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University of Alberta

Functional Significance and Microbial Ecology of the Anterior Hindgut of the American Cockroach, *Periplaneta americana* (Dictyoptera: Blattidae)

by

Ludek Zurek

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Department of Entomology

Edmonton, Alberta, Canada

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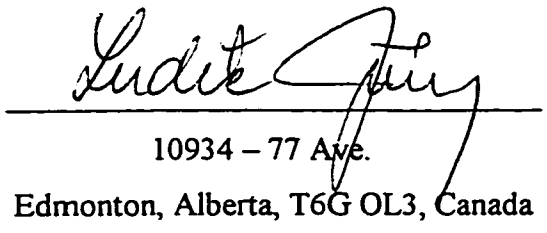

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled "Functional significance and microbial ecology of the anterior hindgut of the American cockroach, *Periplaneta americana* (Dictyoptera: Blattidae)" submitted by Ludek Zurek in partial fulfillment of the requirements for the degree Doctor of Philosophy.

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Abstract

Physiological significance and microbial ecology of the anterior hindgut of the American cockroach, *Periplaneta americana* were examined. Reduction of the hindgut microbial population led to decreased concentration of volatile fatty acids (VFA). Cockroach development was significantly retarded. Negative effects of bacterial reduction were partially compensated by supplementary feeding of VFA in the diet. The anterior hindgut presumably acts as a fermentation chamber and bacterial VFA significantly contribute to cockroach metabolism.

The effects of suppression of methanogenesis with a drug, 2-bromoethanesulfonic acid (BES), on the hindgut ecosystem and development of the cockroach *P. americana* fed either low or high fiber diet were evaluated. Methane production and VFA concentration in the hindgut of cockroaches fed high fiber diet were significantly higher than those of cockroaches fed low fiber diet. Although BES treatment greatly reduced methane production, VFA concentrations in the hindgut, cockroach weight gain and development time were not significantly altered. These results indicate that methanogenic bacteria are not essential for keeping low hydrogen pressure in the hindgut lumen and normal cockroach development. Molecular analysis revealed methanogenic archaea are represented by the genus *Methanobrevibacter*.

Sulphate- and sulphite-reducing bacterial pathways were detected in the hindgut of *P. americana*. Sulphite-reducing bacteria are represented by *Clostridium bifermentans* and *C. celerecrescens*. A high sulphate diet did not affect the profile of VFA in the hindgut, cockroach development and mortality, while numbers of sulphate-reducing bacteria significantly increased. The black band region spread to the anterior part of the

colon. Potential toxic effects of increased hydrogen sulphide production were most likely neutralized by iron supply in the diet.

The filamentous bacterium, *Streptomyces leidynemati*, was isolated from the cockroach hindgut, cultured on artificial media, and metabolically tested. Preliminary tests for antibiotic and cellulolytic activities were negative. Strong chitinolytic activity of *S. leidynemati* was detected.

The diversity of the bacterial symbionts in the digestive tract of *P. americana* was examined. Polymerase chain reaction, cloning and sequencing as well as Checkerboard DNA-DNA hybridisation revealed the greatest phylogenetic diversity of intestinal symbionts resided in the hindgut. New bacterial species for *P. americana* were detected. Sequences of several clones were only distantly related to known bacterial species and genera.

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Chapter 1

General Introduction

1.1. Cockroach ecology and pest status

The American cockroach, *Periplaneta americana* L. (Dictyoptera: Blattidae) is a large insect; the adults measure 35-50 mm in length. Based on fossil evidence, it is probable that this insect has remained basically unchanged in body form for about 280 million years (Robinson, 1996). The American cockroach is an omnivorous and opportunistic feeder; it can feed on virtually any organic material available (Roth, 1981). *Periplaneta americana* originates from tropical Africa but it has been spread by commerce throughout the world (Roth, 1981). Outdoors, *P. americana* can be found in warm and humid habitats and is active at temperatures from 21 to 33°C. It is one of three cockroach species (others are Oriental cockroach, *Blatta orientalis* and German cockroach, *Blatella germanica*) closely associated with human dwellings where food is prepared and stored; they frequent restaurants, grocery stores, bakeries and kitchens. The American cockroach is also commonly present in caves, mines, latrines, sewers, sewage treatment plants and dumps (Roth, 1981).

Because of their movement between sewers and human food materials, cockroaches can transfer pathogens which they carry on their body surface or in the digestive system (Roth and Willis, 1957). Cockroaches have been implicated in disease outbreaks caused by human pathogens such as *Salmonella* sp., *Cryptosporidium* sp., and *Acanthocephala* sp. (Klowden and Greenberg, 1976; Zerpa and Huicho, 1994; Schal and Hamilton, 1990; Bolette, 1987). Several other opportunistic human pathogens, including *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Serratia marcescens*, and *Streptococcus faecalis*

have been consistently isolated from the digestive tract of *P. americana* (Roth, 1981, Paul *et al.*, 1992). In addition, cockroaches in urban environments can be a source of psychological stress and serious medical problems related to allergenic reactions (Kang and Chang, 1985). Furthermore, cockroach exuviae support growth of large populations of the house dust mite (*Dermatophagoides pteronyssius*) which can contribute to bronchial asthma (Schal and Hamilton, 1990; Pola *et al.*, 1988). Development of resistance to chemical insecticides in cockroaches (the American cockroach is resistant to DDT, organo-phosphates, and carbamates) (Beally and Marquardt, 1996), has stimulated research into alternative control methods. These include mechanical and physical control methods, ultraviolet-light exposure, heat treatments, and electronic control devices, none of which are effective or applicable to field situations (Schal and Hamilton, 1990). Biological control agents including parasites, predators and pathogens seem to be more promising (Zervos and Webster 1989; LeBeck 1991, Koehler *et al.* 1992), although the most widespread biological agent used in the management of insect pests, *Bacillus thuringiensis*, is ineffective against the American cockroach (Lee *et al.*, 1984). My research focused on cockroach - microbial interactions provides a better understanding of cockroach physiology and the diversity of its microbial symbionts. It may unveil new microbial isolates that could be manipulated to the detriment of this species and possibly other insect pests.

1.2. Digestive tract and associated microorganisms

The digestive tract of *P. americana* consists of the foregut (pharynx, oesophagus, crop, and gizzard), midgut (caeca, ventriculus), and hindgut (ileum, colon, and rectum)

(Bignell, 1981). While the alimentary canal in general provides a suitable environment for the development and establishment of numerous and diverse microbial communities, the suitability of individual parts of the tract depends on the host physiological activities as well as on adaptations of microorganisms themselves.

The foregut comprises about 50% of the total gut volume. Although lipids may be transported across the foregut wall, the main function of the foregut is food storage and initial digestion (Bignell, 1981). Digestive enzymes are introduced to the crop from the salivary glands and by reflux from the midgut. Lined with a thick chitinous cuticle the foregut is much less permeable to water, ions and organic compounds than other parts of the alimentary canal. The pH of the foregut is lower and more variable than that of the other regions of the gut; values range from 5.4 to 6.5. This variation may be dependent on the nature of the food (Bignell, 1981). Few bacteria have been observed attached to the foregut wall and those observed appeared to be associated with the ingested food. On a volume basis, fewer bacteria have been observed and isolated from the foregut than from other regions of the digestive tract (Bignell 1977a, Cruden and Markowetz 1987).

The midgut, devoid of a cuticular layer, is lined with a microvillus brush border which provides the main site of food digestion and nutrient absorption. The cockroach secretes a variety of digestive enzymes into the midgut lumen, and the end products of digestion are transported back across the midgut epithelium to the haemolymph (Bignell, 1981). The food is separated from the epithelium by a peritrophic membrane that is continuously secreted from a ring of specialized columnar cells at the anterior part of the midgut. Peritrophic membrane, consisting of chitin, protein and mucopolysaccharide, forms a porous lattice with openings about 0.15 μm in diameter. These openings permit

passage of enzymes and other macromolecules, but not bacteria (Bignell, 1981). The peritrophic membrane (PM) extends to the ileum and disintegrates in the colon, probably by action of sclerotized teeth of the valve that separates the ileum and colon. It is possible that PM is further degraded by the hindgut microorganisms (Bignell, 1981).

The function of peritrophic membrane is not completely understood. Terra and Ferreira (1981) have proposed that PM makes digestion in the midgut radially partitioned. Degradation of macromolecules takes place in the endoperitrophic space and hydrolysis of the oligomers occurs principally in the ectoperitrophic space that separates PM from the midgut epithelium. Some terminal digestion is mediated by enzymes bound to the apical cell membrane and/or occurs intracellularly (Terra and Ferreira, 1981). Several other functions of PM have been suggested, including protection against abrasion for the midgut epithelium in the absence of mucous secretions (Richards and Richards, 1977), and acting as a barrier to microbial infection (Mercer and Day, 1952).

The pH of the midgut is higher and more stable than that of the foregut. Values range between 6.3 and 6.7 (Greenberg *et al.*, 1970). The midgut contains significant numbers of bacteria. Cruden and Markowetz (1979) reported 10^8 bacteria per ml that could grow aerobically and $3x10^8$ per ml growing anaerobically on rumen fluid-containing medium. The most common isolates were facultatively anaerobic *Enterobacter agglomerans*, *Klebsiella oxytoca*, and *Citrobacter freundii*. Bracke (1977) observed filamentous microorganisms attached to the peritrophic membrane.

The hindgut is composed of the short muscular ileum, a large sac-like colon, and the rectum. Since the rectum is known to transport ions and water actively, the function of the entire hindgut is thought of as osmoregulation (Bignell, 1980). However, the

functional significance of the anterior part of the hindgut particularly, the colon, is unknown. The colon is well developed in *P. americana* and represents about 30% of the volume of the entire digestive tract. Morphology and ultrastructure of the colon wall of *P. americana* have been described in detail by Bignell (1980) and it is consistent with tissues maintaining the potential for an active transport. The colonic fluid is hypo-osmotic to the primary urine and haemolymph and in addition contains lower concentrations of amino acids than that of the midgut content (Wall and Oschman, 1970). With the exception of the extreme posterior end where the colon narrows to join the rectum, the wall is thinner than that of the ileum and the rectal pads. The epithelium consists of a single extensively folded layer of the columnar cells surrounded from the haemolymph side by layers of muscle and connective tissues (Bignell, 1980). Cuticle lining the luminal side of the epithelium bears numerous cuticular spines projecting up to 60 μm into the lumen (Elzinga and Hopkins, 1995). The thickness of cuticle ranges from 1 μm at the anterior part of the colon to 3 μm at the extreme posterior end. Since the peritrophic membrane breaks up as the ileal content enters the colon, the presence of the spines and the extensive folding cause the gut contents to firmly adhere to the colon wall (Bignell, 1980). The columnar cells are 15-25 μm in height and 6-12 μm wide with the apical part comprising numerous parallel folds of the plasma membrane with inserted mitochondria. Apical infoldings are dilated at the tip, forming substantial extracellular spaces up to 0.8 μm wide. Cytoplasm of the epithelial cells contains numerous microtubules, mitochondria and free ribosomes (Bignell, 1980). This contrasts with the rectal pads where the mitochondria are almost exclusively confined to the cytoplasm bordering the plasma membranes (Noirot and Noirot-Timothee, 1976). Overlying

chitinous cuticle is extensively folded to follow the contours of the colonic epithelium (Bignell, 1980). Despite the existence of a large literature on the structure of the integumentary cuticle (Neville 1975, Richards 1978), little information is available on the cuticle lining the foregut and hindgut. A common feature is that both, integumentary and alimentary cuticle, are largely composed of a chitin – protein composite procuticle that is overlain by a non-chitinous epicuticle. Only valves and triturating surfaces of the gut lumen are formed by sclerotized (tanned) cuticle (Bignell, 1984). Epicuticle shows regular thinning, producing dome-like protrusions of the cuticular surface into the gut lumen. These features might facilitate uptake of organic materials (Bignell 1980, 1984). Pore canals are absent in the alimentary cuticle, reflecting the fact that the wax layer is not secreted. At the posterior end of the colon, the wall thickens and the columnar epithelium is replaced by smaller, flattened cells and cuticular spines shorten to 3 μm (Bignell, 1980). The colon contains strong ouabain-insensitive ATPase activity and only a small fraction of ouabain-sensitive ATPase. Since inactivation of ouabain is a property of Na^+, K^+-activated ATPases, this indicates a relatively minor role for the colon in ionic regulation. It leaves, however, open the possibility for other functions requiring an active transport, possibly absorption of organic solutes (Bignell, 1980). The pH of the colon is higher than that of the foregut and midgut, and ranges from 6.7 to 6.8 (Greenberg *et al.*, 1970). The redox potential of the colon ranges between –84 and –240 mV, suggesting a low oxygen tension that may approach anaerobic conditions (Warhurst, 1964).

There is clear evidence that the hindgut provides more suitable conditions for the establishment of microbial populations than that of the foregut and midgut. Several studies showed a numerous and diverse population of bacteria (Bignell 1977a, Bracke *et*

al., 1979, 1978, Cruden *et al.*, 1979, Cruden and Markowetz 1980, 1981, 1984, 1987, Foglesong *et al.*, 1975, 1984). In addition, several species of archaea (Gijzen *et al.*, 1991, Hackstein and Stumm, 1994), protists (Koura and Kamel 1992), and oxyurid nematodes (Adamson and Noble, 1992) have also been reported from the cockroach colon.

The composition of microorganisms reflects a low oxygen tensions in the colon. Dilution series on rumen fluid–containing media indicated that 10^8 bacteria could grow aerobically, while over 10^{10} grow anaerobically (Bracke, 1977). The common facultatively anaerobic bacterial isolates are similar to those reported from the other insects including crickets (Ulrich *et al.*, 1981), crane fly larvae (Klug and Kotarski, 1980) and termites (Schultz and Breznak, 1978). The most common facultatively anaerobic isolates on a variety of selective and non-selective media are *Citrobacter freundii, Enterobacter agglomerans, Klebsiella oxytoca, Serratia* sp., and *Streptococcus* sp. (Becker *et al.*, 1982; Cruden and Markowetz, 1979, 1984). These isolates may scavenge oxygen diffusing to the lumen through the colon wall (Cruden and Markowetz, 1987).

Strictly anaerobic bacteria isolated from the hindgut are more numerous and more varied (Cruden and Markowetz, 1987). Species isolated on non-specific media include *Acidaminococcus fermentans, Bacteroides sp., Bifidobacterium sp., Butyrivibrio sp., Clostridium sporogenes, C. bifermentans, Coprococcus sp., Eubacterium moniliforme, Fusobacterium varium, Lactobacillus sp., Peptococcus variabilis, Peptostreptococus productus, Propionibacterium avidum,* and *Ruminococcus* sp.. Spirochetes are commonly observed in the cockroach hindgut, however they have not been isolated or identified (Cruden and Markowetz, 1987). Most of these isolates are also a part of natural

microbiota of termites, oriental and giant cockroaches (Holdeman *et al.*, 1977), and mammalian intestinal tracts (Holdeman *et al.*, 1977, 1983). Microscopic observations have revealed that many bacteria are attached to cuticular spines and the hindgut wall and are not removed by washing. Some rod-shaped bacteria divide longitudinally which insures continuous attachment. Some bacteria are attached to the surface of unidentified filamentous bacteria producing a web-like array of the colon microbiota (Cruden and Markowetz, 1987). Many bacterial morphotypes observed by transmission or scanning electron microscopy have not been isolated, cultured or identified (Bracke, 1977). Cruden and Markowetz (1981) detected fourteen microbial morphotypes in their transmission electron microscopy survey. They reported that microbiota of three wall associated regions (the anterior and posterior colon, and black band region at the extreme posterior end of colon) are different from that of the hindgut lumen. The three wall fractions were also significantly different from each other. The dark black band has been observed in both laboratory-reared and in wild cockroaches. Chemical analysis of this region detected ferrous sulphide, which might indicate very low redox potential; elements, including iron, sulphur, aluminum and copper also have been detected. Numbers of bacterial spores are significantly higher in this region than in other parts of the alimentary canal (Cruden and Markowetz, 1987). The significance and specific metabolic activities in this region are not known.

Representatives of the Archaea domain are also represented in the hindgut lumen. Free-living methanogenic archaea have morphology similar to the genus *Methanospirillum* as reported by Cruden and Markowetz (1981). Additionally, Gijzen *et al.* (1991) observed methanogenic archaea morphologically similar to the genus

Methanobrevibacter, endosymbiotically associated with the protist *Nyctotherus ovalis*. These protists commonly inhabit the hindgut lumen. (These authors used the term - bacteria for methanogens. Since 1994, methanogenic microbes are considered in a separate domain called Archaea).

The hindgut bacterial microbiota is also very diverse metabolically. Fermentation of carbohydrates and amino acids (Cruden and Markowetz, 1987), degradation of carboxymethylcellulose (Cruden and Markowetz, 1979), degradation of cellulose and hemicellulose (Bignell, 1977b), chitinolytic activity (Cruden and Markowetz, 1987), nitrogen-fixation (Cruden and Markowetz, 1980), uric acid degradation (Becker *et al.*, 1982), methanogenesis (Gijzen and Barughare, 1992), and acetogenesis (Kane and Breznak, 1991) have been reported. Volatile fatty acids, primarily acetate, propionate, and butyrate arise from the fermentation processes (Bracke and Markowetz, 1980; Cazemier *et al.*, 1997) and their transport from the hindgut lumen to the haemolymph is feasible (Bracke and Markowetz, 1980). A significant reduction of hindgut anaerobic population by metronidazole, an antibiotic, had no effect on metabolism of adult *P. americana*, while development of subadult cockroaches exposed to the drug from hatching, was significantly retarded (Bracke *et al.*, 1978). The mechanism for the potential contribution of the hindgut microbiota to cockroach metabolism, was not examined however. There have been no studies of the effects of the gut microbiota on insect gut development. Flatt *et al.* (1958) and Sanders *et al.* (1959) showed that development of the rumen mucosa of calves was stimulated by volatile fatty acids (especially butyric, propionic and lactic acid) produced by activities of the ruminal

microbiota and that such stimulation could be simulated by externally supplied fatty acids in the diet.

Several species of ciliated protists, including *N. ovalis*, *Balantidium ovatum*, and *B. blattarum* are a part of the natural hindgut microbiota of *P. americana* (Koura and Kamel, 1992). *Nyctotherus ovalis* has been shown to possess cellulolytic activity and contributes to cockroach metabolism (Gijzen and Barughare, 1992). The mechanism of this contribution is unclear but the ciliates' ability to degrade cellulose is likely important. Several species of oxyurid nematodes including, *Leidynema appendiculata*, *Hammershmidtiella diesingi*, and *Thelastoma* sp. commonly found in the hindgut lumen, do not appear to have any effect on metabolism and survival of *P. americana* (Bignell and Mullins, 1977, Adamson and Noble, 1992). Hoffman (1953) observed a filamentous bacterium *Streptomyces leidynemati* (Actinomycetales) attached to the surface of oxyurid nematodes in the hindgut lumen. No obvious effects on nematode physiology have been reported. Attempts to isolate and cultivate the *S. leidynemati* on artificial media in that study were not successful however.

Studies conducted over a period of more than forty years clearly show that the hindgut of *P. americana* contains a large population of microorganisms that are morphologically, phylogenetically and metabolically very diverse, yet only a small portion has been identified and characterised. There is evidence that the hindgut is permeable to organic solutes and the ultrastructure of the hindgut wall indicates that an active transport in this tissue is feasible.

1.3. Microbial diversity of intestinal symbionts

In contrast to plants and animals, the diversity of microorganisms is largely unknown. Over the last decade, studies have revealed that metabolic and phylogenetic diversity of microorganisms is far greater than previously believed. It is conservatively estimated that less than 1% of the bacterial species and less than 5% of fungal species are currently described (Tiedje, 1995). One of the most exciting breakthroughs in the characterisation of microorganisms from natural communities comes from techniques used to analyse nucleic acids of bacteria present in any community (Pace, 1996). In this approach, which does not require cultivation of the organisms, nucleic acids are extracted from the environment, and their ribosomal RNA or rDNA are obtained, amplified by polymerase chain reaction (PCR) and PCR product is cloned and sequenced to identify the organism. This method has helped to unveil large numbers of unknown or previously unidentified organisms. Phylogenetic identification of an isolate and its relationship to known and cultivable organisms then might provide new clues for formulating suitable nutritional medium and laboratory culturing (Pace, 1996). When successful, consequent cultivation of microorganisms greatly enhances our ability to determine the full complement of genetic and physiological characteristics of newly isolated species. With the exception of human intestinal microbiota, hindgut of some termite species, and bovine rumen, very little is known about diversity and functional significance of microorganisms associated with the digestive tracts of either vertebrates or invertebrates (Kane and Pierce, 1994). In many microbial ecosystems, the functionally active unit is not a single species or population but a consortium of many types of organisms living in

close symbiotic association. It is important, nevertheless, to identify individual types of microorganisms in order to uncover the contributions of each (Pace, 1996).

The overall objective of this thesis is to evaluate the physiological significance of the anterior part of the hindgut and its bacterial symbionts for metabolism and development of the American cockroach, *Periplaneta americana*. Evidence for the contribution of bacterial volatile fatty acids (VFA) to cockroach development is presented. Examination of physiological significance and phylogenetic diversity of methanogenic symbionts in the cockroach hindgut is also provided. Furthermore, two bacterial metabolic activities, sulphate- and sulphite-reduction, have been detected from the cockroach hindgut ecosystem for the first time. Sulphite-reducing bacteria are phylogenetically identified and the potential of sulphate-reducing bacteria for the biological control of cockroaches is assessed. Also, the actinomycete, *Streptomyces leidynemati*, living on the surface of oxyurid nematodes in the cockroach hindgut, has been successfully cultured on artificial media and metabolically tested. In addition, a molecular analysis approach is employed for the first time to assess phylogenetic diversity of the intestinal microbial community of the American cockroach.

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Chapter 2

Contribution of the Colon and Colonic Bacterial Volatile Fatty Acids to Metabolism and Development of the American Cockroach

2.1. Introduction

In spite of numerous studies on microbial composition and ultrastructure of the anterior hindgut (ileum and colon) of *P. americana* (Bignell, 1977a, 1980; Fogglesong *et al.*, 1975; Bracke *et al.*, 1979; Cruden and Markowetz, 1984, 1987, Gijzen *et al.*, 1991, 1994), its role in cockroach metabolism and development is poorly known. Several authors have suggested that the microbial community in hindgut of the cockroach *P. americana* contributes to nutritional and developmental processes of the host (Bignell, 1980; Bracke and Markowetz, 1980, Bracke *et al.*, 1978; Gijzen and Barughare, 1992), however direct evidence is lacking.

