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SPIROCHAETES OF THE RAT GASTROINTESTINAL TRACT: ECOLOGY AND CHARACTERIZATION

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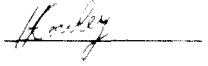
A Thesis Submitted to the Faculty of Science,
University of the Witwatersrand, Johannesburg
for the Degree of Doctor of Philosophy.

Johannesburg, 1986

DECLARATION

I the undersigned, HEATHER MAIREAD COWLEY, declare that the results presented in this thesis represent my own unaided work and that they have not been submitted for any degree in any other university.

DATED at JOHANNESBURG on this 5th day of MARCH, 1986.



ABSTRACT

The spirochaete population of the conventional rat gastrointestinal tract was studied with the objective of elucidating their taxonomy and ecology. Four morphologically distinct spirochaetes, with ultrastructure consistent with that described for *Treponema* genus, were identified. Transmission and scanning electron microscopy, light microscopy and fluorescent antibody tracing revealed that these spirochaetes inhabited the large intestine (caecum and colon), where they were associated with lumen contents and the mucosal epithelium, even penetrating inoffensively into the lamina propria. These bacteria colonized the infant gut during the fourth week of life, and thereafter formed part of the adult autochthonous flora. Development of suitable culture media and selective isolation techniques, successfully yielded axenic cultures of spirochaetes with morphologies similar to those seen *in situ*. One of these morphotypes was taxonomically characterized and compared to *T. hyodysenteriae* and *T. innocens*. These bacteria had an obligate serum requirement, grew anaerobically at 37-40°C and had fastidious peptone requirements. Growth in liquid culture medium yielded a generation time of 19 hours. As cultures aged, non-viable granules were produced. The isolates fermented galactose, inositol, inulin, lactose, mannose, and xylose; hydrolysed aesculin; digested gelatin and were weakly β haemolytic. Acetic acid was the major metabolic end-product produced. Isolates had a G+C mol ratio of 30.7%. Analysis of lipids indicated that phosphatidylglycerol was the predominant phospholipid, while phosphatidylserine, phosphatidylcholine and cardiolipin were also detected. Glycolipids, of which monogalactosyldiglyceride was the major component, contained galactose as the sugar moiety. Octadecenoic acid (18:1) and n-hexadecanoic acid (16:0) were the major fatty acids. Results indicate that these isolates are distinct from any other recognized *Treponema* species.

Some of the material presented in this thesis was presented at the Third International Congress of Gastrointestinal Microecology, Boston, United States of America, 1983; the abstract of which is published in Hill, M. J.; Borriello, S.P.; Hardie, J.M.; Hudson, M.J.; Lysons, R.J. and Tabaqchali, S. (Eds). 1984. Models of Anaerobic Infection. Martinus Nijhoff, Dordrecht.

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LIST OF ABBREVIATIONS

ATP	adenosine triphosphate
LPS	lipopolysaccharide
ATCC	American Type Culture Collection
NCTC	National Collection of Type Cultures
GIT	gastrointestinal tract
NEC	neonatal necrotizing enterocolitis
SPF	specific pathogen free
SEM	scanning electron microscopy
TEM	transmission electron microscopy
TLC	thin layer chromatography
GLC	gas liquid chromatography
PS	phosphatidylserine
PC	phosphatidylcholine
C	cardiolipin
PG	phosphatidylglycerol
MGDG	monogalactosyldiglyceride
ADF	anaerobic diluting fluid
PBS	phosphate buffered saline
FCS	foetal calf serum
TSBA	trypticase soy blood agar
TSB	trypticase soy broth
RGCA	rumen fluid-glucose-cellobiose-agar

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LIST OF CHEMICAL SUPPLIERS

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CHAPTER 1

INTRODUCTION

1.1 GASTROINTESTINAL MICROECOLOGY

1.1.1 General

Ecological principles applicable to any ecosystem pertain to the microbial flora of the gastrointestinal tract (GIT). In the normal adult animal, each habitat within the gut is colonized by an autochthonous (indigenous) flora, which occupies a niche in that habitat and thereby contributes to the economy of the system (Savage, 1977b). Allochthonous (nonindigenous) organisms may be found in any habitat, but normally these are transients and contribute little to the system, although a habitat vacated by its autochthonous flora may be temporarily colonized by these microbes (Savage, 1983). Similarly, severe disruption of the gastrointestinal ecosystem, may result in autochthonous microbes invading habitats to which they are allochthonous (Savage, 1977b). Bacterial transients are derived from food and other ingesta of the animal, from proximal gastrointestinal regions into more distal ones, and even from lower intestinal habitats into higher ones in coprophagous animals (Savage, 1983). Gastrointestinal autochthonous organisms as defined by Inman and Takeuchi (1982) and Savage (1977b) are:

- capable of anaerobic growth,
- always present in normal adults,
- colonize particular areas of the tract,

- colonize their ha[illegible] during succession in infant animals,
- maintain stable populatio[illegible] levels in climax communities in normal adults,
- may associate intimately with the mucosal epithelium in the colonized areas.

1.1.2 Succession

In its pristine condition, the GIT of a newborn animal is sterile and is subsequently colonized by microbes in a characteristic sequence. Complex interactive mechanisms involving the animal, its environment and diet, and the microbes themselves regulate the course of successional events and ultimately, the population levels and geographic distribution of the climax communities (Savage, 1977b). Pioneer populations are derived from the mother during the birth process. Primary colonizers of the gut in most suckling infants are members of the *Lactobacillus* genus, closely followed by the facultative anaerobes *Escherichia coli* and *Streptococcus faecalis*. These bacteria may assimilate oxygen in the gut and thereby prepare the gut for colonization by obligate anaerobes. Profound transitions in the gut population occur when the infant begins to consume solid food; the population levels of the obligate anaerobes increases progressively and when weaning has been completed, these bacteria are at adult climax levels. Some components of the microflora may colonize their habitats only after weaning (Savage, 1977b). The increase in strict anaerobes is accompanied by a concurrent decline in the facultative population, which may be associated with the production of toxic, anaerobic metabolic end products such as volatile and non-volatile short chain fatty acids and the production of hydrogen sulphide.

1.1.3 Regulation

The autochthonous microbiota exerts forces to maintain the *status quo* of the community and to exclude allochthonous colonization. The maintenance of low oxidation-reduction potentials, the production of metabolic end products (eg. volatile fatty acids), nutritional competition and the production of bacteriocins and antibiotics may all interact to exclude invaders and regulate the composition and localization of microbial communities in the GIT (Clarke, 1977). Factors generated by the host such as pH, stasis, bile acid production, epithelial turnover and diet, may also have regulatory effects on the gut microflora (Draser and Barrow, 1985; Gordon and Pesti, 1971; Savage, 1977b). By deconjugating bile acids, stimulating peristalsis, and inducing secretory IgA and lysozyme production, the gut microbes themselves alter host physiological responses and thereby contribute to the regulation of the gut microflora (Draser and Barrow, 1985). By sharing host mucosal or blood-group antigens, autochthonous bacteria may escape recognition by the hosts' immune system (Foo and Lee, 1974) and this could be of particular advantage to those bacteria that colonize the host epithelial surface. Phagocytosis by paneth cells, macrophages and polymorphonuclear leucocytes located in the mucosa may also regulate the microbial flora on the epithelium and within the crypts (Davis *et al* ., 1972b; Erlandsen and Chase, 1972; Savage, 1977b; 1983).

1.1.4 Microbial habitats

Microbial habitats in the GIT are distributed vertically from the oesophagus to the anus, and horizontally from the center of the lumen to the depths of the crypts. Each habitat provides different environmental or nutritional conditions suitable for its' microbial colonizers. Habitats within the gut are usually of microscopic di-

mension (microhabitats) and may occur in the lumen (on the surfaces of solid materials eg. foodstuffs); on the epithelial surface (in the mucus or on the microvilli), in the mucosal crypts (Savage, 1983). Major areas in the mammalian "straight-tube" gut embrace the oesophagus, stomach, small intestine and large intestine. Variations to this scheme are displayed by herbivores, that have either a rumen, where the stomach is enlarged and ramified into compartments, or a caecum; a blind sac located at the end of the small intestine and the beginning of the large. In both cases the undigestable plant material is delayed in transit and exposed to microbial degradation from which the animal derives nutritional benefit. The relative stasis of the caecum, rumen and large intestine enables the development of a lumenal microbial flora in these areas. Due to the constant movement of the mucus and lumenal contents through gut, some microbial types have evolved special structures for adhering to the glycocalyx or epithelial cell membranes. These microorganisms associate with the epithelium by stable reactions which may involve specific molecular interactions between microbial surfaces and the epithelium or mucous glycoproteins (Savage, 1980b).

The gram positive cocci and rods that adhere to the epithelium of the oesophagus and stomach of various animals, the *Clostridium* species that adhere to the mouse colon and the spiral bacteria which adhere to the caecum and colon in rats and mice, may attach by filaments containing acid polysaccharides which extend between the bacteria and the epithelial glycocalyx (Lee, 1980; Savage, 1978; 1980b; 1983), and which may be plasmid coded (Savage, 1984). Other microorganisms alter the architecture of the mucosal surface with which they associate, and are physically associated with the glycocalyx or underlying membrane of the epithelial cell. The filamentous organisms that colonize the epithelium of the small

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intestine of mice and rats, adhere by a specialized holdfast inserted into a socket in the microvillus membrane of the epithelial cell (Dobbins, 1984; Inman and Takeuchi, 1982; Savage, 1980b). At the area of attachment, the epithelial cell membrane is intact, but the microvilli around the site are attenuated and fused. The cytoplasm adjacent to the site contains fewer particles than that more remote from the attachment site. An electron dense plaque is present in the cytoplasm adjacent to the site of attachment (Dobbins, 1984; Inman and Takeuchi, 1982). These changes have been interpreted as a stabilization of the attachment site by a sol-gel transformation in the epithelial cell membrane and even perhaps the adjacent cytoplasm. The rod shaped bacteria present on the colonic epithelium of rats, and the spirochaetes and spiral bacteria intimately associated with the epithelium of man and monkeys, may induce similar changes in epithelial cells (Savage, 1980b). Freeze fracture studies of these bacteria have shown membrane specialization in the outer membrane of both microbes and the area of attachment (Neutra, 1980; 1984).

1.1.5 Microbial niches

Microorganisms are found in particular habitats because they are best fitted genetically and biochemically to fill niches in that habitat. The niche of every microbial species in the gastrointestinal ecosystem may never be defined with precision and in most cases such information has to be inferred from *in vitro* studies of available nutrients, types of end-products produced and the stability of the community in the habitat (Savage, 1977b). The interactions occurring between microbes in each habitat of the tract dictate how energy flows through the system. The anaerobic nature of the GIT suggests that gut microorganisms must have the capacity to generate energy by mechanisms not involving oxygen as the terminal electron

acceptor. Most species accomplish this by fermentation, but some fix hydrogen and carbon dioxide to form methane; a process which requires low oxidation - reduction potentials and consequently only operates in the large bowel, caecum or rumen.

Substrates for fermentation are derived either from dietary intake or from endogenous products generated by the animal. Mucin, enzymes produced by the host and desquamated epithelial cells serve as important carbon, energy and nitrogen sources for some gastrointestinal microbes (Hoskins, 1984; Savage, 1977b; Prins, 1977). Ammonia derived from ureolysis and deamination of amino acids is however the major nitrogen source for gut bacteria. Volatile and non-volatile fatty acids produced as end-products of microbial fermentation, contribute to the nutrition of other bacteria as well as the animal host. Vitamins E, K and many of the B group, are also synthesized in the large bowel by *Escherichia coli*, *Klebsiella aerogenes* and *Bacteroides fragilis*, but unless the host practices copography, these are lost in the faeces and are probably of little benefit to the host (Draser and Barrow, 1985). Microbial digestion of ingested food occurs in both the rumen and caecum, where microbial processes yield end products that are absorbed and utilized by the animal. Most of the nutritional requirements of ruminants are met by microbial fermentation, while microbial activity in the herbivore caecum may supplement the available energy by providing 25-35% of the animals' nutritional needs (Savage, 1977b).

