6 Collembola as a Habitat for Microorganisms

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6.1 Introduction – Diversity and Activity of Collembola

Collembola (springtails) are microarthropods that can range in size between 0.2 mm and 1 cm, with most having an average length of 1–5 mm. To date, approximately 7500 different species have been described, and there is no doubt that Collembola are among the most abundant groups of arthropods on Earth (www.tolweb.org). Traditionally, taxonomy has placed Collembola in the group of insects, but recent classification places them in a class alone under the Superclass Hexapoda. Collembola are regarded as a phylogenetically old group, with an age of almost 400 million years. They are probably the oldest Hexapoda alive. Their position in the phylogenetic tree is still under debate as there is recent evidence, based on molecular and morphological data, that Collembola are actually more closely related to crustaceans then to insects, which would mean that the Superclass Hexopoda is actually not monophyletic (Nardi et al. 2003; Bellinger et al. 2004). Within the class of Collembola, however, the systematic classification seems to be, at least roughly, in good accordance with new data from molecular phylogeny (Frati et al. 1997; Park 2002; D'Haese 2003). In a recent classification presented by Hopkin in his book about Collembola (Hopkin 1997), three orders can be differentiated: i.e., (1) the *Arthropleona*, with 15 families and more than 5,500 species, (2) the *Neelipleonea*, with only one family and 25 species, and (3) the *Symphyploeona*, with two families and almost 900 species.

Most Collembola live in soil or leaf litter covering the soil surface. Epedaphic species are adapted to living in the litter layer; hemiedaphic species colonise mainly the upper organic layers of soils; and euedaphic species have adapted to living in the soil matrix, but typically not deeper than 10 cm below the soil horizon (Hopkin 1997). Epedaphic Collembola tend to be pigmented, whereas the euedaphic Collembola are often non-pigmented. The geographical range of Collembola is enormous, as they

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are found in all imaginable climatic regions, from the Antarctic or Arctic environment to the tropical belt (Rusek 1998). In most terrestrial ecosystems, Collembola are found as an important part of the soil mesofauna, which also includes mites, nematodes, enchytraeids, millipedes, earthworms, ants, small gastropods, isopods, or larvae of insects. Densities of 10^4 to 10^5 specimens of Collembola per m^2 are not unusual for many soils and, in such soils, Collembola are an important part of the terrestrial food web. On the one hand, they feed on different organic materials and, on the other, they serve as prey, especially for predatory mites and spiders (Hunt et al. 1987; Bilde et al. 2000; Agusti et al. 2003; Bonte and Mertens 2003).

Together with mites, Collembola are often the major constituents of soil microarthropods. Microarthropods, as a functional group of ecological importance, initiate the degradation of organic material, e.g., that accumulates in the litter layer, and thereby ultimately enhance the cycling of carbon and nitrogen in soil (Filser 2002). By chewing on organic substrates, such as dead plant material, and subsequently passing the substrates through the gut, microarthropods restructure the organic material and facilitate microbial degradation (Rusek 1998). Collembola feed on fungal mycelia and some other organic substrates, among them animal remains (Hopkin 1997; Rusek 1998). *Folsomia candida* (Isotomidae, Entomobryoidea, Arthropleona), one of the most laboratory-reared Collembola species, was even shown to feed on nematodes (Lee and Widden 1996). Other Collembola can feed on living plants at certain environmental conditions and thereby cause some problems in agriculture (Sievers and Ulber 1990; Bishop et al. 2001). On the other hand, Collembola can consume plant pathogenic fungi or stimulate growth of mycorrhiza and thereby support plant health (Lussenhop 1996; Nakamura et al. 1992; Gange 2000; Sabatini and Innocenti 2000, 2001).

The feeding preferences of different Collembola species have been investigated in both laboratory studies and by analysing the gut of field-collected specimens. In the laboratory it could be demonstrated that different Collembola have different feeding preferences, e.g., for certain species of fungi or other microorganisms (Visser and Whittaker 1977; McMillan 1976; Bardgett et al. 1993; Chen et al. 1995; Kaneko et al. 1995; Thimm and Larink 1995). There are indications that Collembola can smell food or detect carbon dioxide gradients (Bengtsson et al. 1991; Hedlund et al. 1995) and both attributes probably serve to differentiate food in terms of palatability. In breeding stocks, e.g., with *F. candida*, the specimens can be fed with autoclaved baker's yeast or pea puree, but it can be observed that the animals also feed on their own faeces, on the exuvia they generate by moulting, and even on dead specimens, if the opportunity is given (Borkott and Insam 1990; own observations).

Analyses of the gut contents of field-collected Collembola frequently show a high diversity of different material, indicating that many Collembola

are actually omnivores and less selective than suggested by food preference assays in the laboratory. Numerous substrates have been detected in the gut contents from single specimen; e.g., fungal hyphae, collodial material, plant material, fungal spores, pollen, bacteria and animal remains (Chen et al. 1996). The composition of the contents of the gut material varies between species from the same habitat, but it also varies within a species. Seasonal changes, which affect the availability of different food sources, as well as environmental factors, including pollutants, account for these variations (McMillan 1975; Anderson and Healey 1972; Ponge 2000; Gillet and Ponge 2003). It should be noted that the uptake of different foods may not only affect processes associated with digestion and mineralisation, but also with dispersal. Dispersal of ingested substrates may be beneficial or harmful, depending on whether mycorrhizal spores, which eventually stimulate plant growth, or pathogenic fungi, which destroy plants, are transported to root surfaces (Williams et al. 1998; Dromph 2001). Collembola not only move actively by jumping – they can also be dispersed over long distances by wind (Hopkin 1997). Inevitably, the dispersal of Collembola also means that the gut contents (spores, pollen, microorganisms) are dispersed. Recently this aspect has drawn our attention with regard to the importance of Collembola in unintentionally disseminating genetically engineered microorganisms (Tebbe 2003) or plant pathogenic bacteria (Hildebrand et al. 2001; for more details, see Sects. 6.3, 6.4, and 6.5).