The cockroach colon is a large sac-like organ representing about 30% of the volume of the entire alimentary canal (Bignell, 1980). This organ contains a dense and extremely varied bacterial population which consists of both facultative and obligate anaerobes (Becker *et al.*, 1972; Bignell, 1977a; Bracke *et al.*, 1979; Cruden and Markowetz, 1987). Many of these bacteria are firmly attached to the cuticular wall and to the surface of unidentified filamentous bacteria producing a web-like array of the colon microbiota (Cruden and Markowetz, 1987). Production of volatile fatty acids (VFA) arising from fermentation of sugars and amino acids and their passive transport through the cuticular layer of the colon wall to the haemolymph have been reported (Bracke and Markowetz, 1980). However, specific elimination of the anaerobic hindgut microbiota with metronidazole had no effect on metabolic processes in adult *P. americana.* In contrast, stunting (retarded weight gain, longer generation time, underdeveloped gut) was observed in

subadult animals maintained from hatching on the antibiotic drug (Bracke *et al.*, 1978). Diet composition has been shown to affect both gut microflora and production of organic acids (Kane and Breznak, 1991). Bignell (1977b) proposed that cellulose and hemicellulose are potentially of nutritional significance in *P. americana* and that hindgut is an important degradation site. Degradation of carboxymethyl cellulose in the hindgut aws subsequently demonstrated (Cruden and Markowetz, 1979).

The present study describes profiles of volatile fatty acids in the colon during cockroach development and their alteration with metronidazole treatment. The effect of metronidazole on cockroach development (weight gain, development time) was examined. Supplementary feeding of VFA to compensate for the impact of metronidazole was provided. The effects of reducing the microbial population in the hindgut by using sterile rearing conditions on cockroach development were also examined.

2.2. Materials and methods

Rearing colonies: Colonies of *P. americana* L. have been maintained for many years in our department. Each colony is housed in a 15 liter aquarium containing tiers of wooden shelves resting on a sawdust substrate. Diet consists of Purina dog chow and oat meal (1:1 ratio) and water *ad libitum*.

2.2.1. Experiment I (reduction of intestinal microbiota by antibiotic treatment)

Experimental colonies: Oöthecae were removed from rearing colonies and newly hatched cockroaches were exposed to used sawdust substrate for 24 hours to acquire their intestinal microflora. After this treatment insects were placed in 8 liter plastic containers supplied with fresh sterile sawdust bedding and a single wooden shelf. These colonies were reared in an

environmental chamber maintained at 27° C, 75% relative humidity and 10L : 14D light regime. All colonies were provided with identical quantities of food and water. Water was supplied in 30 ml glass tubes with cotton stoppers. Containers were sterilized by bleach (10%); bedding, shelves, food, water, water containers and cotton were autoclaved before use.

Experimental conditions: In an initial experiment, newly hatched insects were divided into two unsexed groups of sixty each. The control group was fed a measured amount of sterile food and water. The test group was fed the same quantity of food, but its drinking water contained metronidazole. In a subsequent experiment, a third colony was added. It received the same amount of metronidazole, plus supplements of VFA (acetic acid, 1.66 mg/ml and propionic acid, 0.33 mg/ml of drinking water). Drinking water in all colonies was replaced every seven days.

Antibiotic: Metronidazole [1- (2–hydoxyethyl)–2-methyl–5–nitroimidazole] acts specifically against anaerobic microorganisms by being reduced intracellularly by a phosphoroclastic reaction. The reduced form of the drug binds to the bacterial DNA, causing an overall disruption of DNA replication and transcription (Edwards, 1977, Church *et al.*, 1996). Metronidazole has no effect on intracellular bacterial symbionts in the cockroach fat body. Microscopic examination of the fat body has revealed no differences between mycetomes of metronidazole treated (for 164 days) and control cockroaches. The cells were the same size and all were packed with large bacterial rods, many of which were apparently dividing (Bracke *et al.*, 1978). Metronidazole (Sigma Chemical Comp., St. Louis, Mo.) was incorporated into drinking water at a level of 400 μg/ml.

Weight gain: The fresh body weight of each cockroach was measured at 30, 60, 90, 120, 150, 180 days after hatching.

Analysis of volatile fatty acids: Every thirty days after hatching, specimens were selected at random from each colony and used for analysis. At thirty and sixty day time points six specimens were used; at subsequent time points four specimens were analyzed. After chilling, cockroaches were cut open in Ringer's solution bath (Appendix 1) and the colon ligated, dissected out and weighed. For a preliminary analysis of the entire cockroach gut, the foregut, midgut and colon were individually ligated and analyzed separately for VFA. Each sample was made from one or two colons homogenized in fifty µl of sterile water, five µl of phosphoric acid and one µg of 4-methyl valeric acid (internal standard) and centrifuged at 11,310 x *g* for 20 min at 4° C. Supernatant was collected and used for analysis by gas-liquid chromatography. Analysis was done on Varian 3400 GC with Stabilwax-DA Crossbonded Carbowax - PEG column (30m x 0.25mm ID x 0.5µm film thickness, Restex Corporation, Bellefonte, PA) programmed from 100°C to 170°C at 10°C/min. Injector and detector (FID) were maintained at 200°C. Helium was used as a carrier gas at 20 psi head pressure and at 30 ml/min as a make-up gas, air at 300ml/min and H_2 at 30 ml/min. Data were analyzed on Shimadzu EZChrom Chromatography Data System (Shimadzu Scientific Instruments, Inc., Columbia, MD). Concentration of VFA was converted to µg of VFA per gram (wet weight) of the gut region.

Analysis of methane: Gas chromatography was used to measure methane production by intact insects according to a standard method (Gijzen *et al.*, 1991). Eight to ten randomly chosen insects were closed in glass bottles sealed with a rubber stopper and a screw cap and used for analysis at 30, 60, 90, 120, 150, and 180 days after hatching. Bottles were incubated

at room temperature for one hour and then analyzed for methane content. The procedure was performed twice (over a twenty-four hour period for each sample) to accommodate possible daily fluctuations.

Scanning electron microscopy. Each insect was chilled and dissected to remove the colon. After five minutes' fixation in 2% paraformaldehyde, a longitudinal incision was used to open the colon. Content and fixative were removed by rinsing with Ringer's solution. Tissue was postfixed in Bouin's fixative, dehydrated, and critical point dried. Specimens were examined with Cambridge S 250 scanning electron microscope and photographed.

2.2.2. Experiment II (reduction of intestinal microbiota without using antibiotics)

Experimental colonies: Surfaces of oöthecae from rearing colonies were sterilised by immersion in bleach (10%) for 15 min. Newborn cockroaches were divided into two colony groups of fifty-six each. Insects were placed in 8 liter plastic containers supplied with fresh sterile sawdust bedding and a single wooden shelf. These colonies were reared in an environmental chamber maintained at 27°C, 75% relative humidity and 10L : 14D light regime. All colonies were provided with identical quantities of food and water. Water was supplied in 30 ml glass tubes with cotton stoppers.

Experimental conditions: To reduce hindgut microbial symbionts without using antibiotics, containers, shelves, shavings, glass tubes, cotton, water and food were sterilised by autoclaving or by bleach (10%) before use. The control colony received used substrate (1.24 grams) from rearing colonies for establishment of the natural gut microbiota. The treated colony received the same amount of used but autoclaved substrate from rearing colonies. No additional steps were taken to keep this colony sterile.

Weight gain: The fresh body weight of each cockroach was measured at 30, 60, 90, 120, 150, 180 days after hatching.

Bacterial cell count: At 180 days, four randomly selected cockroaches from each colony were cooled, their hindguts were dissected and contents pooled and transferred to tubes containing 10 ml of potassium phosphate buffer. These tubes were consequently used for dilution series to 10^{-10} and each dilution tube was plated in triplicate on the nutrient agar (Bacto – Nutrient agar, Difco, Detroit, MI, USA) and incubated in either aerobic or anaerobic conditions for two and three weeks, respectively. The direct count of the colony forming units (CFU) (Russek and Colwell, 1983) was used for the bacterial cell count. Colonies with different morphologies from each treatment were recorded.

Statistical analysis: For both experiments, statistical significance of differences between means of the body weight was assessed by Student's *t* test.

2.3. Results

2.3.1. Experiment I (antibiotic treatment)

Preliminary analysis of the entire cockroach gut revealed that the highest VFA concentrations occurred in the colon. Only acetic and propionic acids were detected in the other parts of the gut, and these were at clearly lower concentrations (Table 2.1). These findings are consistent with studies reporting the most dense bacterial colonisation in the hindgut followed by the midgut and the foregut (Cruden and Markowetz, 1987; Bignell, 1977a).

Only colonic VFA were considered in further experiments, although bacterial populations in other parts of the gut were likely affected by antibiotic treatment. During

cockroach development, concentrations of acetic and propionic acid were highest at first thirty days then diminished slightly and remained relatively constant (Figure 2.1 - 2.2). Concentrations of acetic acid were highest, followed by propionic, isovaleric, and butyric acids (Figure 2.1-2.3). Traces of isobutyric, valeric, and caproic acids were also detected in most extracts (Figure 2.3).

Metronidazole treatment reduced the cockroach weight gain over the entire growth period. The greatest difference, 32.8%, was observed at 60 days of age (Table 2.2). Adult body weight was reduced by 13.2%, and development time was extended by 14 days (186 days in total) in comparison with the control colony. The observation by electron microscopy of colonic bacterial microflora after metronidazole treatment confirms results of the examination made by Bracke *et al.* (1978). Although bacterial microflora was drastically reduced (filamentous bacteria, an abundant type in the control colony, were completely eliminated) (Figure 2.4 - 2.5), a small population of predominantly cocci remained in the colon (Figure 2.6 - 2.7). Cuticular spines projecting into the colonic lumen were underdeveloped and lacked normal dense bacterial colonisation (Figure 2.7). Concentrations of VFA were extensively reduced. Acetic acid was lowered by 60.1 %, and propionic acid was reduced by 85.5 % (Figure 2.1 - 2.2). Isovaleric, butyric, valeric, isobutyric, and caproic acids were completely eliminated (Table 2.1).

Supplementary feeding of VFA partially compensated for the effects of the antibiotic treatment. A significant difference in weight gain between the cockroaches treated with metronidazole and the cockroaches treated with metronidazole and VFA supplements was observed in 60, 90, and 120 days. Cockroach adult body weight was increased by 9.6% (Table 2.2) and development time was shortened by 8 days (178 days in total). Scanning

electron microscopy revealed that metronidazole in acidic solution (pH 4.2) remained an effective antibiotic in the colon. Bacterial populations in cockroaches with metronidazole and VFA supplements were similar to those of cockroaches treated solely with metronidazole.

Methane production was consistently very low (average: 0.012 μmol per gram of insect fresh weight per hour) throughout cockroach development and was not significantly different among experimental colonies (data not included). Cockroaches used in this study lacked populations of *Nyctotherus ovalis*, protozoans that are known to harbour methanogenic bacteria as endosymbionts (Gijzen *et al.*, 1991). Methane production in these cockroaches presumably originated from free-living methanogens not suppressed by the antibiotic treatment.

During VFA analysis, two peaks tentatively identified as furfural (2-furaldehyde) and 5-hydroxymethyl furfural were detected (Figure 2.8). These two toxic compounds were produced in the gas chromatograph injection port (xylose disolved in water was injected to the GC port resulting to formation of the same two peaks) presumably by heating of pentoses and were not the natural component of the cockroach gut content. A similar observation has been made during VFA's analysis of the alfalfa silage and the colon contents of pigs (M. Fenton, personal communication)

2.3.2. Experiment II (initial sterile conditions)

Weight of cockroaches reared in initially sterile conditions was significantly lowered in comparison to those of the control colony. Significant differences were detected in all

weight measurement periods (Table 2.3). The adult body weight of cockroaches from treated and control colonies was 833.7 mg and 921.7 mg, respectively (Table 2.3).

Direct bacterial cell count of CFU at the end of cockroach development revealed clear differences in the abundance and diversity of the hindgut microbiota between the control and treated colony (initial sterile conditions). Nutrient agar plates inoculated with hindgut contents from control cockroaches contained 8.8×10^9 and 4.6×10^9 bacteria per ml growing aerobically and anaerobically, respectively. Inoculum from cockroaches grown in initially sterile conditions revealed 2.9×10^8 aerobic bacteria and 1.7×10^8 anaerobes per ml of hindgut content. Differences were observed in the diversity of bacterial morphotypes. Seven and five different morphotypes were observed in the control colony under anaerobic and aerobic conditions, respectively. Treated cockroaches had only three morphotypes growing anaerobically (all present in control colony) and three aerobic morphotypes (two present in control colony).

2.4. Discussion

Volatile fatty acids can act as oxidizable substrates and biosynthetic precursors. Acetate is an important oxidizable substrate for termites and xylophagous cockroaches (Odelson and Breznak, 1983), and an important precursor for the biosynthesis of termite fatty acids (Blomquist *et al.*, 1979; Mauldin, 1982), terpenes (Prestwich *et al.*, 1981) and, along with propionate, cuticular hydrocarbons (Blomquist *et al.*, 1979). Acetate and propionate also can be incorporated into the carbon skeleton of juvenile hormone in many insect species (Schooley *et al.*, 1973; Peter and Dahm, 1975; Tobe and Feyereisen, 1983; Jennings *et al.*, 1975).

The incorporation of acetate to cuticular and internal hydrocarbons in *P. americana* has been reported (Blomquist *et al.*, 1975; Conrad and Jackson, 1971). *Periplaneta americana* is one of few insect species with the ability to synthesise long-chain polyunsaturated fatty acids (Blomquist *et al.*, 1982). Louloudes *et al.* (1961) reported incorporation of acetate to palmitoleate, linoleate, linolenate, oleate, and palmitate. The incorporation of propionate into branched hydrocarbons in *P. americana* also occurs (Conrad and Jackson, 1971). Experiments presented by Halankar *et al.* (1985) revealed that the major pathway of propionate metabolism is the conversion of propionate to acetate and then its incorporation into hydrocarbons. There are no reports of metabolism of butyrate, isovalerate and other VFA for *P. americana.*

Although the effect of metronidazole on cockroach weight gain was not completely neutralised, weight increased with VFA supplements. The cockroach daily water intake is not known and it is difficult to calculate the amounts of VFA necessary to compensate accurately for the effect of metronidazole treatment. The high concentrations of VFA in drinking water could alter food and water consumption, thereby affecting cockroach weight gain. Rapid transport of VFA from colonic lumen may further complicate calculations. Results of an ultrastructural study and stereological analysis of the colon wall of *P. americana* (Bignell, 1980) suggest the active transport of organic solutes may occur in this tissue.

The contribution of hindgut bacterial symbionts has been also shown without using antibiotics. Although cockroaches grown in initially sterile conditions acquired bacterial symbionts during development, the abundance and diversity of these bacteria remained lower that that in cockroaches with natural intestinal bacterial symbionts. After 180 days

(the end of development) weight gain of treated cockroaches remained significantly lower. It is important to underscore that only one broad-spectrum medium (nutrient agar) has been used for cell counts. Growth of some bacteria might not to be supported by this medium. Nevertheless, these results indicate clear alteration and reduction of the hindgut bacterial symbionts by initial sterile rearing conditions and consequent retarded weight gain and development time.

Since the American cockroach has little or no ability to metabolise pentoses (Bignell, 1981) and lacks the ability to synthesise carbohydrates from lipids, it is likely that *P. americana* benefits from bacterial fermentation of monosaccharides (including pentoses), amino acids and possibly soluble disaccharides and oligosaccharides entering the hindgut. As a major site of bacterial colonisation and attachment, the colon of *P. americana* presumably functions as a fermentation chamber. Although *P. americana* is not strictly dependent on the products of fermentation of its intestinal symbionts, it is likely that VFA's contribute to cockroach development as oxidizable substrates and biosynthetic precursors. The food transit time is longest in the hindgut (Snipes and Tauber, 1937), and the hindgut tissue undergoes substantial peristaltic and antiperistaltic movement (Cook and Reinecke, 1973). Large populations of bacteria attached to the cuticular spines gain deeper access to the colonic lumen without being discharged with faeces. The cuticular spines presumably also act as blades, helping to homogenise the content of the colon and advance the fermentation process. Taken together, these factors support the hypothesis that the colon of *P. americana* plays an important role in the cockroach physiology and development.

According to Gijzen and Barughare (1992) and Gijzen *et al.*, (1994) the hindgut anaerobic protozoan *N. ovalis*, which harbours methanogenic bacteria as endosymbionts,

and possesses cellulolytic activities, plays a major role in cockroach metabolism. In this study, colonies were devoid of this protozoan, as substantiated by very low methane production. Adult body weight in the control colony was similar to weights reported for colonies without *N. ovalis* population (Gijzen and Barughare, 1992). The reduction of anaerobic bacterial populations reduced insect weight gain and extended development time, suggesting a significant contribution of anaerobic bacterial microflora to cockroach metabolism and development. It is possible that, when a population of *N. ovalis* is present, bacterial microflora play an important role by additional conversion of intermediates of cellulose degradation to volatile fatty acids.

Insects are not generally able to synthesise cholesterol and depend entirely on food to supply this essential nutrient or in cases of many phytophagous species, its precursors (phytosterols) (Bignell, 1981). Contribution of intestinal bacterial flora to the metabolism of sterols was reported for the German cockroach *Blattela germanica* (Clayton, 1960). While the quantitative requirement of *P. americana* for cholesterol is not known, the diet contained 0.17mg of cholesterol per gram of food, which is presumably sufficient to cover the cockroach needs.

A changing dependence on the products of microbial metabolism suggests that nutritional requirements of immature cockroaches differ from those of adult animals. Subadult cockroaches may depend more heavily on products of their intestinal symbionts due to high metabolic rates during growth and development. The significance of bacterial VFA probably increases substantially in time of food deficiency. The hindgut content is retained in the lumen and all substrates, including cellulose and chitin are degraded by microorganisms to VFA which become available to the host. Intestinal symbionts probably

help to maintain a broad nutritional versatility among omnivorous cockroaches. This versatility may well contribute to making this group one of the most successful of insect species.

Figure 2.1 Profiles of acetic acid from colon contents during development of *P. americana* from control and metronidazole treated colonies.

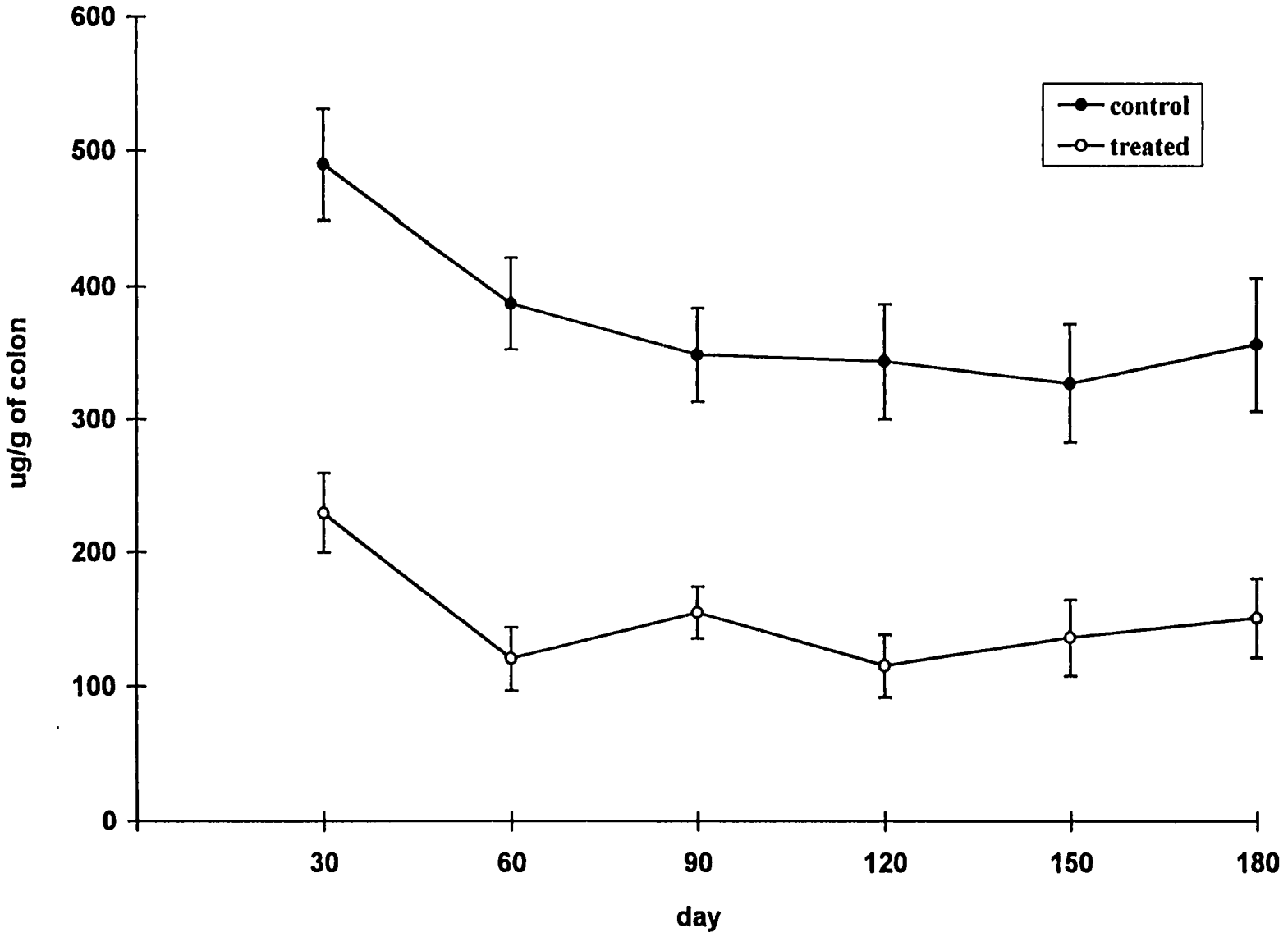

Figure 2.2 Profiles of propionic acid from colon contents during development of *P. americana* from control and metronidazole treated colonies.

