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Symbiosis with bacteria enhances the use of chitin by the springtail, *Folsomia candida* (Collembola)*

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Summary. The relationship between *Folsomia candida* and chitin-degrading microorganisms was studied. On chitin agar, 10^{10} bacteria were isolated per g faeces, and 3.8×10^{11} bacteria per g gut contents, 1/3 of them showing a clear (chitin-free) zone around the colony. The most abundant chitin-degrading bacteria were *Xanthomonas maltophilia* and *Curtobacterium* sp. To determine the bacterial contribution in the use of chitin by *F. candida*, a feeding experiment was carried out. *F. candida* were fed with chitin, either amended with or without tetracycline as an inhibitor of bacteria. When tetracycline was omitted, the biomass of *F. candida* was increased compared to those fed chitin with tetracycline. However, this result was observed only when the food replacement intervals were long enough to allow bacterial colonization before ingestion of the food. In a food-selection experiment, a preference for chitin colonized with microorganisms as opposed to sterile chitin was found. The results indicate that a mutualistic symbiosis of *F. candida* with chitinolytic microorganisms is likely to enhance chitin degradation. This relationship is not only intra-intestinal but also involves an extra-intestinal phase.

Key words: Soil – Symbiosis – Chitin degradation – Collembola – *Folsomia candida* – *Xanthomonas maltophilia* – *Curtobacterium* spp.

Microorganisms are the key factor of litter catabolism in soil. However, the soil mesofauna may be regarded as a catalyst, enhancing energy and nutrient fluxes. The feeding activities of animals increase the surface area of substrate exposed to microbial attack and their intestines serve as a most favourable microhabitat for microorganisms. Through grazing, the soil mesofauna may also maintain fungal and bacterial populations in an active growth stage, and probably effects qualitative changes in the soil microflora (Anderson and Bignell 1980; Bakonyi 1989). The soil mesofauna also acts as a vehicle for transporting microorganisms towards metabolizable substrate, and thus may be called a mobile microhabitat.

Inside the digestive system, growth conditions for the microorganisms largely resemble that of a continuous culture. Symbiotic relationships between animals and microorganisms may range from competition to mutualism (Lewis 1985), but the distinction between different relationships is often not obvious. However, clearly mutualistic symbioses have frequently been observed. These symbioses often involve microbial specialists for degrading certain organic compounds, such as cellulose or chitin (Wallwork 1970). Parle (1963) reported that cellulolytic and chitinolytic bacteria and actinomycetes are abundant in earthworm gut contents. However, his data suggested that enzymes produced by the worm rather than the microorganisms were the main agents hydrolysing cellulose and chitin. Wallwork (1970) supposed that symbiotic bacteria might be important for chitin degradation in Collembola. However, he did not give supportive data.

Among soil arthropods, Collembola are one of the most abundant groups, feeding mainly on detritus, fungi, and bacteria. We found that *F. candida*, a widespread representative of the Collembola, was feeding on its own chitinuous exuvia. Some other springtail species did not appear to feed in this way. Hence, we hypothesized that *F. candida* has an effective mechanism for the use of chitin, possibly involving microbial symbionts. The present study was designed to investigate this possible relationship.

Materials and methods

Culture of F. candida

We studied springtails derived from a laboratory culture that had originally been isolated from an agricultural soil (Luvisol). Plastic culture boxes (19×19×5 cm) containing 400 g sieved (< 2 mm) and heat-treat-

* Dedicated to the late Prof. Dr. W. Kühnelt

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ed (105 °C, 24 h) soil were kept in the dark at 15 °C. The soil water content was adjusted to 22%. The cultures were supplied weekly with dried (105 °C) *Urtica dioica* powder, Biosol® (a product containing dried fungal mycelium; Biochemie GmbH, A-6250 Kundl, Austria), and dry yeast. Only uniform size-classes of *F. candida* were used in the experiment.

