannosidase; 20 = α-fucosidase.

phosphatase, butyrate esterase, leucine aminopeptidase, acid phosphatase, and β-glucosidase. All but one strain produced caprylate esterase-lipase and β-galactosidase. Alkaline phosphatase was the only enzyme produced in high concentration (≥ 30 nmol) by all strains.

There are some differences in our results compared to those obtained from isolates from Spain (Alonso *et al,* 1990) which could be due to variations in strains, methods, or the API ZYM kits used. For example, only one of our unmated strains approached the levels of phosphoamidase that were found in Spanish strains, and α-mannosidase was not produced by 3 of our strains. Also, our strains did not produce α-glucosidase, whereas 78.3% of Spanish strains produced detectable amounts of 10 nm.

Predominantly mycelial inocula of some fungal species produce the same enzymes, although in reduced amounts, as spore suspensions (Bridge and Hawksworth, 1984; Gilliam *et al,* 1989). However, in the *A apis* strains that we tested, valine aminopeptidase was produced by unmated but not by mated strains. Also, phosphoamidase and *N*-acetyl-β-glucosamidase were produced by all or most, respectively, of the unmated but only one of the mated strains. Thus, these 3 enzymes may be useful for identification of mycelia of *A apis* associated with honey bees. Of these, va-

line aminopeptidase may be the most useful marker since few molds associated with honey bees produce this enzyme (Gilliam *et al,* 1989). β-Galactosidase and α-mannosidase may be candidate marker enzymes for identification of both mated and unmated *A apis* strains since they were produced by most strains tested but are rarely produced by bee-associated molds (Gilliam *et al,* 1989). Overall, + and - strains gave similar results, but more unmated strains, particularly + ones, must be evaluated.

In this study, cultures were incubated for the minimum time required for all strains within a group (unmated or mated) to produce sufficient inocula for the API ZYM test strips and for mature spore cysts to be present in mated strains. This required 9 d of incubation for unmated strains and 2 wk for mated strains. We recognize that enzyme profiles may be affected both by growth times and the type of inoculum. However, this does not negate the possible use of enzymes as markers for *A apis* from bees and hive products. Plating would be required to isolate molds for testing, and the same incubation times that we used should produce sufficient inocula and reproducible results.

We shall use these methods to test additional *A apis* strains, particularly those from other geographical areas and from other bee species as they become available, to determine whether the enzymatic profiles obtained in the present study are indicative of most *A apis* strains and to test further the feasibility of using marker enzymes to identify unmated *A apis* strains and to distinguish *A apis* from other molds commonly associated with bees.

Résumé — Activité enzymatique de souches d'*Ascosphaera apis,* champignon entomopathogène de l'abeille (*Apis mellifera* L). Le champignon *Ascosphaera apis*, responsable du couvain plâtré chez l'abeille, existe à l'état de mycélium (+ ou –) et de formes fécondées sporulées. À l'aide du système API ZYM, nous avons déterminé et comparé les profils enzymatiques de 15 souches (soit non fécondées +, non fécondées -, soit fécondées et sporulées avec des sporocystes). Ces profils peuvent être utiles pour identifier des souches mycéliennes, pour détecter des différences de souches et pour déterminer le rôle des enzymes extracellulaires dans la virulence d'*A apis*. Des méthodes de préparation d'inoculums pour les tests ont été mises au point, car les méthodes existantes pour les moisissures ne donnaient pas satisfaction. Toutes les souches ont produit de la phosphatase alcaline, de la butyrate estérase, de la leucine aminopeptidase, de la phosphatase acide et de la β-glucosidase. La valine aminopeptidase a été produite par les souches non fécondées mais non par les souches fécondées. La phosphoamidase et la N-acétyl-β-glucosaminidase ont été respectivement produites par toutes ou presque toutes les souches non fécondées, mais par une seule des souches fécondées. Ces 3 enzymes peuvent donc être utilisés pour identifier les souches mycéliennes d'*A apis* associées aux abeilles. Parmi eux, la valine aminopeptidase est peut-être le marqueur le plus utile, puisque peu de moisissures associées aux abeilles la produisent. Pour la même raison, la β-galactosidase et l'α-mannosidase, produites par la plupart des souches testées, sont des candidats comme enzymes marqueurs pour les souches fécondées et non fécondées d'*A apis*.