1.1.6 Microbial flora

The microbial flora of those regions of the gut which are relatively static and of neutral pH, is extremely dense and diverse, while fewer and more specialized bacteria are associated with areas of the tract that have a faster flow rate and lower pH. The stomach of

non-ruminant animals contains less than 10^1 organisms/mℓ and most of these are not obligately anaerobic Drasar and Hill, 1974). The autochthonous microflora of the stomach is generally attached to the mucosa, and in most animals is comprised of lactic acid bacteria (lactobacilli and streptococci) and yeast of the *Candida* genus (Savage, 1983). In addition, some helical organisms have been reported to be associated with the gastric secreting-epithelium of monkeys, cats and dogs (Savage, 1980b). In the small intestine, population densities attain levels of 10^4-10^5 organisms/mℓ in the duodenum and 10^6/mℓ in the distal ileum (Drasar and Hill, 1974). In the duodenal region of the small bowel, gram-positive, facultative bacteria predominate, but in the ileum, equal numbers of aerobes and anaerobes are found (Drasar and Hill, 1974). This flora is more diverse, and is comprised of rods, cocci and helical bacteria. Segmented, filamentous bacteria adhere to the mucosae via specilized holdfasts in the mid and lower regions of the small bowel in many animals including rats, dogs and chickens. Enormous microbial populations develop in the lumen of the caecum and colon, and many genera and hundreds of species have been isolated, most of which are strict anaerobes and include members of the *Clostridium*, *Eubacterium*, *Bacteroides*, *Veilonella*, *Fusobacterium*, *Lactobacillus* and *Streptococcus* genera (Clarke, 1977; Savage, 1977b). Due to the complex interactions between bacteria in this habitat, many representatives of the autochthonous microflora, have not been axenically cultivated *in vitro*. Other anaerobic bacteria colonize habitats on the large bowel mucosa, and these include fusiforms belonging to the *Clostridium*, *Eubacterium*, *Fusobacterium* and *Bacteroides* genera, helical bacteria and spirochaetes (Savage, 1983).

1.2 SPIROCHAETALES

1.2.1 General description

The *Spirochaetales* order is comprised of helical prokaryotes characterized by unique cellular anatomy and distinctive motility. Although members share a number of structural similarities, they vary widely in their metabolism and natural distribution. Spirochaetes are gram-negative, slender, flexuous, helically coiled organisms, 3 to 500μm long and 0.1-3.0μm wide (Johnson, 1977; Smibert, 1973). The morphological features that serve to define and differentiate spirochaetes from other organisms include a coiled protoplasmic cylinder that houses the genetic and cytoplasmic elements and which is bounded by a cytoplasmic membrane-cell wall complex. Wound around the protoplasmic cylinder are filamentous periplasmic flagella, while an outer membranous "sheath" envelopes the protoplasmic-periplasmic flagella complex (Fig. 1).

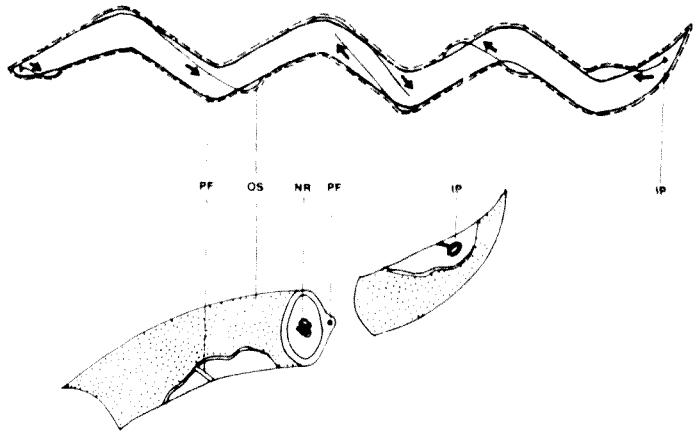

Fig. 1. Diagrammatic representation of a typical spirochaete as interpreted from electron micrographs. (After Holt, 1978.) Abbreviations: PF, periplasmic flagella; OS, outer sheath; IP, insertion pore; NR, nuclear region.

Canale-Parola (1984) in the latest edition of Bergey's Manual of Systematic Bacteriology recognizes five genera as members of the *Spirochaetales* order. These genera, *Cristispira*, *Treponema*, *Spirochaeta*, *Borrelia* and *Leptospira*, are differentiated according to the criteria listed in Table 1 (refer section 1.2.7).

1.2.2. Outer Envelope

The outer envelope is a flexible, triple-layered membranous structure that completely surrounds the microorganism (Canale-Parola, 1978; Pillot and Ryter, 1965). The viability of the spirochaete is dependent on an intact outer sheath, and damage of the membrane mediated by antibodies and complement, or chemical agents, results in cell death (Johnson, 1977). Chemical analysis of the outer sheath has revealed that it contains proteins, lipids (predominantly in the form of phospholipids) and carbohydrates, with the proportion of these components varying between genera (Johnson, 1977; Holt, 1978; Masuda and Kawata, 1982). Lipopolysaccharide (LPS), structurally and functionally similar to that in gram-negative bacteria, has been detected in several species of spirochaetes (Jackson and Zey, 1973; Neussen *et al.*, 1983), although other investigators present evidence that disputes this contention (Baseman *et al.*, 1979; Boehringer *et al.*, 1984; Penn *et al.*, 1985).

The outer membrane has a fine structure which varies from being smooth and non-textured in *Spirochaeta stenostrepta* (Holt, 1978) to a morphologically complex layer. A non-cultivable oral spirochaete associated with ulcerative gingivitis has been described as having a "pinstriped" sheath (Listgarten and Socransky, 1964), while the sheaths of *Spirochaeta aurantia*, *Treponema microdentium* and the oral treponeme E21 are arranged in polygonal subunits

(Holt, 1978; Masuda and Kawata, 1982). The outer envelope of *Borrelia* species lacks structural detail (Holt, 1978).

There are no known functions ascribed to the outer sheath (Holt, 1978), but suggestions that it could play a role in cell motility have been made (Canale-Parola, 1978). The outer sheath may be analogous to the outer membrane of gram-negative bacteria (Canale-Parola, 1984; Joseph *et al.*, 1973; Masuda and Kawata, 1982), in which case it could function in chemotaxis (Canale-Parola, 1978), hydrolytic enzymatic processes (Holt, 1978), as well as transport of impermeable components into the protoplasmic cylinder (Canale-Parola, 1978).

Since the outer sheath is the most external layer of a spirochaete, host defence mechanisms are probably directed against this membrane (Johnson, 1977), although Penn *et al.* (1985) have evidence which refutes this. Hamsters immunized with purified sheath material, are protected against lethal doses of *Leptospira interrogans canicola* while swine, previously exposed to homologous *T. hyodysenteriae* strains are resistant to reinfection (Joens *et al.*, 1983). Pathogenic *Borrelia* and *Treponema* species are also immobilized and killed by antibody-complement systems, probably reacting with sheath components (Johnson, 1977). Wasserman antibodies formed during the early stages of syphilis are produced against cardiolipin moieties present in the outer membrane of a number of treponemes (Jackson and Zey, 1973) and are thus relatively non-specific. Species-specific sheath polysaccharide antigens have been detected in several *Treponema* species and could be used in a classification system similar to that used in the identification of *Salmonella* serotypes (Jackson and Zey, 1973). Recently, four serotypes of *T. hyodysenteriae* were identified (Mapother and Joens, 1985).

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1.2.3 Protoplasmic Cylinder

The protoplasmic cylinder encompasses the cell wall, the cytoplasmic membrane and the enclosed cytoplasmic contents (Holt, 1978). Treponemes devoid of their cell envelope retain their spiral shape; a property that has been attributed to the composition of the rigid peptidoglycan in the cell wall (Jackson and Zey, 1973), which even retains its coiled configuration when in a purified state (Canale-Parola, 1978; Joseph *et al.*, 1973). The direction of coiling could be a stable identifying characteristic, since *Treponema denticola*, *Treponema phagedenis* and *Treponema refringens* are all right-handed helices, while *Treponema pallidum* and *Treponema paraluis-cuniculi* are left-handed helices (Stepan and Johnson, 1981). The spiral configuration of spirochaetes is lost if they are treated with lysozyme, penicillin or other agents that affect the integrity or synthesis of the peptidoglycan layer (Canale-Parola, 1978; Joseph *et al.*, 1973). As in other prokaryotes, the cell wall contains both muramic acid and N-acetyl-glucosamine (Jackson and Zey, 1973), although with the exception of *Leptospira* species, the spirochaete peptidoglycan is distinctive in that ornithine replaces the diaminopimelic acid and lysine components (Johnson, 1977; 1981; Joseph, *et al.*, 1973; Umemto, *et al.*, 1981). Unlike other gram-negative bacteria, the peptidoglycan of *T. pallidum* (kazan) lacks immunoadjuvanct activity in guinea pigs, although it does activate human complement (Umemota, *et al.*, 1981).

The peptidoglycan layer is joined to an inner cytoplasmic membrane, which is morphologically similar to that found in other bacterial cells (Holt, 1978). The cytoplasmic membrane of spirochaete cells has never been purified or characterized (Holt, 1978), although Kawata (1967a) has reported the presence of a_2 and b cytochromes in this membrane.

The cytoplasm of spirochaetes contains ribosomes and mesosomes (Hovind-Hougen, 1976a; Pillot and Ryter, 1965; Smibert, 1973). Aggregates of fine fibrils or microtubules have also been observed in the cytoplasm of several spirochaetes (Hovind-Hougen, 1976a), with six to eight of these fibrils associated together in a species-dependent, variable number of clusters (Holt 1978). These fibrils may be of taxonomic value since they have been observed in most members of the *Treponema* genus, but not in leptospires or borrelias (Hovind-Hougen, 1976a; 1976b; Canale-Parola, 1984).

Nuclear regions can be seen as delicate strands in less dense areas of the cytoplasm (Listgarten and Socransky, 1964; Pillot and Ryter, 1965; Smibert, 1973). Extranuclear plasmid DNA, recently detected in pathogenic *Borrelia* species and *T. pallidum*, has caused concern about potential emergence of extrachromosomally mediated resistance to antibiotics (Hyde and Johnson, 1984; Norgard and Miller, 1981). The presence of bacteriophage-like particles in *Treponema*, *Spirochaeta* and *Leptospira* cultures, further suggests that genetic exchange between spirochaetes could exist, although no such system has as yet been recognized (Harwood and Canale-Parola, 1984; Lewin, 1960).

1.2.4 Periplasmic Flagella

Periplasmic flagella (periplasmic fibrils, axial fibrils) are structurally and chemically similar to prokaryotic flagella, comprising of two basic structural components, a shaft or filamentous portion and an insertion apparatus (Holt, 1978). The shaft varies from 15-25nm in diameter and consists of a core surrounded by a non-striated sheath (Canale-Parola, 1978; Joseph and Canale-Parola, 1972; Hovind-Hougen, 1976a). The insertion apparatus is 40nm wide (Listgarten and Socransky, 1964) and is differentiated into a

proximal hook together with a number of insertion discs (Holt, 1978). A single pair of rings is found in *Borrelia*, *Spirochaeta* and *Treponema* species, while leptospires (with the exception of *Leptospira illini* (Faine and Stallman, 1982)) have a series of discs similar to those present on the flagella of gram-negative bacteria (Johnson, 1977). Chemically, the periplasmic flagella are comprised of protein with a similar amino acid composition to that of eubacterial flagellin (Bharier and Allis, 1974; Holt, 1978). The structural similarities between periplasmic fibrils and bacterial flagella, suggest that these organelles have similar or analogous functions. Furthermore, drugs such as serotonin and atropine sulphate, which affect the motility of flagellated bacteria, also inhibit the movement of spirochaetes, inferring that a common mechanism of motility may be involved (Canale-Parola, 1977).

The periplasmic flagella are inserted into a depression in the protoplasmic cylinder near the terminal end of the cell and extend towards the opposite pole of the cell, usually overlapping at the cell center (Holt, 1978). The number of fibrils per cell varies from two to hundreds, with *Leptospira* and *Spirochaeta* species having only one periplasmic fibril inserted at each pole of the cell, *Treponema* species containing one to ten axial fibrils originating at each pole and *Borrelia* species with ten to twenty periplasmic flagella (Smibert, 1973). Members of the *Cristispira* genus have more than a hundred periplasmic fibrils aggregated together to form the "cristus" or lateral ridge characteristic of this genus (Smibert, 1973).

1.2.5 Motility

Although many attempts have been made to interpret the mechanisms of spirochaete movement, little is known about the motility of these

prokaryotes. Spirochaetes exhibit three types of movements in liquid environments; translational motion, rotation about their long axis (corkscrewing) and flexing, which includes a number of motions such as bending of the cell near the ends or the middle, looping, lashing or vibrating (Canale-Parola, 1978). Frequently after flexing, the cell will resume travel in a new direction.

Translational movement of spirochaetes is enhanced in viscous environments (Greenberg and Canale-Parola, 1977; Lee, 1980) and the ability to move through such environments may confer selective advantages on those spirochaetes inhabiting areas such as the gingival crevice, mucosa epithelium, crystalline style of molluscs, termite gut contents and rumen fluids (Canale-Parola, 1978). Furthermore, some spirochaetes exhibit a positive taxis towards viscous milieux (Petrino and Doetsch, 1978).