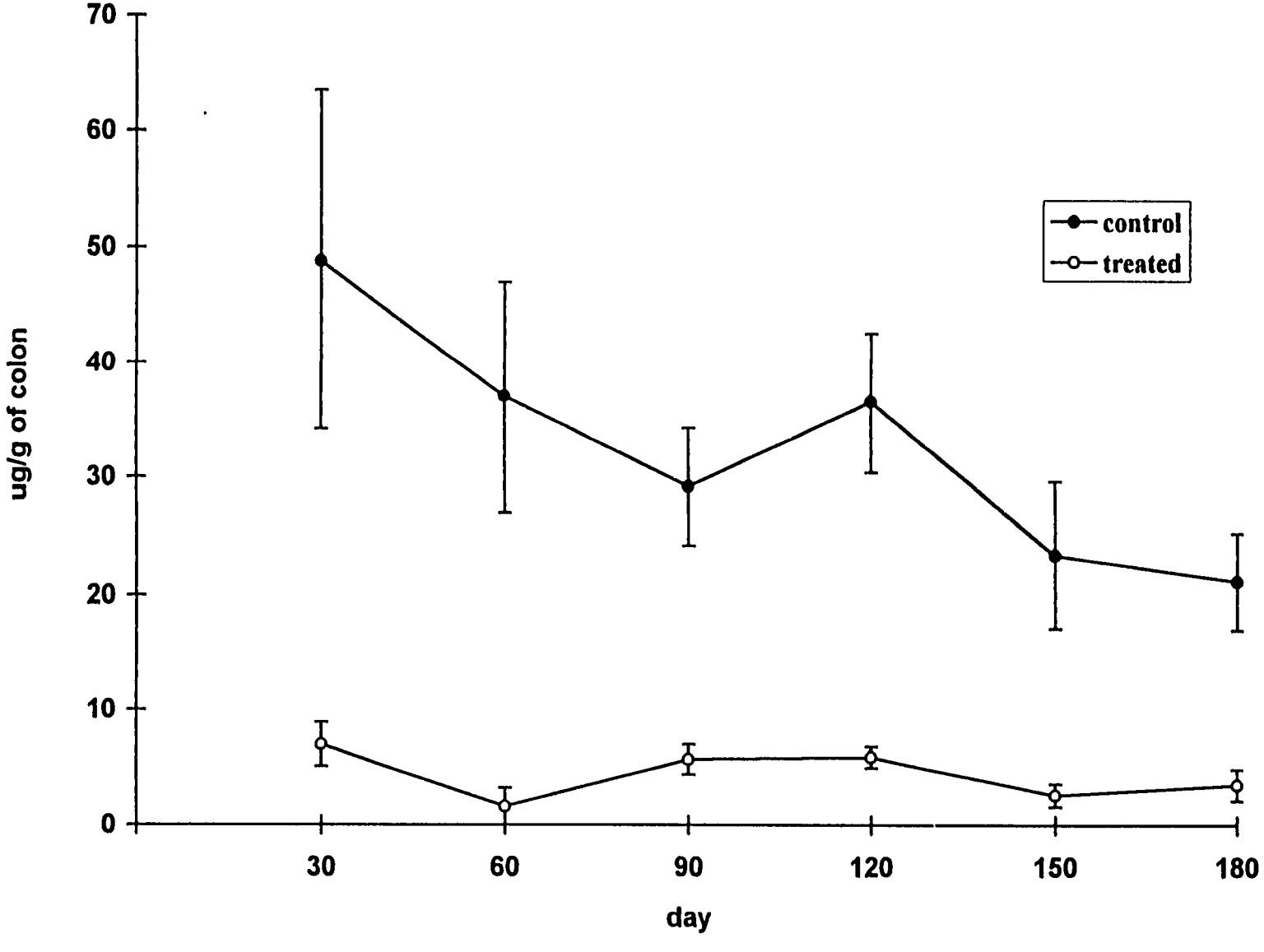

Figure 2.3 Profiles of isovaleric and butyric acids from colon contents during development of *P. americana* from control colony (eliminated from treated colonies).

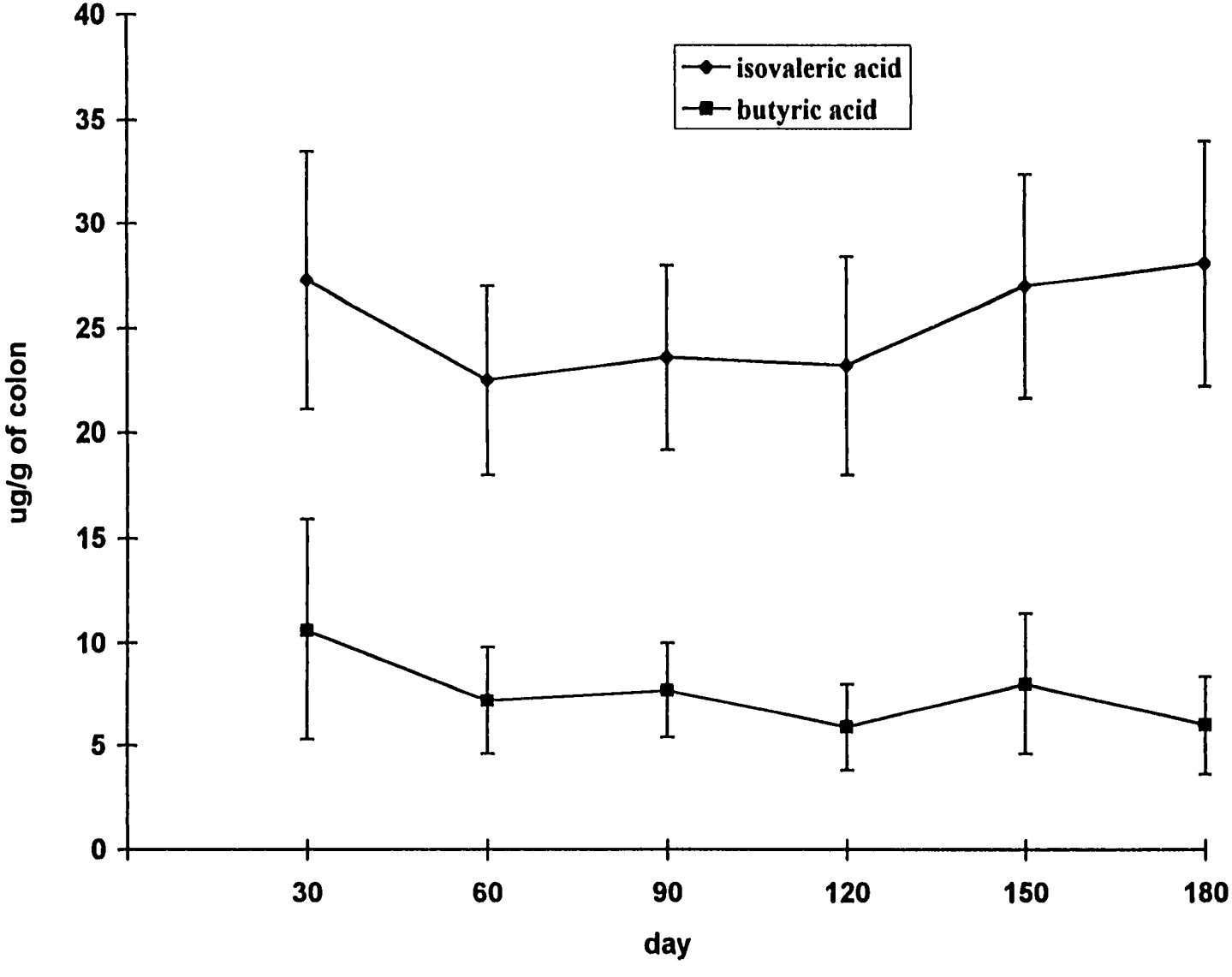

Figure 2.4 Scanning electron micrograph of the anterior colon wall of *P. americana* from the control colony. Filamentous bacteria attached to the wall are predominant. (Bar = 200 μm).

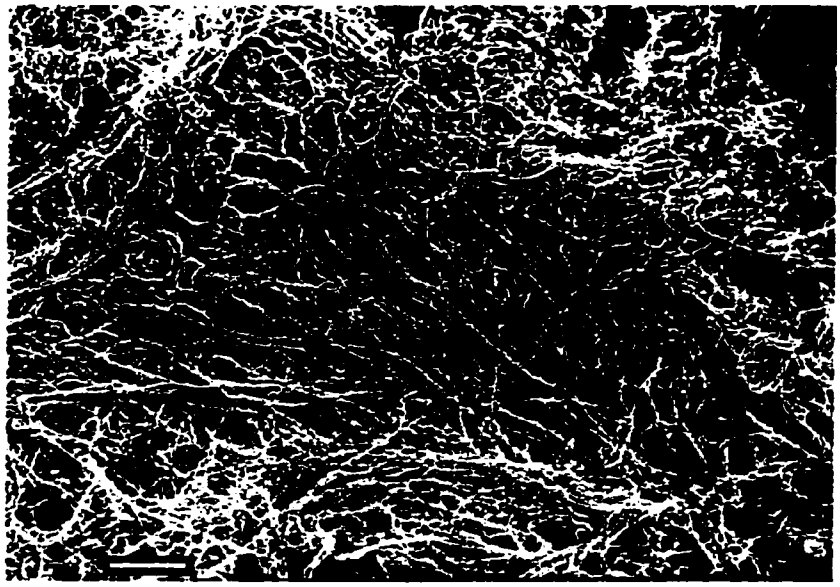

Figure 2.5 Scanning electron micrograph of the anterior colon wall of *P. americana* from the metronidazole treated colony. Filamentous bacteria are completely eliminated. (Bar = 200 μm).

Figure 2.6 Scanning electron micrograph of the anterior colon wall of *P. americana* from the control colony. Bacterial rods are attached to the cuticular filaments. (Bar = 2 μm).

Figure 2.7 Scanning electron micrograph of the anterior colon wall of *P. americana* from the metronidazole treated colony. Cuticular spines are underdeveloped and lack bacterial colonisation (Bar = 2 μm).

Figure 2.8 Gas-liquid chromatograph of volatile fatty acids from the colon content of *P. americana* showing furfural and 5-hyroxymethyl furfural.

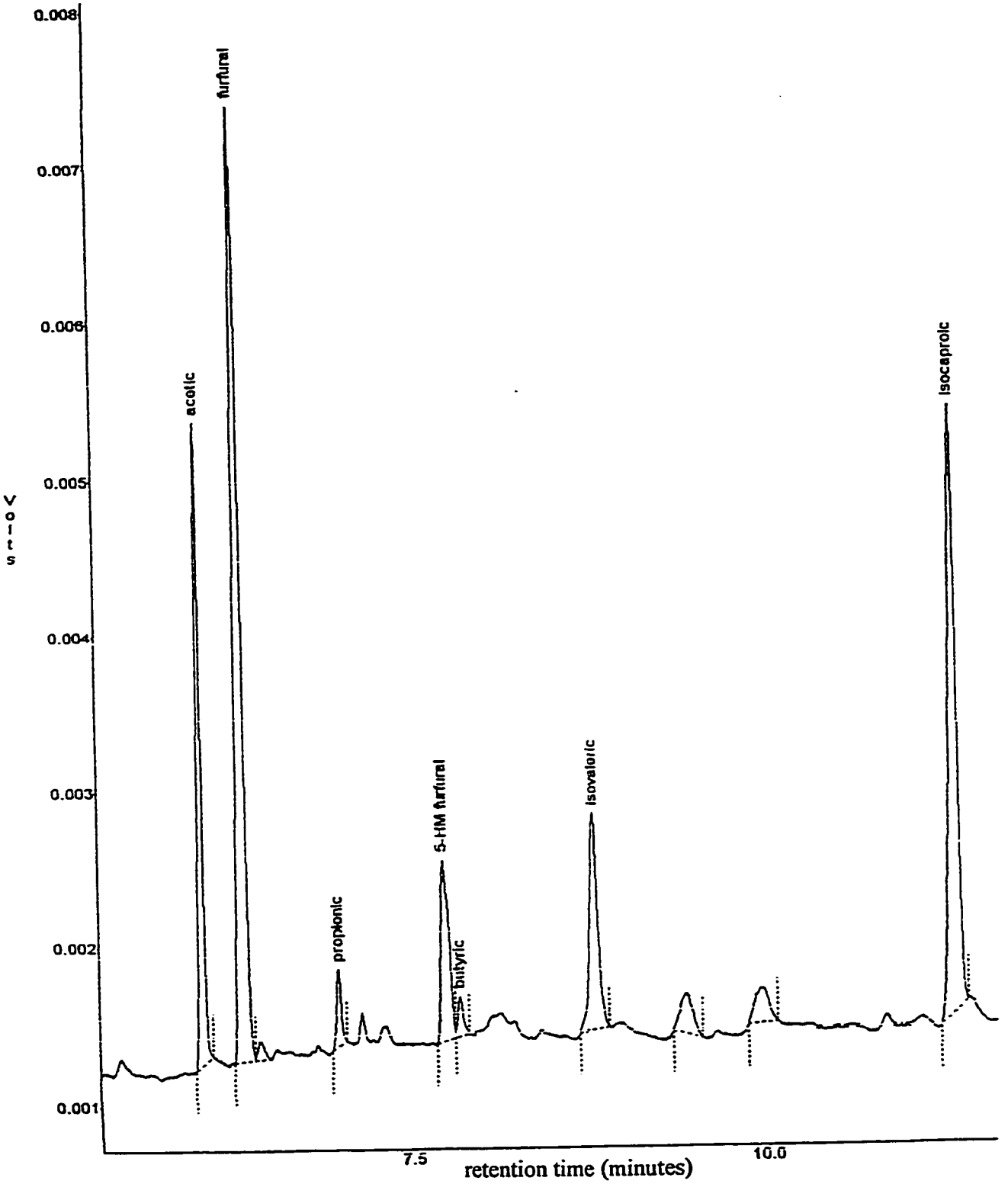

Table 2.1 Concentrations of volatile fatty acids (in μg per gram of the gut region ± SEM) in different regions of the gut of subadult (60 - 90 days of age) *P. americana.*

Acid	Foregut	Midgut	Hindgut (colon)	No. of animals
acetic	43.2 ± 5.2^{a}	110.3 ± 13.2^{b}	343.3 ± 43.2^{c}	4
propionic	2.2 ± 0.34^{a}	5.2 ± 0.89^{b}	36.5 ± 6.1^{c}	4
isovaleric	ND	ND	23.2 ± 5.2	4
butyric	ND	ND	5.9 ± 2.1	4
isobutyric	ND	ND	< 3.0	4
valeric	ND	ND	< 3.0	4
caproic	ND	ND	< 3.0	4

(ND - not detected; SEM - standard error of mean)

^{a,b,c,} - means with different superscript differ (Student t- test, $P < .05$)

Table 2.2 Weight gain of cockroaches *P. americana* exposed to different treatments from hatching.

	Metronidazole			Control			Metronidazole + VFA's		
Age (days)	No. of animals	Avg.wt. (mg)	SEM	No. of animals	Avg.wt. (mg)	SEM	No. of animals	Avg.wt. (mg)	SEM
1	60	3.1^{a}	NM	60	3.1^{a}	NM	60	3.1^{a}	NM
30	59	17.8^{a}	0.4	58	19.8^{b}	0.5	57	18.1^{a}	0.4
60	51	64.1^{a}	2.1	51	95.4^{b}	4.2	49	71.6^{c}	2.8
90	45	186.6^{a}	9.5	45	237.9^{b}	11.9	43	215.4^{b}	8.2
120	41	371.4^{a}	17.3	41	433.2^{b}	18.9	39	419.9^{b}	16.9
150	36	661.9^{a}	32.4	37	748.9^{b}	27.8	35	$733.9^{a,b}$	26.7
180	32	733.0^{a}	35.7	32	844.4^{b}	35.8	30	$803.0^{a,b}$	26.4

$^{a, b, c}$ - means within same age class with different superscripts differ ($P < .05$)
NM - not measured; SEM - standard error of mean

Table 2.3 Weight gain of cockroaches *P. americana* exposed to different treatments from hatching.

	Sterile			Control		
Age (days)	No. of animals	Avg.wt. (mg)	SEM	No. of animals	Avg.wt. (mg)	SEM
1	56	3.1[a]	NM	56	3.1[a]	NM
30	48	21.9[a]	0.5	52	23.8[b]	0.4
60	41	85.9[a]	2.3	47	101.9[b]	3.8
90	38	238.4[a]	8.9	45	281.7[b]	10.5
120	37	468.1[a]	16.7	44	533.2[b]	18.1
150	33	714.02[a]	28.1	39	809.72[b]	25.9
180	31	833.7[a]	33.3	36	921.5[b]	36.5

[a, b] - means within same age class with different superscripts differ ($P < .05$)
NM - not measured
SEM - standard error of mean

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Chapter 3

Methanogenic Archaea in the Hindgut of the American Cockroach: Diversity and Significance for Cockroach Development

3.1. Introduction

The evolution of methane associated with the gut microflora of termites and cockroaches has been known for a long time and is well documented (Bracke *et al.*, 1978; Cruden and Markowetz, 1984, 1987, Gijzen *et al.*, 1991, Kane and Breznak, 1991). The significance of arthropods (primarily termites) for the global methane efflux has been the subject of many studies (Zimmerman *et al.*, 1982, 1984, Zimmerman and Greenberg, 1983, Rasmussen and Khalil, 1983, Khalil and Rasmussen, 1983, Collins and Wood, 1984, Brauman *et al.*, 1992, Hackstein and Stumm, 1994). In contrast, little is known about the significance of methanogenic microbes in gut metabolism and the overall physiology of their hosts. Termites and cockroaches depend on, or benefit from, the end products of the microbial fermentation in the gut (Odelson and Breznak, 1983, Breznak, 1984, Gijzen and Barughare, 1992, Zurek and Keddie, 1996). To enhance fermentation, methanogens function in the gut lumen as an electron sink and keep hydrogen pressure low, while carbon incorporated into methane is lost to the host (Breznak and Switzer, 1986; Breznak and Brune, 1987, Miller, 1995). So far, no study has directly assessed the significance of methanogenic symbionts in the gut ecosystem and possibly for metabolism of their insect hosts.

Since methanogenic microbes are commonly found in anaerobic ecosystems, the presence of methanogens in the gut lumen of various arthropods, especially those that

feed on a cellulose rich diet, is not surprising. Hackstein and Stumm (1994) examined 110 representatives of different terrestrial arthropod taxa for methane production and reported that methanogens occur in four groups: millipedes (Diplopoda), cockroaches (Dictyoptera), termites (Isoptera) and some scarab beetles (Coleoptera, Scarabeidae). These authors suggested that arthropods with these symbionts contribute substantially to atmospheric methane.

The gut of the American cockroach, *Periplaneta americana* L., contains a large and complex microbial population, most of which are located in the colon (the largest part of the hindgut). The colon lumen is anaerobic and carries a dense and extremely varied bacterial population consisting of both facultative and obligate anaerobes (Becker *et al.*, 1972, Cruden and Markowetz, 1987, Bignell, 1977, Bracke *et al.*, 1979; Foglesong *et al.*, 1975). Several species of ciliated protozoa (Koura and Kamel, 1992) and oxyurid nematodes (Adamson and Noble, 1992) also have been reported as common symbionts in the hindgut lumen. Methanogenic archaea, resembling *Methanobrevibacter* spp., endosymbionts of the ciliate *Nyctotherus ovalis*, are restricted to the hindgut (Gijzen *et al.*, 1991). A smaller portion of the methanogenic population, identified as *Methanospirillum* spp., are free-living (Bracke *et al.*, 1979, Gijzen *et al.*, 1991). In this insect, methane is formed primarily from hydrogen gas and carbon dioxide with little or none derived from acetate (Cruden and Markowetz, 1987, Gijzen *et al.*, 1991). Methanogenesis acts as the main electron sink reaction in hindgut fermentation of *P. americana* (Cruden and Markowetz, 1987).

Elimination of the gut anaerobic microflora with the antibiotic, metronidazole, has no effect on metabolism of adults of *P. americana*, while the same treatment significantly

reduces weight gain of subadults, lowers adult body weight and extends cockroach development time (Bracke *et al.*, 1978). Previously, Zurek and Keddie (1996) reported that dietary supplements of volatile fatty acids partially compensate for the effect of metronidazole on cockroach weight gain and development time.

The ciliate *N. ovalis* possesses cellulolytic activity and also contributes to cockroach metabolism and development (Gijzen *et al.*, 1991, 1994, Gijzen and Barughare, 1992). Gijzen and Barughare (1992) presented evidence that elimination of methanogenesis by BES (2-bromoetanesulfonic acid) treatment has no significant effect on numbers of *N. ovalis*, VFA concentrations in the hindgut, and metabolism of adult *P. americana*. In that study adult cockroaches were used. Since only subadults of *P. americana* are negatively affected by the loss or alteration of the gut microflora (Bracke *et al.*, 1978), the significance of methanogenic archaea in the hindgut metabolic activities of subadult cockroaches, cockroach weight gain and development time has not been determined. The present study examines the diversity and functional significance of methanogenic symbionts in the hindgut ecosystem and development of *P. americana*.

3.2. Materials and methods

Animals: a) Rearing colonies: Each rearing colony of *P. americana* L. was housed in a 15 litre aquarium containing tiers of wooden shelves resting on a sawdust substrate. Two types of diet were used: low fiber diet (LFD; 7.2 % crude fiber) consisting of Purina dog chow and oat flakes (1:1 ratio) and a high fiber diet (HFD; 24.8 % crude fiber) consisting of wheat bran cereal and Purina dog chow (4:1 ratio). Water was supplied *ad libitum*.

b) Experimental colonies: Oöthecae were removed from rearing colonies and newly

hatched cockroaches were placed in 8 litre plastic containers supplied with fresh sterile sawdust bedding and a single wooden shelf. Subadults acquired natural gut microflora by exposure of newly hatched cockroaches to old substrate (sawdust mixed with faeces from rearing colonies) for 48 hours and then by keeping two pairs of adults from rearing colonies in each experimental colony for 90 days. Experimental colonies were reared in an environmental chamber maintained at 27°C, 75% relative humidity and 10L : 14D light regime. All colonies were provided with identical quantities of food and water. Water was supplied in 30ml glass tubes with cotton stoppers. Containers were sterilised with bleach; bedding, shelves, food, water, water containers and cotton were autoclaved before use. Newly hatched insects from each diet type (LFD, HFD) were divided into four unsexed groups of fifty each. For each diet formulation, two control groups were fed a measured amount of sterile food and water; two test groups were fed the same quantity of food, but their drinking water contained BES. Drinking water in all colonies was replaced every seven days.

Drug: The drug, 2-bromoethanesulfonic acid (BES), which acts specifically against methanogenic bacteria was used. BES, an analogue of coenzyme M, disrupts the bacterial metabolic pathway leading to production of ATP and methane (Gunsalus *et al.*, 1978). Six mM BES (Sigma B 9008) was added to the drinking water of treated colonies up to 90 days; ten mM BES was used from 90 to 180 days of cockroach development.

Weight gain: The fresh body weight of each cockroach was measured at 30, 60, 90, 120, 150, and 180 days from hatching. Statistical significance of differences between means was assessed by two way Anova-test (0.05 level).

Analysis of volatile fatty acids: Every thirty days after hatching, four specimens were

selected at random from each colony and used for analysis. Cockroaches were chilled, cut open, the colon ligated, dissected out and its weight recorded. Each sample was made from one or two colons homogenised in fifty µl of sterile water, five µl of phosphoric acid and one µg of 4-methyl valeric acid (internal standard) and centrifuged at 11,310 x *g* for 20 min. at 4°C. Supernatant fluid was collected and used for analysis by gas-liquid chromatography. Analysis was done on Varian 3400 GC with Stabilwax-DA Crossbonded Carbowax - PEG column (30m x 0.25mm ID x 0.5µm film thickness, Restex Corporation, Bellefonte, PA) programmed from 100°C to 170°C at 10°C/min. Injector and detector (FID) were maintained at 200°C. Helium was used as a carrier gas at 20 psi head pressure and at 30 ml/min as a make-up gas, air at 300ml/min and H_2 at 30 ml/min. Data were analysed on Shimadzu EZChrom Chromatography Data System (Shimadzu Scientific Instruments, Inc., Columbia, MD). Statistical differences between means were assessed by two way Anova–test (0.05 level).

Analysis of methane: Gas chromatography was used to measure methane production of intact insects at 30, 60, 90, 120, 150, and 180 days from hatching. Eight to ten randomly selected insects from each colony were enclosed either in 24 ml or 130 ml glass bottles sealed with a rubber stopper and a screw cap. Bottles were incubated at room temperature for one hour and then analysed for methane. The procedure was performed twice (over a twenty-four hour period for each sample) to compensate for possible daily fluctuations. Analysis was done on Varian Star 3400 GC with 12 ft. poropak Q 1/8 inch column maintained at 45° C connected to the methanizer (Porter and Volman), consisting of a 2% Nickel oxide column with a continuous supply of hydrogen at 20 ml/min in constant temperature 350° C. Nitrogen was used as carrier gas at 30 ml/min. The injector and

detector (FID) were maintained at 100°C and 400°C, respectively.

