This is to certify that the

dissertation entitled

USE OF NUCLEIC ACID BASED METHODS TO STUDY THE

BACTERIAL COMMUNITY OF THE CRICKET HINDGUT

presented by

Jorge W. Santo Domingo

has been accepted towards fulfillment
of the requirements for

PH.D degree in Microbiology and Public Health

Major professor
James M. Tiedje

Date 3/29/94

USE OF NUCLEIC ACID BASED METHODS TO STUDY THE BACTERIAL COMMUNITY OF THE CRICKET HINDGUT

by

Jorge W. Santo Domingo

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Microbiology and Public Health

1994

ABSTRACT

USE OF NUCLEIC ACID METHODS TO STUDY THE

BACTERIAL COMMUNITY OF THE CRICKET HINDGUT

by

Jorge W. Santo Domingo

The effect of changes from a chow to alfalfa, pulp, or protein-based diets on the composition and metabolic activity of the microbial community inhabiting the hindgut of *Acheta domesticus* was investigated using DNA based- and other microbial culture and process-based methods. A maximum of 90 % of the hindgut bacteria were lysed and a yield of 6 µg of DNA per hindgut was obtained using a DNA extraction method that lyses for both Gram positive and Gram negative bacteria. The polymerase chain reaction method was used to amplify a region of the 16S rRNA gene using gut microbial community DNA. Amplification of this region suggested that DNA extracted from the hindgut was of good quality. CsCl-bisbenzimide gradients of the extracted DNA were used to generate community profiles based on the % G+C of the resident bacterial populations. This method suggested that the cricket is dominated by bacteria of G+C contents between 32 - 57 %. This approach also indicated that changing to pulp or protein diets resulted in a decrease in diversity of hindgut bacteria with different genomic G+C contents. These

structural changes observed were accompanied by a decrease in the rates of hydrogen, carbon dioxide, and volatile fatty acids production.

Fluorescently labeled 16S rRNA-targeted probes were also used to determine the presence of different microbial groups in the hindgut of three species of crickets: *A. domesticus*; *Gryllus* spp., and *Scapteriscus* spp.. Gram-positive bacteria of low G+C, bacteriodes, α-Proteobacteria, sulfate-reducing bacteria, fusobateria, bifidobacteria, enterics, and methanogens represented 27.7, 10.3, 5.6, 4.6, 3.4, 3.0, 1.71, and 0.6 %, respectively, of the bacteria in *A. domesticus*.. Although all groups were present in all crickets, densities of bacteriodes and sulfate-reducing bacteria were significantly higher in *Gryllus* spp. while the lowest densities of bacteriodes were found in *Scapteriscus* spp. In contrast, *Scapteriscus* sp. were found to harbor higher densities of archaeabacteria. Since methane is produced by this cricket microbiota the archaeabacteria may be methanogens. Hybridization studies with eubacterial probes indicated that approximately 90 % of the hindgut bacteria in these crickets is metabolically active and chiefly eubacterial. Fluorescent probes also showed that the densities in sulfate-reducing bacteria, bacteriodes and Gram positive of low G+C content decreased significantly in crickets shifted from a chow to a pulp diet. In contrast, crickets switched to a protein diet showed an increase in sulfate-reducing bacteria. The total number of bacteria remained constant on all diets. These results show that changes in diet can shift the relative abundance of bacterial populations in the cricket hindgut, thus further demonstrating that dietary perturbations can affect the structure and function hindgut microbial community.

NEVER FOLLOW REASON,
MANKIND HAS A VAGUE UNDERSTANDING OF WHAT THAT IS;
BUT ALWAYS FOLLOW THE MESSAGES OF YOUR HEART
SINCE WITHIN YOUR HEART YOU WILL FIND LOVE,
AND LOVE IS AS BIG AS THE UNIVERSE,
THE ONLY REASON FOR LIVING.

To LETTY, WILFRIDO and YOLANDA and to those that fight for friendship and world peace; and to those that fight against all odds.

ACKNOWLEDGMENTS

My most sincere expression of gratitude goes to all the members of my guidance committee, Drs. John Breznak, Michael Klug, Michael Thomashow, and Eldor Paul, and especially, to my advisor Dr. James M. Tiedje. His mentorship goes beyond scientific matters; I was lucky to learn from him. Also, thanks to Dr. Mike Kaufman who's help was invaluable.

I would have never done it without my best friends: Letty, my mother and my father; they were there, always, and served as a source of inspiration, even though they never understood the how and why of my endeavors. Jose and Ines encouraged me during those first cold days in 1987. Rob, Joanne, and Marcos, original members of the Gordo Club; I hope to keep their friendship forever. Drs. Sandra Nierzwicki-Bauer and Ellen Braun-Howl for their help with the fluorescent probes technology. Dr. Dave Harris helped many times; my conversations with him were always fruitful and the source of ideas. Thanks Dave! Thanks to CME's staff: Ann, Annette, Anne G., Lisa, and Robyn for their help and assistance. Sheridan Haack, Dave Odelson, Paul Turner, Yuichi Suwa, Bonnie Bratina, and Thomas Schmidt shared comments and impressions; thanks guys. Finally, thanks to Dr. Terry C. Hazen, now my academic grand-father, for his help and patience in the early days and for introducing me to microbial ecology.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Introduction

Insect genera represent the most numerous group of extant organisms among the zoological classes with more than 900,000 species described (Davies, 1988). These invertebrates exhibit an unparalleled evolutionary radiation within the metazoans that is reflected in the wide spectrum of niches they occupy in many ecosystems. Insects are key players in the transformation of plant compounds like cellulose; thus their importance in global biogeochemical cycles is unquestionable. In addition, their economic impact on agriculture and health is significant world wide (e.g., crops pollinated by insects in the United States are valued at billions of dollars annually; Brusca and Brusca, 1990). Ironically, insects are also responsible for the significant destruction of a great variety of crops (e.g., corn, alfalfa, potato, citrus). Moreover, insects are implicated in the transmission of many human diseases worldwide (e.g., malaria, lyme disease), making them indirectly responsible for millions of deaths, especially in third world countries.

Today's need to understand the biology of insects has become imperative, which in turn explains the birth of different specialized areas within entomology. Nevertheless, one area in entomology that has received sporadic attention by the scientific community is insect microbiology. In the past, work in this field has been inspired by the numerous studies showing the frequent association of insects with microorganisms, and the evidence suggesting that microbial symbionts could contribute to the overall success of insects in nature (Dadd, 1985; House, 1974; Koch, 1967; Buchner, 1965; and Brooks,

1963). While insect microbiology has somewhat regained popularity within the last decade due to the development of insect biocontrol programs, basic ecological questions that deal with the nature of insect-microbial interactions are rarely addressed (Krischik and Jones, 1991).

Historical perspective

The interactions between insects and microbes have long been recognized. In fact, research on insect microbiology dates back to the early 1800's. Many of the first microbial forms recognized to be in association with insects were discovered after the analysis of unique organs, proximal to the fat body of a variety of insects (Buchner, 1965). For example, Leydig observed that yeasts in aphids were associated with what he called "pseudovitellus" (Buchner, 1965). These cryptic structures were later called mycetomes. [Mycetocytes -name coined for insect cells harboring intracellular symbionts- aggregate to form mycetomes.] Specific associations between insects and bacteria was first described by Blochmann when he reported on the presence of bacteria in eggs of ants and wasps (Buchner, 1965).

The early work of Umberto Pierantoni (1910) was crucial in "removing the blindfold from the eyes of some skeptics" and directly induced the description of many examples of symbiosis in Homoptera (Buchner, 1965). The characterization of the microbial entity, however, was poorly described (e.g., bacteria or yeast). Many attempts to identify the bacteria were made, but lack of an accurate

classification scheme for bacteria hampered the correct identification of the symbionts. For instance, Petri (1907) reported on the intestinal bacteria of the fly *Dacus oleae* as the agent of gall formation in olive trees. Originally classified as *Pseudomonas savastonoi*, the gall causing bacterium was later suggested to belong to the Rhizobiaceae family and renamed *Agrobacterium luteum* by Hellmuth (Buchner, 1965).

As documented by Cleveland (1924), Lestes was the first to observe the presence of protozoa in termites. In 1877, Leidy described different genera of protozoa found in *Reticulitermes flavipes*. By the early 1900s it was suspected that protozoa help termites to digest wood, even though Grassi and Foa (1911) contended that "defaunated" termites of the genus *Kalotermes* "possano digerire il legno anche sensa gli speciali Protozoi" (that is, could digest wood even without the unique Protozoa). Years later, Cleveland demonstrated that by eliminating the protozoa (i.e., by incubating the termites at 36^0 C for 24 h) insects died within 20 days, "probably due to their inability to digest wood". Cleveland also showed that termites that regained their protozoa reestablished their ability to live on a diet of pure cellulose, and further indicated that glucose was the product of cellulose fermentation absorbed by the termites. Later studies performed by Hungate, suggested that acetate, and not glucose, was the principal product assimilated by these insects (Hungate, 1939; 1943). These results have since been supported by the work of several investigators (Honigsberg, 1970; Thayer, 1976; Yamin, 1980; Odelson and Breznak, 1983).

The success of Cleveland prompted investigations on the effect of the removal of symbionts from insects. Several of these studies clearly established the nutritional basis for housing microbial symbionts (Pant and Fraenkel, 1954); others only suggested the need for symbionts in order to grow, develop, or reproduce, without an in-depth analysis of the microbiota and its contributions (Buchner, 1965).

Biological perspective

The study of insect microbiology has relevant implications to many areas of research in biology. For example, insects are known to act as vectors of plant pathogens. The transmission of plant pathogens by insects was established in the work of Leach (1926) on the fly *Hylemyia cilicrura*. His research indicated that bacteria (*Pseudomonas fluorescens* and *P. nonliquifaciens*) transmitted through fly eggs were responsible for the destruction of bean, peas, cabbage and many other plants. Yet, in most cases, the mechanism of microbial inoculation or insect responsible for the transmission of plant disease is unknown (Barbosa and Letourneau, 1988). Also, most studies have focused on vectors of agriculturally relevant crops, ignoring the role this phenomenon plays in the destruction of trees indigenous to many countries of the Americas and Europe (Wolfe and Caten, 1987).

As recently postulated by Barbosa (1991), the effect of microbes on the ecology of insects is noticeable in situations where the microbes modify plant resources to increase their suitability to

herbivores. Microorganisms can influence the defensive chemistry of plants in different ways which may become beneficial to the plant, or conversely, to the insect (Krischik and Jones, 1991). On the one hand, plants associated with N_2-fixing bacteria (e.g., legumes) often produce nitrogen-based compounds like alkaloids which are directly utilized as defense mechanisms against herbivores (Johnson and Bentley, 1991; Johnson et al, 1989). On the other hand, microbial symbionts have been suggested to perform the role of detoxifiers of plant allelochemicals, which enables insects to feed on an otherwise unsuitable plant (Dowd, 1991).

The concept of microbial transformation of plant allelochemicals has not been fully explored by entomologists or microbiologists, despite the well documented biodegradative capabilities of microbes (Zeikus, 1984). Microbial biodegradation shows great versatility, from transformation of naturally occurring biopolymers (e.g., cellulose) to partial dehalogenation of highly recalcitrant polychlorinated biphenyls. Moreover, the growth and mutation rates of microbes has allowed them to quickly adapt to their surroundings, and to exploit a great number of niches. Thus, it is reasonable to hypothesize that insect microbes might mediate plant-insect interactions by transforming plant compounds (Jones, 1984).

Erlich and Raven (1964) suggested that the evolution of insect and plant interactions have been dominated, by the development of plant chemical defenses and the herbivore's ability to evolve detoxifying mechanisms. Indeed, some insects have developed enzymatic machinery that allows them to feed on plants which are

unpalatable and even toxic to other insects. While some of these detoxifying mechanisms are intrinsic to the insect (e.g., enzymes like hydrolases that might act on allelochemicals), there is growing evidence suggesting that both ecto- and endo-microbial symbionts could be responsible for transforming biologically active compounds (Dowd, 1992; Scudder *et al.*, 1982; Rosenthal, 1983).

One scenario that might guide the investigation of the role of microbes in this kind of tri-trophic interaction (insect-plant-microbe) relates to the plant's production of allelochemicals in response to microbial invasion (Levin, 1976). Some of these compounds have antimicrobial properties and could affect the palatability of plants to insects as well (Krischik, 1991; McIntyre *et al.*, 1981; Karban, *et al.*, 1987). In these cases, the success of microbial infection will in part correlate with the capacity of microbes to transform plant secondary compounds. Thus, insects that are associated with microbes which could alter the toxicity or palatability of allelochemicals, might improve their likelihood of feeding on plants otherwise distasteful or toxic to them. While this might play a dynamic role in insect feeding behavior evidence supporting this hypothesis is only circumstantial.

The nature of insect-microbe interactions is of special interest to evolutionary biologists. Recently, several studies have illustrated how aphids and their intracellular symbionts represent an interesting example of true obligate symbiosis (Munson *et al.*, 1992; Munson *et al.*, 1991a). Evidence suggesting the host's need to harbor this symbiosis has been presented by studies in which antibacterial treatment that eliminated the microbes led to the insect's death or

sterility. It has been proposed that microbial symbionts synthesize essential amino acids utilized by the host (Baumann *et al.*, 1993).

So far, aphids symbionts have not been cultured outside of their host. Nevertheless, molecular analysis of their 16S rRNA genes have revealed their relatedness to members of the Enterobacteriaceae family and therefore should be considered of proteobacterial origin (Munson *et al.*, 1991b; Unterman *et al.*, 1989). Interestingly, bacteria phylogenetically related to these symbionts are presumed to be free-living. Another significant difference between these symbionts and their close free-living relatives is the dramatic reduction in the number of rRNA operons (e.g., seven in *Escherichia coli* to one in the symbiont). Since similar observations have been made for luminescent bacteria inhabiting fish organs (Haygood and Distel, 1993), it has been proposed that the number of rRNA operons could be used to discriminate between true obligate and nonobligate or transient microbial symbionts (Wolfe and Haygood, 1991). Furthermore, unique regions in the molecular machinery of the aphid's symbiont seem to be regulated by the invertebrate (e.g., chromosome initiation and messenger termination) suggesting eukaryote coordinated control of eubacterial functions. These results combined have recently lead to the hypothesis that aphids symbionts are in a transition state on their way to become an intracellular organelle (Baumann *et al.*, 1993).

One prominent fact regarding wood-feeding insects is that their diet is normally low in nitrogen content (Lipke and Fraenkel, 1956). Their microbial symbionts have been suggested as mitigators of nitrogen deficient diets (Cochran, 1985). Early studies showing the

presence of nitrogen fixers within the termite gut suggested that they play a key role in supplying nitrogen to the insect (French *et al.*, 1976; Potrikus and Breznak, 1977). This hypothesis seemed reasonable since the product of nitrogen fixation (i.e., ammonia) could then be utilized by the insect. However, the levels of nitrogen fixation in some termite species are low and therefore might not contribute significantly to the nitrogen needs of the host (Breznak, 1975). It is now recognized that some species of termites (e.g., *R. flavipes*) have developed means of recycling nitrogen-rich compounds, such as uric acid. Uric acid is known to be a metabolic excretion product in many insects, but only low levels of this compound appear to be excreted by termites. This has been explained by the fact that urolytic bacteria localized in the hindgut of termites transform uric acid into products which could be absorbed by the insect (Potrikus and Breznak, 1980). Similarly, intracellular symbionts in the fat body of cockroaches have been implicated in the transformation of uric acid (Donnellan, and Kilby, 1967). Other insects (e.g. ants, beetles and termites of the *Macrotermes* subfamily) seem to fulfill their dietary nitrogen needs by feeding on fungi, taking advantage of the fact that fungal parts are richer in nitrogen content than wood or plant material (Wood and Thomas, 1989; Quinlan and Cherret, 1979).

Microbial symbionts have been suggested to provide a variety of compounds for different groups of insects (e.g., plant-sap feeders and wood-eating insects, Buchner, 1965). Some of these compounds include several vitamins (e.g., biotin, thiamin), amino acids (e.g., methionine, tryptophan, and cysteine), and enzymes of microbial

origin that seem to complement the host nutritional needs (Dadd, 1985). Bacteria could also be directly used as carbon sources by mosquitoes larvae (Hinman, 1930; Henry, 1962; Walker *et al.*, 1991; Theiry *et al.*; 1991).

Insect gut microbial communities

Many insects harbor high densities of microbes in their gut. A great variety of microorganisms seem to coexist in this habitat (Cruden and Markovetz, 1987). This seems particularly true in wood-eating insects, where the gut simultaneously harbors microorganisms which belong to the *Archaea*, *Bacteria*, and *Eucarya* domains. As in vertebrate systems, facultative and strictly anaerobic bacteria are normally present in the gut of insects.

Historically, microbiological studies have concentrated on the microbial fraction of the insect gut which could be cultured (Kotarski and Klug, 1980). Since culturing techniques are highly selective, these studies do not accurately reflect the complete diversity of microbes that interact with the insect gut, nor do they establish the tangible importance of such microbial populations in the insect-microbiota symbiosis. For example, the presence of members of the *Enterobacterieae* family in insect guts is commonly reported in the literature (Hunt and Charnley, 1981; Charpentier *et al.*, 1978). This is not surprising due to the ubiquity of this bacterial group in many environments and the fact that pure isolates are readily attainable. Yet, to this date there is lack of conclusive evidence supporting the relevance of free living enterics to insects.

Examples do exist, however, where the classical approach of culturing microbes to identify members of microbial communities has proven useful in understanding the role of insect gut communities, as in the case of termites and cockroaches. For instance, the isolation of acetogens, combined with the observation of cellulose degradation by the protozoan population, has strongly supported the model of microbial interactions within lower termites proposed by Odelson and Breznak (1983). In this model, the acetogens serve as hydrogen (H_2) consumers, and through syntrophic interactions they reduce the H_2 tension, allowing microbial processes to become energetically favorable.

It has been suggested that normal gut microbiota prevent pathogenic bacteria from invading the gastrointestinal tract of healthy animals (Tannock, 1984). Seldom has such a role been examined for insect microbiota. Dillion and Charnley (1986) proposed that the gut microbiota of the desert locust, *Schistocerca gregaria*, produces an antifungal toxin that reduces the viability of the fungus *Metafhizium anisopliae*. In termites, only when microbial numbers are drastically reduced by antibiotics, can pathogenic strains of *Salmonella* colonize the insect gut. The proposal that invaders success is low in ecosystems harboring complex microbial communities is a reasonable one. In fact, in many habitats new colonizers seem to be excluded unless favorable conditions select for them (Moody and Mack, 1985), and in many circumstances such conditions need to extend for long periods of time. Thus, insect gut microbial communities could be beneficial to their host by occupying the hindgut (an otherwise susceptible structure to the invasion of

pathogens). While it remains to be determine the ecological importance of these type of studies (that is, how often gut symbionts can prevent insect disease in the wild), this area of research clearly has relevant implications in relation to biocontrol.

With the exception of termites (Schultz and Breznak, 1978), cockroaches (Cruden and Markovetz, 1987), and crickets (Urlich, *et at.*, 1981) examples of studies were an accurate characterization and identification of insect gut symbionts are rare in the scientific literature. In fact, only a few bacteria out of potentially hundreds of species inhabiting insects gut have been identified to the species level. Considering that the number of species of insects used in microbiological studies represents a small fraction of extant insects on Earth and that most insects examined are associated with microbes, it is reasonable to suggest that insects may be a great source of unknown microbial diversity. An example illustrating this notion is the work of Breznak on the isolation of new species of acetogens from the termite gut (Kane and Breznak, 1991; Kane *et al.*, 1991; Breznak *et al.*, 1988). Although the role of these bacterial isolates has not been established, is clear that acetogens are responsible for the production of carbon compounds (e.g., volatile fatty acids) of nutritional importance to their host. Therefore, novel microorganisms with relevant ecological roles might inhabit the insect gut.

In most anaerobic habitats the predominant hydrogen consumers are methanogens or sulfate reducers (Zehnder and Stumm, 1988). Nevertheless, in the hindgut of some species of termites it has been found that this postulate is not always true:

acetogens outcompete more efficient H_2 scavengers (Breznak, 1990). In fact, the gut of several groups of insects could be loosely classified as predominantly methanogenic or acetogenic, although both processes might occur simultaneously in some termite species (Brauman *et al.*, 1992; Kane and Breznak, 1991). Since acetogens are normally outcompeted by methanogens in conditions where methanogenesis could occur, there must be other factors (e.g., mixotrophy) contributing to this incongruency in acetogenic termites.

Despite the importance of gut microbiota to the nutrition of their plant feeding hosts, in most cases the specific role that gut microbes play is uncertain. Moreover, the presence of gut microbiota does not always imply the insect's need for this association since axenic colonies of insects have been successfully reared under laboratory conditions (Daser and Brandl, 1992; Kaufman, 1988). In fact, most gut microbes could be only transient inhabitants in insects only because the gut environment offers them an array of soluble carbon sources normally absent in other habitats. Nevertheless, this does not exclude the possibility that nonobligate symbionts could be beneficial to their host as implied in the studies of Kaufman and Klug (1991, 1990).

Use of ribosomal sequence information in microbial ecology

The inability of culturing techniques to describe *in toto* microbial communities has required the development of methods that do not rely on the culturable status of microorganisms (Holben and Tiedje, 1988). Some of these methods have in common the use

of biological markers (e.g., lipids, bacteriochlorophylls, triglicerides, lipopolysaccharides, and coenzymes F350 and F420) to detect the presence of bacteria in natural environments (Cole and Enke, 1991; Herbert, 1990; Parkes, 1987; White, 1983). While biological markers are suitable to measure microbial biomass, their use in enumerating microorganisms in the environment is questionable.

In recent years, a few function-related gene probes have been developed to monitor bacteria in soil and aquatic ecosystems (Holben and Tiedje, 1988; Holben *et al.*, 1992). This approach eliminates the necessity of culturing microbes. However, such probes target only organisms that carry the gene of rather similar sequence, and thus, in most circumstances they only characterize a small fraction of the microbial community. Moreover, unless the target gene is only found in a limited number of organisms, gene probes seldom reveal which microbe is responsible for the hybridization signal.

An alternative approach for the analysis of environmental communities involves the molecular analysis of genes carrying phylogenetic information and the development of phylogenetic probes for hybridization studies against intact microbial cells or nucleic acids isolated from the microbial communities. The genes of choice belong to the ribosomal RNA (rRNA) family and the most commonly analyzed thus far are the small subunit (16S) and large subunit (23S) rRNA. (Weller *et al.*, 1992). Since ribosomal probes were used in this study the following section will be devoted to describing certain aspects of the rRNA-based technology and its use in microbiological studies.

Three different rRNA populations with sedimentation coefficients of approximately 5, 16, and 23S are found in prokaryotes. Due to their paramount role in all organisms, these molecules have been conserved throughout evolution not only at the primary but also at the secondary level (Gutell *et al.*, 1985). While all three rRNAs have been sequenced for several organisms, most researchers currently focus on the analysis of 16S rRNA, since its size and information content are better suited to construct of phylogenetic trees and thus to determine evolutionary relationships between organisms.

Landmark concepts in modern biology have been revealed through the molecular analysis of the rRNA. Based on sequence information, Woese *et al.* (1990) proposed three major phylogenetic domains (*Archeae*, *Bacteria*, and *Eucarya*), replacing the five kingdom lineages championed by Whittaker (1969). Woese's "clustering" of living forms has been confirmed using the molecular analysis of other evolutionary conserved molecules (e.g., elongation factors Tu and G as well as the α and β subunit of ATPases; Iwabe *et al.*, 1989). A phylogenetic tree obtained using 23S rRNA sequences is also in agreement with this proposal (Schleifer and Ludwig, 1989).

Similar analyses have been used to infer the nature of the first organisms present on Earth (Pace, 1991). Although the sequences of the so-called common ancestor will be difficult (if not impossible) to elucidate, it is now presumed that the closest extant relatives belong to thermophilic archaea of the genera *Pyrodictium*, *Thermoproteus*, and *Pyrobaculum* (Stetter *et al.*, 1990).

The position of many organisms within the major microbial phylogenetic groups has been determined using sequence comparison of the small subunit rRNA (i.e., 16S rRNA-like). Examples of this can be found for both microsporidia, (Vossbrinck *et al.*, 1987), marine diatom (Medlin *et al.*, 1988), extreme thermophiles (Burggraf *et al.*, 1992; Stetter *et al.*, 1987), Gram positive bacteria (Stackebrandt *et al.*, 1987; Stackebrandt *et al.*, 1985), and purple bacteria (Woese et al, 1985; Woese *et al.*, 1984a; Woese *et al.*, 1984b).

Ribosomal sequences have suggested that nuclear and organellar genomes are derived from different lineages, giving support to the endosymbiosis theory (Sankoff *et al.*, 1992). In addition, rRNA sequence comparison maintains that functionally related organelles, although of prokaryotic origin might have different descents: chloroplasts seem to be closely related to cyanobacteria (Giovannoni *et al.*, 1988) whereas mitochondria are affiliated to extant a proteobacteria (Yang *et al.*, 1985).