Several models have been proposed to explain spirochaete motility, and these are described in detail in a review by Canale-Parola (1978). The most comprehensive and accepted model, is that proposed by Berg, who assumes that the protoplasmic cylinder is semirigid, the periplasmic fibrils rotate in a manner analogous to bacterial flagella and that the outer sheath is flexible and involved in motility. In this model, it is proposed that the periplasmic fibrils rotate, and in doing so cause the rotation of the helical protoplasmic cylinder within the outer sheath. A change in the direction of rotation of the fibrils is accompanied by a change in the direction of rotation of the periplasmic cylinder. According to this model, the helical cell rotates about its longitudinal axis and moves along by screwing its way through the liquid environment (Canale-Parola, 1978).

1.2.6 Cell Division

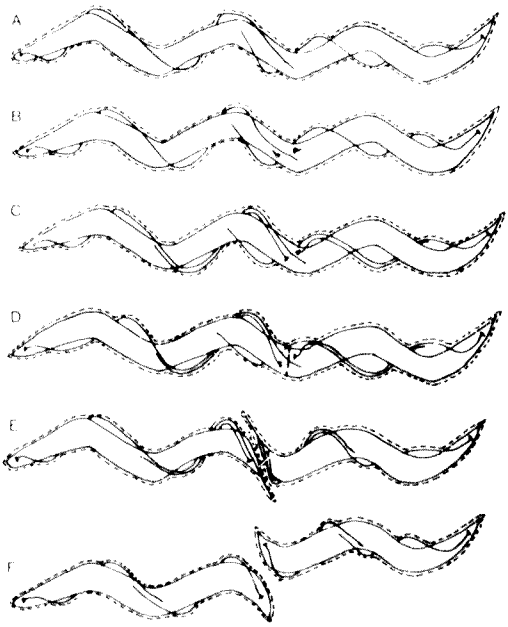

Fig. 2. Diagrammatic representation of spirochaete cell division. A: mature cell; B and C: formation of new periplasmic flagella; D: constriction of protoplasmic cylinder; E: constriction of outer sheath; F: newly divided spirochaetes.

Spirochaetes divide by transverse binary fission (Listgarten and Socransky, 1964). Early in the division process, before any cytolegical signs of cell division, new periplasmic flagella are formed at a region slightly subterminal to the division plane a the center of the mother cell (Holt, 197[illegible]; Hovind-Hougen, 1976). When these fibrils are completely formed, constriction of the protoplasmic cylinder begins along a plane midway between the newly synthesized periplasmic fibrils (Holt, 1978). Outer sheath constriction commences only when constriction of the protoplasmic cylinder is com-

pleted (Hovind-Hougen, 1976; Listgarten and Socransky, 1964). The periplasmic flagella of the mother cell pass across the division plane, and protrude from the newly divided cells. The extending fibrils are surrounded by a sleeve of material continuous with the outer envelope and the organism (Listgarten and Socransky, 1964). These extentions are temporary and are probably degraded by cellular enzymes. The division process is presented diagrammatically in Fig.2.

1.2.7 Classification

In Bergey's Manual of Systematic Bacteriology, two families, the *Spirochaetaceae* and *Leptospiraceae* are recognized as members of the *Spirochaetales* order (Canale-Parola, 1984). The *Spirochaetaceae* family encompasses four genera - *Spirochaeta*, *Treponema*, *Borrelia* and *Cristispira*, while the *Leptospiraceae* family only contains the *Leptospira* genus (Johnson and Faine, 1984). Characteristics of each genus as documented in Bergey's Manual of Systematic Bacteriology are listed in Table 1

This classification scheme is similar to that published in the 8th edition of Bergey's Manual of Determinative Bacteriology (Smibert, 1974). where criteria such as cell-length, relationship to oxygen and occurrence in nature was used to differentiate between genera. The weight given to the environment from which the organism was isolated, means that classification, even to the genus level, is impossible if the habitat from which the organism was isolated is unknown (Smibert, 1976b). Furthermore, since both borrelias and pathogenic treponemes are classified to the species level according to the vectors which transmit them and the disease lesions they cause (Hovind-Hougen, 1976a), it is likely that a particular species may have more than one name. The finding that *T. pallidum* (the

Table 1. Characteristics of Spirochaeta genera as listed in Bergey's Manual of Systematic Bacteriology (Canale-Parola, 1984)

characteristic	Spirochaeta	Treponema	Leptospira	Cristispira	Borrelia
type species	*S. plicatilis*[a]	*T. pallidum*	*L. interrogans*	*C. pectinis*	*B. anserina*
oxygen requirements	facultative to anaerobic	anaerobic or microaerophilic	aerobic	facultative[b]	microaerophilic
energy and carbon sources	carbohydrates	carbohydrates or amino acids	long chain fatty acids or alcohols	ND	carbohydrates
DNA (G+C mol%)	51-65	25, 36-43 and 53	35-53	ND	27-30.5[d]
size width (μm)	0.2-0.75	0.1-0.4	0.1	0.5-3.0	0.2-0.5
length (μm)	5-250	5-20	6-20	30-180	3-20
pathogenicity	no	some species	some species	no	yes
natural habitat	free living in aquatic environments	mouth intestinal tract and genital areas of animals	free-living in water or soil and associated with animals	digestive tracts of mollusc	circulatory systems of animals; transmitted by arthropods

[a] since it's description by Ehrenberg in 1835 this organism has never been axenically cultured and the proposal that *S. stenostrepta* Zuelzer replace *S. plicatilis* as type species has been made (Canale-Parola, 1981a)
[b] Breznak, 1973
[c] Paine and Stallman, 1982.
[d] Hyde and Johnson, 1984
Symbols: ND, not determined

agent of both venereal and endemic syphilis) and *Treponema pertenue* (the etiological agent of yaws) are genetically indistinguishable (100% DNA homology) (Miao and Fieldsteel, 1980), has led to their classification as subspecies of each other (*viz. T. pallidum* subspecies *pallidum* and *T. pallidum* subspecies *pertenue*), while the agent of endemic syphilis has been classified as *T. pallidum* subspecies *endemicum*). The pinta agent has retained its species name of *Treponema carateum*, although it may yet prove to be a subspecies of *T. pallidum* (Canale-Parola, 1984). These findings support the theory that the four treponematoses recognized on pathological and clinical evidence, are caused by one organism with environmental influences dictating the clinical symptoms (Brothwell, 1981). The lack of DNA homology between *T. pallidum* and either *Treponema paraluiscuniculi* (biotype Reiter) or *Treponema refringens* (biotype Nagouchi) (Miao and Fieldsteel, 1978, 1980) together with the large discrepancy in G+C mol% ratio, between the non-cultivatable

pathogenic treponemes (52-53%) and the cultivatable host associated treponemes (43%) suggests that they could belong to different genera.

In addition to the five recognized genera, large spirochaete-like bacteria possessing a sheath, periplasmic fibrils, a helical protoplasmic cylinder and, in addition, flagella-like appendages extending from each pole have been described in the hind-guts of various xylophagous insects (Margulis *et al.*, 1981; To, *et al.*, 1978a). The generic names *Pillotina*, *Hollandina*, *Diplocalyx* and *Clevelandina* have been proposed for these bacteria (Hollande and Gharagozlou, 1967; Margulis *et al.*, 1981). However, since these organisms have not been isolated in pure culture, nor the names validly published, they have not yet been formally assigned to the *Spirochaetales* order (Breznak, 1984). The classification system proposed by Hovind-Hougen (1976a) and modified by To *et al.*, (1978a) does accomodate these spirochaetes. In this system, the spirochaete phylum is subdivided into three classes, the *Spirochaetales*, the *Cristispirales* and the *Leptospirales*. The *Spirochaeta*, *Treponema* and the *Borrelia* genera are members of the *Spirochaetales* class, while the *Cristispira*, *Pillotina*, *Diplocalyx* and *Hollandina* genera are members of the *Cristispirales* class. The *Leptospirales* class is also divided into two genera, the *Leptospira* and the *Leptonema* (To *et al.*, 1978). Although *Leptonema* was considered for inclusion as a second genus in the *Leptospiraceae* family in Bergey's Manual of Systematic Bacteriology, the subcommittee on the taxonomy of *Leptospira* (ICSB) deemed that insufficient information was available for defining this genus (Johnson and Faine, 1984). However, evidence is mounting for the formation of the *Leptonema* genus (Bazovska *et al.*, 1983).

A number of alternative classification criteria for the identification of spirochaetes have been proposed. Listgarten and Socransky (1965) and Hovind-Hougen (1976) have suggested that spirochaetes, and more particularly treponemes, can be taxonomically differentiated according to their electron microscopic morphology. They postulate that criteria such as cell length, cell width, number of periplasmic flagella and fine structure of the outer envelope could be used to classify treponemes to the species level. Although morphological descriptions of this kind may be useful in distinguishing various non-cultivable strains, it does have the limitation that organisms of similar structure could prove to be distinct species when biochemical differentiation becomes feasible.

Smibert (1976b) suggests that the end products of glucose fermentation could be used to identify spirochaetes, since *Borrelia* species produce only lactate as a growth end product, while other genera form a variety of end products from glucose fermentation. He also suggests that the *in vitro* growth requirements for fatty acids could be used for generic classification. *Spirochaeta* species do not require fatty acids for *in vitro* growth, while the other genera have fatty acids requirements.

The abundance of cellular lipids in the *Spirochaetaceae* (14-30% of their dry weight), has led to the proposal that lipid composition and metabolism could be useful taxonomic criteria (Livermore and Johnson, 1974) Suggested parameters for the classification of spirochaetes to the generic level, together with the characteristics of each genus, are presented in Table 2. Since some spirochaetes from the human oral cavity and from pig faeces have a lipid composition and metabolism intermediate between that of the *Treponema* and *Spirochaeta* (Johnson, 1977), these have been included in the table as "rumen-fluid" requiring treponemes, thus differentiating

Table 2. Fatty acid composition and metabolism of the cultivable genera of the *Spirochaetales* order

characteristic	*Spirochaeta*[a]	rumen fluid requiring *Treponema*[b]	serum requiring *Treponema*[c]	*Leptospira*[d]	*Borrelia*[e]
monoglycosyldiglyceride (MGDG)	+	+	+[c]	-	+
phosphatidylcholine	-	-	+	-	+
phosphatidylethanolamine	-	-	D	+	+[d]
β oxidation of fatty acids	-	-	-	+	-
de novo fatty acid synthesis	+	-	-	D	-
desaturation and modification of fatty acids	D	+	-	+[e]	-

[a] Livermore and Johnson, 1974;
[b] Johnson, 1977;
[c] MGDG has not been detected in *T. pallidum* (Matthews *et al.*, 1979);
[d] Johnson, 1981;
[e] Johnson and Eggebraten, 1971.
Symbols: +, positive for characteristic; -, negative for characteristic; D, inconsistency of characteristic.

them from the serum-requiring forms. These treponemes can synthesize long chain fatty acids from short chain molecules such as isobutyric and valeric acids (supplied in rumen fluid) and may represent a separate spirochaete genus. Serum-requiring treponemes could also be differentiated according to their lipid profiles, since available fatty acids may be modified prior to the incorporation of these into the outer envelope. *T. pallidum* contains a greater proportion of palmitic acid (16:0) than testicular tissue, which is rich in fatty acids with twenty carbon atoms and above. Furthermore, *T. hyodysenteriae* (Matthews *et al.*, 1980a) and *T. pallidum* (Barbieri *et al.*, 1981a) may synthesize their own phospholipid and glycolipid components. Lipid profiles would be unsuitable for the characterization of those treponemes that incorporate culture media lipids *en bloc* into their outer membranes (Johnson and Eggebraten, 1971; Livermore and Johnson, 1974).

Classification using fatty acid profiles could also extend beyond the generic level. A study on the cellular fatty acids of oral treponemes, revealed that *T. vincentii*, *T. zuelzerae* and *T. microdentium* all had characteristic lipid profiles (Cohen *et al.*, 1970). *T. pallidum* is the only treponeme that lacks monogalactosyldiglyceride (MGDG), while *T. hyodysenteriae* and *T. innocens* are the only spirochaetes that contain acyl-monogalactosyldiglyceride (Matthews and Kinyon, 1984). *T. hyodysenteriae* and *T. innocens* which were originally considered as biotypes (Kinyon and Harris, 1979), can be differentiated on the basis of alkenyl chain profiles, with 14:0 moieties predominating in *T. hyodysenteriae* and iso-15:0 moieties prevailing in *T. innocens* (Matthews and Kinyon, 1984). Although other aspects of lipid composition, such as sugar moiety in glycolipids and the presence of acylmonogalactoglycerides were originally thought to be of value in the differentiation of these two organisms (Matthews *et al.*, 1980b), critical evaluation revealed discrepancies among strains of both species.