In this chapter we report on own experimental studies that were conducted to investigate the interactions between Collembola and soil bacteria. The objectives of the studies were to learn more about the specific conditions that bacteria face in the gut of Collembola and about the fate of ingested bacterial cells during the gut passage. Would certain bacteria be preferred as food sources and would the composition of the bacterial communities on substrates be affected during a gut passage, e.g., by differential digestion? One specific aspect of our studies also related to the importance of the gut of Collembola as a hot spot for gene transfer between bacteria; other studies were concerned with elucidating the diversity of indigenous bacteria that can be found in the gut or in other compartments of the collembolan body.

Most of our studies were conducted with the previously mentioned, euedaphic, non-pigmented collembole *F. candida* (Fig. 6.1), a species that is ubiquitous and that can easily be kept in laboratory breeding stocks. The species reproduces in the breeding stocks by parthenogenesis, all specimens were female. *F. candida* has a typical morphology for a collembole, with a ventral tube and a springing organ, the *furca*. The furca, a typical feature of Collembola, is held by the tentaculum (a catch) and it is used to make jumps, by snatching out of this tentaculum. In the breeding stocks, which can be maintained in jars with plaster of Paris and charcoal on the bottom

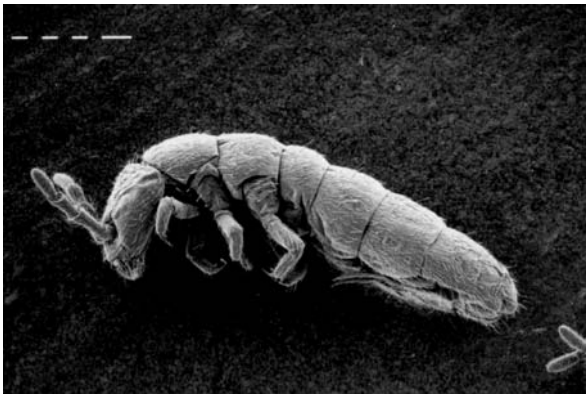

Fig. 6.1. *Folsomia candida*, a euedaphic parthenogenetic species that is can easily be reared in the laboratory, as seen by scanning electron microscopy (SEM) (length of the specimen approx. 2 mm)

(Goto 1960), the females lay their eggs and after 8 to 10 days, the young collemboles hatch. While growing, *F. candida* moults frequently, i.e., it peels off the old cuticula and replaces it by a new cuticula, which is generated under the old one by the epithelium. In contrast to insects, Collembola also regenerate their midgut epithelium during moulting (Humbert 1979). After hatching, *F. candida* moults at intervals of 3 to 10 days. The frequency of moulting decreases during the life of *F. candida* (Snider 1972). After only six moulting stages, the *F. candida* start laying eggs. In our breeding stocks, the specimens reach an age of approx. 10 months when they are kept at 18 °C.

6.2 The Gut of *Folsomia candida* – an Unusual Microbial Habitat That Is Affected by Moulting

The gut of *F. candida* is a rod-like tube, with a small foregut, a large midgut that can increase in size with the amount of food, and a short hindgut. Based on microscopic sections and the direct analyses of lactic acid-treated specimens under the microscope, the volumes of the gut were estimated and the small foregut was found to have a volume of 0.21 nl, the midgut of 6–12 nl, when it was filled, and only 1 nl when it was empty (Thimm et al. 1998). The faecal pellets that were deposited by *F. candida* in the breeding stocks had an approximate volume of 1 nl. With scanning electron microscopy (SEM), a biofilm-like dense layer of mainly rod-shaped bacterial cells could be detected in the region of the peritrophic mem-

brane (Fig. 6.2). The peritrophic membrane is a layer on the gut epithelium which is common in insects and collemboles (Wang and Granados 2001). It is composed of chitinous microfibrills embedded into a proteoglucan matrix (Terra 2001b), and its function is to facilitate the transport of the food bolus through the gut and possibly also to protect the gut epithelium, e.g., from the attack of microbial pathogens (Terra 2001a). Beside this biofilm-like region of the peritrophic membrane, bacterial cells could also be detected in the food bolus (Fig. 6.3), even when sterile food was

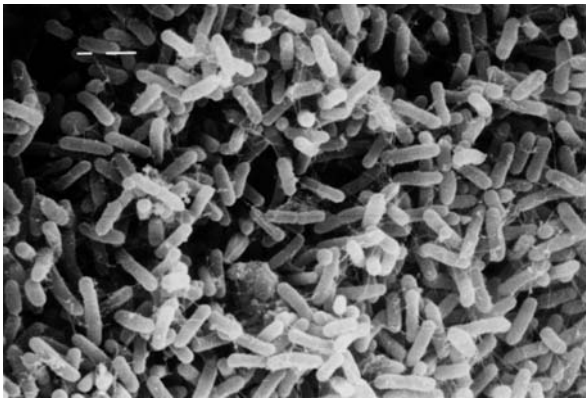

Fig. 6.2. Detection of bacterial cells forming a biofilm-like structure in the peritrophic membrane (PM) as seen with SEM. Note that the mucous layer of the PM is removed by dehydration of the samples, necessary for SEM (figure from Thimm et al. 1998, courtesy of ASM Press). Length of the *left bar*, 1 µm