Numbers of *N. ovalis*: At 180 days, four specimens from each colony were examined for *N. ovalis*. Colons were removed, cut open, and the contents were suspended in 500 µl of sterile water. Ciliates were immobilised by addition of five µl of 70% ethanol and counted visually in dilution series under a dissecting microscope. Statistical differences between means were assessed by two way Anova - test ($P < 0.05$).

Microscopic observation of methanogens: Four randomly selected adult cockroaches from the control colonies were used for observation of methanogens under the phase contrast microscope with UV illumination. Each hindgut was dissected, cut open by longitudinal incision and the contents used for microscopic observation. Methanogenic bacteria were distinguishable by the blue colour caused by the autofluorescent enzyme F_{420} specific for methanogens.

DNA extraction: Two hindguts from adult cockroaches were dissected out aseptically, pooled and homogenised in 500µl of sterile water and centrifuged at 13,000 x *g* for 5 min. Supernatant fluid was discarded and the pellet was used for DNA extraction. Bacterial cells were lysed by the beadbeater (Mini-Beadbeater, Biospec Product) and DNA was extracted by the phenol procedure (Sambrook *et al.*, 1989).

Polymerase chain reaction (PCR): 16S rRNA gene was amplified from extracted genomic DNA by *Taq*Bead™ polymerase (Promega, Madison, Wisconsin) and Archaea-specific forward primer – 1Af (position 1 of 16S rDNA), 5' - TCY GKT TGA TCC YGS CRG AG - 3' and Archaea -specific reverse primer – 1100Ar (position 1100 of 16S rDNA), 5' - TGG GTC TCG CTC GTT G - 3'. Amplification was performed using an Ericomp EZ Thermal Cycler (Ericomp, Inc., San Diego, California).

Cloning and sequencing: *Taq* polymerase - amplified PCR product was inserted into plasmid vector pCR® II - TOPO and cloned into *E. coli* using TOPO TA Cloning Kit (Invitrogen Corporation, Carlsbad, California). 16S rRNA gene of twenty plasmids were submitted for automated sequencing at the Department of Molecular Genetics, Forsyth Dental Center, Boston, MA. Partial sequences (approximately 500 bp) were compared to available 16S rDNA data information using the computer search BLAST (Basic Local Alignment Tool) for GenBank database.

3.3. Results

Volatile fatty acids: a) The findings of VFA concentrations from cockroaches in control colonies fed LFD are consistent with results reported in previous studies (Bracke *et al.*, 1978, Cruden and Markowetz, 1984, 1987, Zurek and Keddie, 1996). Acetic acid was on average detected in the highest concentrations (362 μg/g) and remained relatively constant throughout cockroach development. Propionic and isovaleric acid were detected in lower concentrations (56 μg/g and 47 μg/g, respectively) and fluctuated during cockroach development (Table 3.1). Isobutyric and butyric acid were found in very low concentrations (9.1 μg/g). Traces of valeric and caproic acids were detected in most extracts.

b) VFA concentrations from cockroaches in control colonies fed HFD were clearly higher than those from cockroaches on LFD. Acetic acid was on average highest (580 μg/g) followed by propionic acid (93 μg/g), isovaleric (60 μg/g), butyric (29 μg/g) and isobutyric acid (12 μg/g) (Table 3.2). Valeric and caproic acids were detected in trace amounts.

c) BES treatment had no substantial effect on VFA concentrations in the cockroach colon within the same developmental stage and diet type. Concentrations of acetate and propionate were slightly lowered in all colonies at the end of the cockroach development (Table 3.1 and 3.2).

Weight gain: There was no significant difference in weight between treated and control colonies within each diet type (Table 3.3).

a) The adult cockroach mean body weight on LFD was 923.2 ± 25.0 mg and 898.1 ± 25.2 mg in control and treated colonies, respectively. From 120 days weight gain of cockroaches in treated colonies on LFD was slightly lower but not significantly different from that of control cockroaches (Table 3.3).

b) The adult maen body weight of cockroaches fed HFD was 878.2 ± 31.0 mg and 876.4 ± 36.1 mg in control and treated colonies, respectively.

Development time: Development time was not greatly affected by BES treatment. Development time was 158 ± 12 days in the control colonies and 161 ± 14 days in the BES treated colonies on LFD and 167 ± 19 days in control colonies and 168 ± 23 days in the treated colonies on HFD. No difference in mortality was observed. Food consumption was similar in all experimental colonies.

Microscopic observations: Methanogenic bacteria were distinctly visible under ultraviolet light. Methanogens associated with the *N. ovalis* appeared as a small cocci inside of the protist cytoplasm. They were also present inside of the cyst of *N. ovalis* (Figure 3.1). Free-living methanogens appeared as a short rods attached to the food particles in the hindgut lumen (Figure 3.2).

Methane production: a) In control colonies fed LFD methane production (mean ±

standard deviation) increased from 18.8 ± 6.9 nmol/g/hour at 30 days up to 30.8 ± 8.5 nmol/g/hour at 60 days and then declined slightly to 20.9 ± 6.9 nmol/g/hour at 90 days and remained relatively stable (Figure 3.3). BES treatment suppressed methane production throughout cockroach development. Methane concentration in the treated colonies remained on average 2.9 ± 1.5 nmol/g/hour (Figure 3.3).

b) In control colonies fed HFD, methane production increased from 10.2 ± 2.1 nmol/g/hour at 30 days to 35.9 ± 8.8 nmol/g/hour at 90 days and up to 61.2 ± 27.3 nmol/g/hour at 120 days and then declined slightly (Figure 3.3). BES treated cockroaches fed HFD emitted on average 7.7 ± 4.1nmol/g/hour (Figure 3.3). Considerable variation was detected in methane production among cockroaches from the same colonies within the same developmental stages.

Numbers of *N. ovalis*: Although substantial differences in numbers of *N. ovalis* were observed among individual cockroaches from the same colony, no significant differences among colonies within the same diet type occurred. Hindguts of adult cockroach on LFD contained (mean ± standard deviation) 361.5 ± 214.6 and 329.4 ± 176.3 ciliates in control and BES treated colonies, respectively. Hindguts of adult cockroach on HFD contained 926.8 ± 727.3 and 812.8 ± 586.7 ciliates in control and BES-treated colonies, respectively.

Partial sequence of 16S rRNA gene: Comparison of partial sequence of 16S rRNA gene (1 – 500bp region) of cloned PCR products revealed that all sequenced clones were very similar to each other and to *Methanobrevibacter cuticularis* (95 - 97% homology) (Leadbetter and Breznak, 1996) using information available from Genbank database by the computer search BLAST.

3.4. Discussion

Methanogenic archaea are found in the gut lumen of many cockroach species. Methane production has been detected from *Blatta orientalis, Blattela germanica, Blaberus* sp., *Eublaberus postichus, Gromphadorhina* sp. *Leucophora* sp., *P. americana, P. australasiae, Pycnoscelus surinamensis,* and *Supella supellectilium* (Cruden and Markowetz, 1984, 1987; Hackstein and Stumm, 1994). Also, symbiosis of methanogens and anaerobic protists seems to be widespread. Fenchel and Finlay (1994) have suggested that almost all anaerobic ciliates living in fresh water and in half of the marine species contain methanogenic bacteria as endosymbionts. Methanogens are also associated with termite hindgut flagellates and some free-living amoeboflagellates (Broers *et al.*, 1990, Messer and Lee, 1989). In cockroaches, the ciliate-methanogen association has been reported for *Gromphodorrhina portentosa, P. americana, P. australasiae, P. surinamensis,* and *Blaberus* sp. (Hackstein and Stumm, 1994). Protist-endosymbiotic methanogens are commonly closely associated with hydrogenosome-like organelles. This association probably facilitates interspecies hydrogen transfer, however the functional significance of this symbiosis is not completely understood (Gijzen *et al.*, 1991, Fenchel and Finlay, 1994, Zwart *et al.*, 1988).

Continued function of anaerobic systems generally depends on low hydrogen pressure. Hydrogen plays a key role both as a metabolite and a substrate. Fermenting bacteria produce hydrogen that is consequently metabolised by hydrogen-consuming bacteria (methanogenic, acetogenic, sulphate-reducing). This interspecies hydrogen transfer is an essential interaction in any anaerobic microbial community (Fenchel and Finlay, 1992, 1994). Cruden and Markowetz, (1987) reported that hydrogen gas was not

detectable in the hindgut lumen of *P. americana* and they suggested, since many hindgut isolates produce hydrogen gas in large quantities, that this gas must be rapidly metabolised. However, they did not describe the technique used for H_2 detection. It is possible that today's more sensitive microelectrodes would detect very low levels of hydrogen gas. Nevertheless, methanogenic symbionts most likely do function as the electron sink and keep hydrogen pressure low in the cockroach hindgut by formation of methane from hydrogen gas and carbon dioxide. Acetogenesis in *P. americana* has been detected, however, most of the CO_2 and H_2 is incorporated into methane rather than acetate (Breznak and Switzer, 1986).

Inhibition of methane production may lead to accumulation of H_2 which results in a shift in fermentation to less oxidised VFA (Van Nevel and Demeyer, 1988). Results from this study show that suppression of methanogenesis through cockroach development does not affect VFA concentrations in the hindgut. Cockroach weight gain and development time have not been influenced by a substantial reduction of methanogenesis. It is possible that methane inhibition in the cockroach hindgut leads to changes and adaptation in the microbial community and consequently, removal of H_2 from the hindgut lumen of BES-treated cockroaches is substituted for by other hydrogen-scavenging bacteria, possibly acetogens or sulphate-reducing bacteria. High numbers of sulphate-reducing bacteria, another hydrogen-scavenger candidate (MPN-most probable number on lactate-based medium was 2.4×10^7/ml of hindgut content) were detected in the present study. The identification and examination of their physiological ecology are currently in progress in the laboratory. Hydrogen gas emission was not measured and it is possible that hydrogen gas in BES-treated cockroaches was ventilated from the hindgut to the atmosphere. An

increase of hydrogen gas concentration in the hindgut lumen (depending on the ventilation rate) without any significant effect on steady state of VFA might be possible. Another explanation for the constant concentration of VFA in the hindgut colon would be an active transport system of VFA in the colonic tissue. However, the substantial elevation of VFA concentrations in the hindgut of cockroaches fed HFD makes the presence of an active transport unlikely.

Partial sequence of 16S rDNA of cloned PCR product revealed that all sequenced clones were highly homologous to genus *Methanobrevibacter*. This genus has been suggested for the methanogenic endosymbionts of *N. ovalis* (Gijzen *et al.*, 1991). Since the hindgut of *P. americana* also contains free-living methanogens, further culturing techniques, screening and full sequencing of greater number of clones will be necessary for better understanding of diversity of the cockroach methanogenic symbionts. Fluorescent microscopy revealed that free-living methanogens are represented by very short rods and it is therefore unlikely that they belong to genus *Methanospirillum* as reported by Bracke *et al.* (1979). Methanogens associated with *N. ovalis* appeared as small cocci, morphology indicative of the genus *Methanobrevibacter*.

Findings are consistent with results of Gijzen *et al.* (1991, 1992). Long term exposure to BES (*i.e.* throughout cockroach development) does not affect the size of *N. ovalis* populations. I have confirmed also that *N. ovalis* increases methane production and contributes to the development of nymphs of the *P. americana*. Fluctuations in the methane production of cockroaches of the same age and from the same colony can most likely be attributed to differences in numbers of *N. ovalis*. It is possible that *N. ovalis* is eliminated from the colon during ecdysis and is reacquired by coprophagy which could

account for substantial differences in ciliate numbers. Weight gain during development and adult body weight of cockroaches fed LFD and possessing *N. ovalis* were significantly higher than these of cockroaches lacking this ciliate (Chapter 2). Also, the development time was clearly shortened (by 14 days) in colonies with *N. ovalis*. However, VFA concentrations in the hindgut of cockroaches fed LFD and without *N. ovalis* in the hindgut (Zurek and Keddie, 1996) were not much different from those with complete microflora. It seems that, when feeding LFD, *N. ovalis* does not affect the VFA pool in the cockroach hindgut, unless the VFA are rapidly transported from the hindgut lumen to the haemolymph by an active transport. Undoubtedly, the basis of the contribution of *N. ovalis* to metabolism of *P. americana* remains to be determined.

On the other hand, concentrations of VFA (except isobutyric and isovaleric acid) from cockroaches on HFD in all (control and treated) colonies substantially increased in comparison with those from cockroaches on LFD. It is likely that intestinal microflora contribute to this increase by degrading fiber in HFD and the low nutritional value of HFD is partially compensated by the elevation of bacterial VFA. Consequently, weight gain of cockroaches fed HFD was only slightly lower than that of cockroaches on LFD. Numbers of *N. ovalis* and methane emission by cockroaches fed HFD significantly increased, which is in agreement with previous studies (Kane and Breznak 1991, Gijzen *et al.* 1994).

The importance of VFA may increas substantially during starvation or nutritionally poor diet. The American cockroach can withstand a long period of starvation (up to three months when water is available) (Roth, 1981). In this case the food, including difficult to degrade components of the diet such as cellulose and chitin, is retained in the gut lumen

(Bignell, 1981) and consequently exposed to microbial degradation for a long period of time. The end products of this degradation are then available for cockroach metabolism. It is also likely that the contribution of volatile fatty acids to cockroach metabolism is only one of several aspects of gut microbe-cockroach interaction. Supplementary supply of vitamins and cholesterol, and protection from pathogenic bacteria entering the digestive tract may be other aspects of this symbiosis, especially in wild living animals feeding on diverse food sources.

In conclusion, I have presented evidence that methanogenic symbionts in the hindgut are not essential for normal development of the cockroach, *P. americana*. The results of molecular analysis indicate that methanogens in the hindgut are at least in part represented by the genus *Methanobrevibacter*. Diet composition likely alters the microbial population and consequently concentrations of VFA in the hindgut. The response of the microbial population might allow the host to compensate for changes in the availability of nutrients in the diet. The fate of reducing equivalents (H_2) in the hindgut of BES-treated cockroaches as well as potential presence of an active transport system of volatile fatty acids and other nutrients in the colonic tissue remain for further investigation.

Figure 3.1 *Nyctotherus ovalis* from the hindgut of *P. americana* (top left). The same object under UV illumination showing methanogenic endosymbionts – blue autofluorescence (top right). The cyst of *N. ovalis* from the hindgut of *P. americana* (bottom left). The same cyst under UV illumination showing methanogenic endosymbionts.

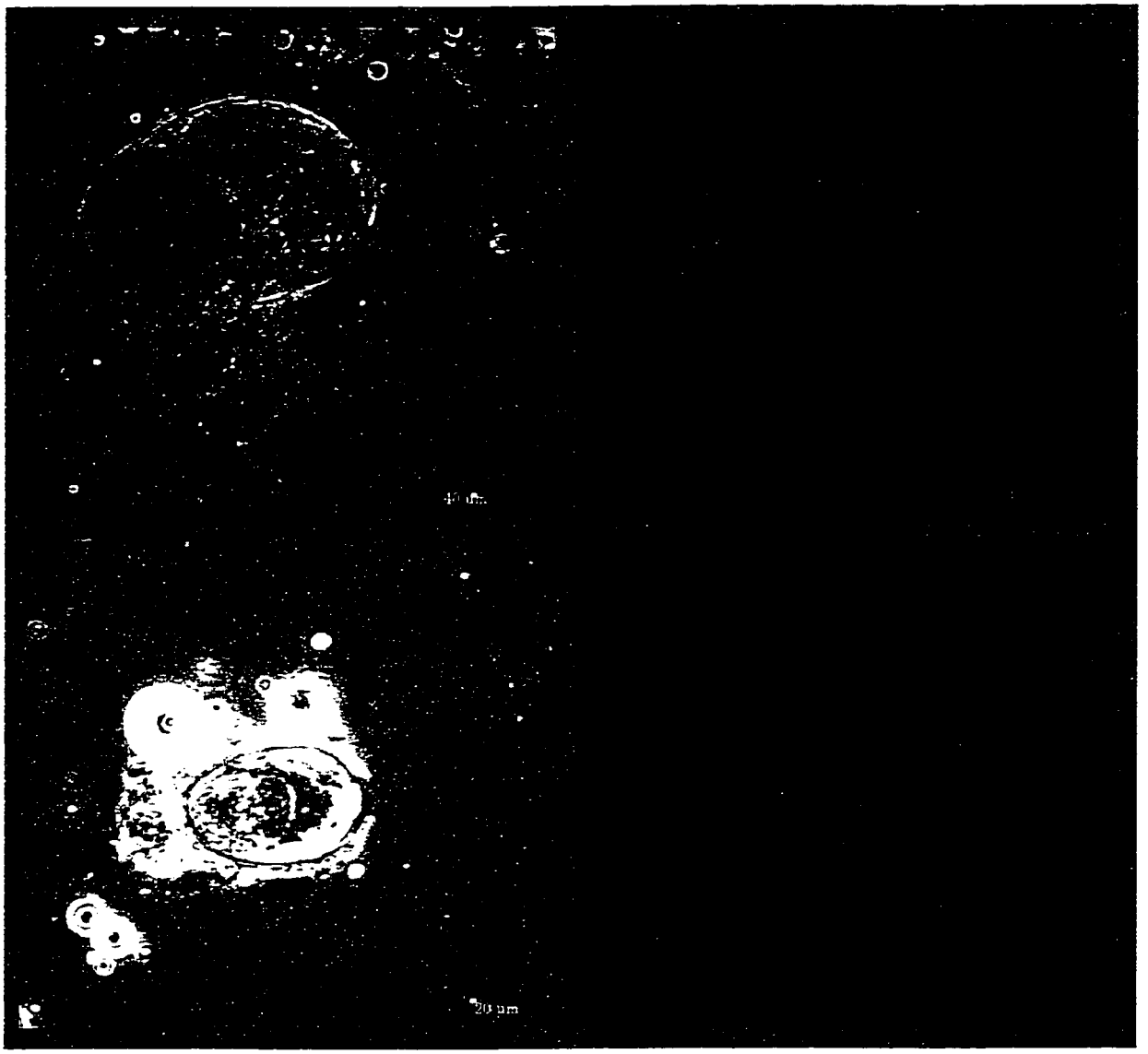

Figure 3.2 Free-living methanogens in the hindgut of *P. americana*. Food particles from the colon lumen (top picture). The same object under UV illumination, showing the blue autofluorescent methanogenic archaea (bottom picture).

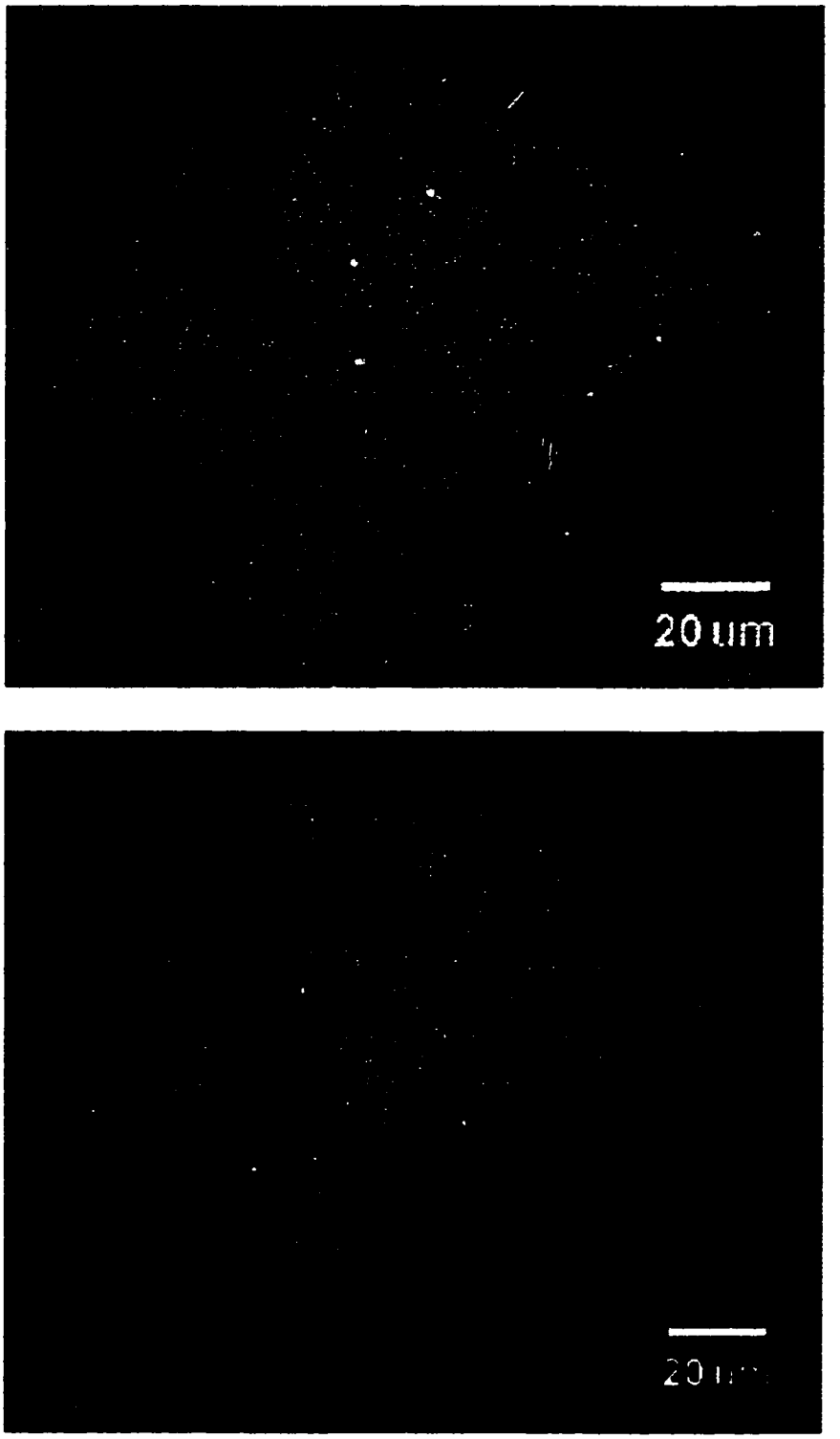

Figure 3.3 Methane production during development of *P. americana* fed high (HFD) and low (LFD) fiber diet in control and BES (bromoethanesulfonic acid) treated colonies. (Bar = standard deviation)

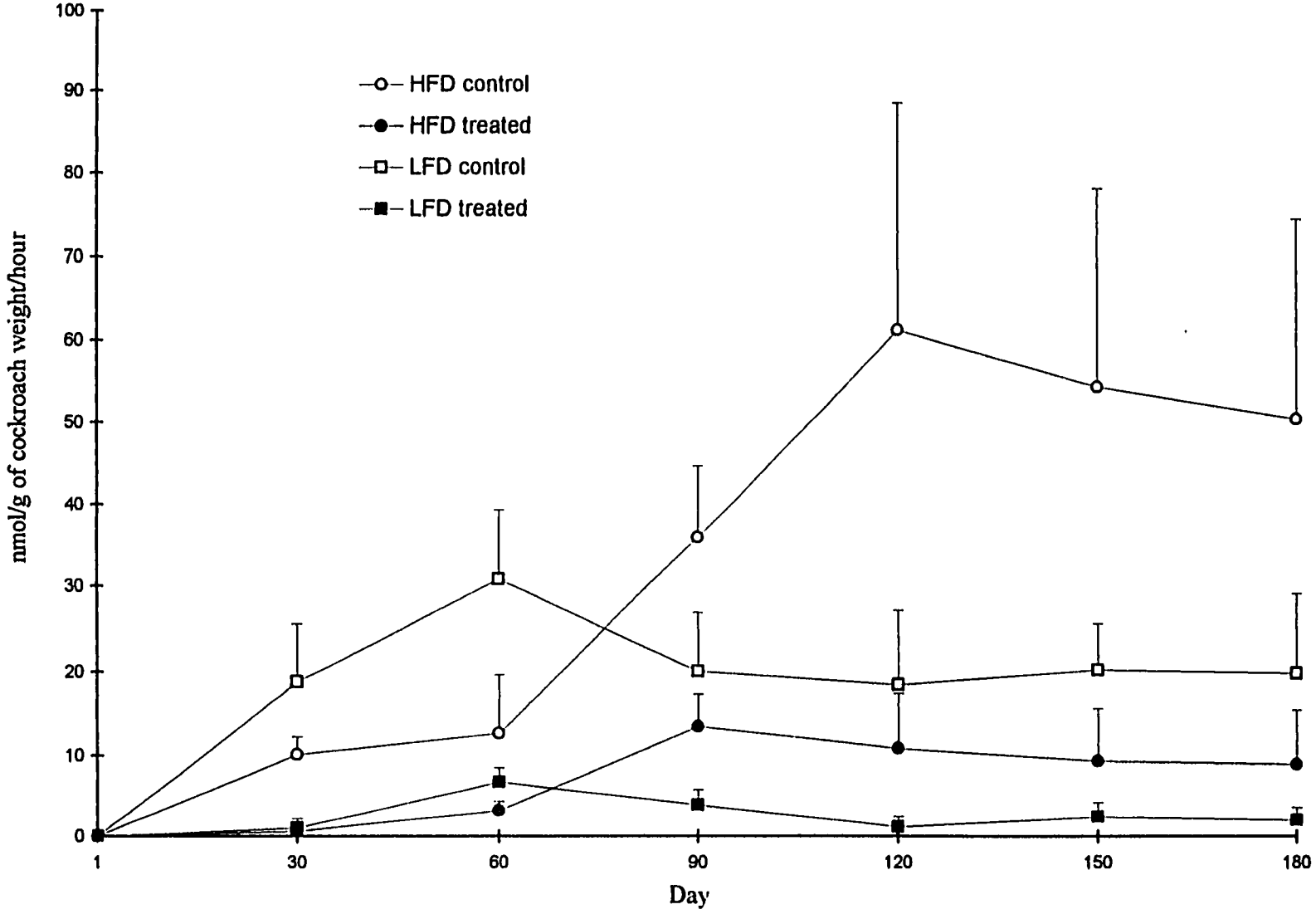

Table 3.1 Profile of volatile fatty acids (μg/g of colon) in the colon during development of the *P. americana* in treated (BES) and control colonies on low fiber diet (LFB).