1.2.8 Metabolism

The metabolism of treponemes has not been extensively studied, although recent interest in these organisms has led to the physiology of the more readily cultivable forms being investigated. Treponemes are diverse in their energy yielding metabolism, with some displaying a great versatility and metabolizing a wide range of substrates, whereas others are exceedingly specific in their nutritive requirements (Johnson, 1981). A group of highly specialized treponemes that only ferment pectin and it's associated monomers have been isolated from the bovine rumen (Ziolecki and Wojciechowicz , 1980) and subgingival plaque (Smibert and Burmeister, 1983; Weber and Canale-Parola, 1984).

Treponemes ferment glucose via the Embden-Meyerhof-Parnas pathway (Canale-Parola, 1977; Cwyk and Canale-Parola, 1979; George and Smibert, 1982; Stanton, 1984). A classical clostridial-type system is generally employed to metabolize pyruvate to acetyl coenzyme A, carbon dioxide and hydrogen (Canale-Parola, 1977; Stanton, 1984), although *T. succinifaciens* and *T. bryantii* reportedly use a coliform-type cleavage system to dissimilate pyruvate (Cwyk and Canale-Parola, 1979; Stanton, 1984) and produce carbon dioxide, hydrogen and acetic acid, together with either ethanol or succinic acid (Smibert, 1976a). *T. pallidum* has a facultative metabolism and under oxygen-limiting conditions, switches from an oxidative metabolism to a fermentative one, in which glucose is fermented by a pathway that utilizes pyruvate as the final electron acceptor, resulting in the formation of lactate (Barbieri and Cox, 1981b). The electron transport system in the Reiter treponeme has been investigated and low redox potential carriers such as rubredoxin, cytochromes a_2 and b and NADH together with electron transport enzymes such as succinic dehydrogenase and fumarate reductase have been identified (George and Smibert, 1982b; Johnson and Canale-Parola, 1973; Kawata, 1967a; 1967b).

Amino acid fermentation is generally restricted to serum-requiring treponemes (Smibert, 1976a) and yields a variety of end products with acetate being the major non-gaseous fermentation product recovered. *T. denticola*, which utilizes a wide range of carbohydrates and amino acids, ferments serine, cysteine and alanine by pathways involving pyruvate as an intermediate (Canale-Parola, 1977) while other amino acids, for example arginine, are dissimilated by a modification of the arginine iminohydrolase pathway, resulting in the production of ammonia, carbon dioxide and proline (Blackmore and Canale-Parola, 1976).

Several spirochaetes, including *T denticola* and *T. succinifaciens*, possess a broad range of purine interconversion enzymes, which could enable the spirochaete to obtain energy, carbon and nitrogen when exogenous growth substrates are not available (Canale-Parola and Kidder, 1982). The wide range of diverse dissimilatory pathways exhibited by *T. denticola* enables it to derive energy from a wide spectrum of substrates. This metabolic flexibility is probably one of the factors that enables the slow-growing treponemes to survive in habitats (such as the oral cavity and the gastrointestinal tract) where a wide range of substrates become available for short periods of time.

1.3. SPIROCHAETES ASSOCIATED WITH THE GASTROINTESTINAL TRACT

1.3.1 Invertebrates

Spirochaetes are associated with the digestive tracts of both vertebrates and invertebrates. The crystalline style and pyloric caecum of molluscs, echinodermes and tunicates harbours numerous spirochaetes, many of which belong to the *Cristispira* genus (Breznak, 1973; Richards, 1978). Spirochaetes are also found in the gut of various insects, particularly xylophagous insects such as termites and certain cockroaches and in some cases their presence is essential for the survival of the host (Eutick, *et al.* 1978). Up to 40% of the total bacteria in the hindgut of *Pterotermes* are members of the *Spirochaetaceae* family (To *et al.*, 1980). Many of these spirochaetes have a unique ultrastructure and are considered as belonging to the *Hollandina*, *Diplocalyx* and *Pillotina* genera (Margulis *et al.* 1981), while other spirochaetes residing in this habitat, have a more conventional morphology and probably do not belong to these genera (Czolij *et al.*, 1985). The invertebrate,

gut-associated spirochaetes may be either free living, sessile on the gut wall, or attached to the surfaces of protozoa. Those that are intimately associated with other cells frequently have one or both ends adapted for this association, enabling them to attach without injury to the host (Bloodgood and Fitzharris, 1976; Corpe, 1980; Margulis *et al.*, 1981). The host cell may also exhibit morphological alterations at the site of spirochaete attachment. The intimate physical association between host cells and some gut associated spirochaetes, and the development of specific structures for attachment, suggests that symbiotic interactions between the insect and the prokaryote may exist.

1.3.2 Vertebrates

Ruminants

In the bovine rumen, population densities of 10^8 spirochaetes/mℓ of rumen fluid are attained (Stanton and Canale-Parola, 1979). These spirochaetes are morphologically and physiologically diverse (Paster and Canale-Parola, 1982). Limited information is available on the contribution made by these spirochaetes to the metabolic processes taking place in the rumen (Breznak, 1973; Pasteur and Canale-Parola, 1982; Stanton and Canale-Parola, 1980), although their numerical dominance implies that their contribution to the rumen metabolic activities is quantitatively significant (Stanton, 1984). Although no cellulolytic spirochaetes have ever been isolated, ruminant spirochaetes frequently utilize the hydrolysis products of plant polymers such as cellobiose and D-glucuronic acid (Stanton and Canale-Parola, 1980) and in the rumen, synergistic relationships between cellulolytic bacteria and spirochaetes such as *Treponema bryantii* probably exist. Rumen spirochaetes may contribute significantly to the degradation of ingested plant material

(Harwood and Canale-Parola, 1984) since many are able to degrade and ferment polymers such as xylan and pectin (Paster and Canale-Parola, 1982).

Monogastric animals

The autochthonous spirochaetes of the monogastric gut have not been comprehensively studied. Spirochaetes inhabit the stomach of dogs, humans, and certain old-world monkeys, the small intestine of rats, and the large bowel of a wide range of animals, where they may be free-living or sessile and attached to the epithelial tissue. Apart from *T. hyodysenteriae*, the proven etiological agent of swine dysentery (Harris *et al.*, 1972a; Kinyon *et al.*, 1977), the pathogenic potential of gastrointestinal spirochaetes is vague. Spirochaetes have frequently been implicated as the etiological agents of various gastrointestinal disorders, particularly as high population densities of these bacteria are found in diarrhoea specimens from various animals including dogs and humans (Pindak *et al.*, 1965; Zymet, 1969). It has been proposed that the preponderance of spirochaetes in the gut may precipitate the onset of diarrhoea by upsetting the delicate balance of the bacterial flora present in this ecosystem (Zymet, 1969). Alternatively, the excessive mucus secretion that frequently accompanies diarrhoea, may induce those spirochaetes that are normally present in the crypts, to be displaced from this habitat, thus contributing to the large numbers of these bacteria observed in diarrhoeal stools. Experimental evidence supporting this contention has been found in rats and dogs, where artificially induced diarrhoea also resulted in increased numbers of spirochaetes in the faeces (Leach *et al.*, 1973). Nevertheless, Zwicker *et al.* (1978) have reported a diarrhoeal condition in guinea pigs in which spirochaetes were implicated as being the causative agent, while Taylor *et al.* (1980) have de-

scribed a spirochaete which is biochemically, ultrastructurally and immunologically distinct from *T. hyodysenteriae* but which is able to induce diarrhoea and colitis in swine.

Intestinal spirochaetosis

"Intestinal spirochaetosis" is a massive spirochaete infestation of the caecal and colonic epithelium, occasionally present in humans (Harland and Lee, 1967), dogs (Turek and Meyer, 1977; 1978), opossums (Turek and Meyer, 1979) and monkeys (Takeuchi and and Zeller, 1972a; 1972b; Cowley and Hill, 1985). In primates, two morphologically distinct spiral bacteria are involved (Neutra, 1980; Takeuchi, *et al.* 1974); the predominant organism having a typical spirochaete morphology, while the other is a flagellated spiral bacterium (Takeuchi and Zeller, 1972b). Two to 9% of human subjects and 13-28% of *Rhesus* monkeys (Harland and Lee, 1967; Minio *et al*, 1973) are reportedly infected. The colonization is spontaneous and can persist for periods of more than 6 years (Harland and Lee, 1967). Although the spirochaetes involved are morphologically distinct in different animal hosts, the infestation is indistinguishable with respect to anatomical sites occupied, the mode of attachment and orientation of the bacteria with respect to the mucosal surface. By associating with the enzyme-containing glycocalyx of the microvilli, the spirochaetes may preferentially utilize nutrients that are normally absorbed, concentrated and degraded in this region (Harwood and Canale-Parola, 1984; Neutra, 1980). Infected cells show non-specific alterations such as interruption of the apical plasma membrane, destruction of microvilli and alteration of the apical cytoplasm (Minio *et al.*, 1973; Neutra, 1980; Takeuchi and Zeller, 1972a; 1972b); changes which could interfere with microvilli function (Antonakopoulos *et al.*, 1982; Cowley and Hill, 1985). The spirochaetes indent the columnar cell surface and

lie in a cylindrical invagination of the host plasma membrane. A small, electron-lucent pit is present between the tip of the organism and the cell membrane (Hovind-Hougen *et al.*, 1982). The bacteria attach exclusively to mature columnar epithelial cells and avoid the surface goblet cells (Takeuchi and Sprinz, 1970), the crypts of Lieberkuhn (Lee *et al.*, 1971; Neutra, 1980), and neoplastically transformed cells (Harland and Lee, 1967; Neutra, 1980), suggesting that mature cell surface components such as glycosylated proteins are required for bacterial adherence (Neutra, 1980). The bacterial membrane specializations suggests that the microorganism may benefit from the association, while the lack of complementary cellular specialization, infers that the host does not benefit from the relationship (Neutra, 1980).

In intestinal spirochaetosis, the bacteria occasionally penetrate beyond the brush border and into the epithelial cytoplasm and lamina propria where they are found either free or partly phagocytosed by macrophages (Antonakopoulos *et al.*, 1982; Harland and Lee, 1967; Minio *et al*, 1973). The presence of spirochaetes in the lamina propria does not induce an inflammatory response (Takeuchi and Sprinz, 1970; Takeuchi *et al.*, 1974). The pathogenic status of this infestation is subject to debate, with several investigators claiming that it is of no clinical significance (Nielsen *et al.*, 1983; Lee *et al.*, 1971; Takeuchi *et al.*, 1974), while others have concluded that it is associated with diarrhoea and rectal bleeding (Douglas and Cruciolí, 1981; Gad *et al.*, 1977), with symptoms depending on the extent and degree of infection (Gad *et al.*, 1977).

Swine dysentery

Swine dysentery is an infectious, transmissible enteric disease that causes severe economic losses (Meyer, 1978). Wild mice have been

demonstrated to harbour *T. hyodysenteriae* (Joens and Kinyon, 1982), and this finding supports previous speculation that these rodents could transmit the disease (Joens, 1980). Infected mice display mucosal lesions similar to those found in infected swine (Joens *et al.*, , 1981; Joens and Kinyon, 1982). Clinically, the disease is characterized by mucohaemorrhagic diarrhoea with inflamed and necrotic lesions occurring mainly in the colonic mucosa t occasionally in the caecum (Clock *et al.*, 1974; Kennedy and ifuss, 1976; Whipp *et al.*, 1979). The spirochaetes that cause dysentery have been cultivated, and Kochs' postulates fulfilled ir ific pathogen free (SPF) pigs, although only mild lesions are pr d in gnotobiotic swine (Lysons, 1984). It is now recognized tha e dysentery is a mixed synergistic infection and that the introduction of *T. hyodysenteriae* into pigs initiates the disease process by causing resident organisms of low pathogenic potential to interact with the spirochaetes and each other and express an inherent pathogenic potential (Lysons, 1984; Meyer, 1978). Organisms believed to be involved in swine dysentery include *Fusobacterium necrophorum*, *Bacteroides fragilis*, *Bacteroides vulgatus* and *Clostridium* species (Joens, *et al.*, 1981; Lysons, 1984; Meyer, 1978; Whipp *et al.*, 1979). The type of opportunists involved, could modify the severity of the disease (Meyer, 1978).