Regions within 16S and 23S rRNA genes that are highly conserved have been used to clone ribosomal genes from microbial DNA extracted from a variety of environments. Sequence analysis of these clones has, in turn, increased our knowledge of the microbial composition of natural environments. For example, Weller *et al.* (1992) retrieved ribosomal sequences of cyanobacteria from hot springs which did not correspond to any of the cultured microorganisms from the same habitat while Liesack and Stackebrandt (1992) have recovered novel sequences from soil ecosystems. These studies seem to support the notion that total

microbial diversity on Earth greatly surpasses the diversity indicated thus far by the culturing techniques.

Another advantage of using sequence analysis of ribosomal genes in microbial ecology is that with the sequence information it is possible to deduce the phylogenetic relationship of the microbes present in natural microbial assemblages without cultivating the organisms or performing time and labor consuming biochemical tests. For instance, the ribosomal sequence of a uncultured fish symbiont revealed its eubacterial origin (Angert *et al.*, 1993). Due to its size, this microorganism was first believed to be an eukaryote, but it is now known as the largest bacterium yet described with an average dimension of 250 x 40 mm.

Ribosomal probes targeting rRNA have been used examine the *in situ* metabolic status of microbial populations (Wallner *et al.*, 1993). This means that not only can these phylogenetic probes potentially determine which microbes are present within microbial communities but also they could indicate which microbes are active (Amman *et al.*, 1990). As the use of this technology becomes routine in microbial ecology, knowledge on the dynamics of populations within natural microbial communities is virtually guaranteed (Pace *et al.*, 1993).

Crickets

Crickets have "imposed upon human consciousness without biting, stinging or destroying" (Walker and Masaki, 1989). Indeed, crickets have rarely been reported as pests to important crops.

Moreover, their musicality, an evolutionary adaptation for propagating the species, has been appreciated since ancient times by oriental cultures. Cricket chirping is species specific, and has been an important tool in ecological studies.

More than 2400 different species of crickets, belonging to 13 subfamilies, are known (Walker and Masaki, 1989). As a group, crickets inhabit a variety of habitats. For example, members of the *Gryllotalpinae* (mole crickets) live in self-made tunnels several centimeters below ground, only exposing themselves to the surface world to eat. In contrast, ground crickets, which belong to the *Nemobiinae* subfamily, are normally found in open grassland areas. Crickets are found in different latitudes, though it is in the moist tropics where the greatest diversity is exhibited.

While feeding behavior in crickets varies greatly, most are believed to be omnivorous. Most of these insects are generally phytophagous, eating predominantly leaf materials, pollen and fragments of grass. Nevertheless, crickets feeding on living prey (e.g., termites), fungal spores, and even opportunistic cannibalistic behavior has been observed. Like other insects, crickets can be selective in what they eat. Water, nitrogen, and mineral content might be important factors that determine the food ingested at different times, but factors like mouth parts, mobility and plant abundance might influence as well.

In contrast to other insects, no intracellular symbionts have been found in crickets. As in other insects, the hindgut of crickets is associated to high bacterial densities (Fig. 1), therefore, favorable conditions for bacterial growth seem to exist. The hindgut of many

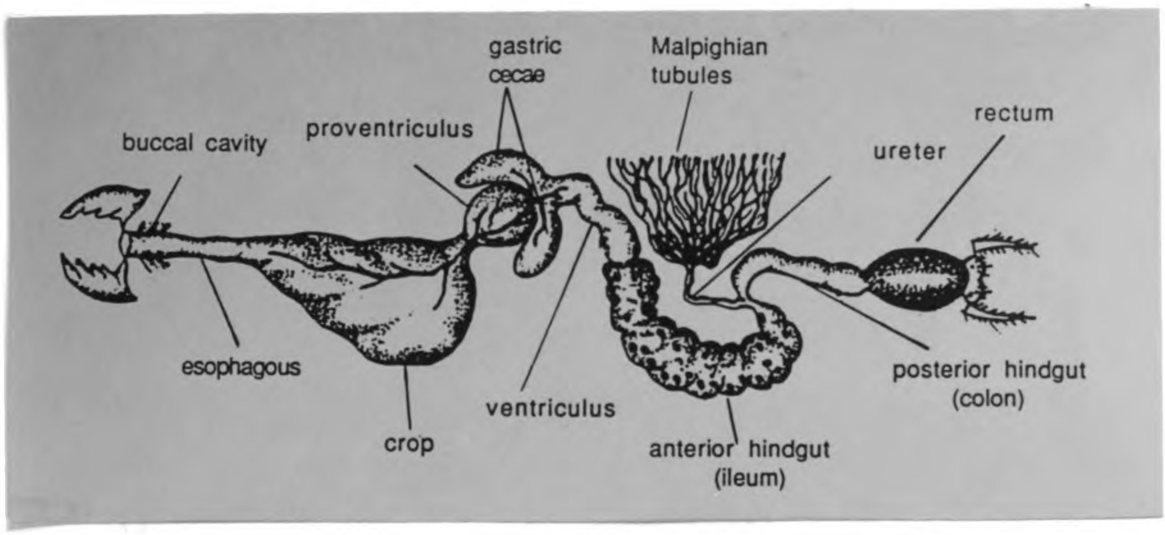

Figure 1. Gryllid digestive tract. From Martoja (1966).

crickets is delimited by two membranes: the gut wall and peritrophic membrane. In some insects these membranes are physically associated; in others, the peritrophic membrane is absent. In crickets, these membranes are normally separated by a space known as the ectoperitrophic space, which is commonly colonized only by bacteria. Transmission electron microscopy has revealed a dense microbial mat in the ectoperitrophic space (Fig. 2). While conclusive evidence is still lacking, the hindgut is presumed to be anoxic due to the presence of a highly active microbial community.

Within all cricket genera, the species most widely studied has been *Acheta domesticus* probably due to the ease of establishing colonies in the laboratory (Fig. 3). *A. domesticus* (commonly known as the house cricket) is an omnivorous cricket of Asiatic origin, now also established in certain European countries as well as in southern and northern states of the United States. This species has commercial value in the United States since it is utilized as a food for many animals kept in captivity (e.g., zoos) as well as bait for fishing. Also, its scientific value has been notable in fields such neurobiology, insect ecology, and insect physiology.

In an attempt to characterize the microbial community in the gut of crickets, Urlich et al (1981) isolated and identified different bacteria from *A. domesticus.* From the 25 isolates characterized, 44 % (i.e., 11 isolates) were shown to belong to the *Enterobacteriaceae* family. Other isolates were identified as *Bacteriodes* sp., *Fusobacterium* sp, and streptococci. The hindgut community of other crickets (e.g., *Gryllus reubens* and *G. velitis*) has been studied by Kaufman (1988). The microbial community of these crickets is

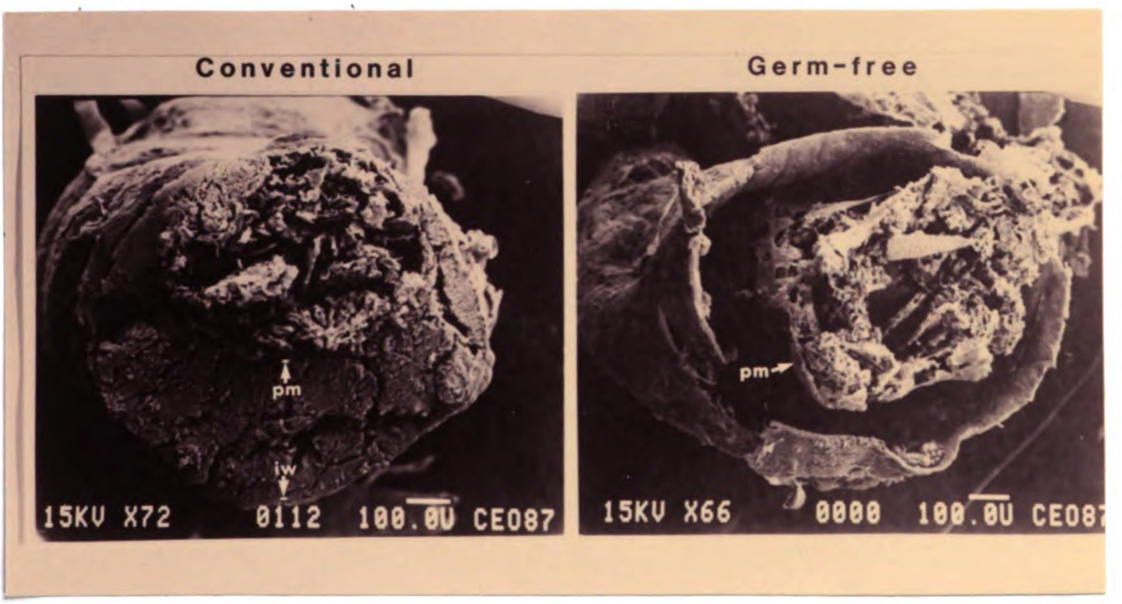

Figure 2. Transverse sections through the anterior hindgut of conventional and germfree *A. domesitcus* showing bacterial biomass located in the ectoperitrophic space between the ileum wall (iw) and the peritrophic membrane (pm).

Figure 3. *A. domesticus*, commonly known as the house cricket.

similar to the house cricket in that the bacterial population seems to be predominantly saccharolytic and acetogenic, although methane evolution has been detected in mole crickets (Kaufman and Santo Domingo, unpublished results). While gryllids chiefly feed on plant material, cellulose does not appear to be an important carbon source for crickets, despite the allegedly cellulolytic activity of cricket gut microbiota reported by Martoja (1966) and MacFarlane and Distler (1982). Since it is assumed that cellulolytic bacteria are normally required to be in physical contact with the polymer, it is improbable that these microbes will be present at all in crickets that harbor their symbionts within the ectoperitrophic space. A critical examination of these controversial findings is needed due to its implication on the understanding of the role of cricket symbionts.

Kaufman (1988) examined the relationship between crickets and their gut symbionts. He found that the absence of gut bacteria did not affect the growth, appearance, and behavior of aposymbiotic crickets. Nevertheless, he argued that the gut microbiota could enhance the digestibility of soluble carbohydrates. This in turn suggests an ecologically relevant role for gut microbes in crickets found in more natural settings.

Scope of this study

Like any other consumer, insects are faced with the problem of finding digestible food that satisfy their nutritional demands for growth and reproduction. Different strategies have evolved in

different groups of organisms to accomplish this, but in general, most consumers can be classified as specialists (and within them we include monophagous and oliphagous organisms) or generalists (e.g., polyphagous and omnivorous organisms). It is uncertain which feeding behavior predominates in insects, nevertheless, research on specialists seem to be more popular, probably due to the economic impact of this group.

Using as a framework a microbiological perspective, insects with a narrow ecological niche (e.g., wood-feeding termites) have also been studied more closely than generalists or omnivorous insects probably because the vital role their microbial symbionts play in the host nutrition. Interestingly, omnivorous insects have also established symbioses with microbes but in most cases it is unknown if the interaction can serve to compensate for the poor quality of their predominant food sources. Moreover, if omnivorous insects could consume a balanced diet by ingesting a wide range of food, it is uncertain if this association with microbes is necessary or beneficial to the host.

It is known that the presence of gut microbiota in crickets is not vital since aposymbiotic colonies could be reared under laboratory conditions. Nonetheless, one intriguing aspect of the cricket gut microbiota is that changes in the host diet could induce changes in the metabolic profiles of the microbial community without affecting the size of it (Kaufman, unpublished results). These biochemical changes can be explained as the emergence of a new microbial community structure. Another reasonable hypothesis is that structure remains intact, but previously inactive enzymes are

induced *de novo*. My goal in this study has been to determine which of the two hypothesis explains the metabolic response of the hindgut community to changes in the host diet. I used DNA based methods that allow me to study the effect of changes in diet on the structure of the community without culturing the different hindgut bacteria housed by the cricket. I have used as a primary model system the hindgut microbial community of *A. domesticus*. The composition of the microbial community of different species of crickets was also examined in an effort to determine the similarities or differences in composition and structure between their gut microbial community.

References

Amman, R. I., L. Krumholz, and D. A. Stahl. 1990. Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. J. Bacteriol. **172**:762-770.

Angert, ER, KD Clements, and NR Pace. 1993. The largest bacterium. Nature **362**:239-241.

Barbosa, P. 1991. Plant pathogens and nonvector herbivores. In *Microbial mediation of plant-herbivore interactions.* P Barbosa, VA Krischik, and CG Jones, eds. pp. 341-382. John Wiley and Sons, Inc., New York, NY

Barbosa, P and DK Letourneau. 1988. Novel aspects of insect-plant interactions. John Wiley and Sons, Inc., New York, NY

Baumann, MA Munson, CY Lai, MA Clark, L Baumann, NA Moran, and BC Campbell. 1993. Origin and properties of bacterial endosymbionts of aphids, whiteflies, and mealybugs. ASM News **59**:21-24.

Brauman, A, MD Kane, M Labat, and JA Breznak. 1992. Genesis of acetate and methane by gut bacteria of nutritionally diverse termites. Science **257**:1384-1387.

Breznak, JA. 1990. Metabolic activities of the microbial flora of termites. In *Microbiology in poecilotherms.* R Lesel (editor). pp. 63-68. Elsevier. New York, NY

Breznak, JA. 1975. Symbiotic relationships between termites and their intestinal microbiota. Symp. Soc. exp. Biol. **29**:559-580.

Breznak, JA, JM Switzer, J-H Seitz. 1988. *Sporomusa termitida* sp. nov., an H_2/CO_2-utilizing acetogen isolated from termites. Arch. Microbiol. **150**:282-288.

Brooks, MA. 1963. Symbiosis and aposymbiosis in arthropods. Symp. Soc. Gen. Microbiol. **13**:200-231.

Brusca, RC and GJ Brusca. 1990. Invertebrates. Sinauer Associates, Inc., Sunderland, MA

Buchner, 1965. Endosymbiosis of animals with plant microorganisms. Interscience Publishers. John Wiley & Sons, Inc., New York

Burggraf, S, GJ Olsen, KO Stetter, and CR Woese. 1992. A phylogenetic analysis of *Aquifex pyrophilus.* System. Appl. Microbiol. **15**:352-356.

Charpentier, R, B Charpentier, and O Zethner. 1978. The bacterial flora of the midgut of two Danish populations of healthy fifth instar larvae of the turnip moth, *Scotia segetum.* J. Invertebr. Pathol. **32**:59-63.

Cleveland, LR. 1924. The physiological and symbiotic relationships between the intestinal protozoa of termites and their host, with special reference to *Reticulotermes flavipes*, Kollar. Biol. Bull. **46**:177-25

Cochran, DG. 1985. Nitrogenous excretion. In *Comprehensive Insect Physiology, Biochemistry, and Pharmacology*, Vol. 4. G A Kerkut and LI Gilbert (editors). pp. 467-506. Pergamon Press, Elmsford, NY

Cole, M. J. and C. G. Enke. 1991. Direct determination of phospholipid structures in microorganisms be fast atom bombardment triple quadrupole mass spectrometry. Anal. Chem. **63**: 1032-1038.

Cruden, DL and JA Markovetz. 1987. Microbial ecology of the cockroach gut. Ann. Rev. Microbiol. **41**:617-643.

Dadd, RH. 1985. Nutrition: organisms. In *Comprehensive Insect Physiology, Biochemistry, and Pharmacology*, Vol. 4. GA Kerkut and LI Gilbert (editors). Pergamon Press, Elmsford, NY, pp. 313-390.

Daser, U and R Brandl. 1992. Microbial gut floras of eight species of tephritids. Bio. J. Linnean Soc. **45**:155-165

Davies, RG. 1988. Outlines of entomology. 7th Edition. Chapman and Hall. London.

DeLong, E. 1992. Archaebacteria in coastal marine environments. Proc. Natl. Acad. Sci. **89**:5685-5689.

Dillion, RJ, and AK Charnley. 1986. Inhibition of *Metarhizium anisopliae* by the gut bacterial flora of the desert locust, *Schistocerca gregaria*: evidence for an antifungal toxin. **47**:350-360.

Donnellan, JF and BA Kilby. 1967. Uric acid metabolism by symbiotic bacteria from the fat body of *Periplaneta americana*. Comp. Biochem. Physiol. **22**:235-252.

Dowd, P. 1992. Insect fungal symbionts: a promising source of detoxifying enzymes. J. Ind. Microbiol. **9**:149-161.

Dowd, P. 1991. Symbiont-mediated detoxification in insect herbivores. In *Microbial mediation of plant-herbivore interactions*. P Barbosa, VA Krischik, and CG Jones (editors). pp. 411-440. John Wiley and Sons, Inc., New York, NY

Ehrlich, PR and PH Raven. 1964. Butterflies and plants: a study in coevolution. Evolution **18**:586-608.

French, JRJ, GL Turner, JF Bradbury. 1976 Nitrogen fixing bacteria from the hindgut of termites. J. Gen. Microbiol. **95**:202-206.

Giovannoni, SJ, S Turner, GJ Olsen, S Barns, DL Lane, and NR Pace. 1988. Evolutionary relationships among cyanobacteria and green chloroplasts. J. Bacteriol. **170**:3584-3592.

Grassi, B and A Foa. 1911. In torno ai protozoi die termitidi. Nota perliminare. Rend. R. Acad. Lincei **20**:725-41.

Gutell, RR, B Weiser, CR Woese, and HF Noller. 1985. Comparative anatomy of 16S-like ribosomal RNA. Prog. Nuc. Acid Res. Mol. Biol. **32**:155-216.

Haygood, MG and DL Distel. 1993. Bioluminescent symbionts of flashlight fishes and deep sea angler fishes from unique lineages related to the genus *Vibrio*. Nature **363:**154-

Henry, SM. 1962. The significance of microorganisms in the nutrition of insects. Trans. N. Y. Acad. Sci. **24**:676-683.

Herbert, RA. 1990. Methods for enumerating microorganisms and determining biomass in natural environments. Meth. Microbiol. **22**:1-40.

Hinman, EH. 1930. A study of the food of mosquito larvae (Culicidae). Amer. J. Hyg. **12**:238-270.

Holben, WE, MA Hood, and JM Tiedje. 1992. Overview: fate and transport of microbes. In *Microbial ecology: principles, methods, and applications*. MA Levin, RJ Seidler, and M Rogul (editors). McGraw-Hill, Inc., New York, NY

Holben, WE and JM Tiedje. 1988. Applications of nucleic acid hybridization in microbial ecology. Ecology **69**:561-568.

Honigsberg, BM. 1970. Protozoa associated with termites and their role in digestion. In *Biology of the termites* Vol. 2. K. Krishna and FM Weesner (editors). Academic Press, New York, NY

House, HL. 1974. In *The Physiology of the Insecta*. Vol. V. M Rockstein (editor). pp. 1-62, Academic Press, New York.

Hungate, RE. 1943. Quantitative analyses on the cellulose fermentation by termite protozoa. Ann. Entomol. Soc. Am. **36**:730-739.

Hungate, RE. 1939. Experiments on the nutrition of Zootermopsis. III. The anaerobic carbohydrate dissimilation on the intestinal protozoa. Ecology **20**:230-245.

Hunt, J and AK Charnley. 1981. Abundance and distribution of the gut flora of the desert locust, *Schistocerca gregaria*. **38**:378-385.

Iwabe, N, K-C Kuma, M Hasegawa, S Osawa, and T Miyata. 1989. Evolutionary relationship of archaebacteria, eubacteria, and eukaryotes inferred from phylogenetic trees of duplicated genes. Proc. Natl. Acad. Sci. USA **86**:9355-9359.

Johnson, ND and BL Bentley. 1991. Symbiotic N2-fixation and the elements of plant resistance to herbivores: lupine alkaloids and tolerance to defoliation.

Johnson, ND, LP Rigney, and BL Bentley. 1989. The short term induction of alkaloids in lupines: difference between N2 fixing and nitrogen-limited plants. J. Chem. Ecol.

Jones, CG. 1984. Microorganisms as mediators of plant resource exploitation by insect herbivores. In *A new ecology: novel approaches to interactive systems.* PW Price, CN Slobodchikoff, and WS Gaud (editors). pp. 53-99. John Wiley and Sons, Inc. New York, NY.

Kane, MD, and JA Breznak. 1991 *Acetonema longum* gen. nov. spec. nov., an H_2/CO_2 acetogenic bacterium from the termite gut, *Pterotermes occidentis.* Arch. Microbiol. 156:91-98.

Kane, MD, A Brauman, and JA Breznak. 1991. *Clostridium mayombei* sp. nov., an H_2/CO_2 acetogenic bacterium from the gut of the African soil-feeding termite, *Cubitermes speciosus.* Arch. Microbiol. 99-104.

Karban, R, R Adamchak, and WC Schnathorst. 1987. Induced resistance and interspecific competition between spider mites and a vascular wilt fungus. Science 235:678-679.

Kaufman, MG, and MK Klug. 1991. The contribution of hindgut bacteria to dietary carbohydrate utilization by crickets (Orthoptera : Gryllidae). Comp. Biochem. Physiol. **98**:117-123.

Kaufman, MG, and MJ Klug. 1990. Microbial community metabolism in the digestive tract of crickets (ORTHOPTERA: GRYLLIDAE): implications for omnivorous insects. In *Microbiology in poecilotherms*. R Lesel (editor). pp. 69-72. Elsevier. New York, NY

Koch, A. 1967. Insects and their symbionts. In *Symbiosis*. Vol. II. SM Henry, ed. pp. 1-106, Academic Press, London

Krischik, VA. 1991. Specific or generalized plant defense: reciprocal interactions between herbivores and pathogen. In *Microbial mediation of plant-herbivore interactions*. P Barbosa, VA Krischik, and CG Jones (editors). pp. 309-340. John Wiley and Sons, Inc., New York, NY

Krischik, VA and CG Jones. 1991. Interactions among insects, plants, and microorganisms: a net effects perspective on insect performance. In *Microbial mediation of plant-herbivore interactions*. P Barbosa, VA Krischik, and CG Jones (editors). pp. 1-6. John Wiley and Sons, Inc., New York, NY

Lawrence, JF. 1989. Mycophagy in the Coleoptera: feeding strategies and morphological adaptations. In *Insect-fungus interactions*. N Wilding, NM Collins, PM Hammond, and JF Webber (editors). Academic Press, New York, NY pp. 1-23

Leach, JG. 1926. The relation of the seed-corn (*Phorbia fusciceps* Zett.) to the spread and development of potato backleg in Minnesota. Phytopathology **16**:32-45

Levin, DA. 1976. The chemical defenses of plants to pathogens and herbivores. Annu. Rev. Ecol. Syst. **7**:121-159.

Liesack, W and E Stackebrandt. 1992. Occurrence of novel groups of the domain bacteria as revealed by analysis of genetic material isolated from an Australian terrestrial environment. J. Bacteriol. **174**:5072-.

Lipke, H and G Fraenkel. 1956. Insect nutrition. Ann. Rev. Entomol. **1**:17-44.

Martoja, R. 1966. Sur quelques aspects de la biologie des orthoptires in relation avec la presence de concentrations microaiennes (bacterial intertinals, rickettsies). Ann. Entomol. Fr. **2**:753-840.

McFarlane, JE and MHW Distler. 1982. The effect of rutin on growth, fecundity, and food utilization in Acheta domesticus (L.). J. Insect Physiol. **28**:85-88.

McIntyre, JL, JA Dodds, and JD Hare. 1991. Effects of localized infection of *Nicotiana tabacum* by tobacco mosaic virus on systemic resistance against diverse pathogens and an insect. Phytopathology 71:297-301.

Medlin, L, HJ Elwood, S Stickel, and ML Sogin. 1988. The characterization of enzymatically amplified eukaryotic 16S-like rRNA -coding regions. Gene **71**:491-499.

Moody, ME and RN Mack. 1988. Controlling the spread of plant invasions: the importance of nascent foci. J. Appl. Ecol. **25**:1009-1025.

Munson, MA, L Baumann, and P Baumann. 1992. *Buchnera aphicola*, the endosymbiont of aphids, contains genes for four ribosomal RNA proteins, initiation factor-3, and the α-subunit of RNA polymerase. Curr. Microbiol. **24**:23-29.

Munson, MA, P Baumann, MA Clark, L Baumann, NA Moran, DJ Voegtlin, and BC Campbell. 1991a. Evidence for the establishment of aphid-eubacterium endosymbiosis in an ancestor of four aphid families. J. Bacteriol. **173**:6321-6324.

Munson, MA, P Baumann, and MG Kinsey. 1991b. *Buchnera* gen nov. and *Buchnera aphicola* sp. nov., a taxon consisting of the mycetocyte-associated, primary endosymbionts of aphids. Int. J. Syst. Bacteriol. **41**:556-568.

Odelson, DA and JA Breznak. 1983. Volatile fatty acid production by the hindgut microbiota of xylophagous termites. Appl. Environ. Microbiol. **45**:1602-1613.

