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Origin and identification of bacteria which produce kairomones in the frass of *Acrolepiopsis assectella* (Lep., Hyponomeutoidea)

E. Thibout*, J. F. Guillot[a], S. Ferary, P. Limouzin[a] and J. Auger

IBEAS, URA CNRS 1298, Faculté des Sciences, Université F. Rabelais, F-37200 Tours, Fax + 33 47 36 69 66, and [a]IUT, Microbiologie, Université F. Rabelais, F-37200 Tours (France)
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Abstract. The volatiles used by the parasitoid *Diadromus pulchellus* to find its host, the leek moth, are produced by the bacteria developing in the frass of the host larvae. The origin and the nature of these bacteria were investigated. Samples were taken from healthy leeks and from infested leeks in the field, as well as from the frass of larvae reared in the laboratory either on the host plant or on an artificial diet. The various species of bacteria identified were cultured in the presence of precursors of leek sulphur volatiles and their volatile emissions were analysed. *Klebsiella oxytoca* and various *Bacillus*, common decomposers of plant matter, were the principal species producing active volatiles which were alkyl disulphides.
Key words. Kairomone production; bacteria; *Klebsiella oxytoca*; sulphur volatiles; frass; parasitoid; *Diadromus pulchellus*; leek moth.

The chrysalids of the leek moth, *Acrolepiopsis assectella* Zell., a specialist phytophagous insect of plants of the *Allium* genus, are parasitized by the specialist ichneumonid *Diadromus pulchellus* Wesm. (Hymenoptera). The locomotory activity of this parasitoid is strongly influenced by the dialkyl disulphides emitted by the larval frass of the leek moth[1]. It has been demonstrated that these kairomones (according to Dicke and Sabelis[2]) are produced by the metabolism of alk(en)yl-cysteine-sulphoxides accumulated in *Allium* species, carried out by bacteria[3]. These bacteria pass through the digestive track of the leek moth larvae and are discharged along with small pieces of the plant. On the same plant, the larva will spin its cocoon on reaching maturity.

The origin and the species composition of the bacteria implicated in the production of the kairomones necessary to *D. pulchellus* in order to find its host were investigated. The nonsymbiotic role of these bacteria in a specialized tritrophic interaction was also analysed according to the species.

Materials and methods

Four solutions were made using sterile distilled water (5 ml) either by washing the leek, *Allium porrum*, or by mixing with the frass of the leek moth:

- solution of healthy leek leaves. The plants were cultivated at Fondettes Agricultural College to the north-west of Tours, France. Two washings were made in the field, on sunny spring days, using between 20 and 30 leeks and taking one leaf per plant.
- solution of frass taken in the field. One sampling was made at the beginning and another at the end of the summer in the same location as above. The frass seen on the leaves of the leek and in the larval mines were collected using a spatula and placed in distilled water.
- solution of frass taken from leek moths reared in the laboratory on leeks. From the leaves used in the rearings, two samplings of frass were made as above.
- solution of frass taken from leek moths reared in the laboratory on an artificial diet.

Two samplings of frass were made, again following the same technique.

The larvae of *A. assectella* were reared using two methods. One stock, which is annually renewed, was maintained on leeks with a photophase of 18 h at 25 °C and a scotophase of 6 h at 18 °C. The other stock used has been reared in the laboratory for several years[4] on an artificial diet containing leek powder, with a photoperiod of LD 14:10 at a constant 25 °C.

The four washings and liquid suspensions of leek or frass were centrifuged at 6000 rpm for 10 min. One ml of each supernatant was plated after dilution of trypticase soy agar (Biomérieux, Marcy l'Etoile, France). After incubation of between 24 and 48 h at 28 °C, the bacteria which had developed were identified by microscopic observation, Gram coloration and biochemical characterization with API 50 CH strips (Api-System, Biomérieux, Marcy l'Etoile, France).

The production of disulphides (DS) was investigated from bacteria isolated from 3 samples; one coming from healthy leek leaves, a second from frass taken in the field and the last from frass taken from rearings made on leeks in the laboratory. The previously identified bacteria were plated on agar supplemented with or

* Corresponding author.