A direct correlation between haemolytic activity and enteropathogenicity of *T. hyodysenteriae* exists (Kinyon *et al.*, 1977; Picard *et al.*, 1979; Saheb, *et al.*, 1981a; 1981b). The haemolysin, which resembles streptolysin S, (Lemcke and Burrows, 1982), is a potential virulence factor that can induce fluid accumulation in ligated rat ileal loops (Saheb *et al.*, 1981b). It has also been established that LPS could be involved in the pathogenesis of swine dysentery (Nuessen *et al.*, 1933). Although *T. hyodysenteriae* adheres to epithelial cells in a manner analogous to other virulent

enteropathogenic bacteria (Knoop *et al*, 1979), it does not induce toxin mediated colonic malabsorption such as is produced by *Vibrio cholerae*, pathogenic *E coli*, *Shigella* and *Salmonella* (Schmall *et al.*, 1983). The virulence factors of *T. hyodysenteriae* and the mechanism by which this organism interacts with other gut organisms to induce diarrhoea, are unresolved.

1.4 AIMS AND OBJECTIVES

Increasing interest in gastrointestinal microbiology, has led to the realization that the gastrointestinal flora can be both beneficial and harmful to the host. Although not as nutritionally important as the ruminant flora, the gastrointestinal microbiota may contribute to the well-being of the host by the synthesis of vitamins, (particularly in coprophagous animals) (Draser and Barrow, 1985; McBee, 1977) and the conversion of indigestable dietary constituents to end-products which the host can utilize (Savage, 1977a). The gut flora degrades potential carcinogens ingested by the host, but is simultaneously responsible for the production of carcinogenic metabolic end products formed from the degradation of bile acids and aromatic amino acids (Prins, 1977). The flora can also assist the host to resist infectious diseases of the GIT through microbial interference activities and prepare the host to react quickly to pathogens by stimulating the production of cross-reacting antibodies (Savage, 1977b). Disturbances of the ecosystem however can lead to the gut microflora causing diseases such as Crohns disease (microbial overgrowth of the small intestine, leading to malabsorption and malnutrition), abcesses, tropical sprue and diarrhoea. If the full importance of the autochthonous flora is to be recognized, it is necessary that all of its constituents are studied both *in vitro* and *in vivo*. This research was undertaken, in order that a better

enteropathogenic bacteria (Knoop *et al*, 1979), it does not induce toxin mediated colonic malabsorption such as is produced by *Vibrio cholerae*, pathogenic *E. coli*, *Shigella* and *Salmonella* (Schmall *et al.*, 1983). The virulence factors of *T. hyodysenteriae* and the mechanism by which this organism interacts with other gut organisms to induce diarrhoea, are unresolved.

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understanding of the role played by spirochaetes in the gastrointestinal ecosystem could be realized.

Treponemes are constituents of the gut autochthonous flora of most animals but although techniques for cultivating these hitherto elusive bacteria have recently been developed, they have not been extensively studied (Harwood and Canale-Parola, 1984). The disease potential of these spirochaetes has not been resolved and the importance of spirochaetes in the GIT ecosystem is unknown, but since they frequently attain high population densities in the more static regions of the GIT, they could play an important role in the functioning of this ecosystem. The rat was chosen as a model for studying these bacteria, since it is a convenient laboratory animal, its gut flora has previously been investigated (Raibaud *et al.*, 1966a; 1966b), and spirochaetes are known to reside in its gut (Davis *et al.*, 1972a; 1972b; Lee and Phillips, 1978; Phillips *et al*, 1978). There have been no reports on the isolation of 'classical spirochaetes' from this animal, although other spiral bacteria from the rat gut have been cultivated (Lee and Phillips, 1978; Phillips and Lee, 1983). Spirochaetes from a mouse caecal epithelium have been grown *in vitro*, but were not extensively studied (Lee and Phillips, 1978). Since it is imperative that gastrointestinal bacteria are not studied in isolation, the project consisted of two phases, the first to establish the ecology of rat gastrointestinal spirochaetes, and the second to characterize these spirochaetes *in vitro* and then attempt to relate their *in vitro* activities to their *in vivo* function. From this work, it is anticipated that insight into the significance of spirochaetes in the gastrointestinal ecosystem of the rat, and perhaps other monogastric animals may be realized.

CHAPTER 2

ECOLOGY OF GASTROINTESTINAL SPIROCHAETES

2.1 INTRODUCTION

That spiral bacteria are constituents of the gastrointestinal autochthonous flora of vertebrates has been inferred since the turn of the century, when these distinctive microorganisms were observed in the digestive tracts of a variety of clinically healthy animals (Macfie, 1916). Since then, spirochaetes have been reported as inhabiting the lumen contents and epithelial surfaces along the entire GIT. Due to their general refractiveness to *in vitro* culture, studies of spirochaetes in the GIT have centered around host distribution and location of spirochaetes with different morphologies. Essentially nothing is known about the physiology and niche of spirochaetes in the gastrointestinal environment (Cwyk and Canale-Parola, 1979) and speculation on their contribution to the functioning of this ecosystem can only be made when these organisms have been extensively studied *in vitro*.

2.1.1 Spirochaetes of the stomach

Helical shaped bacteria with a marked predilection for openings of fundic glands, inhabit the stomach of various animals including dogs (Lockard and Boler, 1970; Pindak *et al.*, 1965; Sedar and Friedman, 1961; Vial and Orrego, 1960; Weber and Schmittdiel, 1962), rats (Doenges, 1939; Savage, 1977a), humans and monkeys (Doenges, 1939; Palmer, 1954). These organisms associate intimately with the brush borders of the gland cells (Savage, 1983) and occasionally penetrate into the parietal cells (Weber and Schmittdiel, 1962). The

presence of lophotrichous flagella-like appendages led to these bacteria originally being classified as *Spirillum* species, but since they also possess periplasmic fibrils, the suggestion that they belong to the *Spirochaetales* has been made (Lockard and Boler, 1970). In recent years, these bacteria have received little attention from microbiologists (Savage, 1983).

2.1.2 Spirochaetes of the small intestine

A highly motile spiral organism resides in the crypts of Lieberkühn of the ileal mucosa of the rat (Hanson, 1970; Phillips *et al.*, 1978). Morphologically, this organism is similar to that which inhabits the gastric mucosa, and consists of a coiled protoplasmic cylinder encircled by periplasmic fibrils that are continuous from one end to the other, and enclosed by a sheath which gives rise to polar, flagella-like appendages (Erlandsen and Chase, 1972). Although not intimately associated with the epithelium, and not found within goblet or undifferentiated crypt cells, this organism has been observed within paneth cells, where it undergoes digestion (Erlandsen and Chase, 1972; Wendelschafer *et al.*, 1973).

2.1.3 Spirochaetes of the large intestine (caecum and colon)

Spirochaetes are normally present in the caeca of dogs (Pindak *et al.*, 1965), pigs (Cwyk and Canale-Parola, 1979), rats (Davis *et al.*, 1972a; 1972b; Leach *et al.*, 1973; Phillips *et al.*, 1978) and mice (Savage *et al.*, 1971), where they are associated primarily with the mucosal layer and are seldom seen in the lumen contents (Davis, 1976; Knehans and O'Dell, 1980; Leach *et al.*, 1973; Savage and Blumershine, 1974). In mice, spirochaetes and flagellated spiral bacteria colonize the mucous layer of the caecum and colon when the animals are three to five days old (Davis and Savage, 1972; Davis

et al., 1973; Savage *et al.*, 1971). These bacteria are among the first inhabitants, and their presence may be necessary for the establishment of conditions that are favourable for the survival of the fusiform bacteria that later colonize this habitat (Davis and Savage, 1972). In the adult animal, the fusiforms and spiral bacteria co-inhabit this mucus layer (Savage *et al.*, 1971; Savage and Blumershine, 1974).

Two of the three morphologically distinct types of spiral bacteria which inhabit the caecal crypts of normal rats have spirochaete morphology in that they possess periplasmic fibrils (Davis, *et al.*, 1972b; Leach *et al.*, 1973). These spirochaetes occasionally penetrate through the epithelium and into the lamina propria, where they are found either in phagocytes or in extracellular locations. The mechanism by which spirochaetes penetrate the epithelium is unknown, but is a spontaneous process and does not appear to have an adverse effect on the host (Davis *et al.*, 1972b). One of these spirochaetes has a "classical"[1] spirochaete ultrastructure, and is found within the mucous granules of the goblet cells, whereas the other, which has polar appendages in addition to periplasmic fibrils, does not penetrate the goblet cells (Davis *et al.*, 1972b).

Treponemes are normally present in the colon of healthy dogs (Pindak *et al.*, 1965), rats (Hanson, 1970) and mice (Savage *et al.*, 1971). In all three animals, the bacterial population at the base of the crypts consists almost exclusively of helical shaped bacteria, while at the mouth of the crypts, the helical bacteria intermingle

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[1] Spirochaete with an ultrastructure such as that described in chapter 1. Used to distinguish from "atypical" spirochaetes in the rat gut which have both periplasmic fibrils and flagella-like appendages.

with fusiforms (Leach *et al.*, 1973; Pindak *et al.*, 1965; Savage, 1983). The rat colonic spirochaetes may attach firmly to cells at the base of the crypts, (Hanson, 1970), while the dog and mice spirochaetes do not adhere to the epithelial cells, even though they are occasionally observed within the goblet cells (Leach *et al.*, 1973).

Although evidence concerning the autochthonicity of spirochaetes in the GIT is inconclusive, there are strong reasons to believe that spirochaetes are autochthonous in the large intestine (caecum and colon), and possibly in other areas of the GIT (Savage, 1983). The aim of this part of the study was to examine the ecology of rat gastrointestinal spirochaetes with a view to establishing their autochthonous status. Habitats in this ecosystem were determined by both light and electron microscopy and estimates of population densities were made using direct microscopic counting techniques. In addition, the sequential colonization pattern by bacteria in infant rat gut was examined in order that all criteria for autochthonicity could be satisfied.

2.2 MATERIALS AND METHODS

2.2.1 Localization of spirochaetes in the rat GIT

Animals

Fifteen conventional and five specific pathogen free (SPF), 250-350g Wistar rats (*Rattus norvegicus*) of either sex were used in this study. Animals were obtained from a central university facility where they were housed in individual wire cages with vermiculite as bedding. They were fed commercial rat pellets (Epol, Johannesburg, South Africa) with the following composition: protein,

180g/Kg; moisture, 120g/Kg; fat, 25g/Kg; fibre, 60g/Kg; calcium, 18g/Kg; phosphorus, 7g/Kg and supplementary trace minerals and vitamins. Water was available *ad lib*. Animals were sacrificed by dislocation of the neck and were dissected immediately.

Sampling sites

Representative samples from both the lumen and epithelial surface were collected from the stomach (keratinized region), duodenal and ileal regions of the small intestine, and distal and proximal regions of the caecum and colon.

Light Microscopy

Sampled lumen contents, were diluted in saline (approximately 1 in 5 dilution), and a film made on a microscope slide. Epithelial microflora was sampled by making a film of scrapings from tissues that had been lightly washed by dipping 10 times into a beaker containing saline, and a further 5 times into clean saline solution. The material was heat fixed and stained by a modified Gram method, in which Ziehl-Neelsen carbol fuchsin was used as a counterstain (Appendix A(1)). This modification was necessary since spirochaetas generally do not stain readily with aniline dyes. Material from the first 5 rats was also stained using the Fontana method (Appendix A(2)). The use of this stain was discontinued since all spirochaetes could be observed using the modified Gram stain which was less capricious. The films were examined for the presence of spiral bacteria using an oil objective lens. Samples fixed in formol-saline (4% formaldehyde in saline) were also examined by phase contrast microscopy. Spiral bacteria were classified according to size and general morphology.

Electron Microscopy - Negative Staining

Diluted lumen contents and scrapings from lightly washed epithelia (*vide supra*) were negatively stained with 2.5% phosphotungstic acid (Appendix B(1)) and examined using a Joel 100S transmission electron microscope operating at 80KeV (Joel, Tokyo, Japan). This enabled spirochaete subpopulations to be identified at the ultrastructural level.

Transmission and Scanning Electron Microscopy

Washed epithelial tissues were fixed in 3% glutaraldehyde in isotonic 0.1M phosphate buffer (pH 7.2) (Appendix B(2)) for a minimum of 24 hours at 4°C. Tissues were cut into smaller pieces (c. $1cm^2$ for scanning electron microscopy (SEM), and $0.5mm^2$ for transmission electron microscopy (TEM)), post-fixed in 1% osmium tetroxide in phosphate buffer, washed and dehydrated in a graded alcohol series (Appendix B(4)). For TEM, tissues were cleared in propylene oxide and embedded in epon-araldite resin (Appendix B(3)). Thick sections (1μm) were stained with toluidine blue solution (Appendix B(5)) and examined with a light microscope. Ultrathin sections were stained with uranyl acetate-lead citrate sequence (Appendix B(6)) and examined using a Jeol 100S transmission electron microscope at an accelerating voltage of 80KeV. For SEM, dehydrated tissue was critical point dried, coated with gold (20nm) and examined using a Joel T20 scanning electron microscope operating at 20KeV.