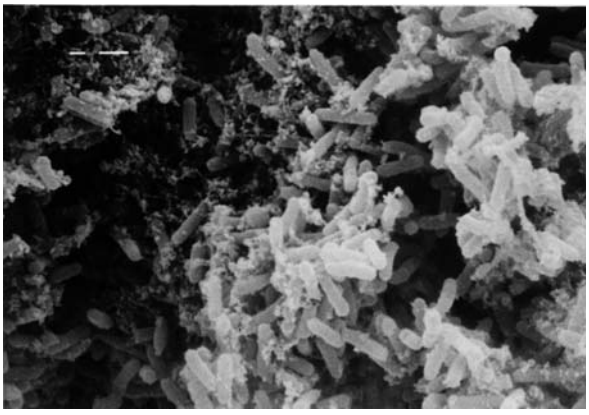

Fig. 6.3. Bacterial cells in food bolus taken from the midgut as detected by SEM. Note that the applied food was sterile and the bacteria originated from the region of the peritrophic membrane, shown in Fig. 6.3. Length of the *left bar*, 1 µm

fed. It is likely that the bacteria located in the region of the peritrophic membrane start to colonise the ingested food and utilise it for growth. Borkott and Insam (1990) detected a total of 4×10^{11} cfu g^{-1} in faeces of *F. candida*, which would correspond, considering the gut volumes determined in these studies, to about 10^6 cfu per specimen. This is about the upper limit of what we detected in our studies. During the moulting cycles, the midgut epithelium is regenerated, the old epithelium, including the peritrophic membrane, is excreted and replaced by a new epithelium that has developed underneath the old one. During the moulting process, the specimens stop feeding, probably because of a transiently non-functional gut. The new epithelium is then coated again by a peritrophic membrane which is excreted from specific epithelium cells in the foregut (Hopkin 1997). Since the peritrophic membrane is densely colonised by bacterial cells, the moulting process results in a dramatic change of the bacterial population in the gut. These changes in bacterial gut colonisation could be quantified by comparing the bacterial cell density in the gut of actively feeding *F. candida* to those that were not feeding (Thimm et al. 1998). Feeding specimens had 1.6×10^4 to 2.7×10^5 cfu per specimen in their gut, as determined by cultivation under aerobic conditions on yeast extract agar. In contrast, the bacterial cell densities in the non-feeding population ranged from 4.9×10^2 to 2.3×10^6 cells per specimen. The high cell numbers were found in specimens immediately before, and the low cell numbers immediately after, the excretion of the old gut epithelium and peritrophic membrane. It can be concluded that the moulting process generates high fluctuation rates and turbulences within the bacterial community and se-

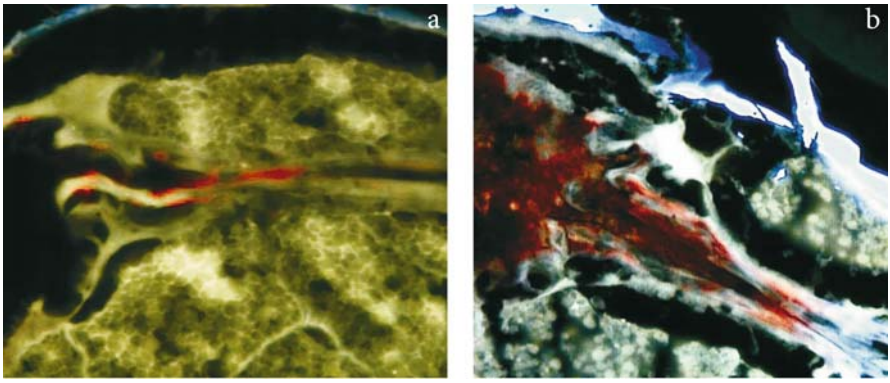

Fig. 6.4. Recolonisation of the gut by bacteria after moulting. **a** Pylorus region with remaining bacteria after the moulting process – a possible starting point for bacterial recolonisation of the gut; **b** growth of bacteria into the hindgut region. Bacterial cells (*red*) were detected by fluorescence in situ hybridisations (FISH) with the 16S rRNA gene probe EUB388, Aargetting all bacteria (Thimm and Tebbe, 2003)

lects for bacteria that are able to rapidly multiply in the gut. The question emerged, from where the bacteria actually recolonise the gut after moulting. Probably, a main pathway for inoculation is faeces, including exuvia, on which *F. candida* normally feeds. Another pathway, however, may come from the opposite direction. We have recently identified structures in the pylorus region, located between the midgut and hindgut, in which bacterial cells were detected even in specimens that had just moulted. Detection of bacterial cells by fluorescence in situ hybridization (FISH) in this region indicated that the bacterial recolonisation of the gut may also start from this point (Fig. 6.4).

6.3 Feeding Preferences of *Folsomia candida* and Fate of Ingested Bacterial Cells

Under natural conditions, *F. candida* seems to be an omnivore, as it feeds on dead organic material as well as on fungal mycelia, nematodes or bacteria. *F. candida*, in fact, is very adaptable to different food sources. To understand the impact of collembolan feeding on the bacterial community structure and diversity in soil, we conducted feeding preference studies with different bacterial species. At the outset of these investigations, it was unclear whether bacteria would be digested, not affected or even stimulated in growth during a gut passage. The feeding preferences for bacteria were tested with a total of twelve different bacterial strains, among them Gram-positive and –negative strains from type-culture collections. These strains were fed to *F. candida* specimens in petri dish-size microcosms offering pairs of choices (Thimm et al. 1998).