Age (days)	Acetate				Propionate				Isovalerate				Isobutyrate				Butyrate			
	Control		BES		Control		BES		Control		BES		Control		BES		Control		BES	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
30	378.8	23.0	410.1	21.5	57.8	3.4	55.8	15.1	35.9	2.1	46.9	27.6	10.1	0.5	9.0	0.4	13.1	5.8	8.7	4.8
60	363.3	96.6	355.8	86.7	65.8	3.8	65.1	32.0	49.5	19.2	24.5	14.7	8.0	5.5	7.6	4.0	8.3	3.7	5.1	2.2
90	362.3	80.3	328.2	62.9	38.4	11.8	37.9	6.5	25.8	11.4	40.4	27.8	6.2	2.7	4.9	1.0	5.2	3.1	3.0	2.5
120	363.3	27.9	386.9	42.9	66.7	7.0	79.8	15.4	58.5	7.3	94.5	50.2	10.2	0.8	12.1	4.7	7.2	6.2	18.9	2.5
150	352.4	35.3	255.4	45.8	54.6	11.6	45.2	7.9	51.6	18.9	18.1	15.8	9.3	1.3	4.3	1.8	8.9	4.3	3.8	2.3
180	352.5	53.3	319.2	39.0	49.9	16.2	34.7	14.6	59.8	19.7	54.7	39.8	7.8	2.2	5.1	1.6	13.1	7.5	8.7	5.0

SD - standard deviation
BES - 2-bromoethanesulfonic acid

Table 3.2 Profile of volatile fatty acids (μg/g of colon) in the colon during development of the *P. americana* in treated (BES) and control colonies on high fiber diet (HFB).

Age (days)	Acetate				Propionate				Isovalerate				Isobutyrate				Butyrate			
	Control		BES		Control		BES		Control		BES		Control		BES		Control		BES	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
30	651.3	58.6	648.9	65.3	95.3	18.6	103.1	27.6	73.9	20.1	67.1	23.2	10.8	6.3	14.2	7.3	26.3	10.4	29.0	11.8
60	628.7	71.1	650.8	41.1	99.8	25.6	123.3	34.1	52.1	16.9	57.9	22.8	8.2	4.7	7.9	4.1	28.2	11.6	31.7	9.9
90	574.3	34.7	451.7	36.9	91.4	14.1	78.5	21.2	89.9	16.2	73.7	18.7	17.3	8.1	16.5	9.3	30.1	8.5	26.2	5.4
120	659.5	44.8	545.1	48.1	113.3	93.5	80.3	46.7	65.1	17.5	53.3	21.6	15.0	10.7	13.9	6.3	40.4	9.1	32.4	11.9
150	477.3	90.2	447.0	82.9	87.1	22.2	76.9	25.6	33.0	11.7	49.9	18.5	11.3	7.4	9.4	3.7	29.8	9.8	22.1	12.3
180	491.1	88.3	463.8	80.4	70.3	24.7	70.8	21.6	47.9	14.1	40.3	19.1	9.7	3.8	10.1	4.5	18.6	11.1	19.9	8.7

SD - standard deviation
BES - 2-bromoethanesulfonic acid

Table 3.3 Weight gain (mean weight) of *P. americana* in (BES) treated and control colonies on low (LFD) and high (HFD) fiber diet.

	Low fiber diet						High fiber diet					
Age	Control			BES			Control			BES		
(days)	No. of animals	Weight (mg)	SEM	No. of animals	Weight (mg)	SEM	No. of animals	Weight (mg)	SEM	No. of animals	Weight (mg)	SEM
1	100	3.1[a]	N.M.	100	3.1[a]	N.M.	100	3.1[a]	N.M.	100	3.1[a]	N.M.
30	86	24.6[a]	0.36	87	25.1[a]	0.36	85	23.0[a]	0.62	86	23.3[a]	0.51
60	78	95.4[a]	20.7	79	91.2[a]	2.04	77	87.1[a]	2.34	78	86.7[a]	2.33
90	70	282.9[a]	8.79	71	281.7[a]	8.45	69	285.7[a]	9.94	69	271.2[a]	9.60
120	62	561.4[a]	18.8	62	523.5[a]	17.48	60	506.5[a]	17.42	61	500.4[a]	16.01
150	54	817.8[a]	30.2	53	767.8[a]	26.34	51	759.8[a]	23.53	53	760.5[a]	22.91
180	46	923.2[a]	25.0	45	898.1[a]	25.61	43	858.0[a]	25.16	45	863.7[a]	36.06

N.M. - not measured
SEM - standard error of mean
BES – 2-bromoethanesulfonic acid
[a,b] - means within same age class and diet with different superscripts differ

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Chapter 4

Sulphate- and Sulphite-reducing Bacteria in the Cockroach Hindgut. Effects of High Sulphate Diet on the Cockroach Development.

4.1. Introduction

Sulphate-reducing bacteria (SRB) are a distinctive group of anaerobic microorganisms (Devereux and Stahl, 1993) unified by a shared ability to carry out dissimilatory sulphate reduction as a principal component of their bioenergetic processes (Singleton, 1993). These organisms use sulphate as a terminal electron acceptor to oxidise both organic and inorganic compounds. Despite this seeming physiological unity, it has been shown recently that these bacteria are morphologically, ecologically, nutritionally and metabolically very diverse (Faque *et al.*, 1991, Widdel and Bak, 1992, Stetter 1992). To date, fourteen genera of bacteria and one genus of archaea with the capacity for dissimilatory sulphate reduction have been reported (Fauque, 1995). Numerous hydrocarbons, short- and long-chain fatty acids, alcohols, amino acids, carbohydrates, and various aromatic compounds serve as energy substrates for metabolism of these microorganisms (Hansen, 1993). Some sulphate-reducing bacteria can grow fermentatively in the absence of sulphate (Singleton, 1993).

The sulphate-reducing bacteria are widely distributed in anaerobic terrestrial and aquatic environments. In association with higher animals, they have been reported from cow and sheep rumen contents (Huisingh *et al.*, 1974, Coleman 1960) and intestines of man and pigs (Gibson *et al.*, 1993, Gebhart *et al.*, 1993). Recently, SRB have been reported also from the digestive tracts of invertebrates, including the hindgut lumen of several lower and higher termites and one species of wood-eating cockroach (Brauman *et*

al., 1990, Trinkerl *et al.*, 1990, Kuhnigk *et al.*, 1996). These SRB may play important roles in the termite hindgut by enhancing acetate formation, removal of oxygen and hydrogen, and by maintaining a low redox potential (Kuhnigk *et al.*, 1996). Pure cultures of SRB are not strict anaerobes but are microaerotolerant and can grow in very low oxygen concentrations (Marshall *et. al.*, 1993). Sulphate reduction is blocked when oxygen is present, however some sulphate-reducing isolates are able to remove oxygen in low concentrations from the microecosystem by respiration. Subsequently, these bacteria use sulphate as the terminal electron acceptor (Kuhnigk *et al.*, 1996). Anoxic conditions or a very low oxygen concentration prevail in cockroach hindguts especially in the center of the hindgut lumen. Accumulation of iron sulphide in the black band region of the colon (Cruden and Markowetz, 1987) indicates the presence of sulphate-reducing bacteria, however these bacteria have never been detected in the cockroach digestive tract.

Although dissimilatory sulphite reduction is probably common in nature, especially among species of the genus *Clostridium* (Dr. F.D. Cook, personal communication), very little information is available in the literature. Harrison *et al.* (1984) reported inducible sulphite reductase from the *Clostridium pasteriamum.* Additions of sulphite to a growing *C. pasteurianum* culture induced this enzyme with considerable formation of sulphide. Although this enzyme was similar to the purified assimilatory sulphite reductase from this species, it was not repressed by cystein while the assimilatory sulphite reductase was suppressed. Furthermore, isolated and purified inducible sulphite reductase from the *C. pasteurianum* was in some aspects (pH optimum, molecular weight) different from other bisulphite reductases from sulphate-reducing bacteria. Given that the genus *Clostridium*

is very ancient, the role of this metabolic pathway might involve a detoxification mechanism for survival in high sulphite concentrations which probably occurred during anaerobic conditions due to volcanic activities several billion years ago (Harrison *et al.*, 1984).

In this study, the presence of sulphate- and sulphite-reducing bacteria in the cockroach hindgut is investigated. Since hydrogen sulphide as the end product of the sulphate reduction is toxic, there might be a potential for utilising SRB in biological control of insect pests including cockroaches by increasing of sulphate concentrations in insect diets and baited traps. The possible exploitation of these bacteria for biological control of cockroaches by enhancement of hydrogen sulphide production is also examined. Two species of sulphite-reducing bacteria have been isolated, cultured and identified.

4.2. Materials and methods

4.2.1. Detection of sulphate-reducing bacteria

Four adult cockroaches randomly selected from rearing colonies, were anesthetised on ice, and cut open by dorsal longitudinal incision. Hindguts were ligated, removed, surface-sterilised, and homogenised in 100 µl of sterile water. Homogenate was then transferred to Butlin's medium (Appendix 1), diluted in series and incubated at room temperature for four weeks. Sulphate-reducing bacteria were detected by the accumulation of black iron sulphide precipitate.

4.2.2. Sulphite-reducing bacteria

Detection and isolation of sulphite-reducing bacteria: Four adult cockroaches randomly selected from rearing colonies, were cooled, and the hindgut was aseptically removed. Hindgut contents were pooled, serially diluted in the phosphate buffer (pH 7.0), and used for inoculation of B_{10} media plates (Appendix 2) containing sodium bisulphite. Inoculation and all other transfers were done in the anaerobic chamber (nitrogen: 85%, hydrogen: 10%, carbon dioxide: 5%) Selected black coloured colonies (indicating sulphite reduction and production of iron sulphide), were transferred into Butlin's medium tubes with and without sulphite. Selected isolates were transferred several times between solid B_{10} medium and liquid Butlin's medium (Appendix 1) and used for DNA extraction and identification.

Identification of sulphite-reducing bacteria: Nucleic acids were extracted (Sambrook *et al.*, 1989) from isolates, 16S rDNA was amplified by polymerase chain reaction (PCR) with eubacterial universal primers: Universal forward - UF: 5'- AGA GTT TGA TYM TGG C – 3'(position 8-23) and Universal reverse primer – UR: 5' -GYT ACC TTG TTA CGA CTT –3' (position 1492) and *Taq* Polymerase (Promega). PCR product was cloned with the cloning kit (Invitrogen) into *E. coli.* Clones were screened by restriction length polymorphism method (RFLP) with restriction endonucleases (Mpn I, Hin III) and visualised by the agarose gel-electrophoresis. Six clones with different digest patterns were sent for sequencing to the Forsyth Institute in Boston, MA, USA. Partial sequences (1 - 500 bp. region) were then compared to the data available from the computer search program BLAST using GenBank database.

4.2.3. Effect of high sulphate diet on cockroach development

Experimental colonies: Oöthecae were removed from rearing colonies and eighty new-born cockroaches were exposed to the old substrate for 24 hours and then divided into two colonies of forty specimens each. Two adults from the rearing colonies were added to each colony to ensure acquisition the natural intestinal microbiota. Cockroaches were placed in 8 litre plastic containers supplied with sawdust bedding and a single wooden shelf. Water was supplied in glass tubes with cotton stoppers. Containers, bedding, shelf, water, and cotton were sterilised by immersion in bleach or autoclaving. One colony of cockroaches received a high sulphate diet consisting of ground Purina dog chow containing 20% (by weight) of calcium sulphate. Control cockroaches were fed with ground Purina dog chow mixed with 20% (by weight) of calcium chloride. Both colonies were provided with identical amounts of food and water. Food was supplied in an open Petri dish to accommodate food consumption measurements.

Weight gain: The fresh body weight of each cockroach was measured at 30, 60, 90, 120, 150, and 180 days from hatching.

Food consumption: Periodically, as food was consumed, it was replaced with fresh diet in all colonies. Any unconsumed diet was weighed and food consumption was recalculated per single cockroach.

Enumeration of sulphate-reducing bacteria: Most probable number (MPN) method (Russek and Colwell, 1983) was used for the count of sulphate-reducing bacteria from the hindgut of cockroaches on high sulphate diet and the control group. Four adult cockroaches at 180 days of development were randomly selected from each colony for SRB enumeration. Cockroaches were chilled, cut open by dorsal longitudinal incision;

hindguts from each treatment were pooled, homogenised, and diluted in phosphate buffer to 1ml final volume. This product was used for inoculation of Butlin's medium and dilution series to 10^{-10}. Tubes were incubated for four weeks in an anaerobic chamber at room temperature. Cell numbers were calculated by MPN count method. Formation of black iron sulphide precipitate was the indicator of sulphate-reducing activity.

Analysis of volatile fatty acids: At 180 days four randomly selected cockroaches were used for the analysis of volatile fatty acids concentrations. Cockroaches were chilled, cut open, the colon ligated, removed and weighed. Each sample was made from one colon homogenised in fifty µl of sterile water, five µl of phosphoric acid and one µg of 4-methyl valeric acid (internal standard) and centrifuged at 11,310 x *g* for 20 min at 4° C. Supernatant fluid was collected and used for analysis by gas-liquid chromatography. Analysis was done on Varian 3400 GC with Stabilwax-DA Crossbonded Carbowax - PEG column (30m x 0.25mm ID x 0.5µm film thickness, Restex Corporation, Bellefonte, PA) programmed from 100°C to 170°C at 10°C/min. Injector and detector (FID) were maintained at 200°C. Helium was used as a carrier gas at 20 psi head pressure and at 30 ml/min as a make-up gas, air at 300ml/min and H_2 at 30 ml/min. Data were analyzed on Shimadzu EZChrom Chromatography Data System (Shimadzu Scientific Instruments, Inc., Columbia, MD).

Detection of hydrogen sulphide emission: Five adult cockroaches were randomly selected from the rearing colonies and individually placed in a sealed glass container with a rubber stopper. After one hour of incubation at room temperature the headspace was analysed by gas-liquid chromatography for presence of hydrogen sulphide. The same conditions were used as those used for the methane analysis (Chapter 3), with the FID detector was replaced by an ECD (electron capture detector).

Examination of morphology of the digestive tract: Two cockroaches from each colony were randomly selected at 180 days of development. The digestive tract (foregut, midgut, hindgut) was removed, ligated and used for morphological observation and photographed under the dissecting microscope.

4.3. Results

4.3.1. Sulphate- and sulphite-reducing bacteria

Detection of sulphate- and sulphite-reducing bacteria: After two to three weeks of incubation in the anaerobic chamber at room temperature, large amount of black precipitate (iron sulphide) were detected in Butlin's medium. This was a clear indication of the presence of sulphate-reducing bacteria. SRB were confirmed by DNA-DNA hybridisation (SRB specific DNA probe) (Chapter 6). The SRB did not form spores, (pasteurisation test) indicating that they did not represent the genus *Desulfotomaculum*. Isolation of SRB on a various artificial media (Postgate B, Butlin's, B_{10} medium) was not successful. Results of cloning and sequencing of 16S rDNA of the whole bacterial nucleic acids content (Chapter 6) in the cockroach hindgut suggest that SRB might be at least in part represented by the genus *Desulfovibrio*.

Sulphite-reducing bacteria formed black colonies on B_{10} medium with sulphite and within two to five days after inoculation produced large amounts of iron sulphide precipitate in Butlin's medium with sodium bisulphite, indicating dissimilatory sulphite-reduction. They were motile, rod-shaped, spore-forming bacteria.

Detection of hydrogen sulphide: The results of gas-liquid chromatography indicated the presence of sulphate-reducing bacteria in the cockroach colon. Although hydrogen

sulphide concentration was not quantified (ECD is not the most suitable detector for this analysis and could be damaged by hydrogen sulphide), its formation was tentatively detected from headspace of all samples used for this analysis.

Isolation and identification of sulphite-reducing bacteria: To ensure purity, black-coloured colonies formed on B_{10} media plates with sulphite were transferred several times between this medium and Butlin's medium with addition of sulphite. Cloned 16S rRNA gene sequences showed that they represent two species of the genus *Clostridium*. Four clones showed very high homology (99%) to *Clostridium bifermentans*, and two clones showed very high homology (98%) *to Clostridium celerecrescens.*

4.3.2. Effect of high sulphate diet on cockroach development

Cockroach weight gain: No significant differences in weight gain from hatching up to 150 days of development between cockroaches fed high sulphate diet and that of the control colony were detected (Table 4.1). At 180 days, however, a significant difference was detected. The adult cockroach body weight was 982.5 mg and 902.9 mg in treated and control colonies, respectively (Table 4.1). Food consumption was slightly higher (8% of total volume) in cockroaches on a high sulphate diet than that of control specimens.

Volatile fatty acids in the hindgut: In the control colony, acetic acid was detected in the highest concentration (231.74 μg/g), followed by isovaleric (30.24 μg/g) and propionic acid (22.73 μg/g). Butyric, isobutyric, and valeric acids were detected in trace concentrations (Table 4.2). No significant difference was found between sulphate-fed and control cockroaches for acetic acid concentration (Table 4.2). In contrast, propionic acid

was significantly lowered (9.43 μg/g) and butyric, isobutyric, and valeric acids were completely eliminated from the colon of sulphate-fed cockroaches (Table 4.2).

Enumeration of sulphate-reducing bacteria: Different diets clearly affected the population of sulphate-reducing bacteria. Numbers of SRB in the hindgut of cockroaches fed high sulphate diet increased more than 7-fold over those of cockroaches fed control diet. The hindgut of cockroaches fed high sulphate diet contained 1.15×10^8 of SRB per ml of colon content. Control cockroaches carried 1.54×10^7 of SRB per ml of colon content.

Morphology of the digestive tract: Microscopic observation revealed differences in the morphology of the digestive tract between treated and control cockroaches. While morphology of the foregut and midgut appeared to be unchanged, the morphology of the colon was clearly affected by high sulphate diet. The black band region of colon of those cockroaches fed high sulphate diet was greatly expanded spreading anteriorly to the ileal region (Figure 4.2). Nevertheless, no tissue abnormalities or signs of pathological effect of sulphide formation were observed. Cockroaches on the control diet had black band region restricted to the posterior end of the colon (Figure 4.1) which is in agreement with previous studies.

4.4. Discussion

High numbers of sulphate-reducing bacteria were detected in the colon lumen of the American cockroach. They are not spore-forming bacteria that exhibit incomplete oxidation of organic compounds. SRB may be common inhabitants of insect digestive tracts (I have detected SRB in the hindgut of other cockroaches, including *Blatella germanica, Blaberus giganteus*, and the cricket *Acheta domestica*). Despite a relatively

low supply of sulphate (drinking water: 46.0 - 65.0 mg/l) SRB have established a large population in the colon of *P. americana*. In the absence of sulphate these bacteria probably grow fermentatively. The extent of sulphate reduction depends on the level of sulphate in the diet. The increase of sulphate concentration in the diet significantly enlarged the population of SRB in the colon and enhanced sulphate reduction (as indicated by expanded black band region of the colon). Sulphate-reducing bacteria do not depend on sulphate and can grow fermentatively, however, when sulphate is available, SRB may rapidly take advantage of this resource and their metabolism becomes energetically more favourable.

With the exception of the last period of development, high sulphate diet had no significant effect on cockroach weight gain and development time. The difference in the body weight at 180 days can be attributed to the increased food consumption on the high sulphate diet. Although the profile of volatile fatty acids was altered in several ways, (reduction of propionate and elimination those VFA present in trace concentrations), the VFA produced in the highest concentrations, acetic and isovaleric acid were not significantly affected by diet containing high levels of calcium sulphate. The only other difference detected was a clear reduction of a furfural peak (formed from pentoses and probably other carbohydrates in the GC injection port) of hindgut contents of cockroaches fed high sulphate diet. While this phenomenon is unexplained, it is possible that the level of pentoses was lower in the hindgut of these cockroaches due to their degradation by sulphate-reducing bacteria.

Although SRB from the cockroach colon were cultured on lactate-based solid (Postgate B), liquid (Butlin's) media, and on the Plate count agar, they could not be

isolated. Apparently they live in a close symbiotic relationship with other lactate-fermenting bacteria, and when separated, SRB cease to grow. The mechanism of this relationship is unknown, however, vitamin and/or carbon interchange is likely to be involved.