2.2.2 Total Counts

The method of Holdeman *et al.*, (1974) was used to determine number of spirochaetes in various sampling areas. One gramme (wet weight) of lumenal contents was placed in 9.0 mℓ of saline, vortexed

and a 0.01mℓ aliquot spread evenly over a 1cm² area marked on a glass slide. The sample was heat fixed, Gr stained (Appendix A(1)) and the number of morphologically distinct spiral bacteria in fifty fields was counted. The total number of each morphotype was then calculated using the formula:

$$x = k\ a\ d\ 100$$

x = total number of each morphotype per gramme of sample

k = number of fields in 1 cm² at 1000x magnification

a = average number of each bacterial morphotype per field

d = dilution factor

For epithelial tissue, 1g of lightly washed epithelium (*vide supra*) was homogenized in a Griffiths tube in 5mℓ of saline, and 0.01mℓ applied to a slide and processed as described above.

2.2.3 Fluorescent antibody tracing

In an attempt to accurately define the habitats of various spirochaete types in the rat GIT, fluorescent antibody tracing of GIT samples was performed. Antibodies to spirochaete types A, B, C and D (which as a result of concurrent work had been cultivated *in vitro*), were raised in New Zealand white rabbits according to the immunization schedule in Appendix C(1 and 2). Immunoglobulins were purified from the serum using the Rivanol method (Walker *et al.*, 1971) (Appendix C(3)), which removes both albumin and α and β globulins to give a chromatographically pure immunoglobulin preparation which is suitable for immunofluorescence. As a control, normal rabbit serum, treated in the same way, was used.

Sections from the ileum, caecum and colon were dissected from two conventional Wistar rats, placed in plastic bags, quenched in liquid nitrogen and stored at -20°C. Thick sections of 0.8μm were cut using a cryostat, fixed in methanol and stained as described in

Appendix C(6) using appropriate antiserum and fluorescein dilutions. To quench autofluorescence, tissue was counterstained with 1% Evans blue BDH) for 5 minutes and washed in phosphate buffered saline (PBS) prior to mounting in phosphate buffered glycerol solution (Appendix C(6)). An alternative approach, using films of lumen contents, and impressions of lightly washed epithelial tissues (*vide supra*) was also used. Epithelial impressions were made by placing a small piece of the washed tissue (lumen side down) onto a clean microscope slide and applying pressure onto the tissue. Films were air dried, fixed in methanol and stained as described in Appendix C(6). Slides were examined with a Leitz epifluorescent microscope fitted with an excitation UG1 Schot filter, a pale yellow barrier filter and water immersion objectives (Leitz, Wetzlar, West Germany).

2.2.4. Succession

The colonization pattern of autochthonous spirochaetes in infant rats was studied in two litters of conventional rats born in the laboratory, and maintained as previously described. Infants from the first litter (litter A) were sacrificed on days 3, 6, 9, 12, 14, 16, 18, 20, 24 and 30, while infants from the second litter (litter B) were sacrificed on days 8, 11, 13, 15, 17, 19, 21, 26, 29 and 33. The infants were killed by cervical dislocation, transported into the anaerobic cabinet where samples from caecum and colon were dissected out for culturing (chapter 3). After removal from the cabinet, sections from the duodenum, ileum, caecum (from day 11 onwards) and colon were placed, without washing, into 3% glutaraldehyde in 0.1M phosphate buffer (pH 7.2) (Appendix B(2)) and stored at 4°C. These samples were processed for transmission and scanning electron microscopy as previously described. Samples for Gram staining (Appendix A(1)) and negative staining - transmission electron microscopy (Appendix B(7)) were also taken.

2.3. RESULTS

2.3.1. Distribution of spirochaetes in the rat GIT

At the light microscope level, at least 8 morphologically distinct helical bacteria could be discerned (Fig. 3), although negative staining - transmission electron microscopy revealed that only 4 of these had a "classical" spirochaete ultrastructure (Types A-D in Fig. 3). Helical bacteria with both periplasmic fibrils and bipolar flagella were also observed (Types E and F in Fig. 3). Type G, a spiral organism typically associated with the epithelial crypts of the ileum, caecum and colon, did not appear to have either flagella or periplasmic fibrils and in transverse section was distinguished by having a characteristic loose outer membrane surrounding the cell. Type H, represents a heterogeneous group of spiral bacteria which at the electron microscope level had one or two flagella extending from one or both ends of the organism; a morphology consistent with that of members of the *Campylobacter* genus. Based on flagellation characteristics, at least three *Campylobacter* species were probably represented, although no attempt was made to recognize different campylobacter types at the light microscope level.

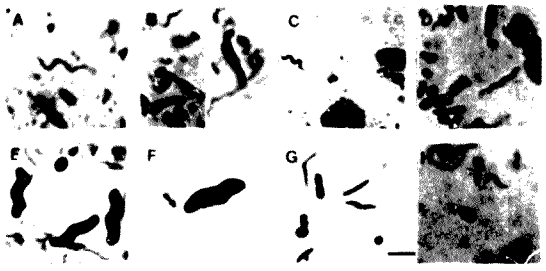

Fig. 3. Light microscopic appearance of helical bacteria in the conventional rat GIT. (Modified Gram stain) Bar=5μm.

Spiral bacteria were only present in the GIT of conventional animals, and were not found in any of the sampled habitats of the five SPF rats examined. Ecologically, the different spirochaete populations varied with respect to population densities attained in different regions of the gut and occupation of particular spatial habitats. A detailed description of the various spirochaete populations within the conventional rat GIT, together with their distribution is presented below. These results were obtained primarily by light microscopy, but were confirmed by negative staining-transmission electron microscopy.

Type A spirochaetes, were loosely coiled "borrelia-like" organisms 10.0±1.57µm in length and 0.44±0.06µm in diameter. Their coils were arranged in a regular manner with each organism having 3-5 coils *c.* 2.5µm apart. These were the largest of the "classical" spirochaetes observed in the rat GIT, and were readily distinguishable from the smaller spiral bacteria. Each organism had 7 or 8 periplasmic flagella originating from each pole and these overlapped at the center of the protoplasmic cylinder to give a 7-14-7 or 8-16-8 periplasmic fibril arrangement. The axial fibriis were *c.* 17nm in diameter, and insertion points were arranged in a line at the poles of the organism. The protoplasmic cylinder terminated in slightly tapered, round ends. Although these organisms were gram-negative with the modified Gram stain, they frequently stained irregularly with undifferentiated areas at regular intervals along the protoplasmic cylinder, giving the organism a "beaded" appearance. These bacteria were observed in all of the rats examined and were localized primarily in the large bowel where they were associated both with the epithelial tissue and the lumen contents. The estimated total population density attained by these bacteria was

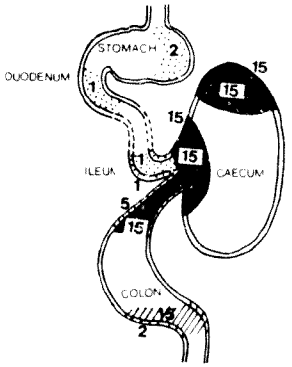

Fig. 4a Schematic representation of distribution and population density of type A helical bacteria in the rat GIT. Key: □ $<10^5$ organisms/g; ▨ 10^5-10^6 organisms/g; ■ $\geq 10^6$ organisms/g. Numbers indicate the number of rats in which this organism was seen in each sampling site; Total number of rats examined = 15.

3 ±2.1 x 10^6 organisms/g of caecal lumen contents, 4.9±0.8 x 10^6 organisms/g of caecal epithelial tissue, 1.6±1.5 x 10^6 organisms/g proximal colon lumen contents and 5.3±3 6 x 10^5 organisms/g of proximal colon epithelial tissue (n=5). The distribution of these bacteria in the rat gut is schematically represented in Fig. 4a.

Type B spirochaetes were 5.8±1.3µm long and 0.33±0.02µm in width. These bacteria were characterized by having an irregular, tight coiling and typically one coil in each organism was of greater amplitude than the other 2-6 coils. The protoplasmic cylinder tapered slightly towards the poles which terminated in round ends. Two periplasmic flagella of 14nm diameter originated at each pole and overlapped at the center, giving a 2-4-2 arrangement. These bacteria were found predominantly in the lumen contents of the large bowel where population densities of 6.6±2.4 x 10^5 and 3.4±0.3 x

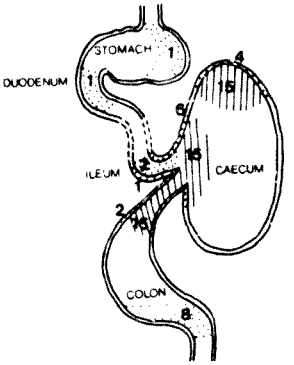

Fig. 4b Schematic representation of distribution and population density of type B helical bacteria in the rat GIT. Key: □ $<10^3$ organisms/g; ▨ 10^3-10^5 organisms/g; ■ $\geq 10^5$ organisms/g. Numbers indicate the number of rats in which this organism was seen in each sampling site; Total number of rats examined = 15.

10^9 organisms/g of lumen contents were recorded for the caecum and proximal colon respectively (n=4). The distribution of these bacteria in the rat GIT is diagrammatically presented in Fig. 4b.

Spirochaete types C and D, were more difficult to distinguish at the light microscope level, since they were of overlapping size and both exhibited tight, regular coiling. The main differentiating criterion for distinguishing these bacteria at the light microscope level were their terminal ends, with type C having tapered, round ends and type D having round, non-tapered ends.

Type C spirochaetes were 6.24±2.3μm in length and 0.34±0.02μm in diameter. Three periplasmic flagella originated from each pole and overlapped in the center to give a 3-6-3 arrangement. Each

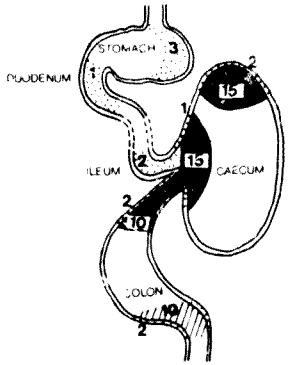

Fig. 4c Schematic representation of distribution and population density of type C helical bacteria in the rat GIT. Key: □<10⁶ organisms/g; ▨ 10⁶-10⁸ organisms/g; ■≥10⁸ organisms/g. Numbers indicate the number of rats in which this organism was seen in each sampling site; Total number of rats examined = 15.

organism had 3-6 regularly spaced coils with each coil being 1.2μm apart although on occasion individuals with 8 coils were seen. These bacteria were present in all of the rats examined, were localized primarily in the lumen contents of the large intestine and attained population densities of 3.1±1.7 x 10^8 and 1.6±1.2 x 10^8 organisms/g of lumen contents in the caecum and proximal colon (n=5). Their distribution in the rat GIT is represented in Fig. 4c.

Type D spirochaetes were the smallest of the spirochaetes, being 4.5±1.4μm long and 0.32±0.08μm wide. They typically had 2 or 4 coils although exceptions were observed. Each coil was 1.5μm apart. The protoplasmic cylinder did not taper as it approached the poles, which terminated in round ends. Each organism had a 1-2-1 periplasmic fibril arrangement. Although observed in 14 out of the

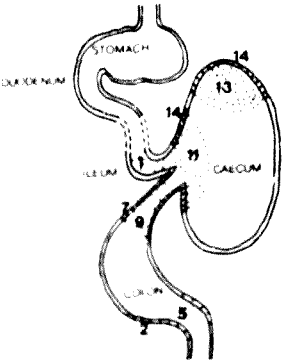

Fig. 4d. Schematic representation of distribution and population density of type D helical bacteria in the rat GIT. Key: □ <10⁴ organisms/g; ▨ 10⁴-10⁵ organisms/g; ■ >10⁵ organisms/g. Numbers indicate the number of rats in which this organism was seen in each sampling site. Total number of rats examined = 15.

15 rats examined these bacteria did not attain high population densities and a mean total count of 4.1±3.9 x 10^5 bacteria/g of caecal epithelial tissue (n=4) and 2.8±1.7 x 10^4 bacteria/g (n=3) of colonic epithelium was recorded. As indicated in Fig. 4d these bacteria were also present in low numbers in the lumen contents.