Table 1. Bacteria identified after washing of leeks or of frass of *A. assectella* in the field or reared in the laboratory on a host-plant or on an artificial diet with leek (D + L).

	Healthy leek	Frass from field	Frass from leek	Frass D + L
Klebsiella oxytoca	●	●	●	
Bacillus macerans	●	●	●	
Bacillus alvei	●	●	+	+
Bacillus licheniformis	○	●	●	+
Bacillus circulans			+	
Bacillus lentus				+
Bacillus laterosporus				+
Bacillus polymyxa			+	
Enterobacter agglomerans			○	+
Pseudomonas aeruginosa	+			
Pseudomonas putida	+			
Serratia marcescens			○	○
Spreptococcus sp.			○	

● Bacteria present producing disulphide after culture with methyl-cysteine-sulphoxide.
○ Bacteria present not producing any disulphide after culture with methyl-cysteine-sulphoxide.
+ The emission of disulphide was not investigated in the identified bacteria.

without 1% S-methyl-cysteine-sulphoxide (MCSO). In the last-mentioned sampling, S-methyl-cysteine (MCS), a possible intermediary in the biosynthesis of MCSO, was also used. Ten plates for each sample were incubated at 28 °C for 48 h in darkness in a closed 5-litre container. The covers of the Petri dishes in each closed container were removed and the volatiles emitted were adsorbed on Tenax for 4 h before gas chromatography (GC).

Bacterial volatiles were identified in culture by GC or GC/electron ionization mass spectrometry (MS) coupling using a Hewlett-Packard HP 5890 A chromatograph and an HP 5989 mass spectrometer. A Hewlett-Packard capillary column 25 m × 0.22 mm ID with HP1 methylsilicone film 0.33 µm thick was used. The chromatography conditions were as follows: on-column injection at 250 °C, temperature program 2 °C/mn from 70 °C to 130 °C then 4 °C/mn to 280 °C, carrier gas He at a flow rate of 0.5 ml/mm. When GC was not coupled with MS, flame photometry (FP) and flame ionization (FI) detectors were used. MS coupling conditions: ionization energy 70 eV, source temperature 176 °C and acceleration voltage 1800 V.

Results

Thirteen species of bacteria were isolated and identified from the solutions analysed (table 1). Two groups can be differentiated within these 13 species according to their frequency and to their ability to produce disulphides from sulphur amino acids. The first group consists of the four bacteria present in at least three of the solutions analysed: *Klebsiella oxytoca*, *Bacillus macerans*, *Bacillus alvei* and *Bacillus licheniformis*. These four bacteria are, moreover, those which are capable of producing disulphides. The second group consists of the nine other bacteria present in at most two solutions and from which the emission of disulphides was not observed (table 1).

Table 2. Volatiles identified by GC/MS in the trapped odour of a methyl-cysteine-sulphoxide supplemented culture of *Klebsiella oxytoca* originating from field *Acrolepiopsis assectella* frass.

Volatiles	Retention times in min	Amounts
DS dimethyl	2.6	+
TS dimethyl	6.8	t
Acetophenon	10.4	+
2-Phenyl-ethanol	12.7	+ +
Undecan	13.6	t
Dodecan	19.2	t
Tridecan	25.1	t
Tetradecan	31.5	t
Indol	22.0	+ + + +

The amount differs in ratio from one to ten for each +. DS = disulphide, TS = trisulphide; t = traces.

When disulphides were present in the bacterial cultures this was due to the bacteria, because a diet containing MCSO on which no bacteria had been cultivated does not emit disulphides. If disulphides were produced from MCSO, as from MCS and emitted by the culture of certain bacteria, only the dimethyl disulphide was observed. The emission was generally accompanied by a very weak emission of dimethyl trisulphide (1/100). In the presence of MCS, *K. oxytoca* and *B. alvei* were, moreover, capable of producing disulphide but in a lesser quantity (1/100) than in the presence of MCSO. In contrast *B. macerans* seemed incapable of metabolizing MCS into disulphide.

The chromatograms of the odours of the bacterial cultures revealed a certain number of other products than sulphur volatiles (table 2). These products have been especially studied in the bacteria which produce disulphides, and particularly in *K. oxytoca* using GC/MS.