From a total of 66 tests, 22 showed significant preferences. Eight different preference classes could be differentiated. The most preferred class contained the type culture strain *Pseudomonas putida* PaW340 and a strain isolated from faeces of *F. candida*. As indicated by 16S rRNA gene sequencing, this strain was also a *P. putida* or a close relative (similarity to the 16S rRNA gene of *P. putida* PaW340, 99.1%). The second class contained two bacterial strains, both isolated from *F. candida* faeces: the Gram-positive isolate, *Arthrobacter citreus* and a close relative of the Gram-negative *Alcaligenes faecalis*. Interestingly, the *A. citreus* was an isolate from a different breeding stock. In fact, the bacteria were kept for nine years in a culture collection (by H. Borkott, Braunschweig) before they were fed to *F. candida* in our studies. The lower preference classes contained different type culture strains but no isolates from *F. candida*. The least preferred classes contained *Corynbacterium glutamicum* and *Bacillus subtilis*. Both species have a potential to live in soil. Also, *Escherichia coli* fell into a low preference class. We

were aware that these choice experiments were not very realistic in regard to natural food sources of *F. candida*, as it is unlikely that *F. candida* finds opportunities to choose between different bacterial species in its natural habitat. On the other hand, the obvious feeding preference of *F. candida* for its own gut bacteria indicated that these bacteria were recognised as beneficial and probably not as a well-digestible food source. Interestingly, the isolate *A. citreus* exhibited chitinase activity and, thus, could possibly act as a gut symbiont, as suggested by Borkott and Insam (1990).

The digestibility of different bacterial species was tested by feeding *F. candida* for one day with strains that had been genetically tagged with the luciferase gene (*luc*) or the gene encoding for the Green Fluorescence Protein (*gfp*). By this means, the bacteria could easily be differentiated from other, indigenous bacteria. The faeces of the specimens were analysed over a period of 56 days in which only sterile food was available for the specimens. *E. coli* cells could only be detected in faeces one day after feeding of the bacteria. The soil bacteria *Sinorhizobium meliloti* or *Pseudomonas stutzeri* were only detected during the first week. In contrast, *P. putida* cells were detected for 20 days and *A. faecalis* even until the end of the experiment (56 days). The data indicated that bacterial species can differ significantly in their capacity to survive or even colonise the gut of *F. candida*.

The "pulse-feed" studies were complemented by studies in which the effect of the gut passage on ingested bacteria was quantified in more detail. Based on feeding colorised food we first determined that the period between ingestion and excretion of food was only 35 min. When we fed *E. coli*, we only detected an average of 4.3 cfu in the gut of each specimen. In contrast, with *S. meliloti* and *A. faecalis*, the other two strains tested, cell numbers were approx. four orders (!) of magnitude higher, indicating these bacteria were not as efficiently digested as *E. coli*. Consequently, due to the efficient digestion, the numbers of *E. coli* cells in faeces were also very low (2 cfu per faecal pellet) and the number of *S. meliloti* and *A. faecalis* were one to two orders of magnitude higher. As estimated from uptake and release rates of the selected strains, *E. coli* populations were reduced 60,000-fold whereas *A. faecalis* was only reduced 500-fold (Thimm et al. 1998). The studies demonstrated that even though the period of time for a gut passage was relatively short, the species specific effects were quite dramatic.

We extended this type of study on the survival of ingested bacterial cells to another Collembola species, i.e., *Protaphorura fimata* (formerly *Onychiurus fimatus*; Onychiuridae, Poduromorpha, Arthropleona). *P. fimata* is a sexually reproducing, non-pigmented, euedaphic species that is less associated with the litter fraction of soils than *F. candida* and that can feed on mycorrhizal fungi as well as on the roots of living plants. In accordance with our studies on *F. candida*, *E. coli* was efficiently eliminated from the gut within two days after feeding, but in contrast to *F. candida*, the soil

bacterium *S. meliloti* was only detectable for two days instead of a full week (Hoffmann et al. 1999). Thus, it can be expected from these results that different Collembola will impose different selective pressures on ingested bacterial cells. Interestingly, we included a strain isolated from the gut of *F. candida*, closely related to *Stenotrophomonas maltophilia*, in the feeding experiments with *P. fimata*, and this strain persisted much longer in the gut after feeding (Hoffmann et al. 1999). We assume that some bacterial species, among them this relative of *S. maltophilia*, developed a certain capacity to survive or even grow in the gut of Collembola. It should be noted that despite some microhabitat preferences, *F. candida* and *P. fimata* can coexist in the same soils and may feed on the same substrates. Possibly, some soil bacteria may have evolved to utilise gut passages, as they occur in most soils with microarthropods, to grow and maintain their populations. *S. maltophilia* may be good example of such an ecological adaptation (see also Sect. 6.6).

6.4 The Gut of Collembola: a Hot Spot for Conjugative Gene Transfer Between Bacteria

Gene transfer between microorganisms in soil has become an issue of public interest in the context of the debate on the environmental risks associated with the deliberate or accidental release of genetically engineered microorganisms. Two mechanisms for gene transfer were considered to be potentially most important for soil: transformation and conjugation. Transformation is the process in which bacterial cells take up cell-free DNA and incorporate it by recombination into their own genome. Conjugation is a process by which self-transferable, mobile genetic elements (plasmids) which carry genes for transfer, replication and possibly other properties, are transferred from a donor to a recipient strain. The transfer can be of narrow-host range, between only closely related bacteria, or it can be of broad-host range, between more distantly related species. The detection of a gene transfer event normally requires the expression of the transferred gene in the new host organisms.