Type E. For the purpose of this report, the spirochaete-like bacteria possessing both periplasmic fibrils and flagella have been grouped together as type E spirochaetes, although if width of these bacteria is a valid differentiation criterion, then two types were probably present in the rat GIT (Fig. 3 e and f). These bacteria were readily recognized at the light microscope level by virtue of their distinctively large size. The most predominant of the two types were 0.95±0.21μm in diameter, and 7.5±2.5μm in length, while

the other type was 1.5±0.3μm in diameter and 8.1±2.3μm in length. These organisms had 2-4 gradual turns with each coil being approximately 2.5μm apart. The ends were round and blunt, and tufts of flagella-like appendages extended from each pole. In addition, thick periplasmic fibrils of 40nm in diameter wound around the protoplasmic cylinder. The wider of the two bacteria seemed to possess 14 of these fibrils whereas the other had 9 such fibrils. These bacteria frequently stained unevenly with the modified Gram stain. As indicated in Fig. 4e these bacteria were frequently seen in the ileum where a density of 2.4±2.1 × 10^6 organisms/g of lumen contents was attained (n=3). They were also consistently found in the large intestine, being present in all the conventional rats examined. The population densities in these sampling regions were 6.8±4.5 × 10^6/g caecal lumen contents, 1.0±0.9 × 10^7/g caecal epithelium, 4.1±2.6 × 10^6/g colon lumen contents and 8.2±1.9 × 10^6/g colonic epithelial tissue (n=5).

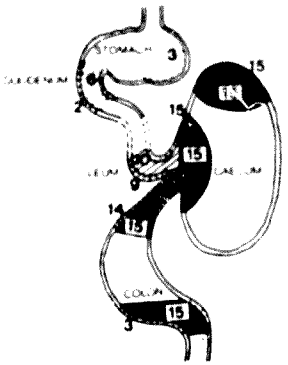

Fig. 4e. Schematic representation of distribution and population density of type E helical bacteria in the rat GIT. Key: □ <10^6 organisms/g, ▨ 10^6-10^8 organisms/g, ■ ≥10^8 organisms/g. Numbers indicate the number of rats in which this organism was seen in each sampling site. Total number of rats examined = 15.

2.3.2 Fluorescent antibody tracing

Although antisera were prepared against 4 morphologically distinct spirochaetes, all were non-specific in that they cross-reacted with spirochaetes of other morphologies. The antisera however were specific, in that they did not react with other eubacteria. Spirochaetes could not be detected in thick sections of gut tissue, since excessive autofluorescence of both tissue and lumen contents obscured specific staining. Spirochaetes were however seen in films of lumen contents and impressions of gut epithelia tissue (Fig. 5), stained with immune but not normal (control) sera. The distribution of spirochaetes in the rat GIT as determined using fluorescent antibody tracing was similar to that determined using Gram stained preparations and negative staining TEM, although the spirochaete with the 3-6-3 periplasmic fibril arrangement (type C), was more frequently seen associated with epithelial tissue than suggested by other techniques. Interestingly, all the antisera reacted with the large "atypical" spirochaetes (types E and F), further suggesting that these are members of the *Spirochaetales*. Cross-reactivity against the undulating membrane of a protozoan species was also noted (Fig. 5e).

2.3.3. Scanning electron microscopy

By scanning electron microscopy, four morphologically distinct spiral bacteria were recognized as being associated with the mucosal epithelium of the caecum and colon (F 7 and 8) where they were loosely affiliated with the Although not in intimate contact[1] with the underlying spirochaete type was

[1] Physical contact in which the architecture of the mucosal cells is altered.

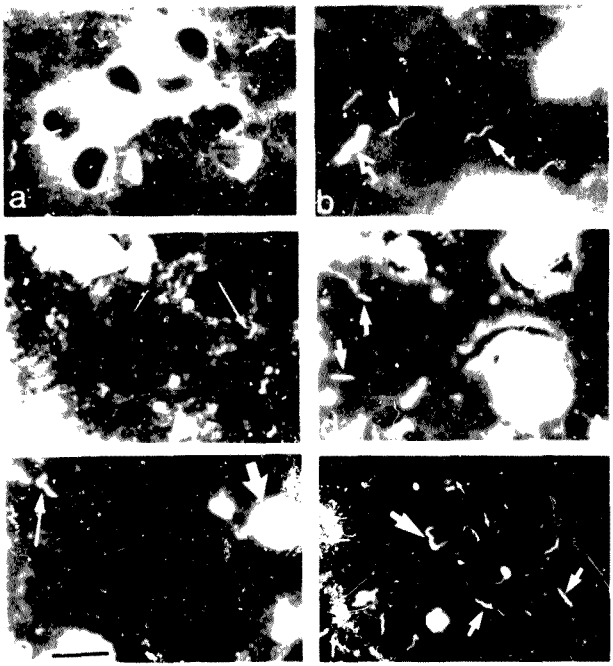

Fig. 5. Fluorescent antibody staining of spirochaetes (arrows) in conventional rat large intestine. The non-specificity of the antisera is evident.
a. caecal epithelium (1:15 antiserum). Type A spirochaetes.
b. caecal epithelium (1:13 antiserum). Type A (closed arrow) and Type E spirochaetes (open arrow).
c. colonic epithelium (1:39 antiserum). Type C spirochaetes.
d. caecal epithelium (1:29 antiserum). Type D spirochaetes.
e. caecal lumen (1:15 antiserum). Type A spirochaetes. Also present are protozoa with fluorescent undulating membrane (large arrow).
f. caecum lumen (1:23 antiserum). Type A (large arrow), B (small arrow) and D (medium arrow) spirochaetes are represented.

observed in close proximity to the microvillus surface. Despite difficulties in accurately defining bacterial types with SEM, the dimensions of the organism seen in Fig. 6, suggest that it is one of the smaller, "classical" spirochaetes, while the loose, irregular coiling infers that it could be a "type B" spirochaete. Whereas spiral bacteria such as this were rarely observed in SEM preparations, a helical organism with a diameter and morphology similar to type A spirochaete was frequently observed within the mucous layer

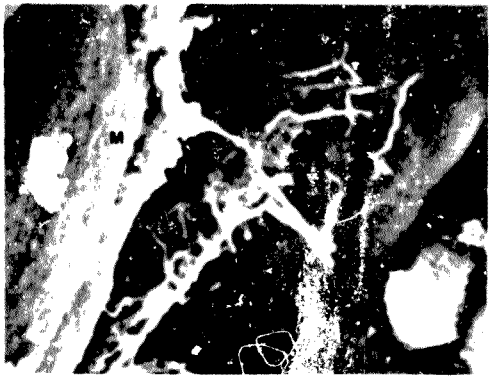

Fig. 6. SEM of conventional rat caecum ... spirochaetes (S) on microvillus (MV) surface and beneath the m... layer. Bar: 5μm

(Figs. 7 and 8, small arrows). Although prevalent on the large intestine epithelium, these type A spirochaetes were never observed in aggregates (microcolonies) but were always seen as individual cells intermingled with the diverse epimural flora.

The most conspicuous and most numerous of the spiral bacteria observed on the mucosal epithelium by SEM, were the large spiral bacteria possessing both periplasmic fibrils and flagella-like appendages. These protruding flagella are evident in Fig. 8 (open arrow), while the thick periplasmic fibrils can be seen winding around the organism in Fig. 9 (arrows). These bacteria were never seen in microcolonies, but were frequently associated with aggregates of fusiform bacteria (Fig. 10). Although primarily located in the caecum and colon, these organisms were also observed on the ileal mucosa. On rare occasions spiral procaryotes with a diameter similar to the smaller of the type E bacteria were observed in the mucous layer of the large colon (Fig. 8).

2.3.4. Light and transmission electron microscopy

Light microscopy revealed the presence of spiral bacteria in the crypts of the ileum, caecum and colon (Fig. 11). Transmission electron microscopy, showed that four morphologically distinct spiral bacteria resided in this habitat. All four morphotypes were present in the caecal and colonic crypts, but only one type was consistently observed in the crypts of the ileum. Crypts were never inhabited by all four bacterial types and usually, one bacterial strain predominated in any one crypt, with low numbers of another type also being present.

Of these four bacteria, only one was a "classical" spirochaete (Fig. 12, 13 and 14) and was similar in morphology to the type A

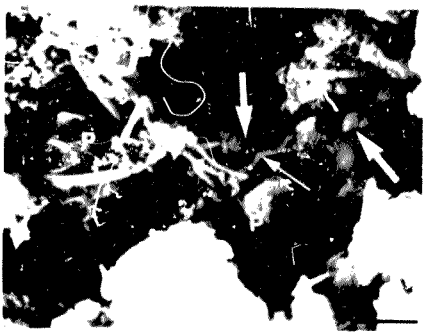

Fig. 7. SEM of conventional rat caecum illustrating two morphologically distinct spiral bacteria. Large arrows: Type E spirochaetes; small arrow: Type A spirochaete. Two types of protozoa (P) are also evident. Bar 5μm.



Fig. 8. SEM of conventional rat colon (proximal region) showing three morphological types of spiral bacteria embedded in the mucous layer (M). The large white arrow depicts the larger Type E spiral organisms, while the small white arrow depicts the smaller Type E spiral organism. The blackarrow points to a type A spirochaete. Protruding polar appendages from one of the type E spiral bacteria are evident (open arrow). Bar 5μm.



Fig. 9 Large spiral organism (type E) embedded in mucous layer (M). Arrows indicate parallel ridges on outer surface of the organism. F, fusiform bacteria. Bar=1μm



Fig. 10 Large spiral organism associated with microcolony of fusiform bacteria (F). M, mucous layer. Bar=1μm

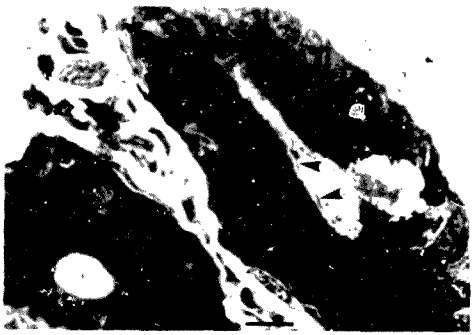

Fig. 11. Toluidine blue staining of thick section of rat caecum, showing spiral bacteria (arrows) within crypt. Bar=10μm.

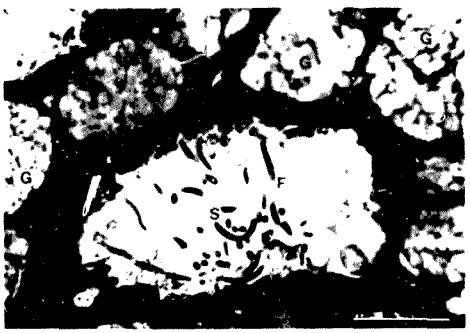

Fig. 12. Spirochaetes (S) and flexuous fusiform bacteria (F) localized in crypt of rat caecum. The crypt is surrounded by goblet cells (G), which have not been invaded by these bacteria. Bar=5μm.

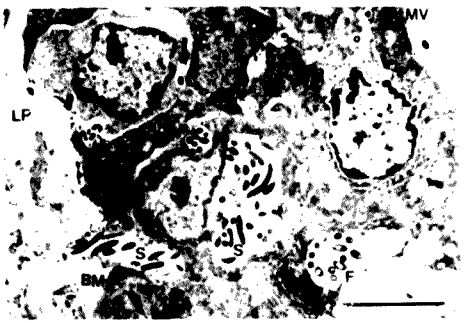

Fig. 13. Cluster of spirochaetes (S) within epithelial cells and penetration of these bacteria through the basement membrane (BM) and into the lamina propria (LP). Note the close proximity of the spirochaetes to the nuclear membrane, lack of membrane surrounding the vesicles containing the bacteria and the single spirochaete in the adjacent cell (arrow). Fusiform bacteria (F) within an adjacent goblet cell and a spirochaete located near the microvillus border (MV) are also visible. Bar=5μm.

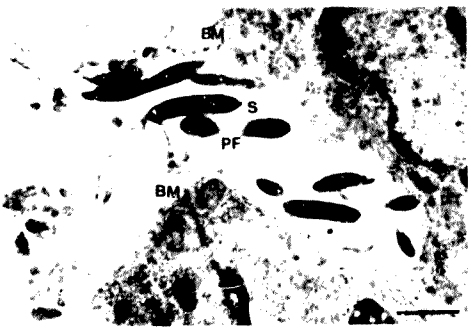

Fig. 14. Longitudinal section of spirochaetes (S) penetrating the basement membrane (BM). The periplasmic fibrils (PF) are clearly visible. Bar=1μm.

spirochaete. These microorganisms were 0.4μm in diameter and possessed seven periplasmic fibrils originating from each pole. Although these bacteria resided in the lumen of the crypts, they were frequently seen within the epithelial cells, and even penetrated through the basement membrane into the lamina propria (Fig. 13 and 14). Within the epithelial cells, the spirochaetes were generally aggregated in clumps, but on occasion, single spirochaetes were observed in the cells (Fig. 13). The bacteria did not appear to be enclosed in a membrane-bound vesicle (Fig. 15) and furthermore did not appear to undergo degeneration while within the cells. Spirochaetes were never observed within the cell nuclei, but were frequently seen in close proximity to the nuclear membrane (Fig. 13). No degenerative changes of the cellular cytoplasmic components was observed.