Pace, NR, ER Angert, EF Delong, TM Schmidt, and GS Wickham. 1993. New perspective on the natural microbial world. In *Industrial microorganisms: basic and applied molecular genetics.* RH Baltz, GD Hegerman, and PL Skatrud. American Society for Microbiology. Washington, D.C. pp. 77-83.

Pace, NR. 1991. Origin of life - facing up to the physical setting. Cell **65**:531-533.

Pant, NC and G Fraenkel. 1954. Studies on the symbiotic yeasts of two insect species, *Lasioderma serricorne* F. and *Stegobium paniceum* L., Biol. Bull. **107**:420-443.

Parkes, RJ. 1987. Analysis of microbial communities within sediments using biomarkers. In *Ecology of microbial communities.* M Fletcher, TRG Gray, JG Jones (editors) Symp. Gen. Soc. Microbiol. **41**:147-178.

Pierantoni, U. 1910. L'origine di alcuni organi d'*Icerya purchasi* e la simbiosi ereditaria. Bull. Soc. Nat. Napoli. **23**:34-56

Potrikus, CJ and JA Breznak. 1980. Uric acid-degrading bacteria in guts of termites [*Reticulitermes flavipes* (Kollar)]. Appl. Microbiol. Environ. **40**:117-124.

Potrikus, CJ and JA Breznak. 1977. Nitrogen-fixing *Enterobacter agglomerans* isolated from guts of wood-eating termites. Appl. Environ. Microbiol. **33**:392-399.

Quinlan, RJ and JM Cherret. 1979. The role of fungus in the diet of the leaf-cutting ant *Atta cephalotes* (L). Ecol. Entomol. **4**:151-160

Rosenthal, GA. 1983. Biochemical adaptations of the bruchid beetle, *Caryedes brasiliensis* to *L*-canavanine, a higher plant allelochemical. J. Chem. Ecol. **9**:803-811.

Sankoff, D, G Leduc, N Antoine, B Paquin, BF Lang, and R Cedergreen. 1992. Gene order comparisons for phylogenetic inference: evolution of the mitochondrial genome. Proc. Natl. Acad. Sci. **89**:6575-80

Schleifer, KH and W Ludwig. 1989. Phylogenetic relationships among bacteria. In *Hierarchy of Life.* B Fernholm, K Bremer, and H

Jörnvall (editors). Elsevier Science Publishers, Amsterdam, pp. 103-117.

Schultz, JE and JA Breznak. 1978. Heterotrophic bacteria present in the hindguts of wood-eating termites [Reticulitermes flavipes (Kollar)] Appl. Environ. Microbiol. **35**930-936.

Scudder, GGE and J Meredith. 1982. The permeability of the midgut of three insects to cardiac glycosides. J. Insect Physiol. **28**:689-694.

Stackebrandt, E, W Ludwig, M Weizenegger, S Dorn, TJ McGill, GE Fox, CR Woese, W Schubert, K-H Schleifer. 1987. Comparative 16S rRNA oligonucleotide analyses and murein type of round-spore-forming bacilli and non-spore-forming relatives. J. Gen. Microbiol. **133**:2523-2529.

Stackebrandt, E, H Pohla, R Kroppenstedt, H Hippe, and CR Woese. 1985. 16S rRNA analysis of *Sporomusa*, *Selenomonas*, and *Magasphaera*: on the phylogenetic origin of Gram-positive eubacteria. Arch. Microbiol. **143**:270-276.

Stetter, KO, G Fiala, G Huber, R Huber, and A Segerer. 1990. FEMS Microbiol. Rev. **75**:117-124.

Stetter, KO, G Lauerer, M Thomm, and A Neuner. 1987. Isolation of extremely thermophilic sulfate reducers: evidence for a novel branch of archaebacteria. Science **236**:820-824.

Tannock, GW. 1984. Control of gastrointestinal pathogens by normal flora. In *Current perspectives in microbial ecology.* Proceedings of the third international symposium on microbial ecology. MJ Klug and CA Reddy (editors), American Society for Microbiology, Washington, D.C., pp. 374-381.

Thayer, DW. 1976. Facultative wood-digesting bacteria from the hindgut of the termite *Reticulitermes herperus*. J. Gen. Microbiol. **95**:287-296.

Thiery, I, L Nicolas, R Rippka, and N Tandeau de Marsac. 1991. Selection of cyanobacteria isolates from mosquito breeding sites as a potential food source for mosquito larvae. Appl. Environ. Microbiol. **57**: 1354-1359.

Urlich, RG, DA Buthala, and MJ Klug. 1981. Microbiota associated with the gastrointestinal tract of the common house cricket, *Acheta domesticus*. Appl. Environ.. Microbiol. **41**:246-254.

Unterman, MB, P Baumman, and LD McLean. 1989. Pea aphid symbiont relationships established by analysis of 16S rRNAs. J. Bacteriol. **171**:2970-2974.

Walker, ED, DL Lawson, RW Merrit, WT Morgan, and MJ Klug. 1991. Nutrient dynamics, bacterial populations, and mosquito productivity in tree hole ecosystems and microcosms. Ecology **72**:1529-1546.

Walker, TJ and S Masaki. 1989. Natural history of crickets. In *Cricket behaviour and neurobiology*. F Huber, TE Moore, and W Loher (editors). Comstock Publisher Associates, Ithaca, NY, pp.1-32.

Wallner, G, RA Amann, and W Beisker. 1993. Optimizing fluorescent *in situ* hybridization with rRNA targeted oligonucleotide probes for flow cytometric identification of microorganisms. Cytometry **14**:136-143.

Weller, R, MM Bateson, BK Heimbach, and ED Kopczynski. 1992. Unculturable cyanobacteria, chloreflexus-like inhabitants and spirochete-like inhabitants of a hot spring microbial mat. Appl. Environ. Microbiol. **58**:3964-3469.

White, DC. 1983. Analysis of microorganisms in terms of quantity and activity in natural environments. In *Microbes in their natural environments*. JH Slater, R Whittenbury, and JWT Wimpenny (editors). Symp. Soc. Gen. Microbiol. **34**:37-66.

Whittaker, RH. 1969. New concepts of kingdoms of organisms. Science **163**:150-160.

Woese, CR, O Kandler, and ML Wheelis. 1990. Towards a natural system of organisms: proposal for the domains Archaea, Bacteria, and Eucarya. Proc. Natl. Acad. Sci. USA **87**:4576-4579.

Woese, CR, WG Weisburg, CM Hahn, BJ Paster, LB Zablen, BJ Lewis, TJ Macke, W Ludwig, and E Stackebrandt. 1985. The

phylogeny of purple bacteria: the gamma subdivision. System. Appl. Microbiol. **6**:25-33.

Woese, CR, E Stackebrandt, WG Weisburg, BJ Paster, MT Madigan, VJ Fowler, CM Hahn, P Blanz, R Gupta, KH Nealson, and GE Fox. 1984a. The phylogeny of purple bacteria: the alpha subdivision. System. Appl. Microbiol. **5**:315-326.

Woese, CR, WG Weisburg, BJ Paster, CM Hahn, RS Stanner, NR Krieg, HP Koops, H Harms, and E Stackebrandt. 1984a. The phylogeny of purple bacteria: the beta subdivision. System. Appl. Microbiol. **5**:327-336.

Wolfe, CJ and MG Haygood. 1991 Restriction fragments length polymorphism analysis of genetic divergence among the light organ symbionts of flashlight fish. Biol. Bull. 181:135-143.

Wolfe, MS and CE Caten. 1987. Populations of plant pathogens: their dynamics and genetics. Blackwell Scientific, Oxford.

Wood, TG and RJ Thomas. 1989. The mutualistic association between Macrotermitinae and Termitomyces. In *Insect-fungus interactions*. N Wilding, NM Collins, PM Hammond, and JF Webber (editors). Academic Press, New York, NY, pp. 69-92

Yamin, MA. 1980. Cellulose metabolism by the flagellate *Trichonympha* from a termite is independent of endosymbiotic bacteria. Science **211**:58-59.

Yang, D, Y Oyaizu, GJ Olsen, and CR Woese. 1985. Mitochondrial origins. Proc. Natl. Acad. Sci. USA **82**:4443-4447.

Zehnder, AJB and W Stumm. 1988. Geochemistry and biogeochemistry of anaerobic habitats. In *Biology of anaerobic microorganisms*. AJB Zehnder (editor) John Wiley and Sons, Inc. New York, NY pp. 1-38.

Zeikus, J. G. 1984. Metabolic communication between biodegradative populations in nature. In *Microbes in their natural environments*. JH Slater, R Whittenbury, and JWT Wimpenny eds. Cambridge University Press. London.

Chapter 1. Comparison of different DNA extraction procedures to isolate nucleic acid from the hindgut bacterial community of the house cricket, *Acheta domesticus*.

ABSTRACT

Due to the heterogeneous growth requirements of bacteria, the use of culture techniques is of restricted value to microbial ecologists studying natural microbial communities. Since nucleic acid methods circumvent the problem of culturability, DNA extraction procedures were evaluated in an effort to determined which was better suited for the analysis of the microbial community inhabiting the cricket hindgut. The procedures tested differed primarily in the biomass required for the recovery of the nucleic acid (number of hindguts needed) and the chemicals used for bacterial cell lysis. The number of cells lysed by the methods ranged from 32.3 to 90.8 % and the DNA yields ranged from 0.72 to 6.29 μg per gut. In all cases the DNA extracted was of good quality as suggested by the ratio of the absorbance at 260 and 280 nm. Successful amplification of a region of the 16S rRNA gene from this DNA by the Polymerase Chain Reaction (PCR) method further demonstrated the acceptable quality of the DNA extracted. The results in this study suggest that DNA based technology can be used to analyze the cricket microbial community.

The hindgut of the house cricket, *Acheta domesticus*, harbors a dense microbial community within the ectoperitrophic space (Urlich *et al.*, 1981). Preliminary microbiological characterization has indicated that this community is dominated by a number of different anaerobic as well as facultative Gram negative, rod or rod-coccoid shaped bacteria capable of fermenting simple sugars (Urlich *et al.*, 1981). Unlike other insects, neither fungi nor protozoa are part of the cricket gut microbiota (Kaufman, 1988). This bacterial community seems to benefit their host by extending the range of carbohydrates utilized by the animal (Kaufman and Klug, 1991). While several studies have indicated the occurrence of certain bacterial groups within the cricket gut (Urlich *et al.*, 1981; Martoja, 1966), knowledge regarding the dynamics of such microbiota remains virtually nonexistent.

In recent years, nucleic acid based methods have become popular in studies of natural microbial communities (Atlas *et al.*, 1992). One obvious advantage of such methods is that they do not rely on the physiological nor cultural status of the individuals making up the microbial community. Extraction procedures have been developed to maximize the recovery of DNA from natural samples such that most members of the microbial community are represented in the nucleic acid fraction (Tsai and Olson, 1992; Tsai and Olson, 1991). Nevertheless, in most cases the efficiency of the DNA extraction procedure is evaluated on the basis of DNA yields and consequently it is virtually unknown if the nucleic acid extracted

belongs to only those microbial populations within the community studied that are sensitive to the cell lysis treatment.

To the author's knowledge there is no published record of a study undertaking the task of evaluating the efficiency of DNA extraction procedures from insect microbial communities. Here, I report the first attempt to isolate DNA from cricket gut microbial communities.

Materials and Methods

Crickets. House crickets (*A. domesticus*) were reared in the laboratory in a 12 hours daylight cycle, at 28^0 C, 70 % humidity. Animals were normally on a diet consisting on a commercially available cricket chow and water *ad libitum*. For diet experiments, adult crickets were placed on petri dishes with autoclaved water and one of three different diets: alfalfa, protein or chow. After five days under these conditions, hindguts were removed as described below. *Gryllus reubens and G. velitis* (provided by M. Kaufman Kellogg Biological Station) were grown under conditions similar to *A. domesticus* using a chow diet as the primary food source.

Preparation of hindguts. Hindguts were surgically removed from adult crickets using fine forceps with the help of a dissecting microscope, resuspended in 1.5 ml autoclaved phosphate buffer saline (PBS, pH 7.2) and homogenized using tissue grinders. To physically separate bacteria from the eukaryotic tissue, homogenized hindgut samples were briefly centrifuged at 11,200 x *g* using a microcentrifuge. Supernatant was collected and centrifuged for 5

min. at 6,800 x *g* to concentrate the cricket bacterial community. Approximately 82 % of the hindgut bacteria, as determined by microscopic counts, was recovered using this approach. This bacterial pellet termed "bacterial fraction" was used for the different analyses.

Different media (e.g., Trypticase Soy Agar, TSA and Brain Heart Infusion agar, BHI), different pH and different incubation temperatures were tested to determine whether culturing conditions recover a significant fraction of the bacteria inhabiting the cricket hindgut. Incubations were performed under aerobic conditions, or alternatively under anaerobic conditions in an anaerobic glove box at room temperature under an CO_2/N_2 atmosphere.

Large scale bacterial community DNA extraction. The bacterial fraction of 25 hindguts was combined in order to isolate large quantities of hindgut microbial community DNA. A similar method to that developed by Holben *et al.* (1988) was used to extract genomic bacterial DNA from the hindgut community. The following modifications were adopted. A final refractive index of 1.3880 was used for the cesium chloride (CsCl) - ethidium bromide (EtBr) gradients. Gradients were centrifuged for 18 h at 198,000 x *g* in a Sorvall ultracentrifuge. A total of 3 ml containing the chromosomal DNA fraction was removed from the gradient using 5 ml syringes with 18 gauge needles. Further purification of genomic DNA by a second CsCl-EtBr gradient was not necessary. Additional manipulations for the isolation of DNA were performed as recommended by the original authors with the exception that DNA was air dried and resuspended on TE (Tris 10 mM, EDTA 1 mM, pH

8.0). The concentration of DNA was determined by optical absorbance at 260 nm (OD_{260}). The large scale method of Ausubel *et al.* (1987) was also employed to extract DNA from the cricket community.

Small scale bacterial community DNA extraction. DNA was extracted from the bacterial fraction of two hindguts following a small scale DNA extraction procedure described by Ausubel *et al.* (1987). Also, the following modified version of a method developed by Visuvanathan *et al.* (1989) was evaluated. The bacterial fraction was resuspended in 400 µl of TE and incubated at 65^0 C for 30 min. Sodium azide (2 % w/v; Sigma, St. Louis, MO) and subtilisin (Carlsberg protease; 50 µl of 100 mg/ml stock solution; Sigma) were added and incubated at 37^0 C for 12 h. Lysozyme (100 ug/ml) was added and samples were then incubated for 5 h at 50^0 C. To lyse the bacterial cells sodium dodecyl sulfate (SDS, 10 % w/v) and pronase (50 µl from 50 mg/ml stock solution) were added and further incubated at 37^0 C for 12 h. Proteins and lipids were then removed by five phenol/chloroform/isoamyl (25:24:1) extractions following a hexadecyltrimethylammonium bromide pretreatment (CTAB; Ausubel *et al.*, 1987). To removed RNA from the nucleic acid pool, the organic phase was then incubated with RNase (Sambrook *et al.*, 1989), followed by an additional phenol/chloroform extraction. Bacterial DNA was precipitated using 0.6 vol of isopropanol, resuspended in TE, and stored at -20^0 C.

DNA extraction efficiency. As an index of cell lysis efficiency of the different DNA extraction methods, viable plate counts and direct microscopic counts were made before and after cell

lysis treatments. Viable bacterial densities were determined as culturable plate counts by spreading aliquots of different dilutions of the bacterial fraction on half strength TSA plates. Plates were incubated aerobically at 30^0 C and colonies were counted after 48 h.

The acridine orange direct count (AODC) method was used for direct microscopic counts (Hobbie *et al.*, 1977). Bacterial fractions were stained with acridine orange (0.01 % final concentration; Sigma) for 5 min. and filtered through a Irgalan-black prestained 0.2 µm polycarbonate membrane (Nuclepore). Excess of stain was removed using 25 % isopropanol. Membranes were then placed on a microscope slide and stored at 4^0 C in the dark until ready for microscopic observation. Fluorescing cells from 10 randomly picked microscope fields were counted using an 63X oil objective on a Leitz Orthoplan 2 epifluorescence microscope (Leitz, FRG).

To further evaluate the efficiency of DNA extraction the yields obtained with each procedure were compared to the total DNA fraction as determined by Burton (1956). Hindguts were homogenized and the bacterial fraction collected as described above. Hindgut bacterial fractions were then resuspended in TE (6 ml) and split into three fractions for bacterial enumeration, bacterial genomic DNA extraction, and total nucleic acid content analysis using the diphenylamine method. For the latter method nucleic acid was extracted as suggested by Burton (1956). The bacterial pellet was resuspended in 5 ml of 0.25 M perchloric acid ($HClO_4$) and shaken in iced water for 30 min. Samples were then centrifuged for 1 min. at 6,800 x *g* and the supernatant removed. Cells were incubated at 70^0 C for 30 min. in 3 ml 0.5 M perchloric acid and centrifuged at 23,000

x *g* for 5 min. Supernatant was carefully removed and store at 4^0 C until ready for analysis. To maximize DNA recovery, an additional acid extraction was performed after which the supernatants were combined. To determine the concentration of nucleic acids, 2 ml of diphenylamine reagent was added to 1 ml of the supernatant and incubated at 30^0 C for 16 h. The optical absorbance was measured at 600 nm and DNA concentrations were calculated with the help of a standard curve of known concentrations of salmon sperm DNA.

The Polymerase Chain Reaction (PCR) was used to amplify regions within the small ribosomal subunit gene using primers that align to well conserved regions of the 16S rRNA gene of prokaryotes (Barry *et al.*, 1990). The sequence for the set of primers used was the following: AGATTTGATCATGGCTCAG and CCACTGCTGCCTCCCGTAG which correspond to positions 8-27 and 342-360 of *Escherichia coli*. Hindgut community DNA and isolate DNA was used as the template for PCR. The following PCR protocol was used: 30 cycles of 3 min. 94^0 C (denaturation), 2 min. 48^0 C (elongation), 3 min. 72^0 C (extension). A Perkin Elmer-Cetus DNA thermal cycler was used for the PCR amplification. The amplification product was separated by gel electrophoresis to determined its size.

RESULTS

Culturable bacteria from the cricket hindgut. Microbiological studies were performed to determine which fraction of the hindgut bacterial community could be cultured. When different media were evaluated, it was found that the highest

densities of bacteria were obtained with TSA and BHI agar plates incubated aerobically (Table 1). Although BHI produced the highest viable counts, TSA allowed the growth of a greater diversity of hindgut bacteria as determined by the colony morphotypes. BHI viable counts however, represented only 11 % of the hindgut microscopic counts. It was also found that dilution of BHI or TSA media had the general effect of decreasing the number of culturable bacteria.

The effect of different media pH on the aerobic viable counts was assessed using TSA and BHI (Table 2). The results showed that a pH close to neutrality allowed the growth of more bacteria. Predominant colony morphotypes grew at pH values between 5 and 9. By contrast, pH values below 5 and above 9 inhibited growth of hindgut bacteria. Comparable aerobic counts were obtained at temperatures from 24 to 37^0 C, although incubation at 24^0 C for at least 48 h was necessary to obtain the highest numbers of culturable bacteria.

Comparison of DNA extraction methods. The ratio of optical absorbance at 260 and 280 nm of community DNA extracted by the different methods indicated was that the DNA was relatively pure; environmental DNA tends to be impure compared to that extracted from pure cultures (Table 3; a ratio between 1.8 to 2.0 is assumed to reflect DNA of high purity). All methods generated community DNA of high molecular weight (> 40 kb), albeit significant mechanical degradation (i.e., DNA shearing) was evident. Community DNA was in all cases susceptible to digestion with several restriction endonuclease enzymes (e.g., *BamH* I, *Pst* I, and *Hpa* I).

Table 1. Comparison of different media and incubation conditions on plate counts method

Medium	Aerobic[a]	% of microscopic counts	Anaerobic[a]	% of microscopic counts
Pseudomonads	13.5	0.54	27.5	1.10
MacConkey	5.50	0.22	8.50	0.34
R2A	26.5	1.06	30.0	1.20
10% R2A	6.50	0.26	24.5	0.98
TSA	126	5.04	92.0	3.68
10% TSA	84.0	3.36	29.0	1.16
1% TSA	26.0	1.04	10.0	0.40
0.1% TSA	22.0	0.88	13.0	0.52
BHI	276	11.0	90.0	3.60
10% BHI	144	5.76	50.0	2.00
1% BHI	56.0	2.24	4.00	0.16

a = CFU x 10^6

Table 2. Effect of pH and temperature on aerobic plate counts[a]

	pH 4	pH 5	pH 6	pH 7	pH 8	pH 9	pH 10	24^0 C	30^0 C	37^0 C
TSA	< 1	44.5	87.5	91	78	85	3	65.5	89	78.5
BHI	< 1	32	98	113	76	93	< 1	89	104	98

a = CFU x 10^6

Table 3. Comparison of different DNA extraction methods

Method	% Cells lyzed per gut[a]	% Cells remaining culturable per gut[b]	DNA yield[c]	OD 260/280	% of hindgut bacterial DNA recovery[d]
Ausubel*	32.3	42.0	0.717	1.24 - 1.60	11.1
Holben*	44.9	27.7	1.91	1.33 - 1.55	19.5
Visvanathan**	90.8	< 0.1	6.29	1.32 - 1.64	33.8
Ausubel**	37.8	39.0	0.929	1.25 - 1.51	12.3

[a] as determined by acridine orange direct counts before and after the cell lysis procedure

[b] as determined by TSA plate counts before and after the cell lysis procedure

[c] as determined by OD_{260}; OD_{260} of 1 = 50 µg

[d] DNA yield / theoretical DNA yield; theoretical DNA yield based on 1 x 10^8 *Escherichia coli* cells = 1 µg genomic DNA

* large scale DNA extraction

** small scale DNA extraction

Different quantities of bacterial community DNA per gut were extracted with each of the methods used in this study (Table 3). The highest DNA yield per gut (6.29 µg / gut) was obtained with the modified version of the method developed by Visuvanathan *et al.* (1989). Also, more bacteria were lysed (90.8 %) when this method was used. A considerable fraction (i.e., > 32 %) of the culturable bacteria resist the lysing steps of the other extraction procedures. Since it was evident that the Visuvanathan method is more efficient for the extraction of DNA from the microbial community of *A. domesticus*, it became the method of choice for the rest of this study.

The primers used for the PCR reaction, commonly used for cloning and sequencing of 16S rRNA genes, align to regions believed to be highly conserved in eubacteria (Lane, 1991). An amplification product of the expected approximately 350 bp was obtained with bacterial community DNA from different species of crickets as the template in the PCR method (Fig. 1). The same size product was obtained with genomic DNA from different bacteria, including several hindgut isolates (data not shown). An intense hybridization signal with an eubacteria specific ribosomal probe (also known as "panprobe", Jones *et al.*, 1989) further supported the authenticity of the PCR product (i.e., 16S rDNA). Furthermore, restriction fragment length polymorphisms obtained for different hindgut bacteria were the same when using as a ribosomal probe PCR products generated with community DNA or hindgut isolates DNA as the template in PCR. (data not shown).

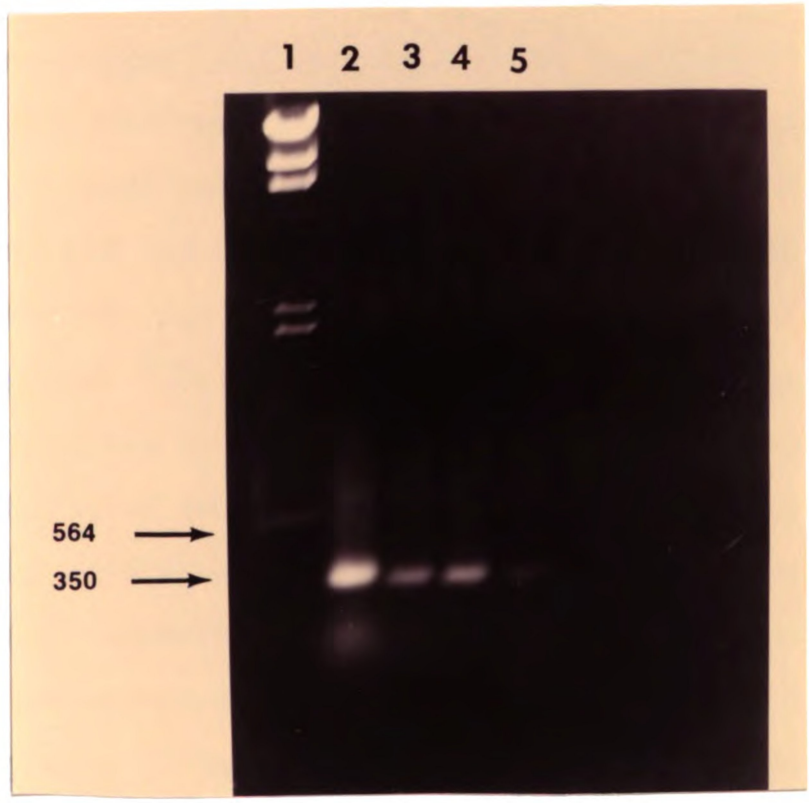

Figure 1. Agarose gel electrophoresis of PCR products using 16S rRNA primers (see text). DNA from the hindgut community of A. domesticus (lane 2), G. rubens (lane 3), G. velidis (lane 4), and the hindgut isolate Bac 4 (lane 5) were used as templates in the PCR methods. Lane 1, λ/HindIII size markers.