Early studies on gene transfer in soil revealed no or only low rates of conjugative plasmid transfer from a donor to a recipient in bulk soil (Ramos-Gonzalez et al. 1991; Smit et al. 1991). However, transfer rates in rhizospheres were much higher, probably because of the presence of metabolically active recipient cells (van Elsas 1992). In analogy to the rhizosphere, we suspected that the gut of invertebrates, and especially that of earthworms (Thimm et al. 2001) and Collembola could also be a "hot spot" for gene transfer. The high number of bacterial cells observed in the

gut of *F. candida* indicated that sufficient recipients were present in the gut, and both the population dynamics in response to the moulting cycles and the release of nutrients by digestion of food indicated that these gut bacteria were in fact metabolically active. In order to demonstrate bacterial gene transfer in the gut of Collembola under laboratory conditions, *E. coli* cells with different types of plasmids were fed to *F. candida* in microcosm experiments (Hoffmann et al. 1998).

We chose *E. coli* strains with self-transferable conjugative plasmids of broad or narrow-host range. In addition, *E. coli* strains with mobilisable plasmids were included. Mobilisable plasmids are only transferred to a recipient if another "mobilising" plasmid is present. These mobilising plasmids can either be in the donor cell itself, or it can be provided by a third partner, a mobilising strain, in a so-called triparental mating. Finally, we also included an *E. coli* strain with a plasmid (pUC18) that was not efficiently mobilisable. We suspected that this plasmid would possibly be transferred by transformation and not conjugation. A transfer of this type was demonstrated to occur under certain conditions in mineral water as a substrate (Baur et al. 1996) and thus it was not unlikely that it would also occur in the gut of *F. candida* . The experimental set-up for gene transfer studies was as follows. A total of 50 or 100 specimens of *F. candida* were fed in one arena (petri dish) with agar that was inoculated with the respective

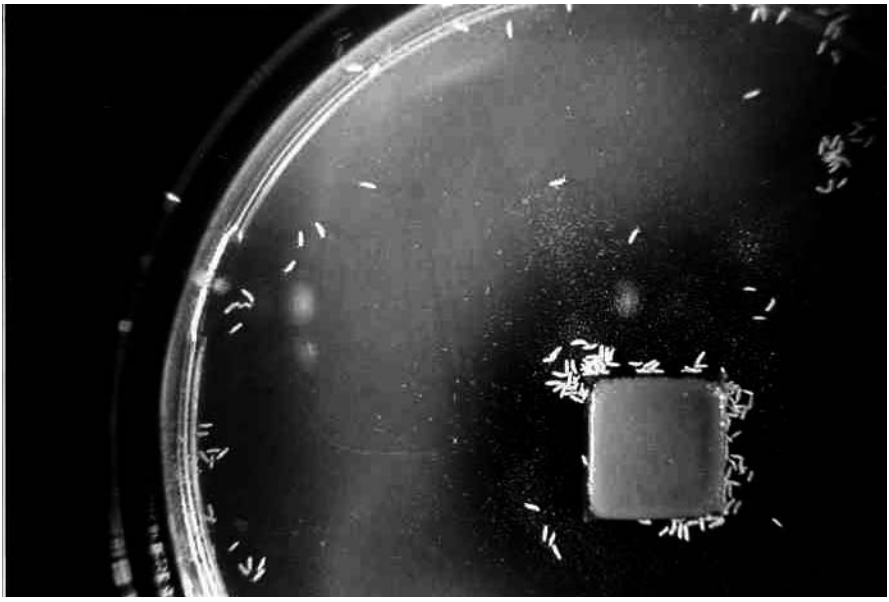

Fig. 6.5. Petri-dish microcosm with *F. candida* feeding on donor bacteria, placed on an agar cube. Note the faecal depositions (*white dots*) on the agar surface

donor strains placed on an agar cube (Fig. 6.5). After feeding for several hours, the specimens were transferred to a new arena without food, but with an agar-surface that contained the antibiotic nalidixic acid, an inhibitor of conjugative gene transfer (Hane 1971). The antibiotic was chosen to prevent conjugative gene transfer in the faeces to show whether gene transfer would take place in the gut. The faeces that were deposited within a period of up to 24 h was then collected and analysed for the presence of donor bacteria, recipients and transcipients (transconjugants or transformants). In order to follow the transfer of plasmids, we chose plasmids that carried a marker-gene (*luc* or *gfp*) and a gene encoding for an antibiotic resistance. The inhibition of the growth of donor cells was achieved by cultivating on an agar with benzoic acid as a sole source of carbon. In preparation for those studies we found that, in contrast to *E. coli*, most gut bacteria of *F. candida* could utilise benzoic acid for growth.

Despite their low survival rate in the gut, several *E. coli* cells could in fact transfer their plasmids to indigenous bacteria of *F. candida*. We expected transformation to be important, because the digestion of the donor cells would possibly result in the release of significant amounts of DNA which would have been available for transformation. However, we could not detect transfer of the non-mobilisable plasmid. In contrast, conjugative transfer of self-transferable narrow- and broad-host range plasmids to indigenous bacteria were detected. Mobilisable plasmids were only transferred when the mobilising genes were located in the donor cell, but not by triparental mating with mobilising genes or plasmids provided by the bacterial community in the gut. Such mobilisation by other bacteria had been shown to occur in soil amended with manure (Götz and Smalla 1997). In our studies, the transfer rates of the broad-host range plasmid RP4, expressed as the transconjugants-to-donor ratio in the faeces, were in the region of 1×10^{-1}. This was as high as rates can be measured under optimised laboratory conditions in filter-mating. The results of our studies underlined the importance of the feeding activity and gut microhabitat conditions of *F. candida* for promoting conjugative gene transfer.