Isolation and enumeration of chitinolytic microorganisms

Isolation from faeces. Approximately 800 springtails were placed in soil Petri dishes and fed for 1 week with Biosol. Then the animals were removed and placed on cellulose–acetate filter paper. After 12 h, 5 mg faeces was sampled and suspended in 1/4 concentrated Ringer's solution. Dilution series were prepared, and 0.2 ml of each dilution was streaked on double-layer chitin agar plates (0.7% chitin; Lingappa and Lockwood 1962). Three replicates were made at each dilution. The plates were incubated at 28 °C for 1 week.

Isolation directly from the gut. To avoid contamination, the springtails were disinfected as follows. First, to immobilize them, the animals were handled in a CO_2 atmosphere. Then the animals were placed in 5 ml wetting agent, shaken, and treated with ultrasound (90 kHz, 10 s). Next, they were pipetted into 70% ethanol (10 s), and returned to Ringer's solution. This procedure was repeated three times. The springtails were tested for surface sterility by being placed on agar plates. The animal was then placed on a microscope slide that was cooled to avoid desiccation. The abdomen was opened laterally, and the haemocoel fluid was removed with blotting paper. The body cavity was repeatedly flushed with Ringer's solution and dried with blotting paper. The hindgut was cut, and the gut content squeezed out with a preparation needle. Then 1 µl Ringer's solution was added, and the suspension was siphoned with a capillary. The filled capillary was weighed and dilutions were prepared as above.

Feeding experiments

The experiments began with 0.01 – 0.03 g of springtails, corresponding to 150 – 450 animals. They were placed in glass Petri dishes on a 3 mm thick layer of plaster-of-Paris mixed with charcoal. Water was added until saturation. The substrates used are shown in Table 1.

The substrates were mixed with agar (2%) as a carrier, autoclaved (121 °C, 15 min), and poured into dishes in 3 mm layers. After the agar had solidified, strips (40×2×3 mm) were cut out and placed on parafilm (used to prevent diffusion) in the cultivation dishes. Yeast extract (Difco) was added to provide micronutrients and vitamins, and chlortetracycline-HCl (Sigma) was used to inhibit bacterial growth. In one experiment, the substrate was replaced after 4 days, and in another it was replaced every day. The weight of the animals was recorded, every day for 6 days.

Food selection experiment

This experiment was carried out to determine food preferences among sterile and microbially colonized substrates. Chitin agar strips, either sterile or inoculated with *F. candida* faeces or cultures of *Xanthomonas maltophilia* and *Curtobacterium* sp., were placed in plaster/charcoal Petri dishes. Twenty animals were placed in each of three replicate dishes, and their location was recorded at regular intervals for 4 days.

Table 1. Substrates used for the feeding experiment

Substrate	Chitin (%)	Yeast extract (%)	Tetracycline (%)
A	–	0.1	–
B	1.75	0.1	–
C	1.75	0.1	0.05

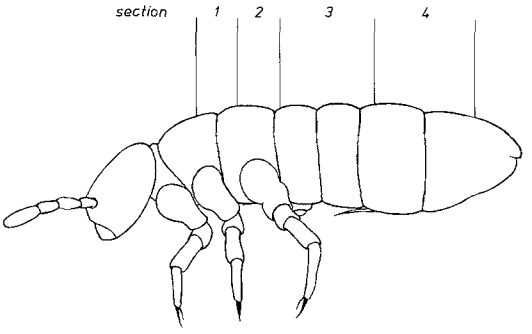

Fig. 1. Cross-sections through *Folsomia candida* for the assay of intestinal chitinase activity

Assay for intestinal chitinase activity

Agar (1.5%) plates containing dyed carboxymethylchitin (1%) were used for this enzyme diffusion test. Chitinase hydrolyses the chitin macromolecule, and the monomers containing the dye may diffuse along the concentration gradient, resulting in a clear zone surrounded by a violet ring (G. Wolf, 1989, personal communication). To inhibit microbial growth, half of the test plates were amended with sodium azide.