DISCUSSION

Culture techniques only recovered a small fraction of the hindgut community (i.e., less than 12 %, Table 1). This strongly suggests that most of the bacteria in the cricket hindgut will not be detected in plate counts. Hence, it was concluded that culturing techniques will fail to completely describe the effect of dietary perturbation on the cricket hindgut community. This is not surprising since it is well known that culturing techniques are highly selective (Sorheim *et al.*, 1989; Atlas, 1985). These results prompted the evaluation of nucleic acid methods as an approach to answer ecological questions regarding the cricket hindgut communities.

Several methods to extract DNA from the cricket hindgut bacterial community were evaluated. The methods tested have been previously used to recover small or large scale quantities of DNA from soil or from bacterial pure cultures (Holben *et al.*, 1988; Ausubel *et al.*, 1987). In general, the results showed that DNA isolation following the Visuvanathan *et al.* (1989) method produced the highest yields. The lower yields of DNA recovered by the other extraction procedures could be explained by their inefficiency in lysing hindgut bacteria. It was also observed that large scale DNA extraction procedures were less efficient than small scale ones (Table 3). One possible explanation is that the concentration of organic material that coextracts with the nucleic acid in the large scale procedures might have been higher, affecting the precipitation of nucleic acid. Alternatively, high concentrations of organic material

might have inhibited the action of proteases and thus prevented bacterial cell lysis.

An obvious disadvantage of large scale DNA extraction procedures in the study of insect hindgut communities is the need to combine several insects guts in order to have enough bacterial biomass so that DNA could be visualized (when illuminated with ultraviolet light) and recovered from the CsCl-EtBr gradients. For *A. domesticus* this is not an impossible endeavor (about 25 hindguts are normally needed for large scale methods); however, for insects with a smaller gut (e.g., termites), a much greater number of animals would be needed. In practical terms, this reduces the number of questions that can be addressed in any given experiment. Moreover, large scale procedures do not allow the researcher to examine the variability in microbial composition of individuals under the same diet.

The differences in DNA extraction efficiency observed for the methods used in this study suggest that while one method might be efficient for one set of natural samples (e.g., soil) it might not be for another (such as insect guts). For instance, organic content of the samples is known to affect the yields of extracted DNA from environmental samples (Atlas *et al.*, 1992; Tsai and Olson, 1992). Also, the composition of the microbial community will most certainly have an impact in the yields of DNA recovered. DNA extraction procedures are selective (i.e., some bacteria resist lysis) which in turn means that the DNA sample does not precisely represent the *in situ* bacterial community. The fact that the yields of different DNA extraction methods are significantly different should not be

overlooked when analyzing microbial communities, especially since genetic information missing might belong to important members of the microbial community.

Since most of the methods for nucleic acid extraction from environmental samples have been developed for gram negative bacteria, an alternate method, originally developed to extract DNA from a wide range of diverse bacteria, including aerobic and anaerobic gram positive and gram negative bacteria, was considered (Visuvanathan *et al.*, 1989). To effectively lyse the bacterial cells, it relies on a heat shock pretreatment, followed by treatments of a high concentration of lysozyme, detergent, two different proteases and longer incubation periods than in commonly used procedures. It should be noted that in initial attempts plate counts suggested that more than 96 % of the culturable bacteria of *A. domesticus* survived or resisted the lysing steps of this method. The long incubation times were suspected of allowing the bacteria that resisted the lysis treatments to multiply, which accounted for the high viable counts. Similarly, the AODC decreased less than 20 % of after the lysis procedure. Sodium azide (0.1 % final concentration) was then added after the heat pretreatment. This additional step appeared to remidiate the problem since the AODC of the hindgut community decreased 90 % and no culturable bacteria were detected (i.e., > 0.01 %) post cell lysis treatment.

The amplification of DNA sequences using the PCR method is now routine in molecular biology. Nevertheless, successful amplification of DNA by PCR in part depends on the quality of the template. For example, organic contaminants could coprecipitate

with nucleic acid (e.g., polysaccharides and humic acids) and interfere with DNA polymerases. Therefore, attaining a reliable amplification product from the hindgut community underlines the quality of the extracted DNA.

References

Ausubel, FM, R Brent, RE Kingston, DD Moore, JG Seidman, JA Smith, K Struhl. 1987. Current protocols in molecular biology. Greene Publishing Associates and Wiley-Interscience. New York, NY

Atlas, R. M. 1985. Diversity of microbial communities. Adv. Microb. Ecol. **7**:1-47.

Atlas, RM, G Sayler, RS Burlage, and AK Bej. 1992 Molecular approaches for environmental monitoring of microorganisms. Biotechniques. **12**: 706-717.

Barry, T R Powell, and F Gannon. 1990. A general method to generate DNA probes for microorganisms. Biotechnology **8**:233-236.

Burton, K. 1956. A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of DNA. Biochem. J. **62**:315-323.

Hobbie, JE, RJ Daley, and S Jasper. 1977. Use of Nuclepore filters for counting bacteria by fluorescent microscopy. Appl. Environ. Microbiol. **33**:1225-1228.

Holben, W. E. and J. M, Tiedje. 1988. Applications of nucleic acid hybridization in microbial ecology. Ecol. **69**:561-568.

Holben, WE, JK Jansson, BK Chelm, and JM Tiedje. 1988. DNA probe method for the detection of specific microorganisms in the soil bacterial community. Appl. Environ. Microbiol. **54**: 703-711

Jones, CL, CJ Sprecher, and JW Schumm. 1989. An oligonucleotide probe to assay lysis and DNA hybridization of a diverse set of bacteria. Anal. Biochem. 181:23-27.

Kaufman, MG. 1988. The role of anaerobic bacterial metabolism in the nutrition of crickets (Orthoptera : Gryllidae). Ph.D. Thesis, Michigan State University, East Lansing, MI USA

Kaufman, MG, and MK Klug. 1991. The contribution of hindgut bacteria to dietary carbohydrate utilization by crickets (Orthoptera : Gryllidae). Comp. Biochem. Physiol. **98**:117-123.

Lane, D. 1991. 16S/23S rRNA sequencing. In *Nucleic acid techniques in bacterial systematics.* E Stackebrandt and M Goodfellow (editors). John Wiley and Sons, New York, NY. pp. 45-68.

Martoja, R. 1966. Sur quelques aspects de la biologie des orthoptires in relation avec la presence de concentrations microaiennes (bacterial intertinals, rickettsies). Ann. Entomol. Fr. **2**:753-840.

Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory Press. NY

Sorheim, R, V. L. Torsvik, and J. Goksoyr. 1989. Phenotypical divergences between populations of soil bacteria isolated on different media. Microb. Ecol. **17**:181-192.

Torsvik, V, J Goksoyr, and FL Daae. 1990. High diversity on DNA of soil bacteria. Appl. Environ. Microbiol. **56**:782-787.

Tsai, YL and BH Olson. 1992. Rapid method for separation of bacterial DNA from humic substances in sediments for polymerase chain reaction. Appl. Environ. Microbiol. **58**: 2292-2295.

Tsai, YL and BH Olson. 1991. Rapid method for direct extraction of DNA from soil and sediments. Appl. Environ. Microbiol. **57**: 1070-1074.

Urlich, RG, DA Buthala, and MJ Klug. 1981. Microbiota associated with the gastrointestinal tract of the common house cricket, *Acheta domesticus.* Appl. Environ. Microbiol. **41**: 246-254.

Visuvanathan, S, MT Moss, JL Stanford, J Hermo-Taylor, and JJ McFadden. 1989. Simple enzymic method for isolation of DNA from diverse bacteria. J. Micro. Methods **10**:59-64.

Chapter 2. Effect of changes in diet on the structure and function of the bacterial hindgut community of crickets

ABSTRACT

The effect of switching to alfalfa, pulp, and protein based diets on the structure and function of the hindgut bacterial community of crickets was monitored using CsCl-bisbenzimide gradients and measuring fermentation products profiles. CsCl-gradients showed that the hindgut community was normally composed of bacteria with a guanine plus cytosine (G+C) DNA content between 32-57 %. The % G+C of the gut community DNA showed that there was a reduction in the diversity of bacterial populations in both pulp and protein fed crickets compared to those fed chow and alfalfa. The bacterial densities, however, were not significantly different for the four diets. The protein-based diet resulted in a decrease in the rate of evolution of volatile fatty acids, even though the ratio of butyrate production to acetate and propionate production was significantly higher in these crickets. Crickets fed pulp and protein diets showed a decrease in hydrogen and carbon dioxide production indicating that those diets also had an effect on the biochemical activity of the hindgut community. These results suggest that changes in the cricket diet can affect the hindgut microbial processes by changing the structure of the bacterial community.

Insect-microbial associations are commonly found through a wide range of different families of insects (Buchner, 1965). Based on the physical association with the insect, microbes can be classified as intracellular symbionts, ectosymbionts, or endosymbionts (Bousch and Coppel, 1974). In most cases these insect-microbe interactions have as a common denominator a nutritional component. For example, ribosomal messenger termination of intracellular symbionts of aphids seem to be regulated by the host while it has been argued that the microbial symbionts provide essential amino acids to the insect (Baumman *et al.*, 1993). In other insects, like *Atta* ants, fungal ectosymbionts are used as a food source while the invertebrate maintains optimal conditions for the microbe to outgrow other fungal species (Cherret *et al.*, 1989). Also, numerous studies have suggested that insect endosymbiont activity is important in complementing the host's diet, which is normally poor in available nitrogen and carbon (Breznak, 1984).

The latter associations have received considerable attention by microbial ecologists particularly because the microbes are found in high densities in specialized areas inside the gastrointestinal tract of many insects. Moreover, some of these insect microbial communities play important ecological roles at a global scale. For instance, microbial symbionts of wood-feeding termites are responsible for cellulose degradation, an abundant but non-labile carbon source for insects. Termites have been suggested to significantly contribute to the global production of methane, an important green house gas; gut microbiota are entirely responsible for the production of this gas (Collins and Wood, 1984; Zimmerman *et al.*, 1982).

Despite the fact that many diverse insect families also harbor gut microbial symbionts, very little is known about the gut microbial communities other than termites (Bayon and Mathlin, 1980; Bracke *et al.*, 1978; Klug and Kotarski, 1980). Gut microbial communities are of a complex nature, not only at the physiological level, but at the structural level. It is believed that dozens or even hundreds of different microbial species coexist at any given point in the gut of ruminants. Likewise, it is conceivable that the guts of many insects will harbor complex and diverse microbial communities made of bacteria with unique physiological requirements, and likely difficult to study through the use of classical culturing methods. Hence, methods that do not rely on culturing microorganisms, like DNA based methods, should allow the microbial ecologists to more accurately examine these communities.

In recent years, only a few studies have investigated how insect microbial communities might react to changes in the nutritional quality of food. Kane and Breznak (1991) noted that the switch to low fiber diets affected the numbers of viable bacteria (i.e., streptococci and lactobacilli) inhabiting the hindgut of cockroaches. On the other hand, studies with crickets have shown that both the number of viable bacteria and direct microscopic counts do not change with changes in the host diet, even though the profile of fermentation products changes (Kaufman, unpublished results). It is unknown whether the new community fermentation profile is a product of shifts in bacterial populations or due to the induction of new enzymatic activity without significant changes in the relative abundance of the established gut microbiota.

These studies were undertaken to understand how the structure and function of the cricket hindgut bacterial community is affected by dietary perturbations. Changes in bacterial community structure were monitored by observing changes in the % guanine plus cytosine (% G+C) using CsCl-bisbenzimide gradients. Rates of production of fermentation products were measured as an index of the metabolic status of the hindgut microbial community. Results suggest that changes in the host diet affect both the structure and function of the insect's microbial community.

Materials and Methods

Cricket diets and gut prepartion. House crickets (*Acheta domesticus*) were reared in the laboratory until adults on a diet of Purina chow. They were then shifted to three main diets containing approximately 48 % of alfalfa-hay, beet-pulp, or protein (casein), 50 % alphacel (indigestible fiber), 1 % of fat (cholesterol) as well as salts and vitamins following Kaufman (1988). The % of easily digestible and water soluble components were 50 %, 51 %, 17 %, and < 1 % for chow, pulp, alfalfa, and protein diet, respectively. Other crickets were maintain in chow for comparison. After 5 days under these diets, the crickets were sacrificed and the hindguts were surgically removed using fine forceps with the help of a dissecting microscope. Hindguts were homogenized using a sterile tissue grinder. A subsample of the homogenized guts was diluted in sterile phosphate-buffered saline in preparation for viable counts. Another subsample was used for microscopic counts.

Viable counts were determined as aerobic plate counts using Trypticase Soy Agar (Difco, Detroit, MI) incubated at 30^0 C for 48 h. The acridine orange direct count (AODC) method was used to determine total microscopic counts (Hobbie *et al.*, 1977). For this method, subsamples from hindgut homogenates were fixed in filter-sterilized 3.7 % formaldehyde phosphate buffered solution and stained with acridine orange (0.01 % final concentration). Samples were filtered through a 0.2 µm pore size irgalan black pre-stained polycarbonate membrane (Costar, Nuclepore Filtration Products, Cambridge, MA) with the help of a vacuum pump. Membranes were then placed on a microscope slide and stored at 4^0 C in the dark until ready for microscopic observation. Fluorescing cells from 10 randomly selected fields were counted using an 63X oil immersion objective on a Leitz Orthoplan 2 epifluorescence microscope (Leitz, Germany). The average number of bacteria from 10 fields was used to calculate bacterial densities.

Bacterial activity measurements. To ensure minimal disruption of the community, hindguts were surgically removed and immediately transferred to vials containing reduced phosphate saline buffer and an anaerobic head space. Triplicates consisting of individual cricket hindguts were used for these analyses. Incubations were also performed under anoxic conditions following a protocol described elsewhere (Lovley and Klug, 1982). The concentration of volatile fatty acids (VFA) was determined by high pressure liquid chromatography. Hydrogen (H_2) and carbon dioxide (CO_2) concentrations were measured using a gas chromatograph.

Rates of VFA, H_2, and CO_2 production were determined over a 3 h period.

Genomic DNA extraction. To separate bacteria from the eukaryotic tissue, the homogenized hindguts were quickly centrifuged (1 sec) at 11,200 x *g* using an Eppendorf microcentrifuge (Model 5415C; Brinkmann Instruments Inc., Westbury, NY). To concentrate the bacterial community, the supernatant was collected and centrifuged for 5 min. at 6,800 x *g*. Genomic DNA was extracted from the bacterial pellet following a modified version of the procedure described by Visuvanathan *et al.* (1989) as it was developed to lyse various gram positive and gram negative bacteria. Bacterial fractions were resuspended on a 400 µl TE (10 mM Tris, 1 mM EDTA) / sodium azide (2 % w/v; Sigma, St. Louis, MO) solution. Subtilisin (50 µl of a 50 mg/ml stock) was added and samples were incubated at 37^0 C for 7 h. Cells were treated with lysozyme (100 µl of 200 mg/ml stock) for 3 h at 50^0 C and then with sodium dodecyl sulfate (SDS, 100 µl of 20 % stock) and pronase (50 µl of 25 mg/ml stock) for 7 h at 37^0 C. Phenol/chloroform extractions were performed following a hexadecyltrimethylammonium bromide (CTAB) pretreatment (Ausubel *et al.*, 1987). The organic phase was then treated with RNaseA to degrade RNA followed by an additional phenol/chloroform extraction. To precipitate the DNA, isopropanol (0.6 vol) was added to the samples and mixed gently before storing the sample for 16 h at -20^0 C. Samples were centrifuged at 14,000 rpm at 4^0 C for 15 min. The supernatant was carefully removed and the DNA pellet allowed to air dry. The DNA was then dissolved in sterile deionized water.

Hindgut community % G+C profiles. A modified version of the CsCl-bisbenzimide method developed by Holben *et al.*, (1992) was used in this study. Approximately 50 µg of bacterial community was added to a CsCl (Boeringer Mannheim Corp., Indianapolis, IN) solution of refractive index (RI) of 1.4000 and adjusted to a final RI of 1.3990. Bisbenzimide was added (10 µl from a 1 mg/ml bisbenzimide stock solution) to the CsCl-DNA solution, mixed well, and transferred to ultracentrifuge tubes. Samples were centrifuged at 110,000 x *g* for 72 h using a TFT65.6 fixed-angle rotor on a Sorvall OTD55B ultracentrifuge (Sorvall Instruments, Wilmington, DE). The density gradients were carefully removed from the rotor and photographed using Kodak 160T Ektachrome color film. Gradients were aliquoted in microcentrifuge tubes as 100 µl fractions using a gradient fractionator (Model 640, ISCO, Lincoln, NE). The RI of each fraction was determined using a Bausch and Lomb refractometer (Model ABBE-3L, Milton Roy Co., Rochester, NY). The DNA concentration of each fraction was determined by UV absorption (OD_{260}). The % G+C of each fraction was determined from the RI using a standard curve of % G+C vs. RI made with genomic DNA of bacteria of known % G+C (*Clostridium perfringens*, 31.0 %; *Enterobacter aerogenes*, 57.5 %; *and Corynebacterium flacumfaciens* 70.4 %; Fig. 1).

Results

Hindgut bacterial densities. The aerobic viable counts of the *A. domesticus* microbial community ranged from 1.3 x 10^8 to 8.5

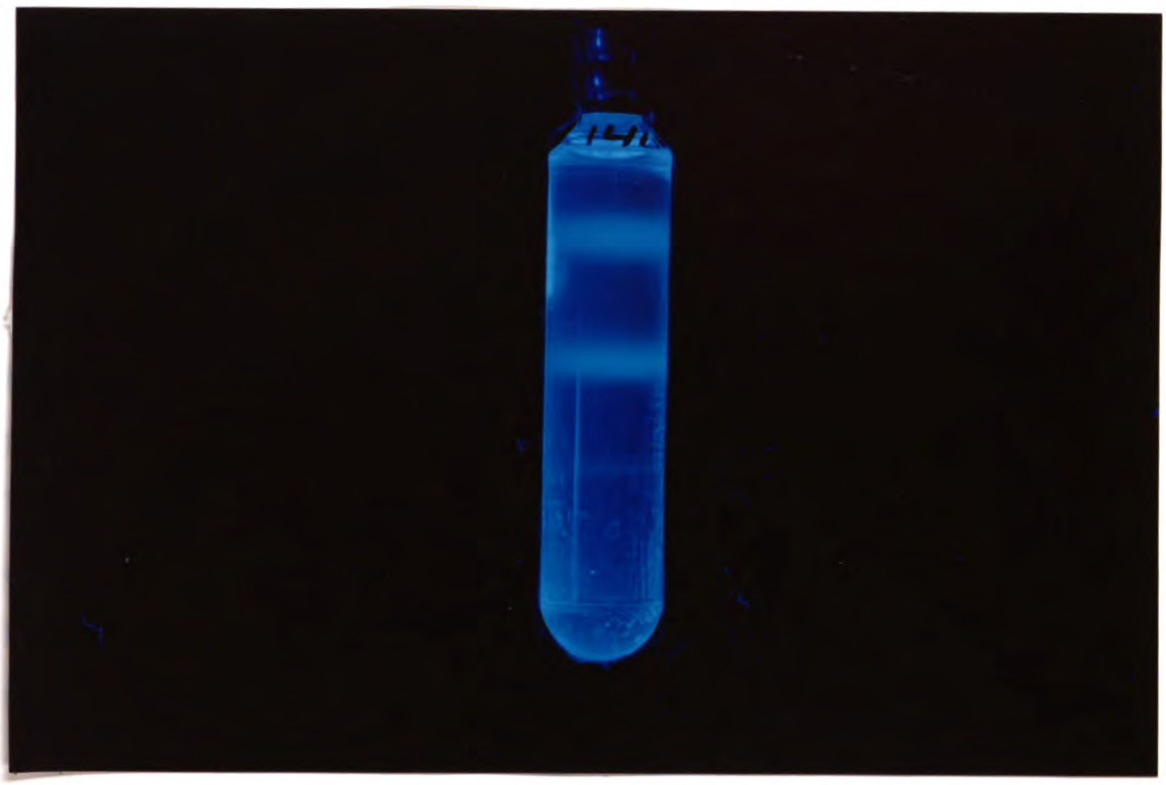

Figure 1. Genomic DNA of *C. perfringens* (31 % G+C), *E. aerogenes* (57 % G+C), and *C. flacumfaciens* (70 % G+C) in a CsCl-bisbenzimide gradient.

x 10^7 cells per gut (Fig. 2). The culturable bacterial fraction averaged about 7 % of the direct microscopic counts. Neither the total culturable bacteria nor the direct microscopic counts differed significantly between diets. These results are in agreement with previous observations (Kaufman, unpublished results).

Community fermentation products. Rates of production of different fermentation products were measured as an index of the metabolic activity of the hindgut community DNA. Rates of CO_2 evolution were significantly less in crickets fed with the protein-based diet (Fig. 3; $P<0.05$). Similarly, less hydrogen was evolved by the hindguts of crickets subjected to pulp and protein diets ($P<0.05$). While the rate of total VFA production for crickets on the protein diet was less than for crickets on alfalfa or pulp (Fig. 4a), the ratio of butyrate to acetate or to propionate was higher in the protein diet than in the other three diets (Fig. 4b).

Community % G+C profile. To further investigate the effect of diet changes on the structure of hindgut bacterial communities, changes in proportions of DNA with different G+C content were examined. Crickets fed on chow and alfalfa exhibited a somewhat homogeneous bacterial community composed of populations with between 32 and 57 % G+C (Fig. 5). However, bacteria of 35 to 45 % G+C content were more abundant in crickets fed alfalfa than in those fed chow. In contrast, the pulp diet resulted in two distinct bacterial populations, one of 35 to 38 % G+C and the other of 43 % G+C. A bimodal distribution was also observed in communities from crickets fed protein but their % G+C content was

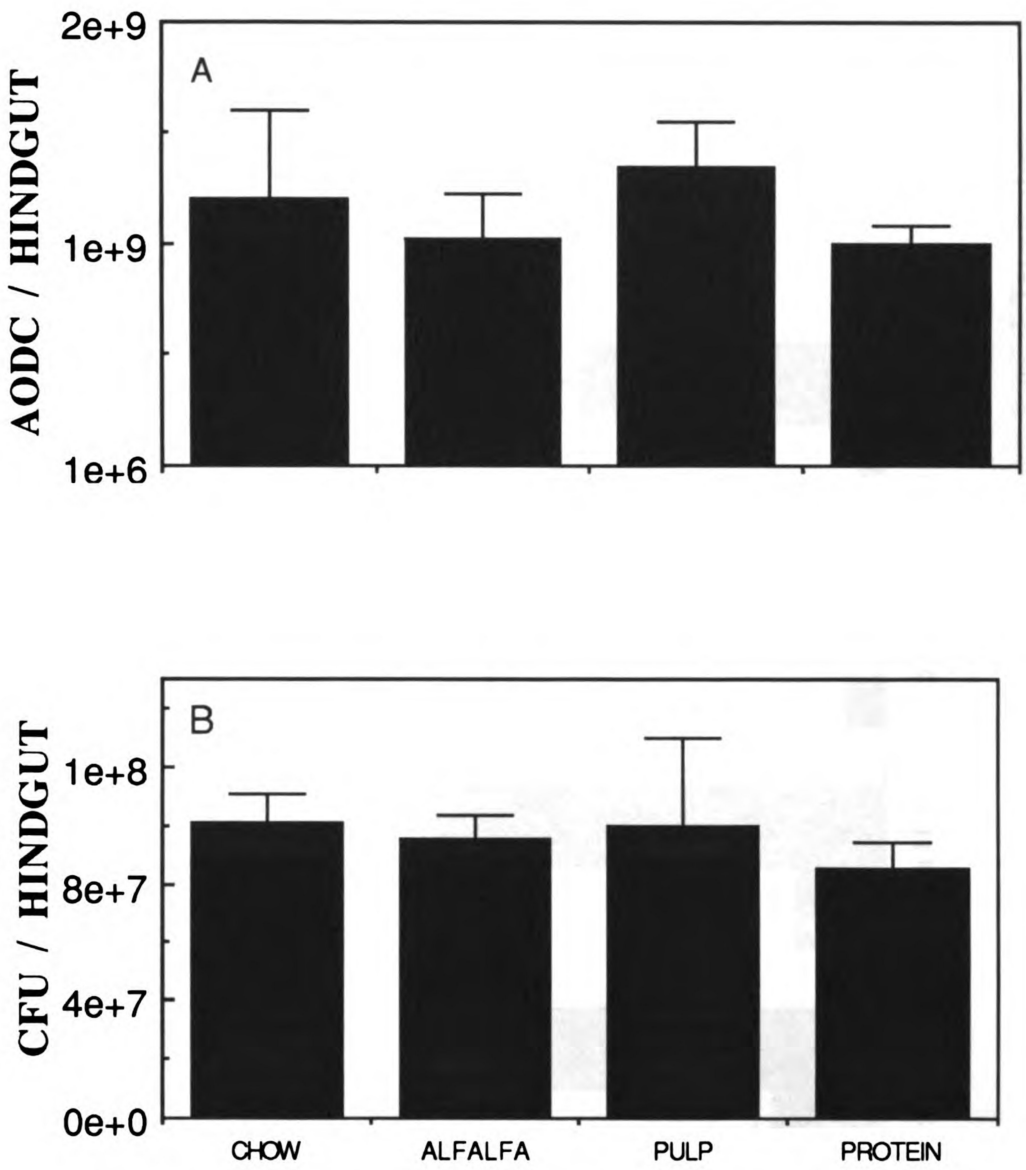

Figure 2. Hindgut bacterial densities of crickets subjected to different diets. (A) Acridine orange direct counts (AODC); (B) colony forming units (CFU) as determine by TSA plate counts.