In order to confirm that the Collembolan gut is a hot spot for conjugative gene transfer we conducted similar studies as described above for *F. candida* with another species, i.e., *P. fimata*. We were interested to see if a Collembola with somewhat different feeding preferences than *F. candida* would also provide suitable conditions of conjugative gene transfer in the gut – and in fact, this was the case (Hoffmann et al. 1999). In contrast to the studies with *F. candida*, however, transfer of narrow-host range plasmids could not be detected. On the other hand, in accordance to the results with *F. candida*, conjugative plasmids and also mobilisable plasmids, with the mobilising genes in the donor cells, were transferred to indigenous gut bacteria. Plasmid mobilisation by indigenous gut bacteria was not detected,

but possibly the threshold of detection was just too insensitive in our studies. We assume that plasmid mobilisation in the collembolan gut is possible, because it is likely that mobilising strains occur in the gut of Collembola, just as they occur in soil or other environments (Smalla et al. 2000).

6.5 Diversity of Microorganisms in the Gut of *F. candida* and Other Collembola

Only a very limited number of studies has looked at the microbial diversity associated with the gut of Collembola. It could be argued that the gut of Collembola is too small, its structure too simple, and the gut passage of ingested material too quick, to allow the development of a specifically adapted or even symbiotic microbial community. In fact, some evidence was collected by high resolution microscopy that at least some Collembola do not possess any intestinal microbial community (Kilbertus and Vannier 1981; Saur and Ponge 1988). An analysis of the gut contents of *F. candida*, fed with hornbeam (*Carpinus betulus*) leaves, however, showed a high abundance of fungal mycelia (Tochot et al. 1982) but (surprisingly) no bacteria were seen. First indications that the gut of Collembola also harbours bacteria and that these bacteria actually contribute to the digestion of food, were reported by Doeksen and Hitchen (cited by W.G. Hale 1967), who cultivated a *Bacillus* sp. from faeces with a capacity to degrade chitin. Chitin is an important substance in the gut as it is a major constituent of fungal mycelia and the cuticules of arthropods. Chitin is also a component of the exuvia (including old cuticules), that are released during the moulting of insects and Collembola, and that many Collembola may feed on. In later studies, Borkott and Insam (1990) found that one third of the culturable bacteria from gut and faeces of *F. candida* showed chitin degrading activity on agar plates. Two of these bacteria were identified; one was *Stenotrophomonas maltophilia* (formerly *Xanthomonas maltophilia*; *Gammaproteobacteria*) and one *Curtobacterium* sp. (*Actinomycetes*, *Actinobacteria*).

In an initial attempt to characterise the diversity of bacteria found in the gut of *F. candida*, we isolated a total of 45 different bacterial pure cultures which had been kept in a breeding stock for several years (Thimm et al. 1998). These isolates could be grouped into 11 different groups according to their Gram-staining, fatty acid profiles, physiological tests and ARDRA (amplified ribosomal DNA restriction fragment length analysis). The abundance of each of these groups ranged from 4×10^2 to 1.2×10^5 cfu perspecimen. Only one group, with an estimated abundance

of 5.3×10^3 cfu per specimen, exhibited chitinase activity. The most abundant group was represented by an isolate most closely related to *Erwinia amylovora* (96.2% similarity of the almost complete 16S rRNA gene). The second and third most dominant groups were represented by isolates related to *Staphylococcus captitis* and to *Pantoea agglomerans*. *E. amylovora* is an important pathogen in orchards as it is the causative agent of the fire-blight disease and we were interested to know if *F. candida* could possibly act as a vector. Feeding experiments with a pathogenic strain of *E. amylovora*, expressing a recombinant *gfp*-marker gene, however, indicated that the pathogen was, in fact, efficiently digested in the gut of *F. candida* (Hildebrand et al. 2001). In the course of this cultivation-dependent detection of microorganisms from the gut of *F. candida*, we only isolated one fungus, which was identified as a cellulose-degrading *Acremonium charticola* (Ascomycetes) (Thimm et al. 1998). However, we did not determine whether this fungus was cultivated from a spore or a mycelium.

A number of different *Proteobacteria* were isolated as transconjugants receiving plasmids from *E. coli* cells (see previous paragraph) (Hoffmann et al. 1998). The *Proteobacteria* comprised one isolate from the Alpha-subclass, related to *Ochrobactrum anthropi* (99.8% similarity of the 16S rRNA gene) and several isolates from the Beta-subclass with isolates related to *Alcaligenes xylosoxidans*, *A. faecalis*, *Comamonas acidovorans* and *Comamonas testosteroni*. Other isolates, among them different *Pseudomonas* species, *S. maltophilia*, or *P. agglomerans*, belonged to the Gamma-subclass. It should be noted that the host-range of the plasmids was responsible for the fact that no bacteria outside of the class *Proteobacteria* could be found. On the other hand, the occurrence of certain bacteria like *S. maltophilia* or *P. agglomerans* in the gut of *F. candida* was confirmed.