We cross-sectioned frozen specimens of *F. candida* at certain points as shown in Fig. 1. With the hind end, the sections were placed on the test agar. After 18 h incubation at 20 °C the diameter of the dye rings was measured.

Statistics

Where appropriate, either a one-way or two-way analysis of variance was carried out, followed by a Tukey test for cell differences.

Results

The chitinolytic bacterial counts were high, with 10^{10} colonies isolated per g faeces, 3×10^9 of which showed a clear zone on the chitin agar. In the gut contents, we counted $3.8\pm2.2\times10^{11}$ bacteria, 1/3 of which showed a clear zone on the chitin agar. These numbers were about five orders of magnitude higher than the numbers of chitinolytic bacteria usually found in soil.

We identified three of the five most abundant chitinolytic bacterial strains in *F. candida* faeces (Table 2). Two of these strains, *Xanthomonas maltophilia* and *Curtobacterium* sp., showed clear zones around the colonies on the agar plates. The other strains did not show clear zones, but showed good growth.

The purpose of the feeding experiments was to reveal whether chitin is used by *F. candida* and whether antibiotic inhibition of bacteria would reduce the weight gain of chitin-fed *F. candida.* The substrate contained yeast extract to provide the animals with micronutrients. In the first experiment, the substrate was replaced at 4-day intervals. Chitin plus yeast extract (substrate B) led to an increase in animal weight (Fig. 2a). When tetracycline was added to the substrate (C), the increase was less pronounced (Fig. 2a). After 4 days, the difference between B and C was significant ($P<0.05$). These results suggest that chitin was used by the springtails and that microorganisms were involved in the process.

When the substrates were replaced at daily intervals, neither produced a biomass increase. At the low yeast-ex-

Table 2. Bacterial strains isolated from *Folsomia candida* faeces and gut contents

Isolate	Pigment	Gram stain	Clear zone on chitin agar	Use of N-acetyl-glucosamine
Xanthomonas maltophilia	Pale yellow	−	Yes	Yes
Curtobacterium sp.	Opaque	+	Yes	ND
Not identified	Opaque	+	No	No
Not identified	Dark yellow	−	No	No
Pseudomonas sp.	Opaque	−	No	ND

ND, not determined

tract concentration used, without an additional C and energy source (substrate A), the springtails lost weight (Fig. 2b). The same result was observed with substrates B and C. The addition of the antibiotic therefore did not show a significant effect.

In the food-preference experiment, the number of animals feeding on the substrates was significantly determined by time and inoculum ($P<0.01$). The analysis of variance also showed a significant interaction between time and inoculum ($P<0.01$). In the initial phase the *F. candida* fed preferentially on the substrate inoculated with *F. candida* faeces (Table 3). However, after about 18 h, considerable feeding activity was observed on the substrate inoculated with *Xanthomonas maltophilia* and, after an even longer time lag, on the substrate inoculated with *Curtobacterium* sp. Feeding activity on the sterile control substrate was observed after 3 days. However, at that time a microscopic examination showed that bacterial colonization of the control substrate was beginning.