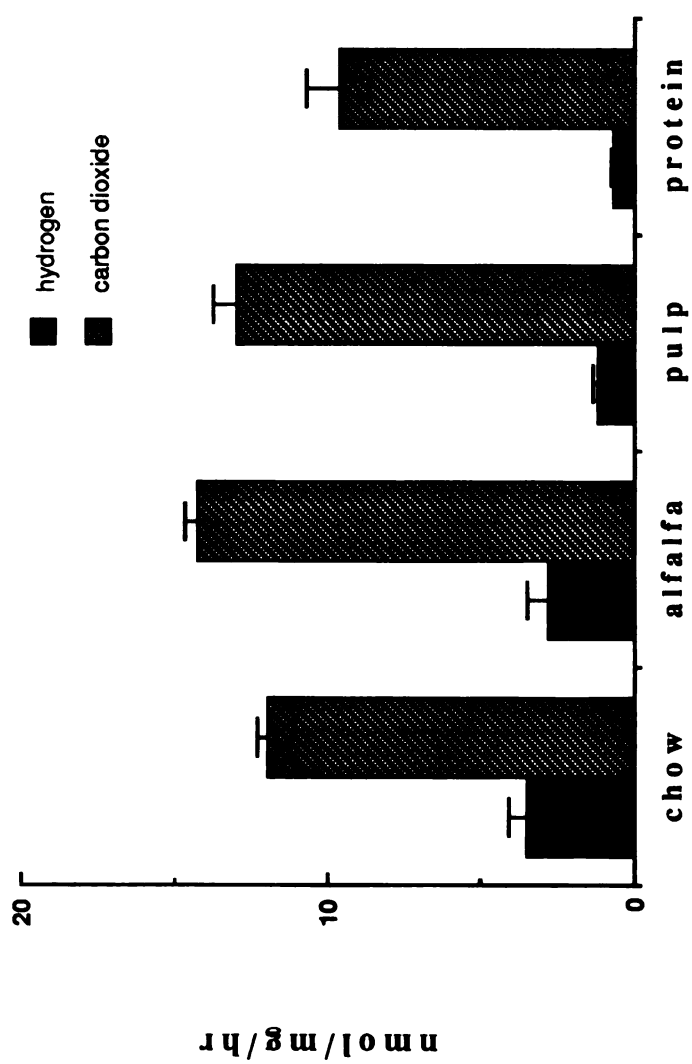

Figure 3. Evolution rates of H2 and CO2 for crickets subjected to different diets.

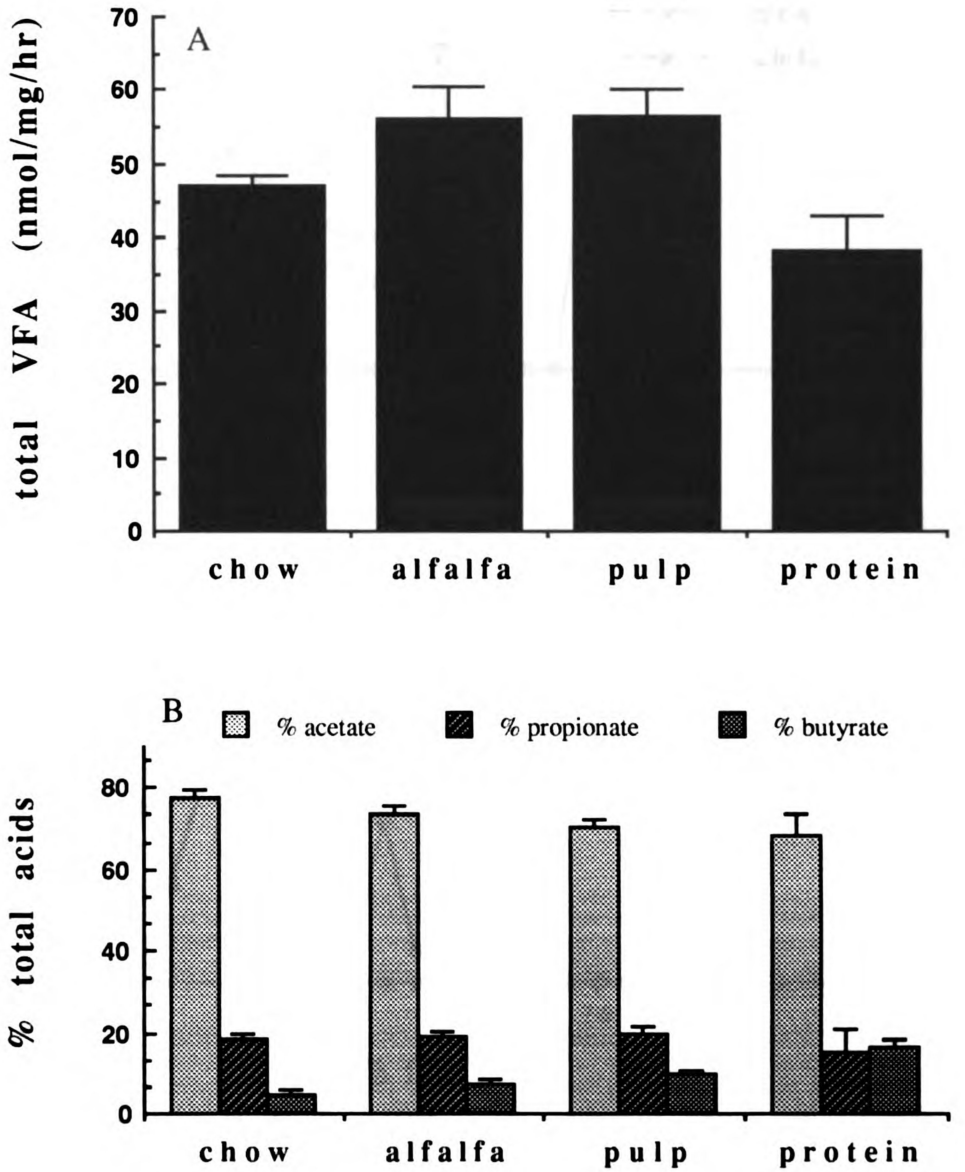

Figure 4. Rates of volatile fatty acids (VFA) in crickets subjected to different diets. (A) Total VFA. (B) Per cent acetate, butyrate, and propinate of the total VFA.

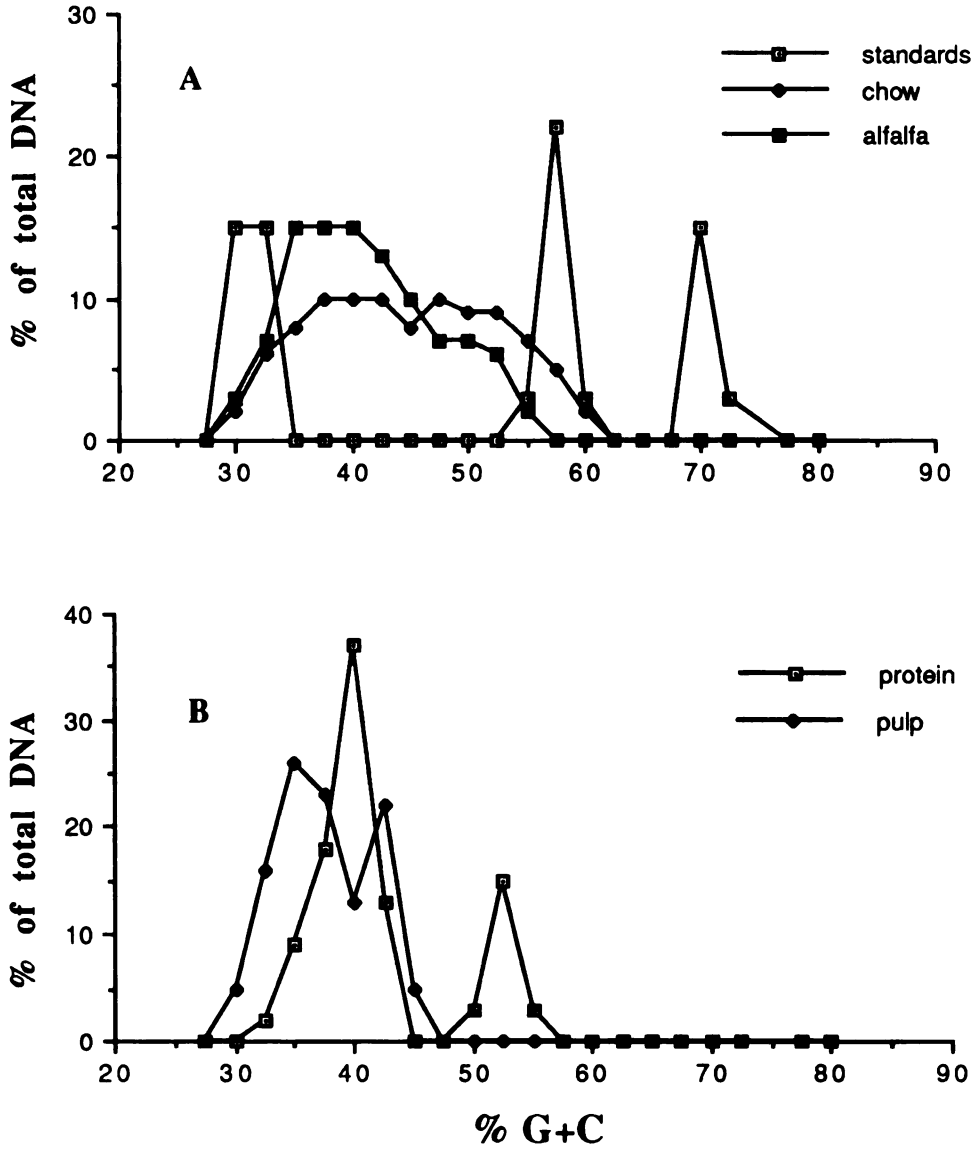

Figure 5. Community % G+C profiles of crickets subjected to different diets. (A) DNA from crickets fed chow or alfalfa; (B) DNA from crickets fed pulp or protein. Standards were DNA from pure cultures of *C. perfringens* (31.0 % G+C), *E. aerogenes* (57.5 % G+C), and *C. flacumfaciens* (70.4 % G+C).

higher; the most abundant populations were those of 37 to 43 % G+C with a secondary population of 50 to 55 % G+C.

Discussion

In order to understand how changes in insect diet affect the structure and function of the hindgut microbial community, the profile of the hindgut bacterial community as determined by the % G+C of its DNA and the rate of fermentation products of crickets was evaluated after the crickets were switched from a chow diet to alfalfa-, pulp-, and protein-based diets. Diet treatments changed both the community structure and the rate of H_2, CO_2, and VFA production while the total microbial population remained constant. Therefore, both the structure and biochemical functions of the cricket hindgut community are altered due to changes in the host diet. This was more evident in pulp and protein diets where the effect was an apparent reduction in the hindgut microbial diversity as suggested by a decrease in bacterial populations with different G+C content and by a concurrent decrease in VFA production.

An increase in the proportion of butyrate in the total VFA pool was observed for crickets fed on the protein diet. Conversely, in mammals the gut microbiota seem to produce more butyrate when fed on high fiber diets (Moir, 1991). The occurrence of butyrate in higher proportions in the protein diet could be due to the type of bacteria favored. For instance, butyrate production is higher in fusobacteria than in other many fermenters (Holt and Krieg, 1984). Indeed, the presence of fusobacteria was detected using

fluorescently labeled ribosomal probes specific for this group (Chapter 3). However, no increase in density of this bacterial group was detected in crickets fed the protein diet over the other diets. It is possible that the protein based diet might have induced an increase in butyrate production by fusobacteria without promoting a significant increase in its density. Alternatively, the rate of butyrate utilization by hindgut bacteria might have been greater in crickets subjected to the other diets.

The % G+C profile presents an alternative method to examine the structure of microbial communities. The principle is based on the preferential binding of bisbenzimide to adenine and thymidine (AT) rich regions. When genomic DNA is centrifuged on CsCl-bisbenzimide gradients, bacteria with different % G+C content will migrate to different positions within the gradient due to the density changes caused by the binding of the dye (Fig. 1). Using this approach, Holben *et al.*, 1992 concluded that water saturation of soils dramatically changed the structure of the soil microbial community. Even though it is reasonable to conclude that two different microbial communities are present if they have different % G+C profiles, limited information related to the functional status of the community is derived from this technique. Since this technique detects changes in the proportions of bacterial populations, this information could be coupled with knowledge of the changes in microbial processes to identify the specific taxonomic groups that respond to dietary perturbations.

Little information on the effect of diets on minor members of the community can be derived from the results in this study. From

use of this technique it seems unlikely that the presence of populations less than 10 % of the total community would be detected. Hence, it is concluded that the results in this study only reflect changes in the numerically abundant bacteria within the hindgut. However, it is reasonable to assume that processes are in part driven by the most abundant group of organisms inhabiting any ecosystem, mostly due to their importance in carbon processing. They in turn, play an active role in the energy flow and in maintaining the dynamics of the ecosystem. Therefore, the assumption that perturbations affecting the most numerically dominant organisms will directly affect the minor components of the cricket hindgut bacterial community is probably valid.

The interpretation given to the results obtained in this study has received further support when using fluorescently labeled ribosomal probes (Chapter 3). These studies have shown that the relative abundance of several phylogenetically related bacterial populations within the cricket hindgut were altered by changes in the host diet. While the ecological significance of these results remains unclear, it could be argued that they imply that insects which have a nutritional dependence on their microbial symbionts might prevent structural and functional changes of their gut microbiota by feeding on a narrow window of diets.

ACKNOWLEDGMENTS

I thank William E. Holben, Dave Harris, and Mary Ann Bruns for their technical advice in the use of CsCl-bisbenzimide gradients. This study was supported by National Science Foundation Grant No. NSF BIR-9120006.

References

Ausubel, FM, R Brent, RE Kingston, DD Moore, JG Seidman, JA Smith, K Struhl. 1987. Current protocols in molecular biology. Greene Publishing Associates and Wiley-Interscience. New York, NY

Baumann, MA Munson, CY Lai, MA Clark, L Baumann, NA Moran, and BC Campbell. 1993. Origin and properties of bacterial endosymbionts of aphids, whiteflies, and mealybugs. ASM News **59**:21-24.

Bayon, C and J Mathelin. 1980. Carbohydrate fermentation and byproduct absorption studied with labeled cellulose in *Oryctes nasicornis* (Coleoptera:Scarabeidae). J. Insect Physiol. **26**:833-840.

Bousch, GM and HC Coppel. 1974 Mutualism between arthropods and microorganisms. In *Insect diseases.* GE Cantwell (editor). Marcel Dekker, Inc. New York, NY

Bracke, JW, DL Cruden, and AJ Markovetz. 1978. Effect of metronidazole on the intestinal microflora of the American cockroach, *Periplaneta americana* L. Antimicrob. Agent Chemother. **13**:115-120.

Breznak, JA. 1984. Biochemical aspects of symbiosis between termites and their intestinal microbiota. In *Invertebrates - microbial interactions.* JM Anderson, ADM Rayner, DWH Walton (editors). Cambridge University Press, London. pp. 173-203

Buchner, 1965. Endosymbiosis of animals with plant microorganisms. Interscience Publishers. John Wiley & Sons, Inc., New York.

Collins, NM and TG Wood. 1984. Termites and atmospheric gas production. Science **224**:84-85.

Cherret, JM, RJ Powell, and DJ Stradling. 1989. The mutualisms between leaf-cutting ants and their fungus. In *Insect-fungus interactions.* N Wilding, NM Collins, PM Hammond, and JF Webber (editors). Academic Press, New York, NY, pp. 93-120.

Hobbie, JE, RJ Daley, and S Jasper. 1977. Use of Nuclepore filters for counting bacteria by fluorescent microscopy. Appl. Environ. Microbiol. **33**:1225-1228.

Holben, WE, D Harris, and E Paul. 1992. Community level analyses: bacteria community profiles based on the % G+C content of the DNA of the component populations. Center for Microbial Ecology Research Findings. pp. 13-15.

Holt, J and NR Krieg. 1984. Bergey's manual of systematic bacteriology. Vol. 1. Williams and Wilkins, Baltimore, MD. pp. 631-636.

Kane, MD and JA Breznak. 1991. Effect of host diet on production of organic acids and methane by cockroach gut bacteria. Appl. Environ. Microbiol. **57**:2628-2634.

Klug, MJ and S Kotarski. 1980. Bacteria associated with the gut tract of larval stages of the aquatic cranefly *Tipula abdominalis* (Diptera:Tipulidae). Appl. Environ. Microbiol. **40**:408-416.

Lovley, DR and MJ Klug. 1982. Intermediary metabolism of organic matter in the sediments of a eutrophic lake. Appl. Environ. Microbiol. **43**:552-560.

Moir, RJ. 1991. The role of microbes in digestion. In *Microbiology of animals and animal production.* JB Woolcock (editor). Elsevier, New York, NY, pp. 29-60.

Visuvanathan, S, MT Moss, JL Stanford, J Hermo-Taylor, and JJ McFadden. 1989. Simple enzymic method for isolation of DNA from diverse bacteria. J. Micro. Methods **10**:59-64.

Zimmerman, PR, JP Greenberg, SO Wandiga, and PJ Crutzen. 1982. Termites: a potential large source of atmospheric methane, carbon dioxide, and molecular hydrogen. Science **218**:563-565.

Chapter 3. Use of Fluorescent Ribosomal Probes to Describe the Cricket Gut Microbial Community.

ABSTRACT

The presence and relative abundance of different bacterial groups within the hindgut of crickets was determined by *in situ* hybridization studies using fluorescent oligonucleotide probes targeting the 16S rRNA. Universal probes hybridized to an average of 98 % of the microbes inhabiting the hindgut of *Acheta domesticus*. The intensity of the hybridization signal suggested that most bacteria are metabolically active. Similar results were obtained for the hindgut community of *Gryllus pensilvaniscus*, *G. reubens*, *Scapteriscus borrelia*, and *S. vicinus*. Ribosomal probes specific for eubacteria indicated that approximately 93, 95 and 88 % of the hindgut microbiota in *A. domesticus*, *Gryllus* sp., and *Scapteriscus* sp., respectively, is of eubacterial origin. Using probes specific for phylogenetically coherent groups, the following bacteria were found: low G+C Gram positive bacteria, alpha proteobacteria, bacteriodes, sulfate reducing bacteria, fusobacteria, bifidobacteria, enteric bacteria and methanogens. These bacterial groups comprised more than 50 % of the microbiota present in *A. domesticus* and *Gryllus* spp., but only 15 % of those present in*Scapteriscus* spp. While the hindgut of the different crickets examined harbor similar bacterial groups, differences in the overall community structure were significant. In addition, hybridization studies showed that the densities of sulfate reducing bacteria increased in crickets fed protein and alfalfa diets while the relative abundance of bacteriodes and low G+C Gram positives decreased in crickets fed a beet-pulp

based diet. These results showed that dietary perturbations changed the structure of hindgut microbial community of *A. domesticus* by altering the relative abundance of the most numerically dominant bacteria in the cricket hindgut.

Our knowledge of microbial communities inhabiting insect guts is fragmentary. Ironically, such microbial communities are important components of processes linked to global carbon cycling (Davies, 1988), production of green house gases (Zimmerman *et al.*, 1982) and transformation of plant compounds toxic to herbivores (Dowd, 1992). Insect gut microbiota also represent an untapped source of novel microbial diversity (Breznak et al., 1988; Kane and Breznak, 1991). In contrast with soil and aquatic environments, the insect gut is a contained ecosystem and therefore a good model to study the assemblage and interactions of complex microbial communities. Despite this fact, only few studies have examined in detail the composition of insect gut microbiota (Bignell, 1984; Schultz and Breznak, 1978).

In the past, several studies have shown that the cricket hindgut harbors a bacterial community, dominated by anaerobic and facultative Gram negative rods (Urlich *et al.*, 1981). These studies have relied on culturing techniques. It is now well recognized that such approaches ignore the bacteria that are difficult to isolate or culture. Recently developed DNA based technology can circumvent some of the shortcomings of the classical methods since they do not rely on the culturability (Sommerville *et al.*, 1989; Steffan *et al.*, *1988*). For instance, DNA gene probes have been used to determine the presence of functionally related microbes in a variety of ecosystems without the need of culturing microorganisms (Holben *et al.*, 1992; Barkay *et al.*, 1989). However, the use of DNA gene probe methodology on environmental samples also has its limitations: high purity DNA is generally required because contaminants that

coprecipitate with the nucleic acid could interfere with the hybridization of the targeted gene. Also, DNA extraction methods are biased against bacteria that are resistant to cell lysis. In addition, lack of hybridization signal might be due to the limited sensitivity of the technology when targeting single copy genes and not to the absence of the targeted microorganisms. Consequently, all these factors need to be taken into consideration when applying gene probes to study natural microbial communities.

The use of *in situ* hybridization with fluorescently labeled ribosomal probes is an approach that overcomes several of the limitations of the gene probe technology (Braun-Howland *et al.*, 1992; Manz *et al.*, 1992; Amman *et al.*, 1990a). *In situ* hybridization is performed without nucleic acid extraction or use of radioactive probes. Also, the detection sensitivity increases several orders of magnitude since the target molecule (i.e., the ribosomal RNA) is intrinsically amplified. Furthermore, due to the physiological role of the ribosomes in all organisms, rRNA-targeting probes also reveals the *in situ* metabolic status of microbes (Poulsen *et al.*, 1993).

Studies using fluorescently labeled ribosomal probes have shown that this approach can provide information related to the composition of natural microbial communities (Manz *et al.*, 1993; Amman *et al.*, 1990b). For example, Wagner *et al.* (1993) have combined different fluorescent phylogenetic probes to visualize the association between different bacterial groups. This methodology has also been used for the detection and quantification of bacteria within complex communities (Amman *et al.*, 1992a). Other studies have used this technology to demonstrated the presence of

intracellular symbionts in the nuclei of ciliates (Amman *et al.*, 1991). In general, phylogenetic probes could provide a more complete assessment of the microbial diversity in different environments.

Here, I use rRNA-targeted, fluorescently labeled oligonucleotide probes to describe the composition of the hindgut microbial community of several species of cricket. I also determined how changes in diet affect the different bacterial groups inhabiting the hindgut of the house cricket, *Acheta domesticus*. These studies demonstrate that the densities of some bacterial groups shift after changes in the host diet.

MATERIALS AND METHODS

Isolation of hindgut community. House crickets (*Acheta domesticus*) were reared in the lab on Purina chow until last molt. Adults were then switched to alfalfa-, protein-, or pulp-based diets or kept on chow as previously described (Kaufman, 1988). After 5 days hindguts were surgically removed, immediately fixed on a 3.7 % solution of formaldehyde in phosphate buffered saline (PBS), homogenized using a sterile tissue grinders, and centrifuged (1 sec at 11,200 x *g* using a microcentrifuge) to remove eukaryotic tissue. Supernatant was collected and centrifuged for 1 min. at 6,800 x *g*. The bacterial pellet was resuspended in PBS and kept at 4^0 C until use.

Mole crickets, *Scapteriscus borrelia, S. vicinus, and* the field cricket, *Gryllus reubens*, were collected in suburban grasslands in North-central Florida by Thomas Walker (University of Florida,

Gainesville). Specimens of *G. pensilvanicus* were collected in a similar habitat but in Central Michigan by Sheridan Haack and hte author. The hindgut bacterial community from these cricket species was obtained and processed following the procedures described above.

Fixed hindgut samples were diluted into filter-sterilized PBS and spotted onto 10-well toxoplasmosis microscope slides (HTC-7, Cel-Line Associates, New Field, NJ) which had been baked at 195^0 C and coated with gelatin as described elsewhere (Giovannoni *et al.*, 1988). Samples were allowed to air dry at 37^0 C and stored desiccated at room temperature until ready for hybridization. Bacteria on slides fixed this way store well for several months.