Although cockroaches fed a high sulphate diet have significantly enlarged population of sulphate–reducing bacteria in the hindgut, accompanied by increased formation of sulphide, no signs of its toxicity and pathological abnormalities in the gut tissues are observed. This could be a result of sufficient amount of metals in the diet, particularly iron, to transform hydrogen sulphide to non-toxic iron sulphide. The considerable enlargement of the black band region of the colon where precipitation of iron and other metals most likely takes place, supports this hypothesis.

In the cockroach hindgut, spore-forming bacteria with the ability to quickly reduce sulphite were detected. Rapid accumulation of iron sulphide in Butlin's medium after addition of sodium sulphite indicates the presence of microbes possessing inducible dissimilatory sulphite reductase. Sequencing of 16S rDNA of the isolate revealed that it represents two species of clostridia, *C. bifermentans* and *C. celerecrescens*. This finding is not unexpected, since inducible sulphite reductase has been previously reported from *C. pasteurianeum*. This enzyme is probably widely spread among other species of this genus. Clostridia are strict anaerobes with fermentative metabolism. Although it was not shown experimentally in this study, they may be able to take energetic advantage of sulphite as an electron acceptor.

In summary, both sulphate- and sulphite-reducing bacteria have been detected in high concentrations in the cockroach hindgut. Sulphite-reducing bacteria were isolated

and identified. Diet induced increase of SRB populations and consequently increase of sulphide formation had no significant effects on the cockroach metabolism and development. Further research is necessary to assess the potential of sulphate- and sulphite-reducing bacteria for the biological control of cockroaches. A reduction of metals, especially iron, together with an increase of sulphite levels in the diet is the next step in this investigation. Composition changes of artificial culture media will be necessary for isolation and subsequent identification and characterisation of sulphate-reducing bacteria from the cockroach digestive tract.

Figure 4.1 Digestive tract of *P. americana* from the control colony showing the black band region (arrowheads) in the posterior part of the colon.

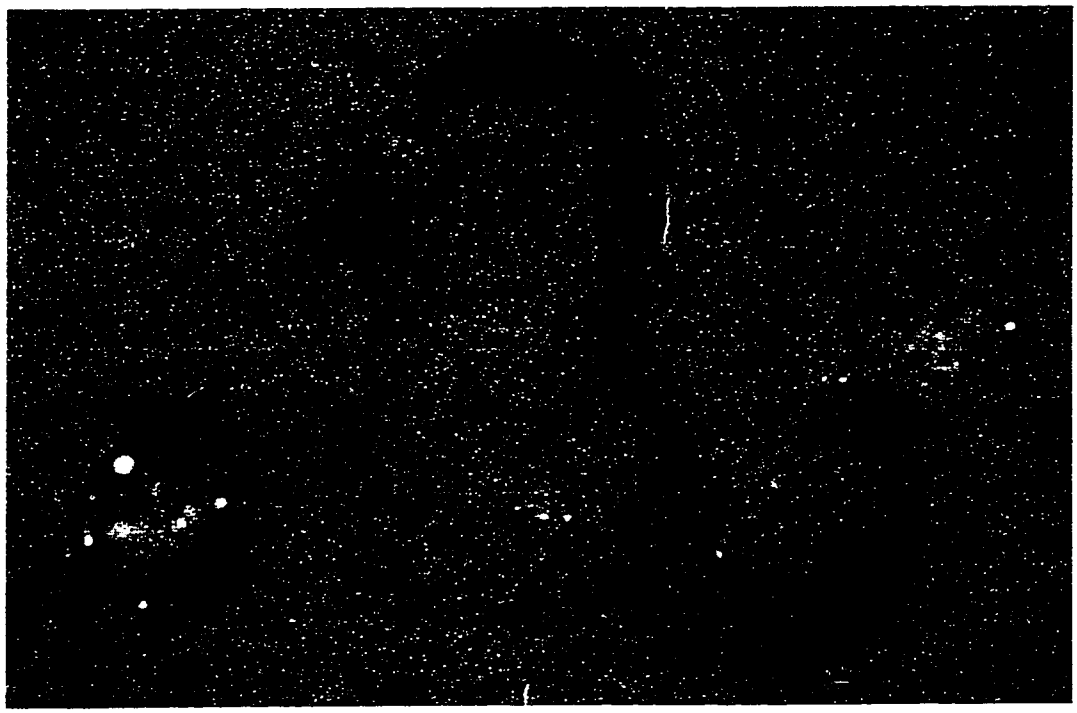

Figure 4.2 Digestive tract of *P. americana* from the colony fed high sulphate diet. The black band region (arrowheads) expanded to the anterior part of the colon.

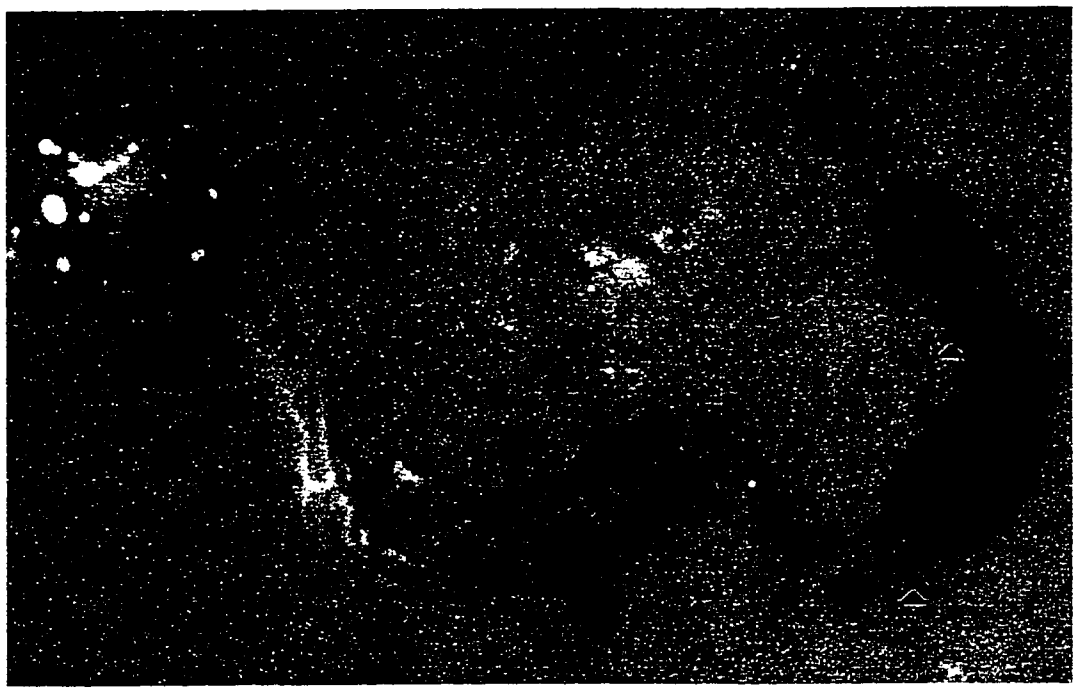

Table 4.1 Weight gain of cockroaches *P. americana* exposed to different treatments from hatching.

	High Sulphate Diet			Control		
Age (days)	No. of animals	Avg.wt. (mg)	SEM	No. of animals	Avg.wt. (mg)	SEM
1	40	3.1[a]	NM	40	3.1[a]	NM
30	35	20.2[a]	0.2	36	18.4[a]	0.4
60	31	106.4[a]	2.3	33	95.9[a]	3.8
90	30	304.2[a]	8.9	29	302.9[a]	10.5
120	30	599.7[a]	16.7	29	556.0[a]	18.1
150	28	841.8[a]	28.1	29	844.6[a]	25.9
180	26	982.5[a]	33.3	27	902.9[b]	36.5

[a, b] - means within same age class with different superscripts differ (Student t-test, $P < .05$)
NM - not measured
SEM - standard error of mean

Table 4.2 Concentrations of volatile fatty acids (VFA) (μg per gram of colon) in the colon of treated and control cockroaches

VFA	High Sulphate Diet		Control	
	Mean	SD	Mean	SD
Acetate	214.41[a]	35.22	231.74[a]	70.11
Propionate	9.43[a]	2.43	22.73[b]	7.83
Isovalerate	34.39[a]	35.29	30.24[a]	34.70
Butyrate	ND	-	5.85	2.20
Isobutyrate	ND	-	3.93	3.32
Valerate	ND	-	1.90	2.84

[a, b] - means within same VFA with different superscripts differ (Student t-test, $P < .05$)
ND - not detected;
SD - standard deviation

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Chapter 5

Isolation and Characterisation of the *Streptomyces leidynemati* (Actinomycetales) from the Surface of Oxyurid Nematodes from the Cockroach Hindgut

5.1. Introduction

The genus *Streptomyces* (Actiomycetales) is represented by Gram positive, aerobic bacteria commonly found in soil and aquatic habitats. The organisms form both an aerial mycelium and a non-fragmenting, branched substrate mycelium (Singleton and Sainsbury, 1993). Conidiospores are usually formed only by aerial mycelium, which fragments, giving a rise to chains of conidiospores. The mode of nutrition is usually chemoorganotrophic; these bacteria can use diverse sources of carbon, including glucose, starch, pectin, lactate, chitin, lignocellulose, and cellulose (Singleton and Sainsbury, 1993, Perry and Staley, 1997, Basaglia *et al.*, 1992, Pasti and Belli, 1985). Some *Streptomyces* species produce compounds with an antibiotic activity, including streptomycin, chloramphenicol, tetracyclin, carbapenems, erythromycin, and other macrolide antibiotics (Singleton and Sainsbury, 1993, Perry and Staley, 1997). Approximately half of the commercially produced antibiotics are derived from this genus. In addition, some are sources of anticancer drugs used in chemotherapy (Perry and Staley, 1997). Despite the importance of this genus of microorganisms, little is known of their ecological roles. For example, it is not known for certain that they produce antibiotics while growing in their natural habitats (Perry and Staley, 1997).

Although digestive tracts of vertebrates as well as invertebrates commonly harbour filamentous bacteria similar to actinomycetes, very few of these symbionts have

been identified to species, even to genus and order. In invertebrates, filamentous bacteria have been reported from millipedes (Schluter, 1980), crane fly larvae (Klug and Kotarski, 1980), termites (Bignell *et al.*, 1979, 1980) and cockroaches (Cruden and Markowetz, 1987; Zurek and Keddie, 1996).

Hoffman (1953) observed a filamentous bacterium attached to the surface of two species of oxyurid nematodes, *Leidynema appendiculata* and *Hammerischmidtella diesingi*, commonly found in the hindgut lumen (Adamson and Noble, 1992). Although Hoffman's attempts to culture the organism on artificial media failed, he described the morphology and habits of this species and named it *Streptomyces leidynemati*. This species stains gram-positive with hyphae (0.7 μm in diameter and up to 280 μm in length) not spirally twisted and having very little branching. No evidence for detrimental effects on the nematode was found. Hoffman (1953) suggested that *S. leidynemati* utilizes the nematode for anchorage and derives its food from the intestinal content of the cockroach.

In this study the *S. leidynemati* from the surface of oxyurid nematodes living in the hindgut lumen of *P. americana* was isolated, cultured on an artificial media and its potential for antibiotic, cellulolytic, and chitinolytic activities was tested.

5.2. Materials and methods

Isolation of *S. leidynemati*: Five adult cockroaches were randomly selected from the rearing colonies, chilled, and the hindgut was exposed by dorsal longitudinal incision. Hindguts were cut open in Ringer's solution bath and adult oxyurid nematodes were aseptically removed with tweezers, rinsed in sterile water and then placed on wet sterile

filter paper in the Petri dish and incubated for six weeks at room temperature. Hyphae growing from nematode tissues were then transferred on plate count agar (PCA) (Difco, Detroit, MI) medium and incubated anaerobically, aerobically and microaerophilically for four weeks. This isolate was used for metabolic testing and microscopic observation.

Antibiotic activity: Five strains of common Gram-positive and Gram-negative bacteria were used to test the antibiotic activity of *S. leidynemati*. *Pseudomonas aeruginosa*, *Escherichia coli* NCIB 8666, *Bacillus subtilis*, *Staphylococcus epidermis*, and *Streptococcus pyogenes* were cross-streaked on PCA medium with *S. leidynemati* and incubated aerobically in the room temperature for two weeks. Growth patterns and signs of antibiotic activity were recorded.

Cellulolytic activity: *S. leidynemati* isolate was transferred to the carbon-free medium (Appendix 2) containing sterile filter paper. Tubes were incubated aerobically at room temperature for seven weeks and signs for cellulolytic activity (degradation of filter paper) were monitored.

Chitinolytic activity: *S. leidynemati* isolate was streaked on the chitin medium (Appendix 2) and incubated aerobically at room temperature for five weeks. Signs of chitin degradation (clearing zones of chitin) were monitored and photographed.

Scanning electron microscopy (SEM): Four adult cockroaches were chilled, cut open by dorsal longitudinal incision, hindguts were exposed, and cut open in Ringer's solution bath. Adult oxyurid nematodes were removed, rinsed in sterile water and placed on cryo stubs. The technique used for sample preparation of the *S. leidynemati* attached to the surface of oxyurid nematodes for SEM was as follows. Samples were fixed to the stubs with OCT (cryo embedding medium). Stubs were submerged in liquid nitrogen (-207 °C)

for 10 min. After freezing, ice was allowed to sublime at room temperature. Samples were gold coated and examined with a JOEL JSM.6301FXV field emission scanning electron microscope (accelerating voltage, 5 kV) and photographed.

5.3. Results

Isolation of *S. leidynemati*: No discernible pattern was observed in the attachment of *S. leidynemati* to the surface of oxyurid nematodes (Figure 5.1 - 5.2). In several cases, the entire nematode surface was covered by bacterial filaments. New growth of *S. leidynemati* was detected on the dead nematode tissues from the third week after transfer to the Petri dish lined with wet filter paper. The bacterium formed aerial mycelium with hyphae up to 300 μm in length (Figure 5.3 - 5.4). Hyphae were aseptically streaked on the plate count agar (PCA) and cultivated aerobically. Growth also occurred under microphilic conditions, however, the bacterium did not grow under anaerobic conditions in the anaerobic chamber.

Antibiotic activity: No signs of antibacterial activity of *S. leidynemati* against strains of *E. coli* NCIB 8666, *Staphylococcus epidermis*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, and *Streptococcus pyogenes* were observed. The colony of *S. leidynemati* grew in a close association with all strains tested without any indication of antagonistic activity.

Cellulolytic activity: In the medium with the filter paper as the only source of carbon, *S. leidynemati* formed several colonies attached to the surface of the filter paper. After an extended period of time (35 days), however, no signs of filter paper degradation were

observed. It is likely that *S. leidynemati* is a very good carbon scavenger and utilised trace amounts of carbon molecules present in water used for the medium.

Chitinolytic activity: Seven days after streaking of *S. leidynemati* on the chitin medium plates, clearing zones of chitin, indications of chitinolytic activity, were observed (Figure 5.5). Chitin was completely cleared under the colonies and for several millimetres around *S. leidynemati* colony (Figure 5.5).

Scanning electron microscopy (SEM): Although the direct freezing by CRYO technique slightly distorted the isolate, SEM has confirmed results of the study published by Hoffman (1953). Hyphae are not spirally twisted and form fragments approximately 3.0 – 4.5 μm long and 0.8 - 1.5 μm wide (Figure 5.6). Limited branching of the hyphae occurs (Figure 5.6).

5.4. Discussion

Filamentous bacteria are common inhabitants of the hindgut of the American cockroach (Chapter 2). Many of them are attached to the hindgut wall producing a web-like array in this microecosystem (Cruden and Markowetz, 1987). Filamentous bacteria were observed attached to ingested pieces of old cuticle (in the hindgut) and in the faeces.

The filamentous bacterium *Streptomyces leidynemati* is attached to the surface of oxyurid nematodes probably to prevent being expelled from the hindgut lumen during defecation. It is possible that this species is also attached to the hindgut wall and cuticular spines although no attempts to isolate it from these sites were undertaken. Results of the test for cellulolytic activity were negative, however it is clear that this bacterium is very good carbon scavenger; it is probably able to use a broad spectrum of carbon sources.

The test for chitinolytic activity has shown that *S. leidynemati* possess strong chitinolytic enzymes. Since cockroaches are both cannibalistic and feed on shed cuticle, the ability to degrade chitin might become an important source of carbon for this bacterium, especially during periods of cockroach starvation. During these periods, the hindgut content is retained in the lumen for an extended period of time (Bignell, 1981) which allows for degradation of complex macromolecules, including chitin. It is also possible that intermediate compounds of the chitin degradation are captured and metabolised by other (fermenting) bacteria. Consequently, the cockroach might indirectly benefit from the chitin degradation by absorption of volatile fatty acids as the end products of the bacterial fermentation. Since only pure chitin has been used for the test, additional studies (activity for the crude cockroach cuticle) are necessary to evaluate the significance of chitinolytic enzymes produced by *S. leidynemati* for the bacterial and cockroach physiology. Further analysis is required to identify these enzymes and their substrate specificity.

Endogenous enzymes with chitinolytic activity secreted by the midgut and associated caeca into the midgut lumen of *P. americana* have been qualitatively detected. These enzymes have not been identified, their substrate specificity and consequently their significance for cockroach metabolism is not known (Waterhouse and McKellar, 1961).

Results of the test for antibiotic activity of *S. leidynemati* against selected bacteria were negative. It is interesting however, that some filamentous bacteria attached to the hindgut wall bear large populations of other microbes (Chapter 2). In contrast, no bacteria have been observed attached to the surface of *S. leidynemati*. Further analysis will be necessary to evaluate the potential antibiotic activity of this species in greater detail.

In conclusion, the filamentous bacterium *S. leidynemati* has been isolated from the surface of oxyurid nematodes from the hindgut of the American cockroach and cultured for the first time on an artificial media. While no strong antibiotic and cellulolytic activities of this bacterium have been detected, it was shown that *S. leidynemati* possess very potent chitinolytic enzyme.

Figure 5.1 Filamentous bacterium *Streptomyces leidynemati* attached to the anterior part of oxyurid nematode from the hindgut of *P. americana.*

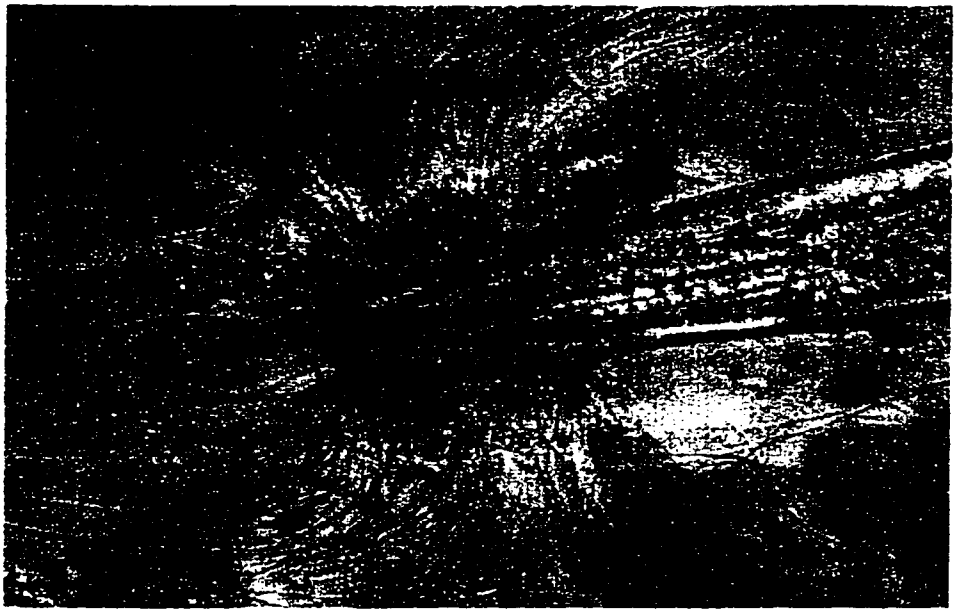


Figure 5.2 Filamentous bacterium *Streptomyces leidy*nemati attached to the middle part of oxyurid nematode from the hindgut of *P. americana.*

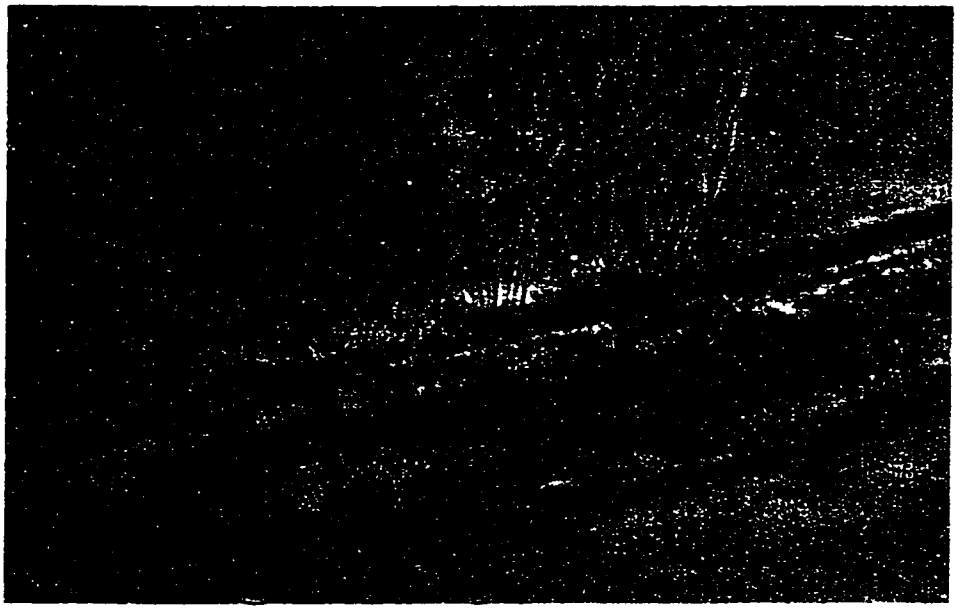


Figure 5.3 *Streptomyces leidynemati* growing from oxyurid nematode tissues on the filter paper (28 days after transfer).

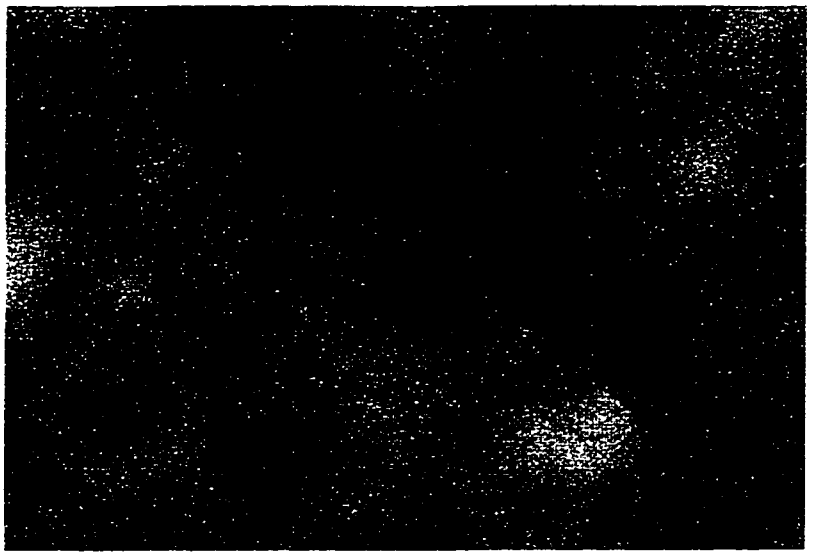


Figure 5.4 Growth of *S. leidynemati* 48 days after the transfer.