In the three *Bacillus* which produce disulphides, no other product was observed in any quantity. In *K. oxytoca* coming from frass collected in the field, the quantities of 2-phenyl-ethanol and particularly of indole

were high (table 2). Moreover, *Enterobacter agglomerans* produced large quantities of 2-heptanone.

Discussion

The four bacterial species, *K. oxytoca*, *B. alvei*, *B. macerans* and *B. licheniformis*, producing disulphides from MCSO, are present in the field, particularly on healthy leeks. The nine other bacterial species incapable of producing disulphides in the presence of MCSO are only observed in the laboratory, with the exception of the two species of *Pseudomonas*. *Pseudomonas* is a genus which is frequently found in leek fields, particularly *Pseudomonas syringae*, the pathogen responsible for a disease of leeks[5], which was not found here.

Certain of the bacteria identified in this work have already been noted as being associated with various insects. *E. agglomerans* is present in the cricket *Melanoplus sanguinipes* reared in the laboratory[6]. Well-known is the case of Diptera in which symbionts have been found. Thus in *Dacus oleae*, *B. licheniformis* has been identified[7] as well as *Pseudomonas putida*[8] and *K. oxytoca*.

K. oxytoca has been noted in the digestive track of *Rhagoletis pomonella*[9] and in that of several species of *Dacus*[10,11]. Finally, in *Delia antiqua*, *K. oxytoca* produces dipropyl disulphide and propyl methyl disulphide from onions containing sulphur amino acids[12]. *K. oxytoca* on the other hand is found naturally in the soil and in numerous plants[13,14] several of which are consumed by man[15]. *Serratia marcescens* was also found in certain *Dacus*[7]. In agreement with other authors[10,11], it can be concluded that it is associated with laboratory-reared insects. In the leek moth, the same must be true for *Bacillus circulans*, *Bacillus lentus*, *Bacillus laterosporus*, *Bacillus polymyxa*, *E. agglomerans* and the *Streptococcus* species which, in contrast to *K. oxytoca* and *B. macerans*, can develop in an artificial diet containing fungicides and antiseptics. It is thus more than likely that the bacteria which in the leek moth frass produce disulphides are very common opportunist bacteria which, like *K. oxytoca*, have a role in the decomposition of plant matter[16]. Thus, in the frass of *A. assectella*, the sulphur amino acids contained in the pieces of leek that passed through the digestive track of the larva would be metabolized by those bacteria which, as a consequence, would emit some disulphides. The frass of *A. assectella* would thus become a good indicator for the parasitoid *D. pulchellus* of the presence of a larva and thus probably of a host chrysalis. If the bacterial flora of the frass of *A. assectella* produces sulphur volatiles used by the wasp, it cannot be considered as symbiotic for the larva of the phytophagous moth. This is in contrast to its role in the cited Diptera which are, in actual fact, saprophages (which eat decomposing matter).

Unlike our earlier work on this subject[3], the bacteria isolated in this study only produce the disulphide corresponding to the sulphur amino acid added to the culture medium. Thus we reported earlier that in the presence of MCSO, the dimethyl disulphide and dipropyl disulphide were observed. Only the dimethyl disulphide was observed now. This difference might be explained by the fact that the latest cultures did not permit the development of all species or those of different strains to those normally found in the frass. Another hypothesis would be that the bacteria were cultivated in isolation and not in a mixture, as in the frass where they can interact and result in a more complex volatile production.

Among the volatile compounds isolated, other than the disulphides, 2-phenyl-ethanol is known to be attractive for the onion fly, *Delia antiqua*. It could be also a product of decomposition[17]. Thus it seems that strong links exist between *Allium*, *K. ocytoca* and the production of disulphides and of 2-phenyl-ethanol. Other volatiles like disulphides, trisulphides, 2-phenyl-ethanol, 2-heptanone, acetophenone (phenyl-ethanone), undecane, dodecane, tridecane or tetradecane have already been observed in cultures of microorganisms[18] and indole is even characteristic of *K. ocytoca*[16].

It could be interesting to examine now if the production by bacteria of several disulphides from only one sulphur precursor is a consequence of the experimental conditions or of the presence of not yet identified bacterial species.

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