***Ascosphaera apis* / enzyme / couvain plâtré**

Zusammenfassung — Enzymatische Aktivität von Stämmen von *Ascosphae-*

ra apis*, ein insektenpathogener Pilz der Honigbiene, *Apis mellifera L. Der Kalkbruterreger des Honigbiene, *Ascosphaera apis*, kommt in Myzelform (+ oder –) vor, und auch als sporulierende Form nach der Paarung. Unter Benutzung des API ZYM-Systems bestimmten und verglichen wir die enzymatischen Profile von 15 Stämmen (entweder ungepaart +, ungepaart - oder sporulierte gepaarte Stämme mit Sporenzysten). Solche Profile können für die Bestimmung von myzelialen Stämmen, die Entdeckung von Unterschieden zwischen Stämmen und die Bestimmung der Rolle von extrazellulären Enzymen bei der Virulenz von *A apis* nützlich sein. Es wurden Methoden zur Vorbereitung des Impfmaterials entwickelt, da sich die bisherigen Verfahren für den Schimmel als wenig zufriedenstellend erwiesen haben.

Alle Stämme erzeugten alkaline Phosphatase, Butyrat-Esterase, Leucin-Aminopeptidase, saure Phosphatase und β-Glucosidase. Valin-Aminopeptidase wurde von ungepaarten, aber nicht von gepaarten Formen erzeugt. Auch Phospho-Amidase and N-acetyl-β-Glucosaminidase wurden von allen oder von den meisten der ungepaarten Formen, aber nur von einem der gepaarten Stämme erzeugt. Deshalb können diese drei Enzyme für die Bestimmung der Myzelformen von *A apis* in ihrem Vorkommen bei der Honigbiene von Nutzen sein. Von diesen könnte die Valin-Aminopeptidase der beste Marker sein, da nur wenige Schimmelpilze der Honigbiene dieses Enzym erzeugen. Aus demselben Grunde könnten möglicherweise β-Galactosidase and α-Mannosidase, die von den meisten geprüften Stämmen erzeugt werden, als Marker-Enzyme sowohl für ungepaarte wie für gepaarte Stämme von *A apis* dienen.

***Ascosphaera* / Enzyme / Kalkbrut / Honigbiene**

REFERENCES

Alonso JM, Rey JM, Hermoso de Mendoza J, Cardenal JA, Antón JM, Naranjo G, Gil MC, Hermoso de Mendoza M (1990) Caracterización enzimática de *Ascosphaera apis* aislada en España a partir de larvas de abeja melífera (*Apis mellifera* L) afectadas por la Ascosferiosis (Pollo Escayolado). *In: 5th Congr Nac Apícola* Don Benito, Spain, 44-46

Bridge PD, Hawksworth DL (1984) The API ZYM enzyme testing system as an aid to the rapid identification of *Penicillium* isolates. *Microbiol Sci* 1, 232-234

Chmielewski M, Glinski Z (1980/1981) Badania nad patogennością *Ascosphaera apis* dla czerwia pszczoły miodnej *Apis mellifera* L. Cześć I. Właściwości biochemiczne *A apis*. *Ann Univ M Curie-Sklodowksa* 35/36, 71-82

Christensen M, Gilliam M (1983) Notes on the *Ascosphaera* species inciting chalkbrood in honey bees. *Apidologie* 14, 291-297

Gilliam M, Prest DB, Lorenz BJ (1989) Microbiology of pollen and bee bread: taxonomy and enzymology of molds. *Apidologie* 20, 53-68

Gochnauer TA, Margetts VJ (1979) Properties of honeybee larvae killed by chalkbrood disease. *J Apic Res* 18, 212-216

Kowalska M (1984) Właściwości biochemiczne *Ascosphaera apis* i *Bettsia alvei*. *Pol Arch Wet* 24, 7-15

Satyanarayana T, Chavant L, Montant C (1985) Applicability of API ZYM for screening enzyme activity of thermophilic moulds. *Trans Br Mycol Soc* 85, 727-730