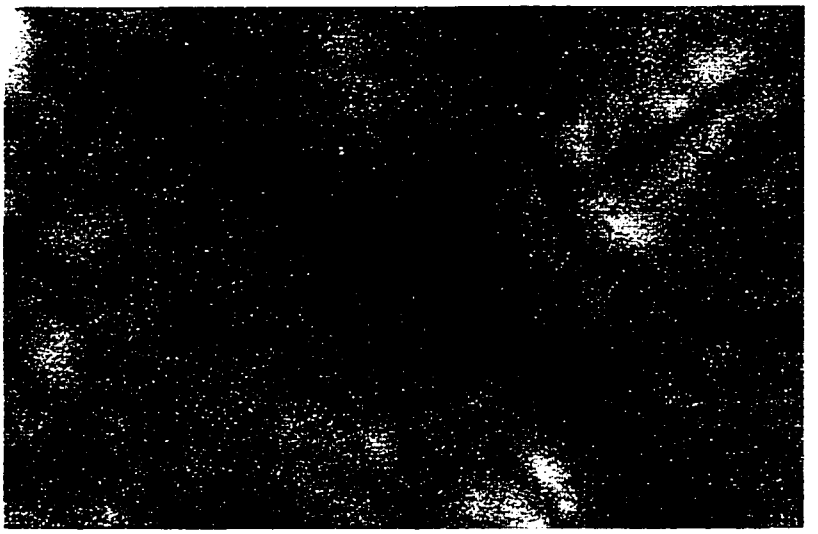


Figure 5.5 *Streptomyces leidynemati* growing on the chitin medium plate. Chitin (white flakes) is completely cleared under the colony and several millimeters around the colony.

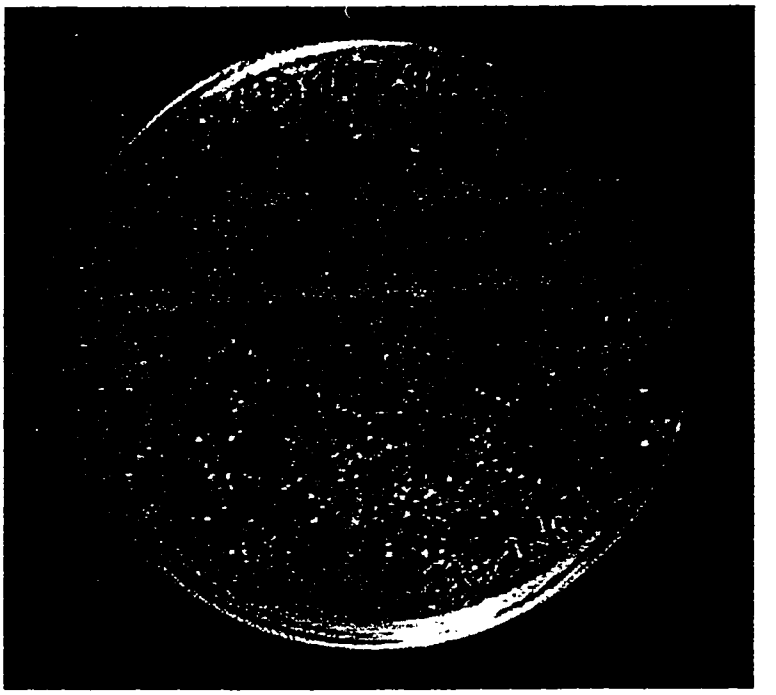
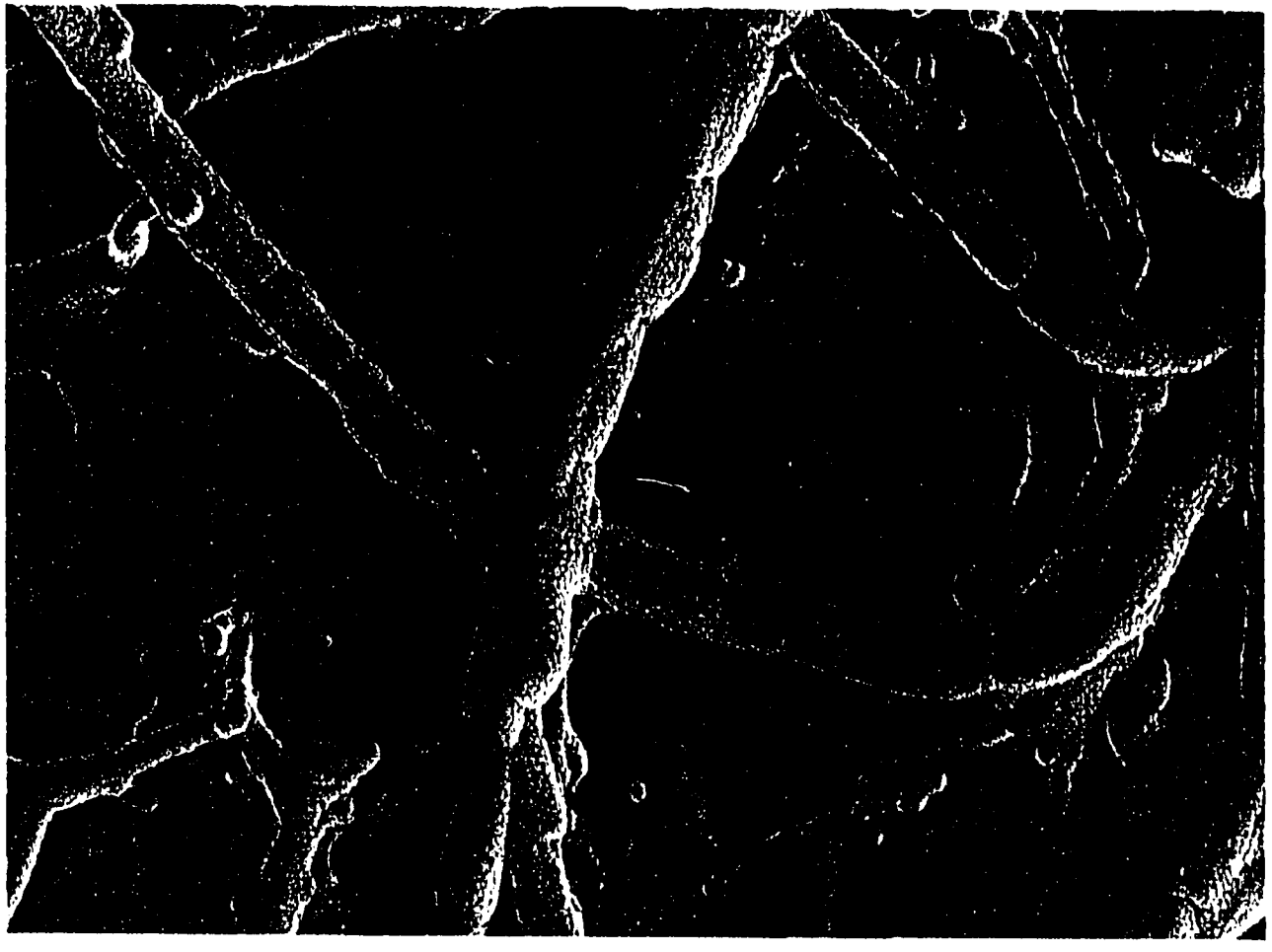


Figure 5.6 Scanning electron micrograph of *Streptomyces leidynemati* from the hindgut of *Periplaneta americana* (Bar = 1 μm)



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Chapter 6

Diversity of Microbial Symbionts in the Alimentary Canal of the American Cockroach (Molecular Analysis)

6.1. Introduction

During more than 3.7 billion years that life has existed on Earth, evolution has led to extensive diversification in microorganisms. Therefore, it is not surprising that microorganisms exhibit tremendous metabolic, genetic, and ecological diversity (Perry and Staley, 1997). To date, only a small fraction of microbial species diversity has been explored and most of the microbial world remains undiscovered (Tiedje, 1995). This diversity is much greater than that of large organisms, even though the number of described species of macroorganisms, particularly insects, greatly surpasses that of microorganisms (Tiedje, 1995). There are many aspects of microbial associations that have just begun to be explored. Microbial symbiosis with animals, plants, and other microorganisms are diverse and common in natural communities. These associations are often essential to the livelihood of both the microbes and their hosts (Kane and Pierce, 1994). Thus, studies of all types of microbial interactions are important to determine the nature of physiological interdependencies between symbiont partners. Knowledge gained yields a better understanding of the function of these diverse ecosytems for human benefit (Pace *et al.*, 1993).

The intestinal tract of higher animals contains one of the most densely structured microbial communities found anywhere in nature. With the exception of microbiota of human colon and bovine rumen, very little is known about microbial symbionts of other

vertebrates, including other farm animals (Hungate 1966, Wilson and Blitchington, 1996, Van Nevel and Demeyer, 1988). Relatively little is known about microbial symbionts in the intestinal tract of invertebrates and lower vertebrates (Cazemier *et al.*, 1997). Isoptera (termites) is the only insect order of which the intestinal microbiota has been extensively studied (Breznak, 1982, Slaytor, 1992, Anklin – Muhlemann *et al.*, 1995). The types of microorganisms residing in the intestinal tracts of most animals is largely unexplored and mechanisms of the host-microbial interactions remain to be investigated.

Knowledge of microbial diversity has been limited because of a traditional obstacle, the vast majority (>99%) of naturally occurring microbes can not be cultured using standard techniques (Amann *et. al.*, 1995). Until recently, there has been no way to describe microorganisms without growing pure cultures.

New techniques, which do not require cultivation of organisms, based on analysis of molecular sequences of highly conserved genes, such as 16S (small-subunit) ribosomal RNA, can be used for phylogenetic identification (Hugenholtz and Pace, 1996). In this approach, nucleic acids are extracted directly from the environment and ribosomal DNA genes are obtained and sequenced to identify the organism. In the simplest variation of this approach polymerase chain reaction (PCR) is used to amplify 16S rDNA from the environment using thermophilic bacterial enzyme - *Taq* polymerase, and rDNA gene primers (universal or specific taxonomic groups) (Hugenholtz and Pace, 1996). The PCR products are then cloned, clones are analysed by restriction endonucleases (restriction fragment length polymorphism – RFLP) (Moyer *et al.*, 1994, Lagaurre *et al.*, 1994) and the selected clones are sequenced and identified. The result is determination of the common phylotypes (phylogenetically identifiable taxa) that occur in the environment

(Hugenholtz and Pace, 1996). The methods for analysing the genetic diversity of complex microbial populations include the denaturing gradient gel electrophoresis (DGGE). DGGE separates different PCR amplified 16S rRNA genes on the basis of their G+C content (Muyzer *et al.*, 1993; Laguerre *et al.*, 1994). The "Checkerboard" DNA-DNA hybridisation that uses oligonucleotide hybridisation probes (nucleic acid probes complementary to specific rRNA or rRNA gene targets) (Socransky *et al.*, 1994) is another method used for assessment of a phylogenetic diversity of complex microbial communities. This technique is reliable for evaluation of relationships at the species level, where ribosomal RNA sequencing lacks the necessary resolving power (Palleroni, 1997, Socransky *et al.*, 1994). Oligonucleotide probes can be fluorescently labelled and specific phylotypes can be visualised, counted, and morphologically characterised by epifluorescent microscopy, *in situ*, directly from the environmental samples (DeLong *et al.*, 1989, Amann *et al.*, 1990, Wagner *et al.*, 1994).

Molecular methods leading to phylogenetic characterisation then provide information for designing an artificial media necessary for culturing of new specific phylotypes. Culturing is essential for the assessment of metabolic and physiological properties of new isolates and their roles in the natural environment (Palleroni, 1997).

In this study two molecular techniques are used for an assessment of genetic diversity of the hindgut microbial symbionts of the American cockroach: 1) amplification of 16S rRNA gene (whole bacterial community) by polymerase chain reaction, cloning, and sequencing; 2) checkerboard DNA-DNA hybridisation technique.

6.2. Materials and Methods

6.2.1 PCR, cloning, and sequencing

Sample preparation and nucleic acid extraction: Two adult cockroaches (male and female) randomly selected from rearing colonies were chilled and cut open by dorsal longitudinal incision. The anterior end (oesophagus), posterior end (rectum) and junctions between foregut, midgut, hindgut were ligated. Three parts (foregut, midgut, hindgut) were separated and surface sterilised by 70% ethanol. Corresponding parts of the digestive tract from each cockroach were then pooled in 0.5 ml Eppendorf tubes, cut open, and suspended in 200 µl of sterile water. Suspensions were vortexed and then pulse centrifuged. Supernatant fluids were transferred to 0.5 ml Eppendorf tubes, while pellets were resuspended in Falcon tubes containing 500 µl sterile water and ultrasonicated for 30 seconds with 3mm tip and 2-3 W output. The vortexed and sonicated suspensions from each part of the alimentary canal were then combined and used for nucleic acid extraction. Cells from each sample (foregut, midgut, hindgut) were lysed by the Beadbeater (Mini-beadbeater, Biospec products) and nucleic acids were extracted by the phenol procedure (Sambrook *et al.*, 1987) and stored in the refrigerator. Extracted nucleic acids were used for PCR, cloning, and checkerboard DNA-DNA hybridisation.

Polymerase chain reaction: 16S rDNA was amplified from each sample by polymerase chain reaction in the EriComp tempcyclers with Universal eubacterial forward primer – UF: 5'- AGA GTT TGA TYM TGG C – 3' (position 8-23) and universal eubacterial reverse primer – UR: 5'- GYT ACC TTG TTA CGA CTT- 3' (position 1492) and *Taq*Bead™ polymerase (Promega, Madison, Wisconsin) (Y = C or T; M = A or C).

Reaction mixtures and cycles are listed in Table 6.1. For each reaction, 5 µl buffer, 4 µl 2.5 mM dNTPs, and sterile distilled water (to a total reaction volume of 50 µl) were added. PCR products were visualised under UV light on a 1% agarose gel containing 0.5 µg/ml of ethidium bromide.

Cloning and sequencing: PCR products were cloned by using TOPO TA Cloning Kit (Invitrogen, Carlsbad, California) into *E. coli* cells. Twenty clones from each gut region containing the pCR®-TOPO plasmid vector with PCR insert were randomly selected and used for further analysis. 16S rDNA from clones was amplified by PCR with Vent (exo)-polymerase and M13 forward: 5'–GTA AAA CGA CGG CCA G–3' and M13 reverse: 5' – CAG GAA ACA GCT ATG AC – 3' primers and Thermopol buffer (Invitrogen, Carlsbad, California) (Table 6.1). Restriction enzyme digestion was performed to analyse selected clones. Each reaction contained 1 µl NEB2 buffer (New England Biolabs), 0.5 µl *HinpI* enzyme (four-cutter restriction endonuclease), 3.5 µl sterile distilled water, and 5.0 µl PCR product. Restricted DNA was analysed by horizontal electrophoresis in 1% agarose gel. Electrophoresis was carried out at 100 mV for 60 min. Gels were stained in an aqueous solution of ethidium bromide (1.0 µl/ml) and photographed under UV illumination. Clones with different digest patterns were sent for sequencing to the Forsyth Institute in Boston, MA. Partial sequences were determined by ABI automated sequencer (Perkin-Elmer, Applied Biosystems Div., Foster City, CA). Sequences (appr. 500 bp) were phylogenetically identified through the computer search BLAST (Basic Local Alignment Search Tool) (Altschul *et al.*, 1990) using comparison to the GenBank database.

6.2.2 Checkerboard DNA-DNA hybridisation

Three DNA samples of the hindgut microbial population were used for this analysis. 16S rDNA was amplified by PCR with *Taq* polymerase and digoxigenin-labelled universal forward primer: UF-Dig: 5'- LAG AGT TTG ATY MTG GC – 3' (position 8-23) and universal eubacterial reverse primer UR: 5'- GYT ACC TTG TTA CGA CTT – 3' (position 1492). Reaction mixtures and cycle conditions are described in Table 5.1. The checkerboard DNA-DNA method is designed for hybridising large numbers of DNA samples against large numbers of DNA probes on a single membrane (Socransky *et al.*, 1994). PCR product from three hindgut samples was run against DNA probes representing ten major bacterial groups: proteobacteria (alpha- , beta- , gamma – groups), enterics, sulphate-reducing bacteria, flavobacteria, Gram-positive bacteria with low G-C content, spirochetes, and two control universal probes (Universal eubacterial probe 341, Universal eubacterial probe 1089) (Table 6.2).

6.3. Results

6.3.1 PCR, cloning, and sequencing

a) Foregut: Restriction fragment length polymorphism (RFLP) with *HinpI* endonuclease of twenty selected clones produced only four distinct patterns. These clones (F1, F2, F3, F4) were sent for sequencing. Partial sequence and phylogenetic identification revealed a high similarity to the two genera of facultative anaerobic bacteria, *Serratia* and *Bacillus* (Table 6.3).

b) Midgut: RFLP of twenty clones originating from the midgut bacterial DNA generated fifteen distinct digest patterns. All fifteen clones were partially sequenced. Phylogenetic

identification revealed that they represent a very diverse bacterial population. Clones M4 and M13 were the same, most likely representing *Serratia marcescens* (>99%, 99% similarity). Sequences of clones M1, M18 were similar to *Clostridium oroticum* (97%), and *Butyrivibrio* sp. (90%) / *Roseburia* sp. (90%), respectively (Table 6.3.). Clone M15 showed very high similarity to the *Pseudomonas aeruginosa* (>99%). Clones M14 and M3 were very similar to the *Desulfovibrio* sp. (90%) and *Bacteroides splanchnicus* (90%), respectively. Sequencing of nine clones (M6 - M12) revealed very low similarity to known organisms in the GenBank database suggesting that these represent new genera (Table 6.3). Sequencing of clone M2 was not successful.

c) Hindgut: RFLP of the hindgut PCR product generated fifteen different digest patterns. Sequencing of these clones revealed that clones H2 and H4 represent most likely *Bacillus amyloliquefaciens* (99%, 98% similarity). Clones H9, H14, H18 were highly similar to *Serratia marcescens* (99, 98, 98%). Clones H11, H13, H7, and H1 were similar to sp. *Xanthomonas* sp. (98%), *Klebsiella planticola* (95%), *Cytophaga* sp. (90%), and *E. coli* (90%), respectively. Clones H5 and H20 showed very low homologies to known organisms in GenBank (Table 6.3). Clone H16 was not successfully sequenced.

6.3.2 Checkerboard DNA – DNA hybridisation:

Three samples of amplified 16S rDNA of the hindgut microbiota were run against ten DNA probes (Figure 6.1, lines 6, 18, 19). This method has confirmed that sulphate-reducing bacteria, flavobacteria, and spirochetes are the components of the hindgut microbial community. The flavobacteria group probe showed the strongest hybridisation against all three hindgut samples. Control Universal probes hybridised well with all

samples (Figure 6.1) (other lines represent samples not relevant to this study). The alpha proteobacteria probe did not work and cross-hybridised at 55 °C with all samples on the membrane. The beta-proteobacteria probe malfunctioned because of low Tm (Thermal melting profile). Surprisingly, the enterics-group probe hybridised very little and only with sample 19. No hybridisation occurred with either Gram+ low GC group probe and Delta-proteobacteria group probe (Figure 6.1).

6.4. Discussion

Phylogenetic identification by analysis of cloned 16S rDNA from the foregut, midgut and hindgut microbiota as well as DNA-DNA hybridisation technique using whole hindgut bacterial population and group-specific oligonucleotide probes have confirmed results of previous studies (Cruden and Markowetz, 1984, 1987, Bracke *et al.*, 1979). The foregut of *P. americana* contained a less diverse microbial community than the midgut or hindgut. Only two species of facultative anaerobic bacteria, *Serratia marcescens* and *Bacillus amyloliquefaciens* were detected by cloning and sequencing of 16S rDNA. Both species are likely common inhabitants of the lumen of other parts of the alimentary canal; both were detected in the midgut and hindgut. High similarity (98-98%) of these clones to sequences in the GenBank database indicates they truly represent these two species. Sequences of clones obtained from the midgut and hindgut bacterial DNA revealed that microbiota of these two regions was phylogenetically very diverse. Some clones showed similarity to genera of facultative and strict anaerobes, including *Clostridium, Butyrivibrio, Fusobacterium, Klebsiella,* and *Bacteroides* that were reported from the cockroach digestive tract in previous studies using the traditional culturing

approach (Cruden and Markowetz, 1987). In addition, several genera, including *Desulfovibrio*, *Pseudomonas*, *Wolinella*, and *Xanthomonas*, indicated by sequences of midgut and hindgut clones, are described here for the first time. Furthermore, sequences of several clones showed very low similarity (81 – 90%) to information available in the GenBank database. These indicate potentially new bacterial genera and species. It is important to underscore that all clones were only partially sequenced (approximately one third of the entire 16S rRNA gene). Therefore, this analysis should be considered as the first step in the investigation of the genetic diversity of cockroach microbial population. Full sequencing of 16S rDNA of clones, especially those with low homology to sequences in the databank, is necessary for the complete phylogenetic characterisation. Furthermore, additional sequencing of a greater number of clones is required for the comprehensive evaluation of phylogenetic diversity of cockroach intestinal symbionts.

In addition, it is essential to consider that the techniques based on analysis of nucleic acids may be subjected to a bias resulting from repeated polymerase chain reaction as well as from differences in microbial cell composition (Palleroni, 1997). For example, it is likely that the first step of the analysis, cell lysis, is affected by differences in microbial cell wall structure and consequently, extracted nucleic acids might underrepresent total microbial DNA population. As a result, microbial cells that have greater tendencies to lyse are likely to dominate in the sample and provide a false assessment of the total diversity of a microbial community.

Checkerboard DNA-DNA hybridisation was only partially successful. While control Universal-eubacterial probes functioned well and hybridised with all samples (with exception of DNA sample 12 - methanogen [archaeal] DNA), Alpha- and Beta-

proteobacteria group probes, did not work properly and these results are not valid. Despite these problems, hybridisation has validated results of cloning and sequencing, and indicated further previously undetected flavobacteria and sulphate-reducing bacteria in the cockroach hindgut. Spirochetes frequently observed (not culturable on known artificial media) in the cockroach hindgut samples (Cruden and Markowetz, 1984, 1987, Zurek, not published) are also detected by the hybridisation technique. Enteric bacteria and G-positive-low GC bacteria groups expected to be present in the cockroach hindgut lumen were not detected. Undoubtedly, results of this experiment should be regarded as preliminary and consequent analysis with redesigned probes and more DNA samples are necessary to make any final conclusions about the diversity of the hindgut prokaryotic symbionts. More specific target probes need to be designed to evaluate phylogenetic diversity of this microbial ecosystem in greater detail. Nevertheless, this technique showed great potential and provides a rapid and useful means to determine the genetic diversity of microbial populations.