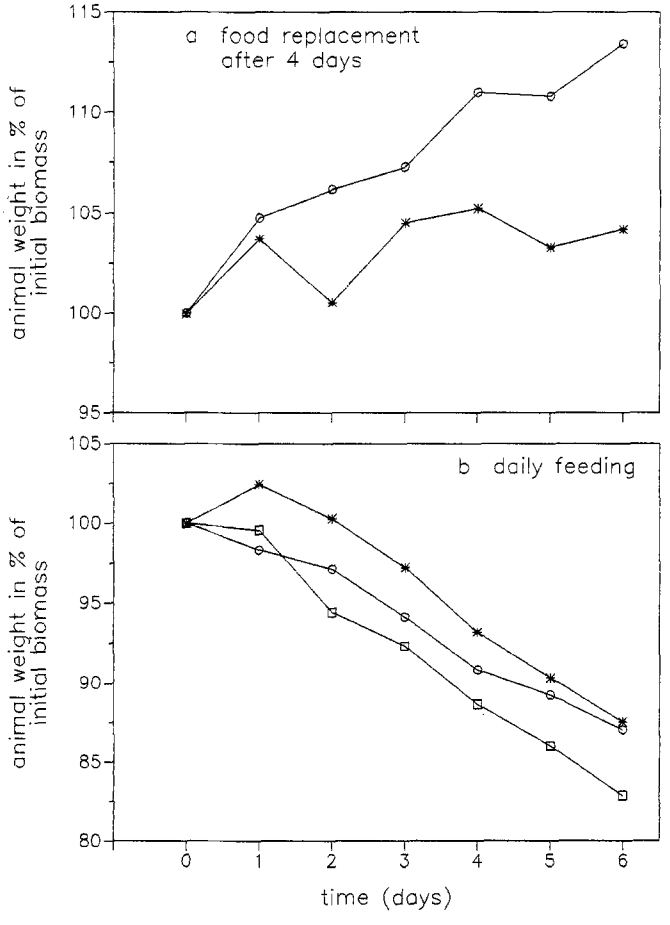

Fig. 2. Biomass changes in *Folsomia candida* fed with various substrates at different feeding intervals. ○——○ chitin+yeast extract, *——* chitin+yeast extract+antibiotic, □——□ yeast extract

Table 3. Food preference experiment: Average number of *Folsomia candida* feeding on chitin agar strips at different times after amendment. The agar strips were either sterile or inoculated with *Xanthomonas maltophilia*, *Curtobacterium* sp., or a mixed culture derived from *Folsomia candida* faeces

Time	Control	Inoculum		
		Xanthomonas maltophilia	*Curtobacterium* sp.	Faeces of *Folsomia candida*
4 h	0	0	0	1.3±0.3
8 h	0	0	0	2.7±0.9
12 h	0	0.3±0.3	0	2.0±0.0
16 h	0	0.7±0.3	0.3±0.2	1.3±0.3
18 h	0	2.0±0.6	0	0.7±0.3
20 h	0	3.3±1.5	0.9±0.2	1.0±0.6
30 h	0	2.3±0.3	2.4±0.5	2.0±0.6
35 h	0	3.3±0.3	2.3±0.3	1.3±0.3
40 h	0.2±0.1	5.7±0.3	2.3±0.7	0
2 days	0.3±0.1	5.1±2.2	1.7±0.4	4.0±1.0
3 days	0.9±0.2	4.3±1.0	2.6±1.5	2.3±1.6
4 days	1.1±2.2	6.0±1.0	4.8±0.3	1.7±0.9

The test on chitinase activity showed that the enzyme was present throughout the intestinal tract. However, the clear zone of sections 2 and 3 was significantly larger than that of section 1, indicating a higher enzymatic activity in the former (Table 4).

Discussion

Compared with the numbers of chitinolytic microorganisms usually found in soil (approximately 10^6), the numbers found in the gut and faeces of *F. candida* were very high and demonstrate the importance of the intestinal microsite for chitin degradation. In soil, actinomycetes are considered most important for chitin degradation. However, chitin degradation in the *F. candida* intestines does not seem to be accomplished by actinomycetes. We

Table 4. Diameter of clear zone (mm) on carboxymethylchitin test agar plates

	Section 1	Section 2	Section 3	Section 4
(1) Agar containing Na-azide (pH 7.0)	2.3 ± 0.5^{a}	3.2 ± 1.0^{b}	3.2 ± 0.5^{b}	$3.0\pm0.7^{a,b}$
(2) Agar without Na-azide (pH 7.2)	2.5 ± 0.4^{a}	3.5 ± 0.7^{b}	4.3 ± 0.6^{b}	$3.3\pm0.3^{a,b}$

Measurements (1) and (2) taken after 1 and 24 h, respectively. Different letters indicate significant differences (Student's t-test, $P<0.05$, $n=3$)

found only very small numbers of viable actinomycete propagules in the intestines (10^3 g^{-1} gut content), perhaps because the feeding activity led to an unfavourable disruption of mycelia.