Recently, a molecular approach independent of cultivation has been used to elucidate further the bacterial diversity found in *F. candida*. By means of PCR, 16S rRNA genes were directly amplified from DNA extracted from *F. candida* specimens (Czarnetzki and Tebbe, 2004a). In addition to the detection of intracellular bacteria (see next paragraph), a number of different 16S rRNA genes, which probably originated from bacteria of the gut, were identified. These 16S rRNA genes were related to *Proteobacteria* of the Alpha-subclass (closest relative: *Paracoccus denitrificans*), of the Gamma-subclass (100% similarity to *S. maltophilia*), of the *Firmicutes* (*Bacillus weihenstephaniensis*; from the *Bacillus cereus* group) and from the *Planctomycetales*, the latter only with as yet uncultured relatives. We assume that the diversity of gut bacteria is much higher than described to date and also that this diversity will be affected by the quality of the ingested food. However, the evidence is accumulating that certain bacteria from soil can utilise the gut passage through *F. candida* or other microarthropods to grow. We suspect that bacteria like *S. maltophilia* or *B. weihenstephaniensis*

are representatives of such a life-style. In a recent review it was suggested that bacteria of the *B. cereus* group utilise the gut of insects to grow and survive in terrestrial habitats (Jensen et al. 2003). Collembola should also be considered in this context.

In a screening experiment, we compared the diversity of 16S rRNA amplified partial sequences from other species than *F. candida*. The SSCP (single strand conformation polymorphism) technique was utilised to compare the amplified products by generating genetic profiles (Schwieger and Tebbe 1998). The profiles of seven different species indicated that different bacteria were dominant in each species(Czarnetzki and Tebbe, 2004a). Interestingly, the patterns of the two closely related species *Mesaphorura macrochaeta* and *Mesaphorura italica* were more similar to each other than to other species. A total of 24 partial sequences were recovered and identified from these profiles, indicating the presence of different members from the group of *Proteobacteria* (Alpha-, Gamma-, and Delta-subclass), *Firmicute*s, *Actinobacteria* and *Bacteroidetes*. The sequences however, were not long enough to allow a more detailed phylogenetic analysis. In several cases, closest relatives as indicated by database searches were 16S rRNA genes from uncultured bacteria from other environments, e.g., soil, wastewater, lake sediment, potato rhizosphere or, in one case, from a tissue of the honey bee (*Apis mellifera*). Again, a sequence related closely to *S. maltophilia* was detected in the DNA of *P. armata*, confirming this species as a common gut inhabitant.

6.6 Collembola Can Harbour the Reproduction Parasite *Wolbachia* and Other Intracellular Bacteria

The long coexistence of arthropods and bacteria for approx. 400 million years, has allowed the development of sophisticated interactions between both groups. Striking examples are the intracellular bacteria, e.g., the endosymbiont *Buchnera* in aphids (Douglas 1998) or the parasite *Wolbachia*, the latter affecting the sexual reproduction patterns of many insects and other arthropods (Stouthamer et al. 1999). In Collembola, intracellular bacteria were first detected by transmission electron microscopy (TEM) in the fat body and ovarial tissue of *F. candida* by Palévody in 1972 (Palevody 1972). In 1999, Vanderkerckhove et al., detected by PCR a 16S rRNA sequence that was closely related to group of *Wolbachia* (*Alphaproteobacteria*) (Vanderkerckhove et al. 1999). In the same study, the authors detected intracellular bacteria in fat bodies and intestinal cells by means of TEM. In our own laboratory, we used the fluorescence in situ hybridisation technique (FISH) with universal gene probes for *Bacteria* on microscopic sections of

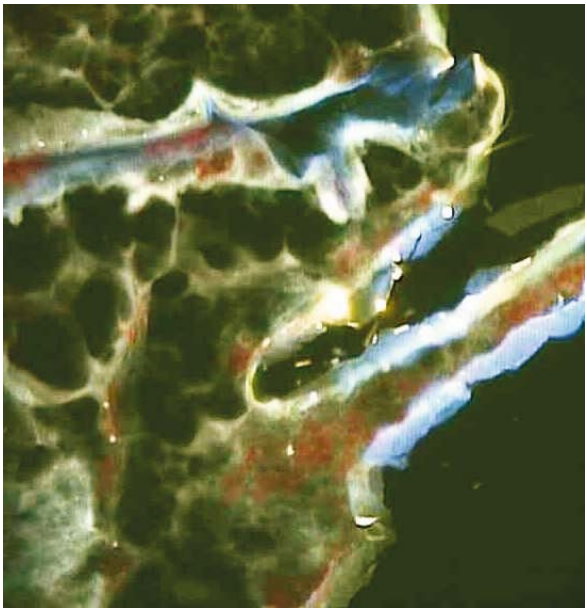

Fig. 6.6. Detection of intracellular bacteria of *F. candida* in the furca and neighbouring regions. The hindgut region and anus are seen in the upper part of the figure, the furca below. Bacterial cells (*red*) were by detected by FISH using the probe EUB388 for *Bacteria*

whole *F. candida* specimens (and not specific tissues) and we found that in addition to the gut, several compartments of the body cavity were colonised by bacterial cells (Thimm and Tebbe 2003).

Intracellular bacteria were detected in fat bodies in different regions and tissues of the body, including the furca (Fig. 6.6), brain and ovaries (Thimm and Tebbe 2003). FISH with a specific gene probe for *Wolbachia*, however, only hybridised with bacteria that were located in the ovarial tissue or the brain region. We concluded that other bacterial species than *Wolbachia* must also be present in the body cavity of *F. candida* (Czarnetzki and Tebbe 2004b). And in fact, recently we detected a 16S rRNA gene related to the intracellular *Rickettsiella grylli* (gamma-Proteobacteria) of other arthropods. We found this sequence by generating clone libraries of 16S rRNA genes amplified from total DNA extracted from *F. candida*. Interestingly, when we compared clone libraries generated from specimens of two different breeding stocks, we found the *Rickettsiella* sequence to be dominant in one stock but completely absent in the other stock. The phenotype of *F. candida* did not seem to affected by the *Rickettsiella* infection. We therefore assume that *Rickettsiella* is a facultative coloniser of *F. candida* and probably a commensal or weak pathogen.