To understand the function and interactions within complex microbial communities requires the cultivation of organisms on artificial media designed to simulate as close as possible natural conditions of the ecosystem. Modern molecular techniques based on the analysis of nucleic acids do not circumvent the need for culturing and do not provide sufficient and reliable information for understanding either physiology of a single phylogenetically identified microorganism or the function of a whole microbial consortium in a community and microecosystem. Molecular methods serve important but limited roles as guides for the isolation and characterisation of new prokaryotic taxa (Palleroni, 1997). Comprehensive descriptions that include not only

molecular data, but also the relevant aspects of the physiology of the organism (polyphasic approach) are necessary to investigate prokaryotic diversity.

In conclusion, this study has shown that the microbial communities in the hindgut of the American cockroach are phylogenetically very diverse. While the molecular techniques used in this study have detected some bacterial genera in the hindgut that were reported in previous studies, several prokaryotic genera have been found as part of the cockroach intestinal microbial ecosystem for the first time. This study has also revealed several microorganisms that are phylogenetically unrelated to the known microorganisms in the GenBank database. These remain to be analysed and characterised further by molecular approaches as well as by culturing techniques.

Table 6.1 Polymerase chain reaction conditions for amplification 16S rDNA for cloning and DNA-DNA hybridisation

PCR reactions mixtures and cycles									
Type of reaction	Buffer	25 mM $MgCl_2$ (µl/rxn)	Polymerase	Forward (fwd) primer	Reverse (rev) primer	Primer volume (µl/rxn)	Template DNA (µl/rxn)	Initial temp. progam	Amplification cycles
PCR of whole DNA population for cloning	10x *Taq*	6	one *Taq* bead	Universal eubacterial (UF)	Universal eubacterial (UR)	1	1	94 °C 5 min.	30x (94 °C, 45 sec., 53 °C 45 sec., 72 °C 90 sec.)
PCR of selected clones	Thermopol (with $MgCl_2$)	-	1 µl Vent (exo)	M13 fwd	M13 rev	1	5	94 °C 5 min	30x (94.5 °C, 45 sec., 53.5 °C 45 sec., 72 °C 2 min.)
Checkerboard DNA-DNA hybridization	10x *Taq*	6	one *Taq* bead	Dig-labeled universal eubacterial	Universal eubacterial (UR)	1	2	94 °C 5 min.	30x (94.5 °C, 45 sec., 53.5 °C 45 sec., 72 °C 100 sec.)

Table 6.2 Oligonucleotide probes used for the "Checkerboard" DNA – DNA hybridisation analyzes of the hindgut microbiota of the American cockroach.

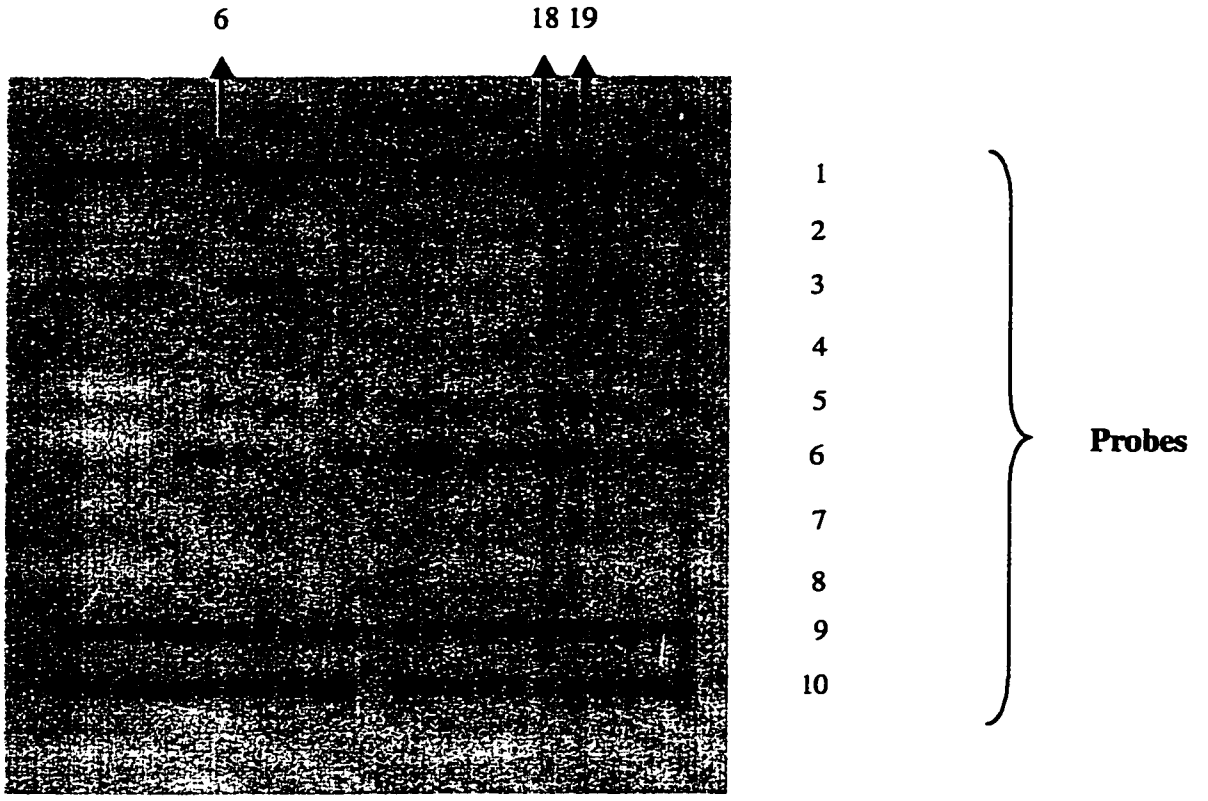
ID	Use	Group	Specific target	Sequence 5'- 3'	Position
Alpha - T	Capture probe	Alpha - Proteobacteria	All Alpha - proteobacteria	20T's - CGRAGTTAGCCGGGGC	500
Beta - T	Capture probe	Beta – Proteobacteria	All Beta - proteobacteria	20T's - TCAATGCTACACGYG	680
Delta - T	Capture probe	Delta - Proteobacteria	All Delta-proteobacteria except SRB	20T's - GCTTKCAWGMAGAGG	96
Flavo - T	Capture probe	Flavobacteria	All Flavobacteria	20T's - TCAGTRCCAGTGTGGGGG	308
Enteric - T	Capture probe	Gamma - Proteobacteria	Enteric group	20T's - CTTTTGCARCCCACTCC	1416
LoGC - T	Capture probe	Gram - positive	Gram positive with low-GC content	20T's - TGTAGCCCARGTCATA	1212
Spiro - T	Capture probe	Spirochetes	All spirochetes	20T's - GACTTGCATGCTTAARACG	45
SRB - T	Capture probe	SRB	SRB, not other Delta - proteobacteria	20T's - CGYGCGCCRCTYTACT	96
Univ – 341T	Capture probe	Universal eubacteria	All Eubacteria	20T's - CTGCTGCCTCCCGTAGG	341
Univ – 1089T	Capture probe	Universal eubacteria	All Eubacteria	20T's - CTCGTTGCGGGACTTAAC	1089

SRB - Sulphate-reducing bacteria

Table 6.3 Phylogenetic identification of 16S rDNA of clones from different parts of the digestive tract of *P. americana*

Foregut			Midgut			Hindgut		
Clone	Identification	% similarity	Clone	Identification	% similarity	Clone	Identification	% similarity
F1	*Serratia marcescens*	98	M1	*Clostridium oroticum*	97	H1	*Escherichia coli*	90
F7	*Bacillus amyloliquefaciens*	99	M2	NGS	-	H2	*Bacillus amyloliquefaciens*	98
F11	*Bacillus amyloliquefaciens*	97	M3	*Bacteriodes splanchnicus*	91	H4	*Bacillus amyloliquefaciens*	99
F12	*Serratia marcescens*	98	M4	*Serratia marcescens*	>99	H5	*Clostridium* sp.	86
(NGS – not a good sequence)			M6	*Clostridium* sp.	81	H7	*Cytophaga* sp.	90
			M7	*Bacteriodes* sp.	87	H8	*Wolinella succinogenes*	>99
			M8	*Clostridium* sp.	85	H9	*Serratia marcescens*	99
			M9	*Thermoanaerobacter thermocopriae*	82	H10	*Bacillus lentus*	92
			M10	*Fusobacterium prausnitzii*	85	H11	*Xanthomonas* sp.	98
			M11	Unidentified termite gut bacterim	85	H13	*Klebsiella planticola*	95
			M12	*Bacteroides* sp.	85	H14	*Serratia marcescens*	98
			M13	*Serratia marcescens*	99	H16	NGS	-
			M14	*Desulfovibrio* sp.	90	H17	*Salmonella* sp.	90
			M15	*Pseudomonas aeruginosa*	>99	H18	*Serratia marcescens*	98
			M18	*Butyrivibrio* sp./ *Roseburia* sp.	96	H20	Gamma proteobacterium	86

Figure 6.1 Checkerboard DNA – DNA hybridisation of the hindgut microbiota of *P. americana* and ten oligonucleotide group probes.



Probes: 1 - Alpha Proteobacteria
2 - Beta Proteobacteria
3 - Enteric group
4 - Delta Proteobacteria
5 - Sulphate-reducing bacteria
6 - Flavobacteria
7 - Gram positive, low GC ratio
8 - Spirochetes
9 - Universal probe 341
10 - Universal probe 1089

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Chapter 7

General conclusion

In industrialised, as well as developing countries, urbanisation has moved humans away from many arthropod pests, but into intimate association with others. The urban environment experiences greater pesticide usage per square kilometer than the agricultural environment (Schal and Hamilton, 1990). Surveys of pest control operators indicate that cockroaches are of the greatest concern in the urbanised environment because of health and aesthetic reasons (Pinto, 1981; Robinson, 1996). The indoor environment consists of a variety of habitats (greenhouses, hospitals, residential settings), each with its own level of pest tolerance and each affected by a specific set of aesthetic, economic and health concerns. Urban pest suppression relies heavily upon multiple applications of broad-spectrum insecticides with little appreciation of the boundaries of the habitat, mobility of the targeted pests, and interaction with natural enemies (Schal and Hamilton, 1990). The harmful effects of insecticides to non-targeted organisms is a great concern and results in more emphasis on research focused on ecology (habitat specificity, food preferences, pheromones) and physiology of urban pests. The examination of biological association of insect pests with other organisms is another factor that might lead to new methods for the integrated pest management.

The American cockroach, *Periplaneta americana* is one of the most important and successful synanthropic pests (Roth, 1981). One of the factors contributing to this success very likely is a great nutritional versatility of this species, resulting to a significant extent from the symbiotic association with microorganisms. The intestinal tract of *P. americana* carries a large and varied microbial population. Much of this

association is mutualistic, both the cockroach and microorganisms benefit from this symbiosis. The most dense and diverse microbial population is carried in the anterior part of the hindgut, the colon. The colon lumen presents for microbes a relatively stable (temperature, pH, oxygen tension) and protected environment as well as continuous supply of nutrients. Cuticular spines increase the internal surface area of the colon accommodating microbial attachment that prevents discharge of bacteria in faeces.

The colon of *P. americana* functions as a fermentation chamber. The lumen of the colon supports a large very diverse population of strictly and facultatively anaerobic microorganisms. Facultative anaerobes likely scavenge oxygen diffusing through the colon wall making the oxygen tension in the centre of the lumen very low and creating a suitable environment for strictly anaerobic fermenting bacteria. Volatile fatty acids (VFA), especially acetic acid, arising from the fermentation contribute to the cockroach metabolism and development (Zurek and Keddie, 1996). This concept is also supported by the colon morphology. Peritrophic membrane breaks up in the anterior part of the colon allowing bacterial attachment to the colon wall. In addition to increasing available surface of the colon wall for attachment, cuticular spines also probably help to mix the colon content (along with the substantial peristaltic and antiperistaltic movement) and permit deeper access of bacteria to the lumen.

The ultrastructure of the colon wall is consistent with tissues maintaining an active transport (Bignell, 1980). The VFA contribution is probably the most significant in periods of cockroach starvation; colon content is retained in the lumen and nutritionally diverse food components including chitin and cellulose are subjected to microbial degradation. Arising VFA are then available to cockroach metabolism. Feeding on

nutritionally poor diet (high fiber diet) results in significant increase of cellulolytic microorganisms and consequently significant elevation of VFA usable for the cockroach (Zurek and Keddie, 1998). It is likely that a rise of VFA concentrations in the hindgut partly compensates for a low nutritional value of the diet. Supplementary supply of vitamins, conversion of phytosterols to cholesterol as well as protection from pathogens entering the digestive tract are other possible aspects of the cockroach microbial association, especially in free-living animals feeding on diverse food sources.

Intestinal symbionts of *P. americana* represent all three domains of life based on comparison of 16S rRNA sequences (Perry and Staley, 1997). A molecular analysis approach and traditional culturing methods show that the Bacteria (Eubacteria) domain is represented by Proteobacteria (gamma- and delta- group), Flavobacteria, Bacteroides, Spirochetes, and G-positive (low GC and high GC-ratio) bacteria. The methanogenic bacteria (genus *Methanobrevibacter*) represent the Archaea domain. Ciliated protists and oxyurid nematodes represent the domain Eucarya.

The complexity of the colon microecosystem of *P. americana* provides this species with a great nutritional versatility. For example, suppression of methanogenesis did not greatly influence the profile of VFA and cockroach development (Zurek and Keddie, 1998). In this case hydrogen gas is most likely consumed by another hydrogen scavenger. (Dr. R. Schauder from the University of Frankfurt, Germany and I have isolated from the hindgut lumen of *P. americana* a new bacterial strain that is able to scavenge hydrogen even in presence of oxygen. Further characterisation is in progress).

In this study, two new bacterial metabolic pathways for the American cockroach, sulphate-, and sulphite reduction, are described. Isolation and identification of the

sulphate-reducing bacteria (SRB) have not been successful yet. SRB are non spore-forming rods and are present in very high numbers in the cockroach colon. It is likely that SRB are widely spread among other insects (I detected SRB from the hindgut of *Blaberus giganteus*, *Blatella germanica* and *Acheta domestica*) and able to grow fermentatively without sulphate. Feeding *P. americana* a high sulphate diet resulted in significant increase of SRB population and expansion of the black band region in the colon. This indicates that hydrogen sulphide is precipitated by iron from the diet to (black) iron sulphide. Consequently, potential toxic effects of hydrogen sulphide were neutralised and cockroach viability was not effected. Sulphite-reducing bacteria were isolated and identified by molecular technique. They are represented by two species of clostridia: *Clostridium bifermentans* and *C. celerecrescens*. The physiological significance of sulphite-reduction for these bacteria remains to be investigated. Feeding of high sulphate and sulphite diet along with elimination of metals from the diet could be the next step in the investigation of a manipulation intestinal microbial symbionts for the biological control of cockroaches.

Microorganisms with potential for degradation of chitin are present in the colon lumen of *P. americana*. Cruden and Markowetz (1987) reported chitin-degrading *Serratia marcescens* from the cockroach hindgut. In this study, the filamentous bacterium *Streptomyces leidynemati* attached to the surface of oxyurid nematodes living in the cockroach hindgut lumen was isolated and cultured. The isolate possesses a strong chitinolytic activity. Recently, J. Dugas (University of Connecticut) and I isolated a chitin-degrading bacterium closely related to *Flexibacter aurantiacus* (Cytophaga group) from the hindgut of *P. americana*. Further characterisation and metabolic testing of this

isolate is in progress. The significance of chitinolytic bacteria for the cockroach metabolism is not known, however, it is likely that the degradation of chitin serves a digestive function related the insect cannibalism and consumption of shed exoskeletons.

The tremendous metabolic diversity of the hindgut microbial symbionts of *P. americana* is further illustrated by my detection of an iron-reducing pathway and reduction of nitrate to nitrite and possibly to nitrogen gas. These pathways remain to be investigated further.

In conclusion, this study shows that the anterior part of the hindgut and its microbial symbionts play a significant role in metabolism and development of the American cockroach, *Periplaneta americana*. It is also evident that the hindgut microbial population is phylogenetically and metabolically extremely diverse. Several new bacterial metabolic pathways for the cockroach intestinal symbionts were detected. In addition, sequences of several clones from the hindgut microbial population indicate undescribed bacterial species and genera.

This investigation is the first step in a more complete understanding of this microecosystem and its great microbial diversity. Further research is needed to uncover complex interactions among microbial populations as well as between microorganisms and the cockroach as a host. Other aspects of this symbiosis, including the potential of the colon tissue for an active transport of the end products of microbial metabolism to cockroach haemolymph remains for further studies. Alteration of microbial populations and their metabolic pathways in the intestinal tract of *P. americana* hold promise for managing cockroaches without using toxic insecticides that are harmful to non-target organisms, including humans.

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Appendix 1

Carbon-free medium for detection of cellulolytic activity

	gram per liter
K_2HPO_4	1.0
$CaCl_2$	0.1
$MgSO_4$	0.3
NaCl	0.1
$FeCl_3$	0.01
NH_4NO_3	2.5

Adjusted to the pH 7.2 – 7.3, the strip of sterile filter paper as a source of carbon was added after autoclaving into each culture tube.

Medium for detection of chitinolytic activity

	gram per liter	
Agar	10.0	(Bacto – Agar, Difco, Detroit, MI, USA)
Peptone	2.0	(Bacto – Peptone, Difco, Detroit, MI, USA)
Chitin (flakes)	20.0	(Sigma, No. C-3641, USA)

Adjusted to the pH 7.0 and autoclaved.

Composition of Ringer's solution used as a bath solution for dissections

	gram per liter
NaCl	9.0
KCl	0.2
$CaCl_2$	0.2

Appendix 2

Composition of Butlin's medium for culturing of sulphate- and sulphite-reducing bacteria

	gram/liter
K_2HPO_4	0.5
NH_4Cl	1.0
Na_2SO_4	2.0
$CaCl_2$	0.1
$MgSO_4$	1.0
Sodium lactate	2.5 ml (60% solution)
Yeast extract	1.0
$FeSO_4$	trace

pH adjusted to: 7.0 - 7.5

Two nails (a source of iron) were added to each tube with 10ml of medium.
For sulphite reducing bacteria, two drops of 5% sodium bisulphite were added.

Composition of B_{10} medium with sulphite for isolation of sulphite-reducing bacteria

	gram/liter
K_2HPO_4	0.8
KH_2PO_4	0.2
$MgSO_4$	0.2
NaCl	0.2
Na_2MoO_4	trace
$CaSO_4$ (sat. sol.)	10ml
Yeast extract	5.0
Peptone	5.0
Ferric phosphate	4.7
Agar	14.0

pH adjusted to: 7.2 – 7.4

After solidification, sodium bisulphite (0.1mM) was added and spread aseptically on the surface of each plate.

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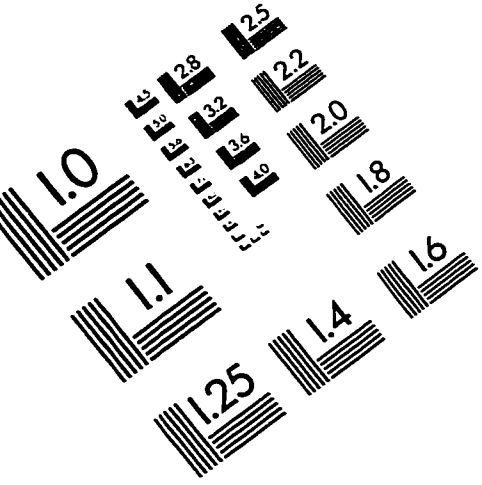

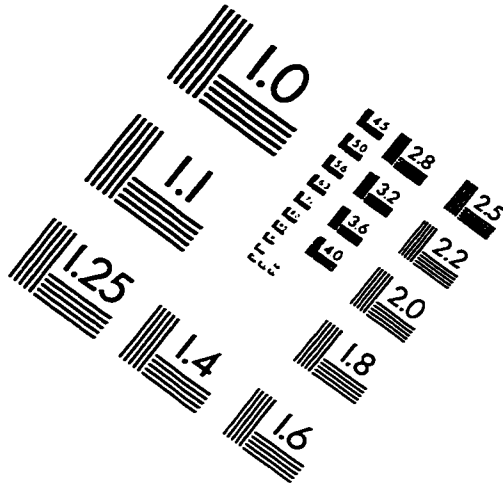

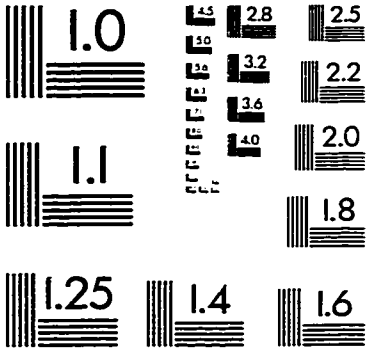

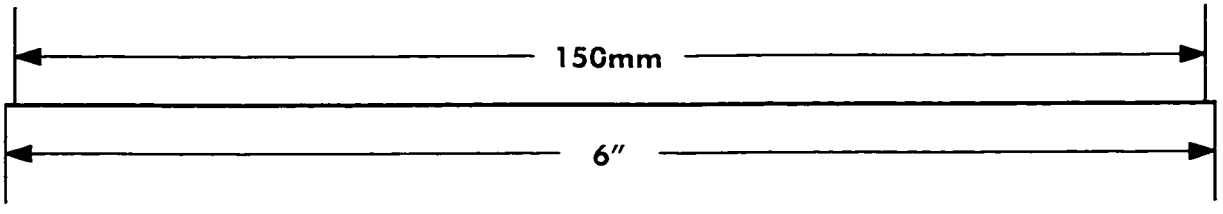

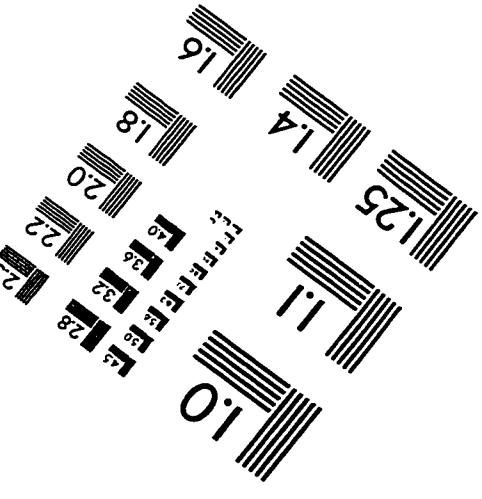

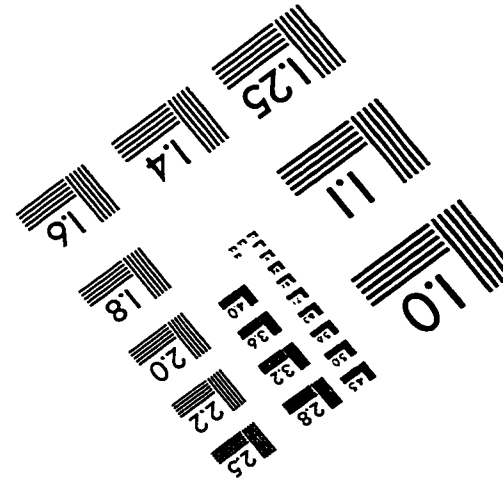

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