The numbers of bacteria in the gut and faeces do not reveal the type of relationship with the host. However, the present data suggest that the bacteria were actively using chitin. There are several possibilities concerning a symbiotic relationship with the *F. candida*. (1) The bacteria in *F. candida* may only compete for the chitin with chitinolytic enzymes. In earthworms, the indigeneous production of chitinolytic enzymes seems to be more important than a symbiotic relationship (Parle 1963). However, nothing is known about the production of chitinolytic enzymes by *F. candida*. (2) If chitinolysis is carried out by the bacterial gut flora and not by *F. candida*, the *F. candida* may obtain intermediate products, such as N-acetylglucosamine liberated through bacterial enzymes. This would be a mutualistic symbiosis.

Replacing the substrate at 4-day intervals produced a biomass increase, but replacing it at daily intervals did not. This implies that: (1) with its own enzymes, *F. candida* does not use chitin effectively enough to rely on it as the sole C and energy source; and (2) intra-intestinal symbiosis is unlikely to be the only relationship between chitinolytic bacteria and *F. candida*. On the substrate that was replaced at 4-day intervals, we observed bacterial and fungal growth. Thus, the *F. candida* feeding on this substrate ingested not only chitin, but also microorganisms and metabolites. This did not occur when the substrate was replaced daily. This observation suggests that a predigestion of chitin before ingestion may be important, possibly along with a steady supply of bacterial inoculum. According to our observations, the intestinal passage takes approximately 2 h. Even with the large numbers of chitin-using bacteria found in the gut, this time is probably too short for thorough use of the chitin. This assumption is supported by results from a chitin assay of the faeces which revealed a high chitin and glucosamine content. Similar conclusions were drawn by Anderson and Bignell (1980), who found that increased bacterial counts in millipede faeces were due to proliferation of indigeneous litter bacteria rather than a population of specific gut symbionts.

The above conclusions are also supported by the results of the food-selection experiments. *F. candida* is apparently not attracted by chitin unless it is inoculated with microorganisms. Our experiment did not reveal whether it was the microorganisms *per se* or the metabolites that attracted *F. candida*. However, it is unlikely that *F. candida* was attracted by olfactory perceptions. A direct (fortuitous) contact with the substrate would be necessary. A coarse orientation response may be induced by a CO_2 gradient, and an experiment showed that in a Petri dish without substrate, *F. candida* gathered around an artificial CO_2 source. Details of the biochemical mechanisms behind the attraction remain to be studied.

It might be argued that tetracycline harms the collembolans, thereby leading to the observed effects of the feeding experiments. However, in preliminary experiments with Biosol and yeast as a substrate, we found that at concentrations $<0.05\%$, tetracycline caused an increase in the *F. candida* biomass. At concentrations $>0.2\%$ *F. candida* reduced its substrate uptake and lost weight. At the selected concentration of 0.05%, these effects were balanced. Thus, in the chitin-feeding experiments, tetracycline most likely inhibited the chitinolytic microflora without affecting *F. candida*.

Selective grazing by *F. candida* on fungi has been demonstrated by Moore et al. (1987). They concluded that selective grazing may have marked effects on ecosystem nutrient cycling and may result in a population shift of fungi. Furthermore, they postulated that mutualism may occur between fungi and Collembola. Our experiments on the use of chitin suggest a mutualistic relationship between *F. candida* and bacteria. The relationship does not seem to be merely casual and fortuitous. There seems to be a mechanism which enables the collembolans to actively seek their symbionts.

Anderson and Bignell (1980) found that feeding by soil mesofauna leads to a shift from fungal to bacterial biomass. This finding may be supported by the data presented here. In the soil, chitin is mainly used by actinomycetes and fungi. However, when *F. candida* feeds on chitin, bacteria thrive. Mechanical disruption and intimate mixing with other soil constituents (e.g. micronutrients) may be more beneficial for bacteria than for actinomycetes and fungi.

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