In contrast to *Rickettsiella*, we detected *Wolbachia* in all of five breeding stocks of *F. candida* analysed (Czarnetzki and Tebbe 2004b). In addition, we found *Wolbachia* by 16S rRNA specific PCR in other parthenogenetic Collembola, i.e., *M. macrochaeta*, *M. italica*, and *P. callipygos*, but we could not detect it in the sexually reproducing species *P. fimata* or *Isotoma viridis*. This indicated that *Wolbachia* may in fact induce parthenogenesis in Collembola. Parthenogenesis is a powerful option in the success of populations in the environment as it allows organisms to multiply more efficiently, e.g., when entering a new ecological niche (Koivisto and Braig 2003). It should be noted, however, that in the case of *Wolbachia* and Collembola, more experimental evidence needs to be collected to confirm this hypothesis. Our own studies, in which we tried to eliminate *Wolbachia* from *F. candida*, were so far unsuccessful (unpublished results). In order to understand better the *Wolbachia*-host relationships, we conducted a phylogenetic analysis of *Wolbachia* with both the 16S rRNA and *ftsZ* genes amplified from *F. candida* of the different breeding stocks and from the other parthenogenetic species. The *Wolbachia* 16S rRNA genes of all *F. candida* breeding stocks was identical and in fact they were also identical to the sequence reported by Vanderkerckhove (Vanderkerckhove et al. 1999). The 16S rRNA sequences of the other Collembola were much more closely related to the *F. candida* sequence than to any sequence from other arthropod hosts. In fact, a monophyletic branch for Collembola could be demonstrated for the phylogenetic tree of *Wolbachia*.

The monophyletic branch of *Wolbachia* from Collembola was also seen in phylogenetic analyses based on *ftsZ* genes (Czarnetzki and Tebbe 2004b). Here, the *Wolbachia* sequences from the different breeding stocks of *F. candida* showed some variations. In summary, our phylogenetic analyses indicated a new Supergroup E for *Wolbachia* in Collembola, Both studies based only on a single sequence from *F. candida* had already postulated that such a supergroup might exist (Vanderkerckhove et al. 1999; Lo et al. 2002). Compared to the approx. 7,500 species that are known in the class of Collembola it is much too early to conclude that supergroup E is an exclusive group for Collembola or that *Wolbachia* of other supergroups have no option to infect Collembola. In fact, we assume that the long coevolution of Collembola and *Wolbachia* make it probable that many such exceptions exist. Our phylogenetic analyses indicated that new supergroup E is a sister group of supergroup A. Supergroup A, like supergroup B, harbours *Wolbachia* from a high diversity of different hosts of the Class *Insecta* and there is no congruence between host and *Wolbachia* phylogeny. Interestingly, the other major group of the soil microarthropods, the mites, which can also be hosts for *Wolbachia*, have no own branch or supergroup in the *Wolbachia* tree. Instead, all yet detected *Wolbachia* sequences belonged to Supergroup B (Breeuwer and Jacobs 1996). In our own study,

a rough estimate of evolutionary rates of the different marker genes (rRNA genes, *ftsZ* genes) indicated that the differences between the Collembola supergroup E and A were much smaller than the phylogenetic distance between Collembola and Insecta (Czarnetzki and Tebbe 2004b). We therefore assume that *Wolbachia* infections took place long after Collembola had diversified. If *Wolbachia* really induces parthenogenesis in Collembola, this would indicate that parthenogenesis is a rather young development during the evolution of Collembola.

6.7 Conclusions

Collembola are a quantitatively and functionally important organisms in most terrestrial ecosystems. Together with mites and some other less abundant groups, they build up the group of microarthropods. Microarthropods enhance the mineralisation and restructuring of organic substrates in soil. Collembola can select for specific food sources, e.g., they prefer certain fungi to others in choice experiments, but many are also quite adaptable to different food sources, which probably explains their high adaptability and success in most soils. The size of the gut of Collembola is very small with a volume of less than 20 nl, as measured for the representative species *F. candida*. The gut passage of ingested food in *F. candida* can last less than 1 h. However, during this passage food is inoculated with bacteria which further enhances the degradation of these substrates. In contrast to some other studies, our own studies indicated that the Collembolan gut can be densely colonised with bacterial cells and that certain bacterial species, like relatives of the type culture strains *Stenotrophomonas maltophilia* or *Bacillus weihenstephaniensis*, have adapted to live in this microhabitat. A precondition for successfully colonising the collembolan gut is that the bacteria resist digestion by the host. Also, successful gut bacteria in Collembola need to grow quickly, since moulting cycles frequently change the total bacterial population by several orders of magnitude within less than a week. The feeding activities of Collembola in the terrestrial ecosystem thus clearly affect both the quality of the organic substrates and the composition of the microbial communities. The high densities of bacterial cells and the microhabitat conditions provide excellent conditions for conjugative gene transfer between bacteria, a factor that should be considered when evaluating rates of bacterial gene transfer in soil. Beside the gut, other compartments of the body of Collembola can also be colonised by bacteria. Two bacteria with an intracellular life-style have been detected so far: one relative of *Rickettsiella grylli* and one belonging to the group of *Wolbachia*. To our knowledge, all *Wolbachia* that have been detected in Collembola are more related to each other

than to *Wolbachia* from other arthropod hosts. The biological importance of *Wolbachia* infections are yet unknown, but as suggested by the hosts that have so far been analysed, such infections may induce parthenogenesis.

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