Development and use of phylogenetic probes. Specificity of the archaeal (291, 635), universal (A+B+C), and eubacterial (342) probes have published elsewhere (Lane *et al.*, 1985). Group specific ribosomal probes for members of the Proteobacteria (α, β, and γ), Gram positive bacteria with low and high % G+C and sulfate reducing bacteria were developed and described in a previous study (Nierzwicki-Bauer, unpublished results). Ribosomal probes specific for *Bacteriodes* sp., *Fusobacteria* sp., *Bifidobacteria* sp. and enterics were developed for this study.

Sequences used for probe development were obtained with the help of computer software (16S rRNA Tool program) generated by Peter Floriani (Rensselear Polytech Institute, Troy, NY). The program compares the 16S rRNA sequence of the bacterial strain or bacterial groups of interest against a library of 16S rRNA sequences from approximately 1200 bacterial species (Belgium 16S rRNA data base;

De Rijk et al., 1992). In essence, the outcome of such software is the identification of a region within the 16S rRNA gene that is specific for the bacterium or bacterial group of interest. Ribosomal probes for broad bacterial groups like subdivisions of the Proteobacteria, enterics, sulfate reducing bacteria, and archeae fulfill the following criteria: 15 to 20 nucleotides long with the minimum sequence mismatch needed to include the maximum number of members of each group. For more cohesive groups like true bacteriodes, fusobacteria, and bifidobacteria, regions used as probes showed perfect homology to all the members of each genus in the database.

The phylogenetic probes were synthesized on a solid support using a DNA synthesizer (Model 391, Applied Biosystems, Inc., Foster City, CA). Tetramethylrhodamine-5 (and 6-) isocyanate (TRTC; Molecular Probes, Inc., Eugene, OR) were coupled to the deprotected oligonucleotides via a six-carbon linker containing a free amino terminus (Aminolink 2, Applied Biosystems) and purified as previously described (Braun-Howland *et al.*, 1992).

Slides containing the previously fixed cells were treated with ethanol/formaldehyde (90:10) and hybridized separately with each probe at a probe concentration of 340 ng/ml. Hybridization and washing conditions have been described elsewhere (Braun-Howland *et al.*, 1992). Total bacterial cells were visualized through the inclusion of either fluorescein-5- isothiocyanate (FITC Isomer I; Molecular Probes Inc., Eugene, OR) or 7-diethylamino-3-(4'-isothiocyanatophenyl)-4-methylcoumarin (CPI, Molecular Probes Inc.) in the hybridization mixture.

Microscopic counts. Cells were visualized using a Zeiss Axioskop epifluorescence microscope (Carl Zeiss, Oberkochen, FRG) equipped with a 100W mercury light source, Omega filters (No. 487915-0 for TRTC; No. 487921-0 for CPI and 487910-0 for FITC, Omega Optical, Brattleboro, VT) and a Zeiss 100X Plan Neofluor oil lens with a numerical aperture of 1.3. Photomicrographs were recorded using an Olympus OM-4 camera (Olympus Optical, Japan) and Fujichrome 100 film (Fuji Photo Film Co., Ltd, Tokyo, Japan).

A minimum of approximately 500 CPI stained cells (i.e., blue fluorescing cells) from 15 combined microscopic fields were counted for each individual hindgut. The % of each bacterial group identified was determined as the number of cells hybridizing to the TRTC-labelled probes (i.e., red fluorescing cells) to the total of CPI-stained cells. Triplicates consisting of an individual cricket (i.e., one hindgut per replicate) were use for each hybridization.

Significant differences between bacterial groups were determined by multiple analysis of variance using the SYSTAT statistics package.

RESULTS

Specificity of phylogenetic probes. Oligonucleotide probes targeting bacteriodes, enterics, archaebacteria, sulfate reducing bacteria, Gram positive bacteria of low and high % G+C were empirically validated by testing against a pool of positive and negative bacterial strains (Table 1). Examples of microorganisms that perfectly match each probe are shown in Table 2. It should be

Table 1. Bacteria that hybridize the different probes

Group	Specificity
Low GC	*Bacillus* spp. and *Lactobacillus* spp.
High GC	actinomycetes, *Nocardia* spp.
alpha	*Agrobacterium* spp., *Nitrobacter* spp,
beta	*Pseudomonas stutzeri*
gamma	*Xanthomonas* spp.
sulfate reducers	*Desulfovibrio vulgaris*
enterics	*Escherichia coli, Klebsiella pneumoniae*
bacteriiodes	*Bacteriodes* spp.
fusobacteria	*Fusobacteria* spp.
bfidobacteria	*Bfidobacteria* spp.
Archeae	extremophiles and methanogens
Eubacterial	most eubacteria
Universal	most *Bacteria*, *Archaea*, and *Eucarya*

Table 2. List of some of the bacterial strains used to test the specificity of the ribosomal probes

Strain	Source
Bacteriodes thetataomicron	Michael Cotta
Bacteriodes ovatus	"
Bacteriodes fragilis	"
Bacteriodes vulgaris	"
Prevotella ruminicola	"
Desulfovibrio desulfuricans	James Tiedje
Desulfomonei tiedjie	"
Klebsiella pneumoniae	Michigan State University, Department of Microbiology
Citrobacter freundii	"
Escherichia coli	"
Serratia marcensces	"
Cytophaga sp.	Sandra Nierzwicki-Bauer
Bacillus subtilis	"
Methanosarcina barkeri	"
Corynebacterium sp.	"
Nitrosomonas sp.	MaryAnn Bruns

noted that several probes will not hybridize with all the members within their particular group or to all the species within the same genus. For example, the probes for Gram positives of low % G+C has no mismatches with most *Bacillus* sp., *Lactobacillus* sp., *Streptococcus*, and *Enterococcus* sp. while only a few *Clostridium* sp. or *Mycoplasma* sp. perfectly match the sequence used for the group probe. Also, the probe for the Gram positives high % G+C group does not hybridize to *Bifidobacterium* sp.; however, a genus specific probe was used to detect this bacterial group.

Due to the lack of bifidobacterial and fusobacterial strains, the empirical validation of these probes could not be done. However, no other bacteria in the database perfectly matches either region selected for the probe. In fact, sequences with the highest complementarity were only found outside of the targeted genera, and these sequences had several mismatches which would not allow for cross-hybridization under the hybridization conditions used in this study.

Bacterial groups present in cricket hindgut. A combination of probes that aligned to well conserved regions of the 16S rRNA gene (i.e., universal probes) was used to increase the sensitivity of the fluorescent probe technique. Hybridization studies with the universal probes showed that more than 96 % of all hindgut microorganisms in the species of crickets examined had the ribosomal content necessary to produce hybridization signals (Table 3, Fig. 1). Moreover, the eubacterial probe hybridized to an average of 96, 95, and 88 % of the *in situ* hindgut bacteria of *A. domesticus* fed on chow, *Gryllus* sp., and *Scapteriscus* sp. (Table 3, Fig. 2),

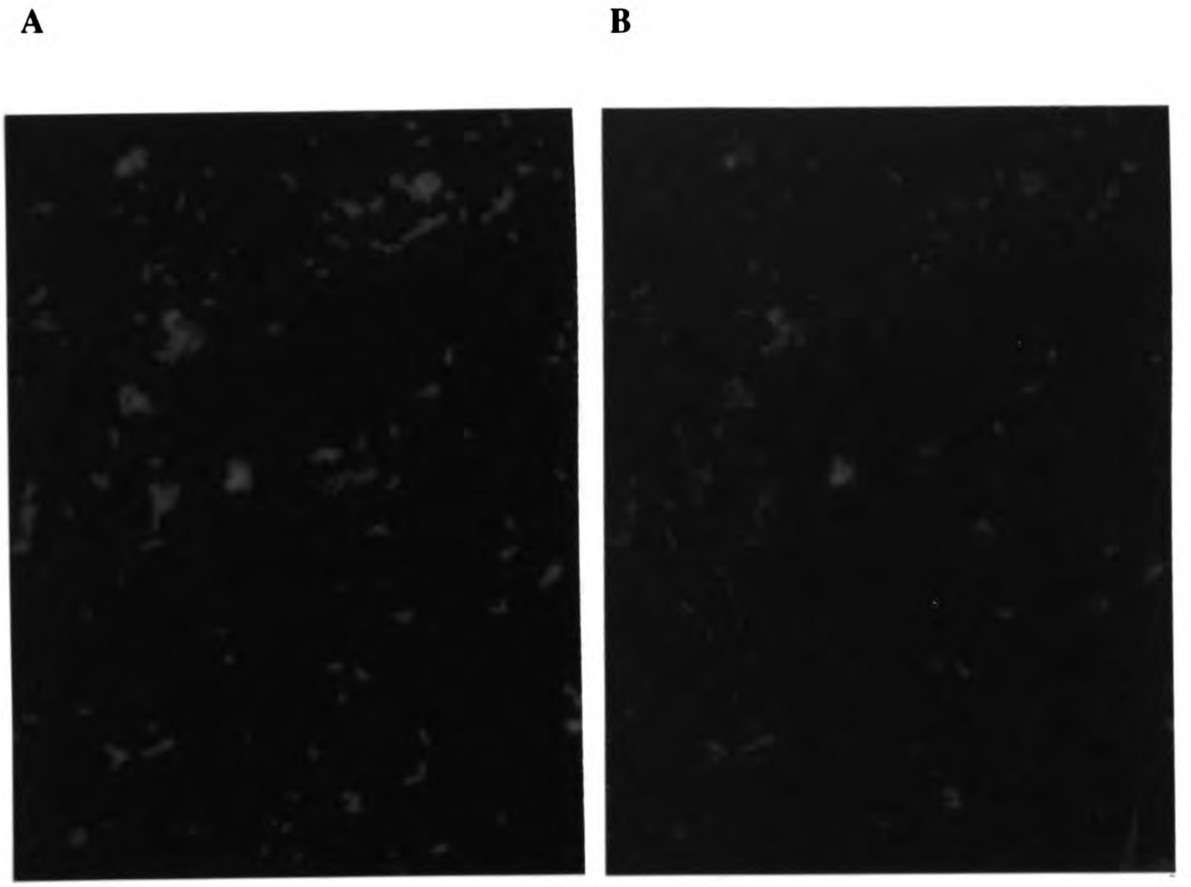

Figure 1. Photomicrographs of *in situ* hybridization of hindgut bacteria using universal probes. (A) Hindgut bacteria stained with coumarin. (B) Hindgut bacteria that hybridized with tetramethylrhodamine- labelled universal probes.

Table 3. Densities of bacteria that hybridized to eubacterial, universal, and all the bacterial group probes combined

Ribosomal probes	*A. domesticus* chow	alfalfa	protein	pulp	*S. borrelia*	*S. vicinus*	*G. rubens*	*G. pensilvanicus*
Universal	1.53×10^9	1.07×10^9	1.02×10^9	1.20×10^9	3.25×10^{10}	3.81×10^{10}	2.21×10^{10}	2.17×10^{10}
Eubacterial	1.50×10^9	9.99×10^8	9.79×10^8	1.15×10^9	2.93×10^{10}	3.46×10^{10}	2.15×10^{10}	2.09×10^{10}
Bacteria identified	8.82×10^8	7.04×10^8	6.12×10^8	3.08×10^8	5.67×10^9	5.24×10^9	1.36×10^{10}	1.11×10^{10}
Direct microscopical counts	1.55×10^9	1.09×10^9	1.05×10^9	1.25×10^9	3.38×10^{10}	3.88×10^{10}	2.25×10^{10}	2.23×10^{10}

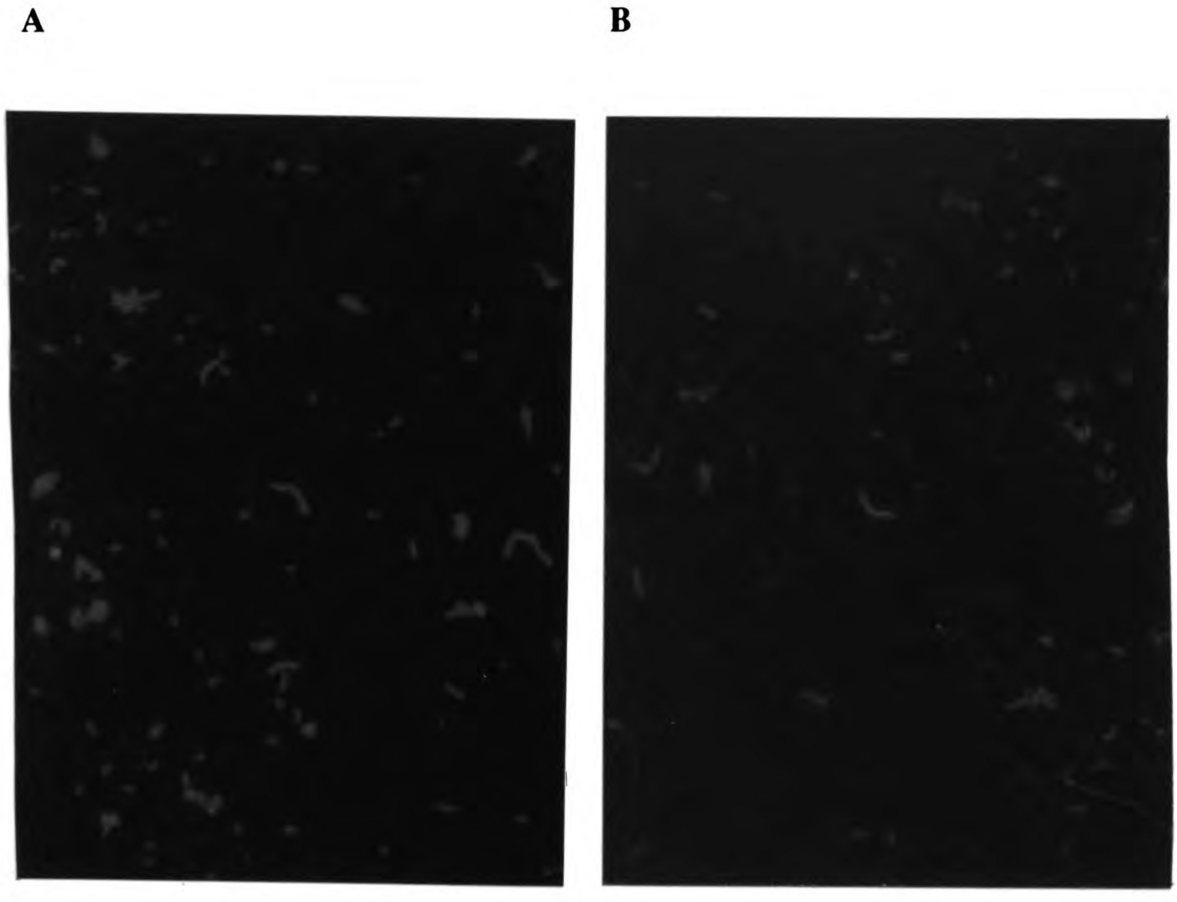

Figure 2. Photomicrographs of *in situ* hybridization of hindgut bacteria using eubacterial probe. (A) Hindgut bacteria stained with coumarin. (B) Hindgut bacteria that hybridized with tetramethylrhodamine-labelled eubacterial probe.

respectively, suggesting that most microorganisms in the crickets studied are eubacteria. Some cells hybridized to the archeal probe which suggested the presence of low densities of archeabacteria.

Sulfate reducing bacteria, bacteriodes, enterics, fusobacteria, bifidobacteria, members of the α subdivision of the Proteobacteria, and Gram positives of low G+C content were detected in the hindgut of *A. domesticus* (Fig. 3). Gram positives of low G+C content and bacteriodes were the numerically dominant groups of eubacteria representing an average of nearly 28 and 11 %, respectively, of the house cricket microbiota. No other bacterial group detected comprised for more than 6 % of the total hindgut microbiota. Enterics and archeae were the least numerous microbial populations, never exceeding 2 %. When the cells fluoresceing to all the group probess were combined, an average of 55 % of the hindgut bacteria of *A. domesticus* and *Gryllus* sp. were identified (Table 3). In contrast, only 15 % of the microbiota inhabiting the *Scapteriscus* sp. hindgut was identified.

To further confirm the presence of bacteriodes and enterics, contents of house cricket hindguts were inoculated in anaerobic Brain Heart Infusion liquid medium (Difco, Detroit, MI). After allowing the cultures to grow for 5 days at room temperature, samples of this enrichment were fixed and hybridized separately with each ribosomal probe. The presence of both bacterial groups was also confirmed. In contrast to the direct staining of hindgut contents, the enterics were dominant in the enrichments, comprising of approximately 45 % of the bacteria seen. Bacteriodes accounted for only 10 % of the cells. In addition, hindgut isolates previously

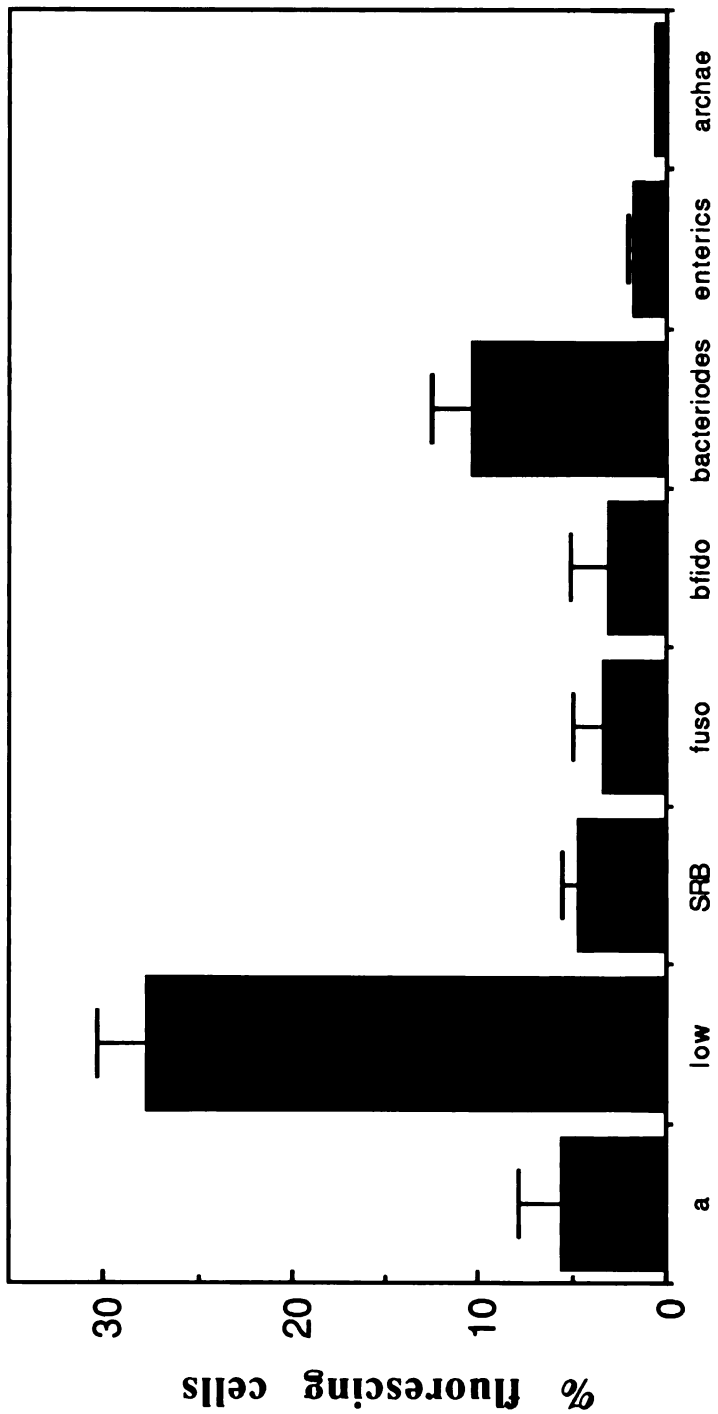

Figure 3. Bacterial groups detected in A. domesticus with the ribosom fluorescent probes.

identified by biochemical methods (FAME and Biolog) as *Citrobacter freundii*, *Klebsiella pneumoniae*, and *E. coli* hybridized to the enteric probe.

Hybridization signals were not detected in the hindgut community with the following probes: β and γ of the Proteobacteria, and gram positives of high G+C content. To determine if these groups were present in densities below the experimental detection limits, the number of cells observed under the microscope was increased to more than 5,000. However, even at this level, no cells were found to hybridize to any of these probes. Since there are approximately 10^9 microbial cells per hindgut, it was concluded that if these bacteria inhabit the gut, their densities must be below 10^6 cells per hindgut.

Effect of dietary treatments on the hindgut microbial community. No significant differences in the relative abundance of bifidobacteria, fusobacteria, enterics, or members of the alpha subdivision of the Proteobacteria were observed for any of the dietary treatments. None of these bacterial groups were present in densities higher than $1.9 * 10^8$ cells per gut, or 7 % of the hindgut bacteria. In contrast, diets had an effect on the other bacterial populations (Fig. 4). For example, archaebacteria were significantly lower in the protein diet ($p<0.05$). In addition, bacteriodes and gram positives of low G+C content were found to be less numerically dominant in the pulp based diet while highest densities of sulfate reducers were obtained in crickets subjected to protein- and alfalfa-based diets. The number of identifiable bacteria decreased significantly for pulp fed crickets (i.e., from an average between 57 and 65 % for the other crickets to an average of 24%; $P<0.01$, Table 3).

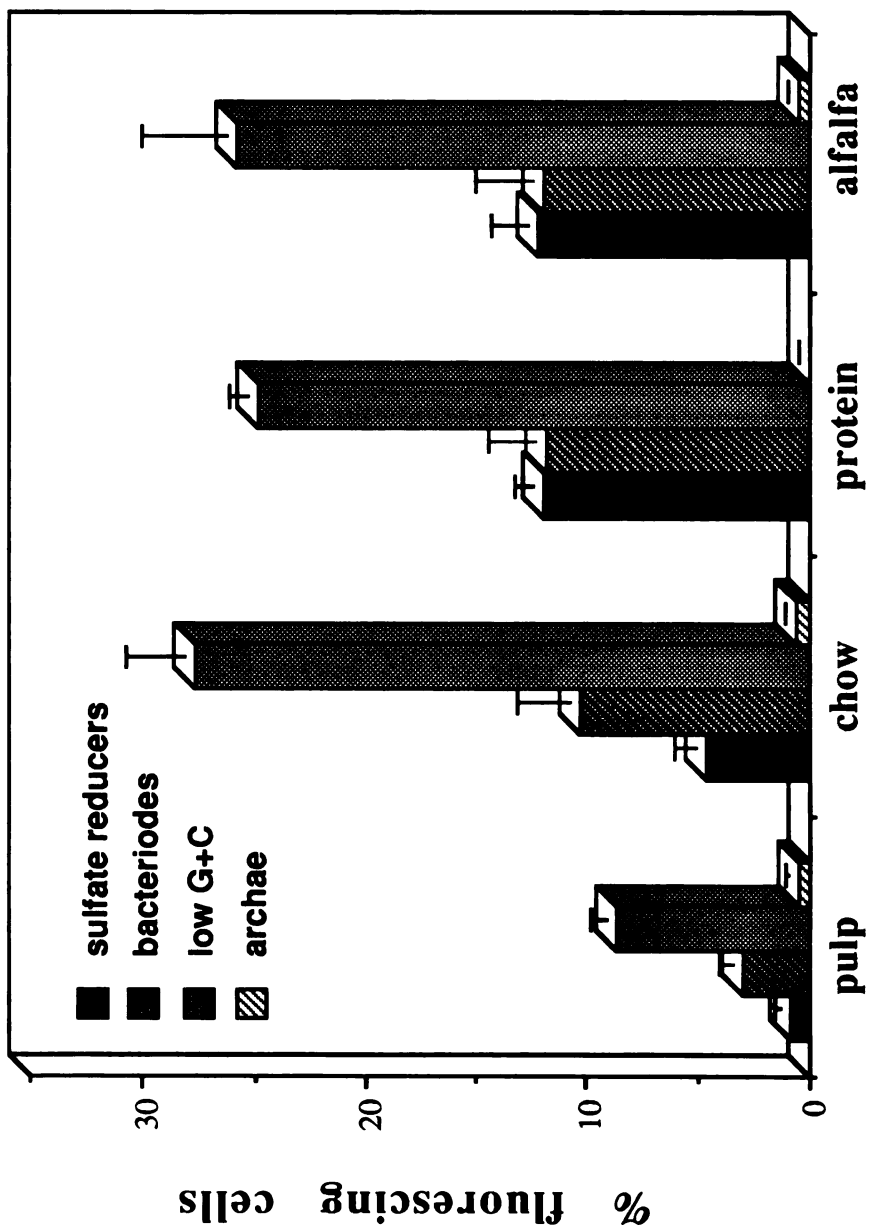

Figure 4. Effect of diet on different bacterial groups in A. domesticus.

Bacterial starvation can not explain the result with pulp since hybridization with the universal probes detected 96 % of the hindgut bacteria. The diets did not change the predominantly eubacterial nature of the hindgut community.

Comparison of the microbial community of different species of cricket. Hybridization studies of the hindgut community of other crickets species were performed in order to determine the extent of the host's influence on the hindgut microbial composition. These crickets species were collected from the wild. All the bacterial groups detected in *A. domesticus* were also detected in *Gryllus* or *Scapteriscus* sp., except for bifidobacteria or fusobacteria which presence was not determined in this study. Differences in the relative abundance of several microbial groups were apparent (Fig. 5). For example, archaebacteria were significantly more abundant ($P<0.001$) in mole crickets (*S. vicinus* and *S. borrelia*). In contrast, gram positives of low G+C content, bacteriodes and sulfate reducing bacteria were less predominant in these cricket species ($P<0.005$).

DISCUSSION

The limitations of the use of culturing techniques to determine the microbial diversity of natural ecosystems are widely recognized. In recent years, however, several studies have demonstrated that rRNA-targeted probes can improve our knowledge about microbial communities since they can detect the presence of not easy to culture microorganisms and simultaneously reveal the phylogenetic identity of members of the community (Manz *et al.*, 1993). Consequently, the

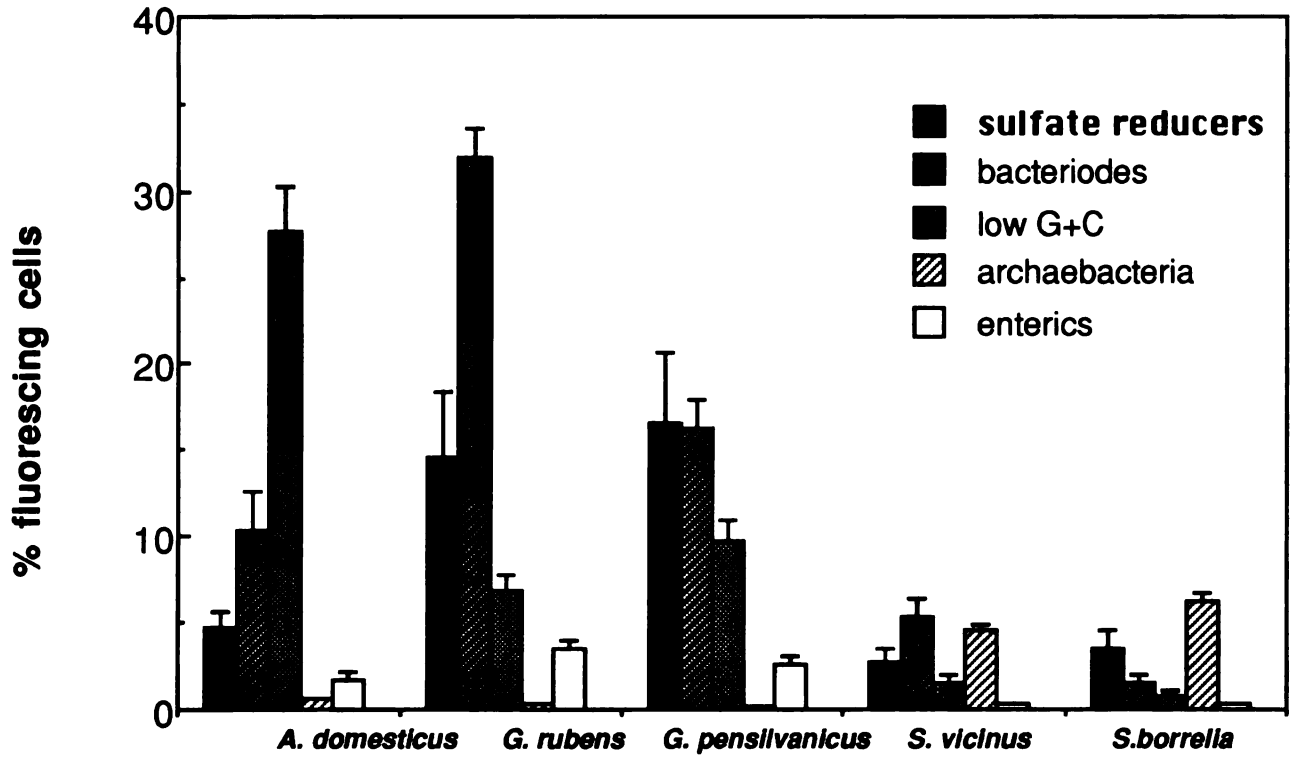

Figure 5. Differences in microbial community structure between different species of crickets. Numbers represent the per cent of the total hindgut bacteria that hybridize to the ribosomal probe specific for each bacterial group. Total microscopical counts were determined as bacteria that stained with coumarin.

goal in this study was to use fluorescently labeled ribosomal probes to identify the bacterial groups normally present in the cricket hindgut. Ribosomal probes have been used to track bacteria within bovine rumen communities (Stahl *et al.*, 1988), mixed culture chemostats (McSweeney *et al.*, 1993) and bioreactors (Amman *et al.*, 1992b), but this is the first attempt of its use to comprehensively describe bacterial communities inhabiting insect guts.

The results with universal and eubacteria probes confirmed the efficiency of the hybridization and qualitatively established that most bacteria within the hindgut microbiota are metabolically active. The universal probes also suggested that phylogenetic probes could potentially identify between 90-98 % of the hindgut bacteria (Table 3). The eubacterial probe indicated that nearly 90 to 96 % of the microorganisms inhabiting the cricket hindgut are indeed eubacteria. This is consistent with the fact that members of the archeal group (i.e., methanogens) do not seem to be major contributors in the cricket hindgut microbiota since the levels of methanogenesis are very low in most cricket species (Kaufman, 1988).

The presence and coexistence of phylogenetically diverse eubacteria within the cricket hindgut was confirmed in this study. The identified bacteria accounted for approximately 60 % of *A. domesticus* and *Gryllus* sp. hindgut community. Nevertheless, the fraction of identifiable bacteria could vary significantly between different species of crickets (Table 3). For instance, in mole crickets only 15 % of the total bacteria were identified. It could be argued that the unidentified bacteria inhabiting the mole cricket gut are not metabollically active, thus containing ribosomal levels below the

detection limits of this essay. Nevertheless, the *in situ* hybridizations signals obtained with eubacterial and universal ribosomal probes suggest high levels of metabolic activity by virtually all bacteria in the hindgut of mole crickets (Fig. 1 and 2). Despite the low levels of bacteria identified in mole crickets, the bacteria identified in *A. domesticus* and *Gryllus* spp. represent a considerable fraction of the cricket community, supporting the use of ribosomal probes to describe complex microbial communities (Wagner et al., 1993; DeLong et al., 1989).

It is conceivable that the unidentified bacteria play important roles within the cricket hindgut microbial community. However, the fact that the bacteria so far identified are present within all crickets examined suggests that they must be important contributors to the microbial processes within the cricket hindgut (e.g., evolution of fatty acids, and fermentation of simple sugars). Arguably, most of these bacterial groups must occupy different niches within the community, hence, these results suggest that microbial communities in the cricket hindgut are complex. Furthermore, different morphotypes were observed within several phylogenetic groups suggesting diversity within bacterial groups (Fig. 6).

Urlich et al. (1981) suggested that the cricket hindgut is dominated by Gram negative bacteria. This finding was based on hindgut isolates. In contrast, the results with ribosomal probes show that microbes phylogenetically related to Gram positive bacteria are numerically dominant components the hindgut community of all crickets examined. Indeed, close to 25 % of the hindgut bacteria of *A. domesticus* gave a typical Gram positive reaction. The fact that

A B

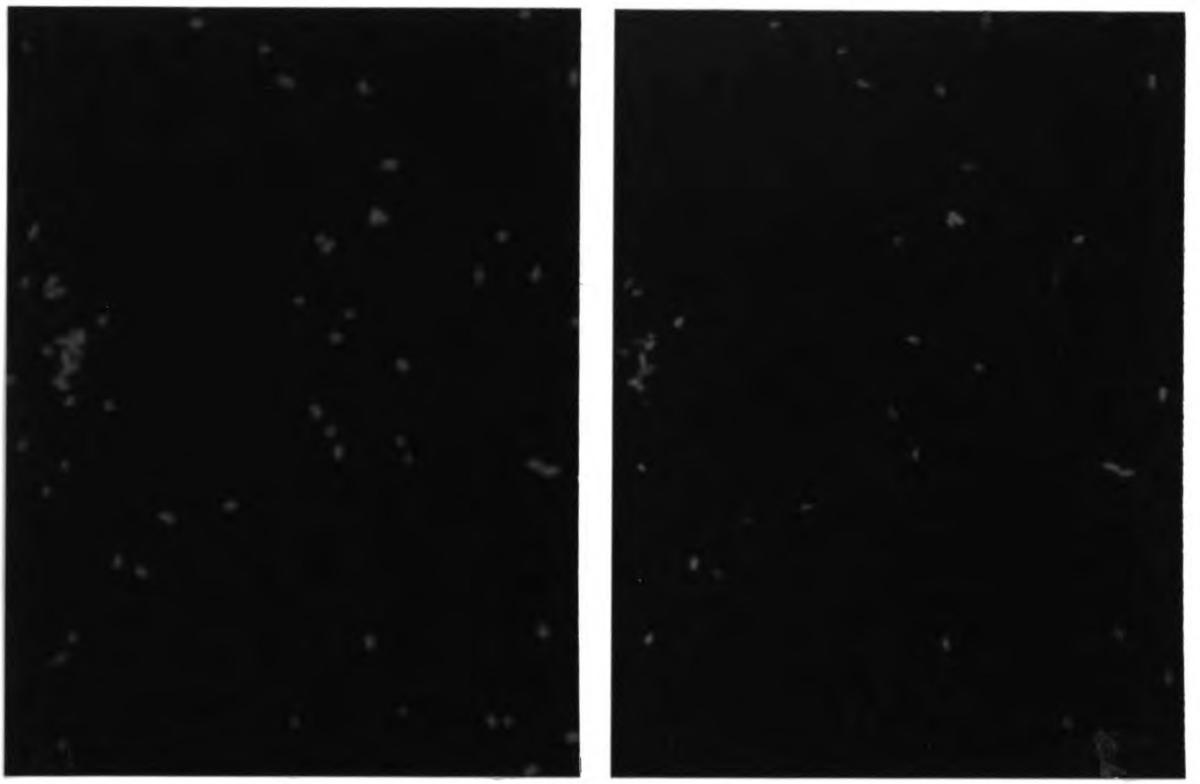

Figure 6. Photomicrographs of hindgut bacterial community hybridized with a bacteriodes specific fluorescently labeled probe. (A) Coumarin stained cells. (B) Hindgut bacteria that hybridized to the probe.

these bacteria might belong to the low G+C group is consistent with the results obtained with CsCl-bisbenzimide gradients where it was determined that bacteria of G+C content between 33 and 50 % dominate the hindgut microbiota of *A. domesticus*.

Despite previous reports on the isolation of *Desulfovibrio* spp. from termites gut, the presence of sulfate reducing bacteria in the hindgut of crickets was surprising since sulfate ions are virtually absent from their dietary regime. It is now recognized however, that sulfate reducing bacteria are present in a wide range of nonsulfidogenic environments (Odom and Singleton, 1993) and it is further argued that they might play relevant roles in interspecies H_2 transfer in termites (Breznak and Brune, 1994). Since sulfate reducing bacteria could represent up to 15 to 20 % of the total hindgut bacteria in *A. domesticus* and *Gryllus* sp., such bacteria should be considered predominant members of these microbial communities. Confirming the identity of the sulfate reducers inhabiting the cricket and if positive, determining their role deserves future consideration.

Interestingly, sulfate reducing bacteria and methanogenic bacteria seem to coexist in the cricket hindgut, which is in contrast with termites where the two major hydrogen consumers are acetogens and methanogens (Brauman *et al.*, 1992). Since sulfate reducers are found in greater numbers than methanogens in the house cricket this suggest that they outcompete the latter group, even though normally the opposite is expected in environments with low concentrations of sulfate, such as the insect gut (Widdel, 1988). Nevertheless, the predominance of sulfate reducing bacteria over

methanogens in the house cricket might be plausible in light of the fact that sulfate reducers have been reported to outcompete methanogens when hydrogen and acetate are available (Widdel, 1988). An alternate hypothesis is that each group utilizes different electron donors, and therefore, there is no direct competition between the groups.

Archaebacteria were more abundant in *Scapteriscus* sp. than in the other crickets. Since methane evolution has been detected in mole crickets (Kaufman and Santo Domingo, unpublished results) it is reasonable to propose that methanogens are responsible for the hybridizing cells seen with the archaebacterial probe. In fact, recent studies with a ribosomal probe specific for methanogenic bacteria has confirmed the presence of this group within the hindgut of mole crickets (Santo Domingo and Nierzwicki-Bauer, unpublished results). Fluorescently labeled polyclonal antibodies have also suggested the presence of hindgut bacteria immunogenically related to methanogens (Conway de Macario, Macario, and Santo Domingo, unpublished results). Assuming that on the average there are 3.9 * 10^{10} bacterial cells per gut, our results suggest that mole crickets could harbor approximately 10^{8} methanogens.

The presence of bacteriodes, fusobacteria, and bifidobacteria in the cricket hindgut it is not surprising since they are normally found in association with the gastrointestinal tract of a variety of mammals (e.g., humans and ruminants; Hungate, 1975; Moore and Holdeman, 1974). In fact, the presence of bacteriodes and fusobacteria in insect guts has been suggested before (Cruden and Markovetz, 1987; Urlich *et al.*, 1981). Since, the ribosomal probe used is specific for

Bacteriodes ovatus, *B. thetathaiomicron*, *B. fragilis*, *B. uniformis*, and *B. vulgatus*, it is concluded that the bacteriodes detected in the hindgut must belong to the *Bacteriodes sensu stricto* (Sha and Collins, 1989; Paster *et al.*, 1994). In contrast, this is the first report, however, suggesting the presence of bifidobacteria in insect guts.

The occurrence of strictly anaerobic bacteria (e.g., sulfate reducing bacteria, bacteriodes, fusobacteria, and bifidobacteria) within this microbial community, supports the hypothesis of the cricket hindgut being of a highly reduced nature. Crickets are similar to termites in this regard; thus, it is probable that the activity of the cricket gut microbiota is responsible for maintaining the anoxicity of this environment (Veivers *et al.*, 1982). These results also suggest that anaerobes, as a group, are a numerically important bacterial group inhabiting the cricket gut. This is supported by the fact that aerobic plate counts commonly correspond to less than 10 % of the total microscopic counts observed in most cricket species.

Given that most hindgut bacteria are metabolically active, there seems to be an abundant pool of available carbon sources for the different bacterial populations inhabiting the cricket hindgut. This is particularly intriguing for *Gryllus* and *Scapteriscus* spp. hindgut microbiota, considering that the diet of these species could also include refractory carbon sources of poor nutritional value, as well as plant allelochemicals potentially toxic to bacteria. In addition, since differences in the community structure were observed between these cricket species, it could be argued that differences in the dietary regimes might be responsible for these structural differences. While more research is needed to validate this hypothesis, it can be

concluded that far from being universal, the composition and structure of cricket gut communities seem to vary between different gryllids species.

It has been previously demonstrated that changes in diets alter the structure and function of the cricket hindgut community (Chapter 2) without significantly affecting the bacterial densities. In those studies the identity of the bacterial populations affected was not determined. In this study, however, phylogenetic probes have suggested that the relative abundance of specific bacterial groups were altered by changes in diet, particularly sulfate reducing bacteria, bacteriodes, and Gram positive bacteria of low G+C content (Fig. 4). Therefore, this study further supports the hypothesis that diet does affect the structure of the cricket hindgut microbial community. While it has not been demonstrated that having a dynamic hindgut community could benefit insects in natural situations, it is reasonable to propose that by shifting the ratio of predominant members, the microbial community could maintain the pool of substrates (e.g., volatile fatty acids) that the cricket could absorb and thus use for growth or reproduction (Kaufman, 1988). This could be achieved if there is functional redundancy within members of the gut microbiota. Since crickets could be reared in the laboratory as germ-free colonies, it is not obvious that carrying a flexible community might be at all times advantageous. In contrast, insects that strictly depend on their microbial symbionts for their survival (e.g., termites) will benefit from the development of a dynamic and resilient gut microbiota that can maintain its functional stability. In the future, it will be interesting to examine the degree

of functional redundancy within the gut microbiota of specialized insects and examine the range of dietary perturbations that such communities can withstand without loss of its functional stability and the fitness of their host.

Differences in the hindgut microbial structure between the different species of crickets examined could be attributable to differences in feeding behavior. However, it remains to be examined if there is a correlation between diet type and the microbial composition of crickets found in nature. For instance, crickets as a group are believed to be omnivorous though predominantly feeding on plant material. While this is true for the species examined in this study, mole crickets are in more direct contact with soil microbial communities, a factor that might influence the composition and structure of their hindgut microbial community. It should also be noted that anatomical differences might also be relevant in the assemblage of the hindgut community. Again, mole crickets are different than other cricket species in that they seem to lack a peritrophic membrane, consequently, gut microbes are in direct contact with the ingested material, while in *A. domesticus* and *Gryllus* sp., hindgut microbes are predominantly exposed to soluble material that penetrates the pores of the peritrophic membrane.

Ribosomal probes indicated the presence of diverse bacterial groups in the cricket hindgut that otherwise will be difficult to isolate and/or identify by culturing methods. This information could be used to select methods that might enhance the opportunity to enrich or isolate microorganisms in pure cultures. In addition, it seems probable that this technique, in combination with analytical

and biochemical methods, will provide information needed to assign functional roles to different bacterial groups within the cricket hindgut microbial community. Finally, fluorescent 16S probes could be used to reveal which members of the hindgut community are attached to the gut wall and peritrophic membrane and which are loosely found within the ectoperitrophic space.

ACKNOWLEDGMENTS

I am very grateful to Drs. Michael Cotta and Gerrit Voordow for kindly providing bacterial strains. Also, thanks to Dr. Bruce Paster for sharing information related to the phylogeny of *Bacteriodes* sp. and Dr. Thomas Walker for providing field and mole crickets. This work was performed in collaboration with Drs. Sandra Nierzwicki-Bauer and Ellen Braun-Howland.

References

Amman, R. I. , B. Zarda, D. A. Stahl, and K. -H. Schlefeir. 1992a. Identification of individual prokaryotic cells by using enzyme labelled rRNA-targeted oligonucleotide probes. Appl. Environ. Microbiol. **58**:3007-3011.

Amman, R. I., J. Stromley, R. Devereux, and D. A. Stahl. 1992b. Molecular and microscopic identification of sulfate-reducing bacteria in multispecies biofilms. Appl. Environ. Microbiol. **58**:614-623.

Amman, R. I., N. Springer, W. Ludwig, H. -D. Görtz, and K. -H. Scheleifer. 1991. Identification in situ and phylogeny of uncultured bacterial endosymbionts Nature (London) **351**:161-164.

Amman, R. I., L. Krumholz, and D. A. Stahl. 1990a. Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. J. Bacteriol. **172**:762-770.

Amman, R. I., B. J. Binder, R. J. Olsen, S. W. Chrisholm, R. Devereux, and D. A. Stahl. 1990b. Combination of 16S rRNA-targeted probes with flow cytometry for analyzing mixed microbial populations. Appl. Environ. Microbiol. **56**:1919-1925.

Barkay, T., C. Liebert, and M Gillman. 1989. Hybridization of DNA probes with whole community genome for detection of genes that encode microbial responses to pollutants: mer genes and Hg2+ resistance.

Bignell, DE. 1984. The arthropod gut as an environment for microorganisms. In *Invertebrate-Microbial interactions*. JM Anderson, ADM Rayner, and DWH Walter (editors). Cambridge Press, New York, NY. pp.205-228.

Brauman, A., M. D. Kane, M. Labat, J. A. Breznak. 1992. Genesis of acetate and methane by gut bacteria of nutritionally diverse termites. Science. **257**: 1384-1387.

Braun-Howland, E. B., S. A. Danielsen, and S. A. Nierzwicki-Bauer. 1992. Development of a rapid method for detecting bacterial cells in situ using 16S rRNA-targeted probes. Biotechniques **13**:928-932.

Breznak, J. A., J. M. Switzer and H. -J. Seitz. 1988. *Sporomusa termitida* sp. nov., an H2/CO2-utilizing acetogen isolated from termites. Arch. Microbiol. **150**:282-288.

Cruden, DL and AJ Markovetz. 1987. Microbial ecology of the cockroach gut. Ann. Rev. Microbiol. **41**:617-643.

Davies, RG. 1988. Outlines of entomology. 7th Edition. Chapman and Hall. London.

DeLong, E. F., G. S. Wickham, and N. R. Pace. 1989. Phylogenetic stains: ribosomal RNA-based probes for the identification of single cells. Science **243**: 1360-1363.

Dowd, P. 1992. Insect fungal symbionts: a promising source of detoxifying enzymes. J. Ind. Microbiol. **9**:149-161.

Giovannoni, S. J., E. F. DeLong, G. J. Olsen, and N. R. Pace. 1988. Phylogenetic group-specific oligodeoxynucleotide probes for identification of simple microbial cells. J. Bacteriol. **170**:720-726.

Holben, W. E., BM Schroeter, VGM Calabrese, RH Olsen, JK Kukor, VO Biederbeck, AE Smith, JM Tiedje. 1992. Gene probe analysis of soil microbial populations selected by amendment with 2,4-dichlorophenoxyacetic acid. Appl. Environ. Microbiol. **58**:3941-3948.

Hungate, RE. 1975. The rumen microbial ecosystem. Ann. Rev. Ecol. Syst. **6**:39-66.

Kane, MD and JA Breznak. 1991. *Acetonema longum* gen. nov., an H2/CO2 acetogenic bacterium from the termite, *Pterotermes occidentis*. Arch. Microbiol. 156:91-98.

Kaufman, MG, and MK Klug. 1991. The contribution of hindgut bacteria to dietary carbohydrate utilization by crickets (Orthoptera : Gryllidae). Comp. Biochem. Physiol. **98**:117-123.

Kaufman, MG. 1988. The role of anaerobic bacterial metabolism in the nutrition of crickets (Orthoptera : Gryllidae). Ph.D. Thesis, Michigan State University, East Lansing, MI USA

Lane, DJ, B Pace, GJ Olsen, DA Stahl, ML Sogin, and NR Pace. 1985. Rapid determination of 16S ribosomal sequences for phylogenetic analyses. Proc. Natl. Acad. Sci. USA **82**:6955-6959.

Manz, W, U Szewzyk, P Ericsson, R Amman, K-H Schleifer, and T-A Stenström. 1993. *In situ* identification of bacteria in drinking water and adjoining biofilms by hybridization with 16S and 23S rRNA-directed fluorescent oligonucleotide probes. Appl. Environ. Microbiol. **59**:2293-2298.

Manz, W, R Amman, W Ludwig, M Wagner, and KH Schleifer. 1992. Phylogenetic oligodeoxynucleotide probes for the major subclasses of Proteobacteria: problems and solutions. System. Appl. Microbiol. 15: 593-600

McSweeney, CS, RI Mackie, AA Odenyo, and DA Stahl. 1993. Development of an oligonucleotide probe targeting 16S rRNA and its application for detection and quantitation of the ruminal bacterium

Synergistes jonesii in a mixed-population chemostat. Appl. Environ. Microbiol. **59**:1607-1612.

Moore, WEC and LV Holdeman. 1974. Human fecal flora: the normal flora of 20 Japanese-Hawaiians. Appl. Microbiol. **27**:961:

Odom, JM and R Singleton. 1993. The sulfate reducing-bacteria: contemporary perspectives. Plenum Press, New York, NY

Paster, B.J., F.E. Dewhirst, I. Olsen, and G.J. Fraser. 1994. Phylogeny of *Bacteriodes*, *Prevotella*, *Porphyromonas* spp., and related bacteria. J. Bacteriol. 176:725-732.

Poulsen, LK, G Ballard, and DA Stahl. 1993. Use or rRNA fluorescence *in situ* hybridization for measuring the activity of single cells in young and established biofilms. Appl. Environ. Microbiol. **59**:1354-1360.

Schultz, JE and JA Breznak. 1978. Heterotrophic bacteria present in hindguts of wood-eating termites [*Reticulotermis flavipes* (Kollar)]. Appl. Environ. Microbiol. **35**:930-936.

Sha, H.N. and M.D. Collins. 1989. Proposal to restrict the genus *Bacteriodes* (Castellani and Chalmers) to *Bacteriodes fragilis* and closely related species. Int. J. Syst. Bacteriol. **39**:85-85.

Sommerville, CC, IT Knight, WL Straube, and RR Colwell. 1989. Simple, rapid method for direct isolation of nucleic acids from aquatic ecosystems. Appl. Environ. Microbiol. **55**: 548-554.

Stahl, D. A., B. Flesher, H R. Mansfield, and L. Montgomery. 1988. Use of phylogenetically based hybridization probes for studies of ruminal microbial ecology. Appl. Environ. Microbiol. **54**:1079-1084.

Steffan, RJ, J Goksoeyr, AK Bej, and RM Atlas. 1988. Recovery of DNA from soils and sediments. Appl. Environ. Microbiol. **54**: 2908-2915.

Urlich, RG, DA Buthala, and MJ Klug. 1981. Microbiota associated with the gastrointestinal tract of the common house cricket, Acheta domesticus. Appl. Environ. Microbiol. **41**: 246-254.

Veivers, PC, RW O'brien, and M Slaytor. 1982. Role of bacteria in maintaining the redox potential in the hindgut of termites and preventing entry of foreign bacteria. J. Insect Physiol. **28**:947-951.

Wagner, M R Amman, H Lemmer, and K-H Shleifer. 1993. Probing activated sludge with oilgonucleotides specific for Proteobacteria: inadequacy of culture-dependent methods for describing microbial community structure. Appl. Environ. Microbiol. **59**:1520-1525.

Widdel, F. 1988. Microbiology and ecology of sulfate- and sulfur-reducing bacteria. In A. J. B. Zehnder Biology of anaerobic microorganisms. Wiley & Sons, Inc., New York, USA

Zimmerman, PR, JP Greenberg, SO Wandiga, and PJ Crutzen. 1982. Termites: a potential large source of atmospheric methane, carbon dioxide, and molecular hydrogen. Science **218**:563-565.

SUMMARY

In the last decade it has become evident that culturing techniques are too limited for the microbial ecologist dealing with the analysis of natural microbial communities. This has had a direct impact in microbial ecology leading to the development of molecular based methods for the analysis of such communities. This influenced my decision on the use of molecular methods to study the structure of the cricket bacterial community.

The results obtained with the CsCl-bisbenzimide gradients and the fluorescent ribosomal probes strongly support the hypothesis that the structure of the hindgut bacterial community could indeed change after a dietary perturbation. Arguably, displacement of some of the numerically dominant microbes by less predominant members of the community could explain the changes in community structure. Although it was not conclusively demonstrated in this study, structural changes most likely had a direct effect in the changes on fermentation profiles observed for the communities of crickets fed on pulp and protein diets.

Future work should be directed towards the identification of the bacteria that are commonly present as the numerically dominant bacteria in the hindgut of *A. domesticus*. It seems reasonable to assume that these microbes will perform some of the important functional tasks within the cricket community. The preliminary identification of some of the groups present in *A. domesticus* with the fluorescent probes should serve as a guide for the isolation of some predominant bacterial groups. Once this has been accomplished,

next step would be to monitor the response of those groups to changes in the host diet. My advice for those who will like to pursue such endeavors is to use fluorescent probes targeting the 16S or 23S rRNA.

To those interested in studying insect gut microbial communities, I must direct a few words of encouragement. Unfortunately, termites is the only insect group that has been studied in any great detail, thus, almost any information on the gut microbiology of a different model system would be a welcome contribution. As defended by Breznak and myself, insects are a great source of unknown microbial diversity with relevant ecological roles, like global carbon processing. Answers to questions addressing this point will prove rewarding. Furthermore, the variety of interactions that have evolved between these two different but equally successful groups of organisms represent a good example of enigmatic biology that when understood will enhance our knowledge of the ecology and evolution of insects and microbes, as well as other living forms.

APPENDIX

Attempts to analyze the gut microbial community by RFLP analysis of 16S rRNA genes.

The approach to be used for the analysis of the effect of a disturbance on the structure of any given microbial community depends on the level of resolution that the investigator wants to achieve. In the previous chapters I successfully used of CsCl-bisbenzimide gradients and rRNA-targeting oligonucleotide probes to study microbial communities. Each method has a different level of resolution that allows the investigator to discriminate between different bacteria. For instance, CsCl-gradients could easily discriminate between bacteria of different G+C content, however, it can not distinguish between bacteria of different phylogeny but similar G+C. In contrast, phylogenetic probes could be developed such that they can discriminate between bacteria carrying different functions (e.g., bacteriodes vs. sulfate reducers), however, they might not discriminate between genera or species that are phylogenetically related. Fingerprints based on restriction fragment length polymorphisms (RFLP) could achieve a finer level of resolution than both CsCl-gradients and *in situ* hybridizations and therefore could complement these methods for studies of microbial communities.

Studies similar to the diet experiments described in Chapter 2 were performed to compare the RFLP patterns of hindgut communities of the house cricket that had been switched to different diets. Community RFLP were generated in Southern hybridization studies using a community ribosomal (16S rDNA) probe. The

following paragraphs describe the techniques and steps used to in these experiments.

The polymerase Chain Reaction (PCR) was used to generate "community" ribosomal probes using the primers and the PCR protocol was described in Chapter 1.. The template for PCR was hindgut bacterial community DNA. Bacterial community DNA (5 μg) from different crickets was digested for 3 h with a given restriction endonuclease enzyme. The DNA fragments were separated by electrophoresis in a 0.7 % agarose gel at 50 volts for 24 h. Nucleic acids were transferred to a Zeta-Probe blotting membrane following the manufacturer's instructions (Bio-Rad Laboratories, Inc., Richmond, CA), after which they were cross-linked to the membrane using a UV Stratalinker (Model 1800, Stratagene, LaJolla, CA). Probes were labeled with α-^{32}P-dCTP (DuPont, NEN Research Products, Boston, MA) using a random primer kit (United States Biochemical Corp., Cleveland, OH). Unincorporated nucleotides were removed from the labeled probe using a Sephadex G50 (Sigma) column. Probes were then boiled for 10 min. and chilled on ice before use. Southern blots were prehybridized for 1 h in a solution containing 7 % lauryl sulfate (SDS, Sigma), 10 mM EDTA, and 500 mM sodium phosphate buffer solution (pH 7.2). Probes were added to the solution and hybridized at 65^0 C for 16 h. Different temperatures were previously tested, e.g., 50, 55, 60, 65, and 70^0 C and the temperature of 65^0 C was selected for the hybridization studies since it reduced cross-hybridization without any apparent lost in signal. Membranes were washed twice in 5 % and then 1 % lauryl sulfate solution at 65^0 C for 30 min. Membranes were exposed to Kodak X-

OMAT AR film (Eastman Kodak Co., Rochester, NY) for 24 h. Films were developed using a Kodak RP X-OMAT Processor (Model M7B).

The ribosomal region amplified is proximal to the 5' end of the 16S rRNA gene which contains stretches of sequences of various degrees of conservation. In theory, this PCR product should exhibit high similarity to the most numerous bacteria within the community. Nevertheless, the probe hybridized to all the hindgut isolates tested to date (data not shown) as well as to many phylogenetically unrelated bacteria (Table 1). Also important is the fact that this probe did not hybridize to the DNA extracted from germ-free cricket hindguts.

The sensitivity of the RFLP methodology was determined by altering the DNA concentration of one of two strains (Fig. 1). It was found that, at best, the RFLP approach can detect bacteria when their DNA concentrations did not differ by more than 100 times. However, the sensitivity is reduced when more complex communities are analyzed. This was observed when DNA from more than two hindgut bacterial isolates were mixed and hybridized against the ribosomal probe (Fig. 2). In this case, several bands unique for each isolate could not be used to discriminate between these hindgut bacteria, while other bands proved to be useful. Even though the use of a different restriction enzyme could have generated an RFLP pattern that would have facilitated the analysis of this artificial community, this would have had to be determined empirically. In reality, since most hindgut bacteria appear to have from 3 to 8 rRNA operons (as determined by their RFLP data; Fig 3), complex communities would

Table 1. Bacteria that hybridizes to the community probe

Bacterial species	Source
Cytophaga XM3	Sheridan Haack
Pseudomonas aeruginosa	David Demezas
P. flourescens	Marcos Fries
P. stutzeri	"
Clostridium thermoaceticum	Lars Ljundahl
Klebsiella oxytoca	Charles Lovell
K. ozanae	"
E. limosum	"
K. pneumoniae	MSU Micro Department
Escherichia coli	William Holben
Bradirhizobium japonicum	"
Luminescent Vibrio	Margo Haygood

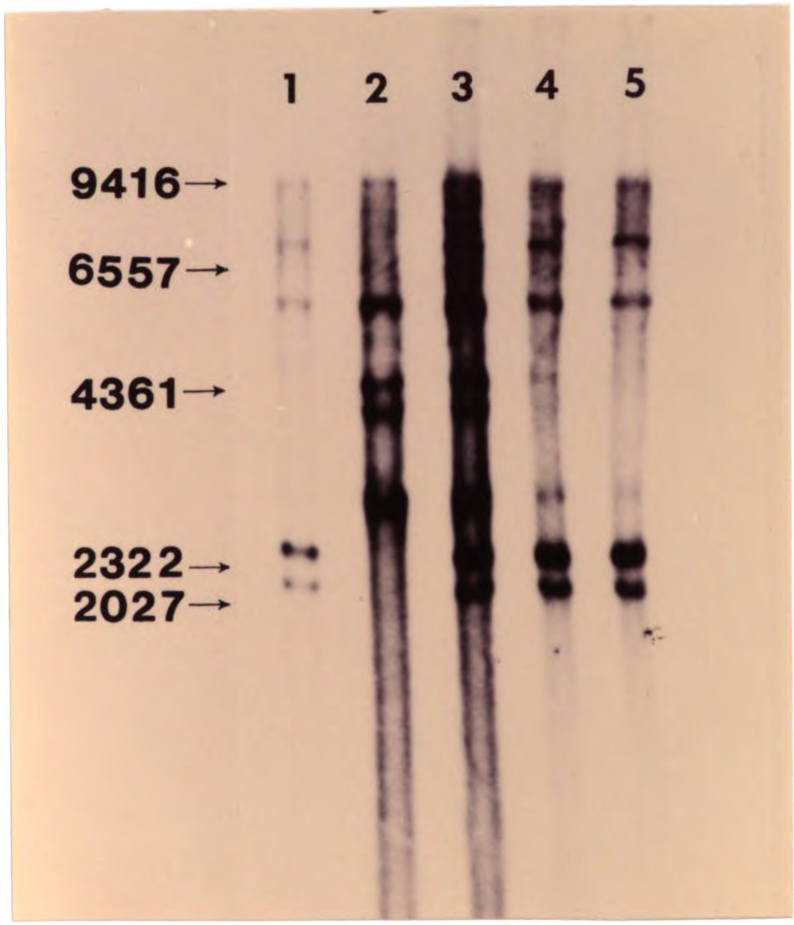

Figure 1. RFLP profiles of genomic DNA from hindgut bacterial isolates AD Raff 13 (lane 1) and AD Raff 28 (lane 28). DNA was digested with BamHI previous Southern transfer. Similar DNA concentrations of each isolates were used on lane 3 (i.e., 1:1). DNA ratio of AD Raff 13 and AD Raff 28 was altered to 1:0.1 (lane 4) and 1:0.01 (lane 5).

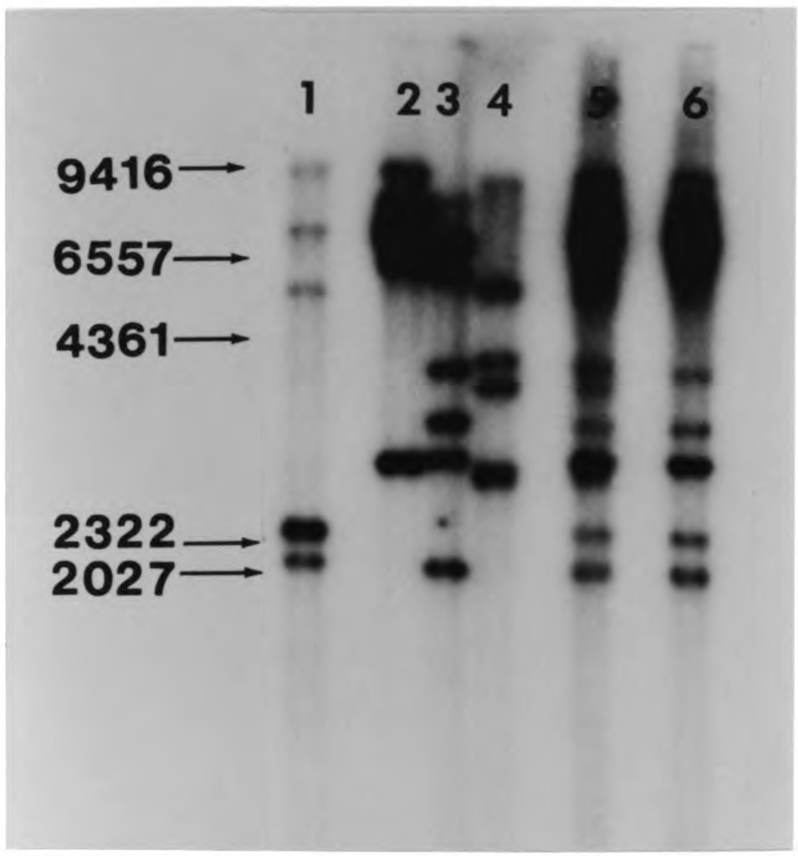

Figure 2. RFLP profiles of genomic DNA from hindgut isolates AD Raff 6 (lane 1), Raff 13 (lane 2), Raff 28 (lane 3), and Bac 4 (lane 4). DNA was digested with BamHI previous Southern transfer. DNA from the four isolates were combined in lane 5. DNA from Bac 4 was excluded in lane 6.

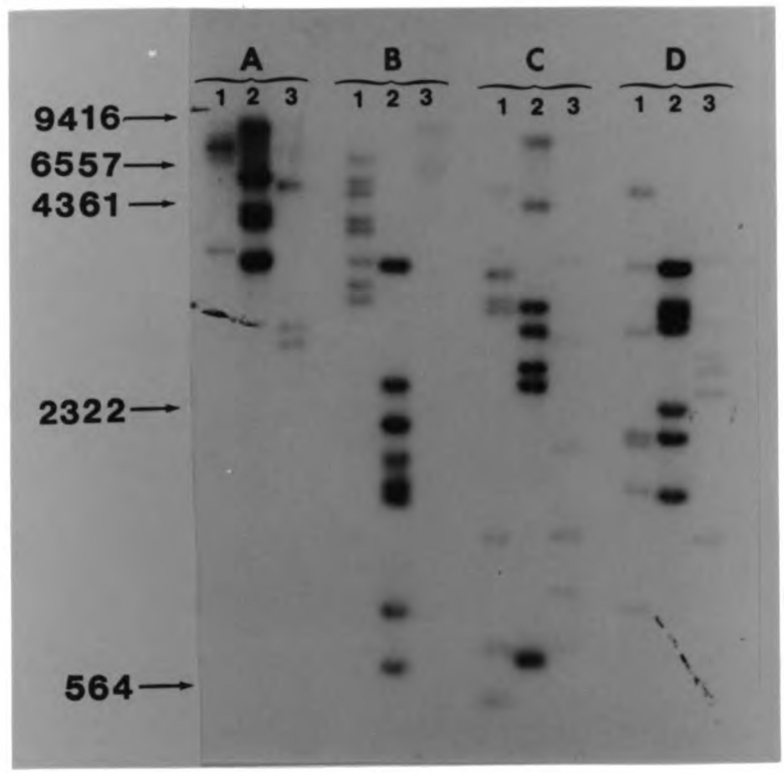

Figure 3. RFLP profiles of genomic DNA of Raff 6 (lane 1), Raff 28 (lane 2), and Bac 4 (lane 3) digested with BamHI (A), BglI (B), EcoRI (C), and PstI (D).

be expected to produce extremely complex RFLP patterns, decreasing the resolution and sensitivity of this approach.

Hybridization of the ribosomal probe to Southern blots of hindgut DNA generated microbial community RFLPs. These RFLPs derive from the dominant members of each hindgut community. Differences in bacterial community fingerprints were observed for each group of crickets subjected to different diets (Fig. 4). However, differences in the fingerprints of crickets fed the same diet were also apparent. To determine if the differences between diets were greater than the differences between individuals on similar diets, a principle component analysis of the RFLP profiles was performed using the Ambis system. Although RFLP patterns for individuals on the same diets clustered, an insufficient number of replicates negated the statistical analysis of this data. Since it was previously demonstrated that diets can alter the structure of the hindgut community, it is tempting to speculate that the RFLP data is suggesting a similar conclusion. For example, although many hybridization bands were shared by crickets subjected to different diets, the relative intensity of some of them was different suggesting, that the structure of the hindgut community was altered by the changes in diet. This was further supported by the presence of unique bands in each of the different community RFLP pattern. Moreover, it is reasonable to speculate that since several bands were shared between several diets suggests that some members of the microbial community were maintained at a constant relative abundance, and probably were not affected by the change in diet. (Fig. 5).

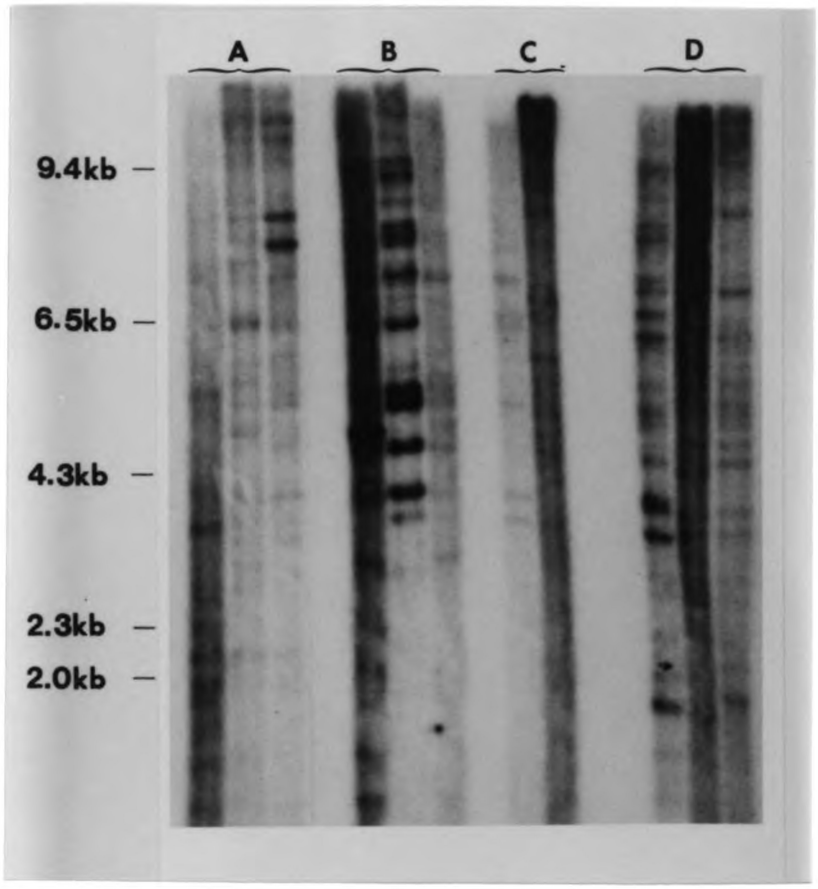

Figure 4. RFLP profiles of hindgut community DNA of different crickets (A. domesticus) subjected to different diets: (A) chow; (B) alfalfa; (C) protein; (D) pulp. Each group represent crickets fed same diet. Ribosomal probe generated using hindgut community DNA as template in the PCR method.

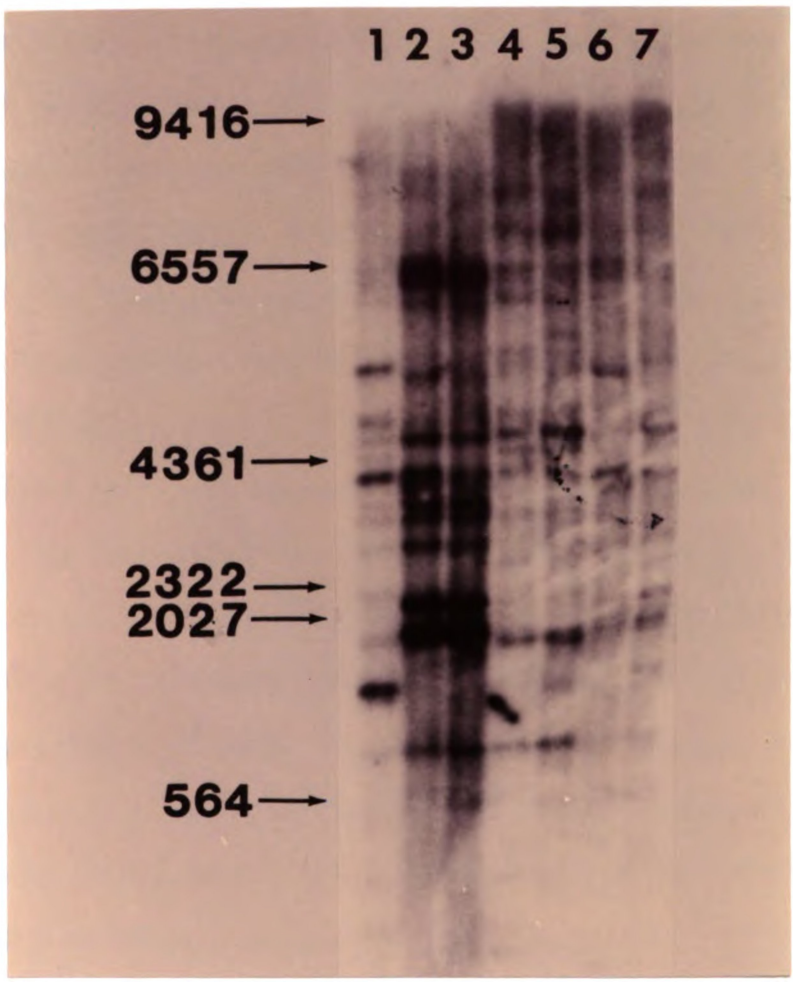

Figure 5. RFLP profiles of genomic DNA from different cricket hindgut communities. Lane 1, *A. domesticus* fed chow. Lane 2 and 3, *A. domesticus* subjected to protein-based diet. Lane 4 and 5, *A. domesticus* subjected to alfalfa-based diet. Lane 6, *G. rubens* fed chow. Lane *G. velidis* fed chow.

Community RFLP profiles revealed discrete bands ranging size from approximately 10 to 0.5 kb (Fig. 5). The number of bands detected per cricket was from 12 to 18. Based on the minimum number of rRNA operons that hindgut isolates seem to have, these band patterns could represent 4 to 6 different bacterial species; however, if the band patterns belong to species with more ribosomal operons, 2 to 3 different species will be represented in the community RFLP.

The relatively low number of bands generated for the hindgut community suggests that such communities are low in diversity. Arguably, these band patterns belong to a small fraction of the members of the hindgut community (i.e., the most numerous bacteria, presumably strict anaerobes). However, even if the individual RFLP's for the aerobic hindgut isolates were to be combined, the result would be a complex community RFLP pattern not suitable for further analysis.

When RFLP patterns of hindgut isolates were compared to community fingerprints, the presence of these isolates was not detected within the community (data not shown). This suggests that these isolates are not members of numerically dominant hindgut bacterial populations. This is further supported by the fact that these bacterial strains have been identified as members of the Enterobacteriaceae family, which were found to represent less than 7 % of the hindgut community (Chapter 3).

Despite the weak statistical design of this experiment, the resolution of hindgut community RFLP was unexpected because previous attempts to generate fingerprints from soil communities

generated smears which made further analysis impossible. The result with soil microbial DNA could be attributed to the high diversity of such microbial communities. For instance, it has been reported that one gram of soil might harbor more than 4,000 different bacterial genomes (Torsvik *et al.*, 1990). Also, the rather poor quality of nucleic acids extracted from soil communities (i.e., highly sheared genomic DNA and the presence of humic acids) might further contribute to the poor resolution of soil microbial community fingerprints. Since community fingerprints were also generated for other species of crickets (Fig. 5) it is potentially possible to use this approach to compare the community composition of different crickets species or to compare the community developed in crickets reared in the laboratory from those caught from the field.

In concluding it should be noted that the approach used here could be used to rapidly examine the effect of perturbations on the structure of the insect gut microbial community. Nevertheless, it does have several limitations: it requires an unbiased DNA extraction (i.e., that will lyse most if not all bacteria within the community); only a small number of bacterial populations can be monitored at the same time; no information related to the identity of the bacterial populations is obtained; and only the dominant populations can be screened. In the present study possible community variation between individual animals on the same diet made it difficult to replicate results. In the future, hybridization studies using probes targeting specific bacterial groups might reduce the number of bands and therefore the complexity of the pattern. In addition, it is of my opinion that this alternate approach will be more useful since it will

provide information on the relative abundance of phylogenetically-related bacterial populations within the cricket hindgut and further infer on the functional relevance of this community. At the present stage of development, however, the method did not give sufficiently reproducible or interpretable results to allow firm conclusions about community structure differences.