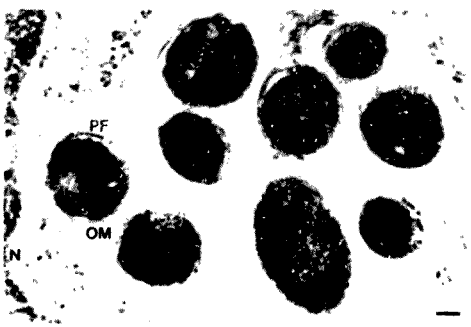

Fig. 15. Transverse section of cluster of spirochaetes in close proximity to the nuclear membrane (N). The periplasmic fibrils (PF) and outer membrane (OM) of the bacteria are clearly visible. Note the lack of membrane surrounding vesicle. Bar=0.1μm.



Fig. 16 Spiral bacteria (S) with distinctive loose outer membrane associated with cryptal microvilli (MV). Bar=1μm.

The second type of spiral organism observed, was localized in the ileal, caecal and colonic crypts, but did not penetrate epithelial cells, nor were they intimately associated with the cells. These bacteria were easily recognized in both transverse and longitudinal sections by a characteristic loose membrane which surrounded the organism (Fig. 16).

The other two helical bacteria observed in the crypts of the large bowel were large bacteria with a distinct scalloped outer structure (Figs. 17, 18 and 19). One of these organisms (Fig. 18) was smaller in transverse section than the other (Fig. 19), had fewer "periplasmic fibrils" and was seldom seen. On the basis of their distinctive ultrastructure and size, these were identified as type E spiral bacteria.



Fig. 17 Transverse section of crypt showing spiral organism with loose outer membrane (S) in addition to organism with distinctive scalloped ultrastructure (P) localized close to microvilli (MV) Bar=2um.

2.3.5. Succession

Spirochaetes were comparatively late colonizers of the rat gastrointestinal tract, being first observed on day 20. Prior to this both light and electron microscopy of the sampled habitats revealed that the microflora consisted predominantly of gram-positive rods and cocci, with minor populations of yeasts and gram-negative rods (Fig. 20 and 21). Up to day 11, the flora of the entire gut was simple, but after this, became increasingly more complex. This diversification of the flora was associated with the appearance and subsequent increase in caecal size. The colonization pattern by spirochaetes in the two litters was almost identical and throughout this study, spirochaetes were never observed in the stomach, duodenum or ileum.

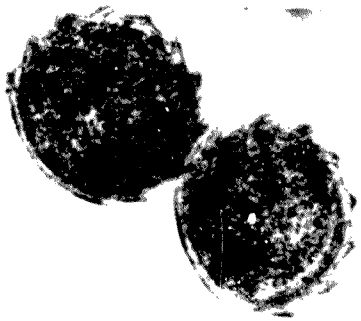

Fig. 18. Transverse section of smaller of the two type E spirochaetes observed in the crypts of the large intestine. 9 thick periplasmic fibrils wind around the protoplasmic cylinder. Bar=0.1μm.

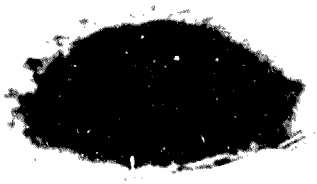

Fig. 19. Transverse section of larger of the two type E spirochaetes in crypts of rat large intestine. 13 thick periplasmic fibrils wind around the protoplasmic cylinder. Bar=0.1μm.

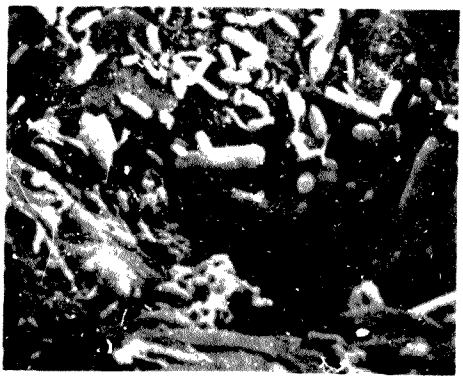

Fig. 20. Rods and cocci on caecal epithelial surface of 17 day old infant rat. (SEM). Bar=5μm

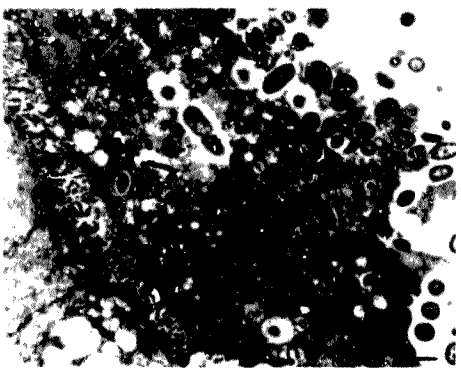

Fig. 21. Thin section of caecal epithelium of 17 day old infant rat depicting bacterial flora within electron dense matter adjacent to intact microvilius (MV) surface. Bar 1μm

Although 2 types of helical bacteria were first observed in the large bowel on day 16, neither of these were members of the *Spirochaetaceae*. Negative staining indicated that one of these initial colonizers had a polar flagellum extending from each end of the organism and was thus ultrastructurally similar to members of the *Campylobacter* genus. These bacteria initially colonized lumen habitats, being seen in the colonic lumen on day 16 and in the caecal lumen, on day 17. Epithelial colonization was first observed on day 20 (Fig. 22) and by the following day these organisms had attained high population densities on the caecal epithelium (Fig. 23). Electron microscopy of this tissue revealed that in localized areas, massive bacterial penetration of the epithelium had occurred (Fig. 24), and that monotrichously flagellated helical bacteria were among the invading microorganisms (Figs. 25 and 26). Although the numbers of these bacteria subsequently declined, they were consistently seen both on the epithelium and in the lumen contents throughout this study.



Fig. 22. Rods and small helical bacteria (arrows) on colonic epithelium of a 20 day old conventional rat (SEM). Bar=5μm

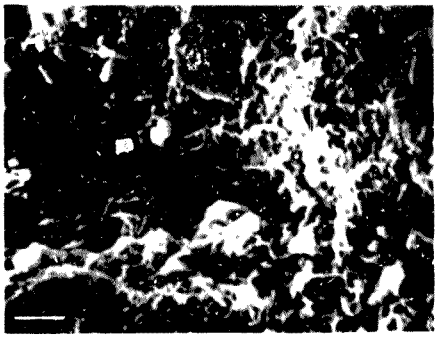

Fig. 23. Massive colonization of caecal epithelium by small helical bacteria on 21 day old conventional rat (SEM). Bar=5μm.

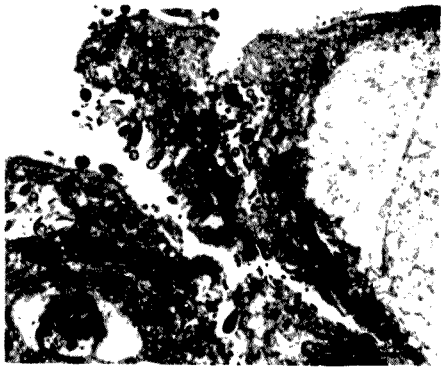

Fig. 24. Low power TEM of caecal epithelium of 20 day old conventional rat depicting bacterial infiltration and damage of epithelial cells. Arrows indicate that spiral bacteria are among the inv[illegible]g bacteria. Intact microvilli (MV) are evident on an adjacent cell. Bar = 5.m.

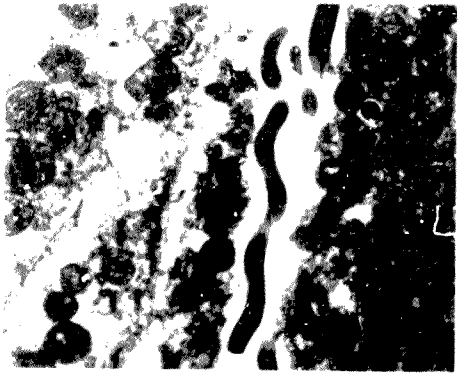

Fig. 25. Spiral bacteria within caecal epithelial cell of 20 day old conventional rat. Bar=1μm.



Fig. 26. High power of spiral bacteria within caecal epithelial cells, the polar flagellum extending from each organism suggests that these are of the genus *Campylobacter* bacteria. Bar=0.2μm.

The bacteria with the characteristic loose outer membrane were seen first on day 16 in the caecum, day 18 in the colon and within epithelial cells on day 19 (Figs. 27 and 28). By day 26, these bacteria had almost exclusively colonized the colonic crypts (Figs. 29 and 30). These organisms initially associated with epithelial cells, and were only observed in lumen contents after day 24.

The large, spiral, bacteria with both periplasmic fibrils and flagella-like appendages, were the earliest of the spirochaete bacteria to colonize the rat gut, being first seen on days 19 in the large intestine and day 21 in the caecum. These bacteria were never seen invading epithelial cells.

The large, loosely-coiled, multifibrillar, borrelia-type spirochaetes (type A), were first seen in both the caecum and colon on day 20 in litter A and day 21 in litter B where they were observed on the epithelium and in the lumen contents. The caecal crypts of the infant sacrificed on day 26 were packed exclusively with these organisms (Figs. 31 and 32) although a similar colonization was not observed in the infant sacrificed on day 29, where only moderate numbers of spirochaetes were seen intermingling with a diverse epimural flora (Fig. 33). Transverse sections of massively colonized tissue confirmed the spirochaete nature of these bacteria (Figs. 34-38) by revealing the presence of 7 periplasmic fibrils (Fig. 35). Despite occasional penetration into goblet cells (Fig. 36), infant gut tissue was not invaded by these spirochaetes. In heavily infested crypts, erosion of the brush border occurred (Fig. 36) but in



Fig. 27. Loosely-coiled intermediate size spiral organism (arrow) on the large intestine epithelium of a 18 day old conventional rat (SEM). Bar=5um.



Fig. 28. Helical bacteria with characteristic outer membrane within caecal epithelium of 19 day old conventional rat. Bar=1um.

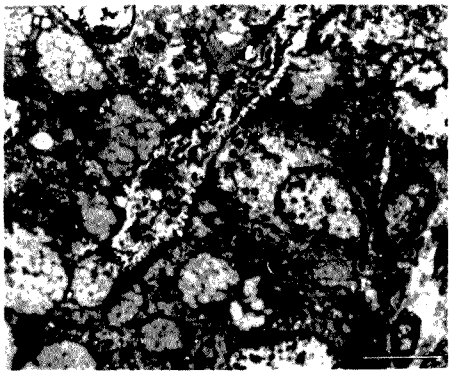

Fig. 29. Low power TEM of colonic crypt of 26 day old conventional rat depicting mass of spiral bacteria within the crypt. Bar=5μm.



Fig. 30. Enlargement of Fig. 29 showing the spiral organism with the loose outer membrane packing crypts of colonic epithelium of 26 day old conventional rat. The integrity of the microvillus surface is evident. Bar=1μm.

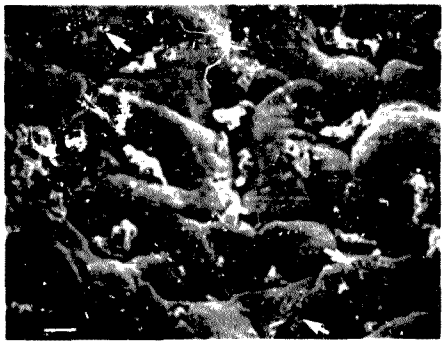

Fig. 31 Low-power scanning electron micrograph of caecal epithelium of a 26 day old conventional rat depicting the extent of colonization by the large, loosely-coiled spirochaete. Each crypt is colonized by a mass of these bacteria. Some dusquamation of the epithelial surface (arrows) is evident. Bar=20um

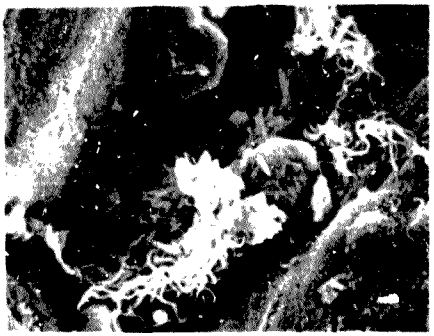

Fig. 32 Enlargement of a crypt of Fig. 31, showing mass of loosely coiled spirochaetes packed into each crypt. Bar=5um.

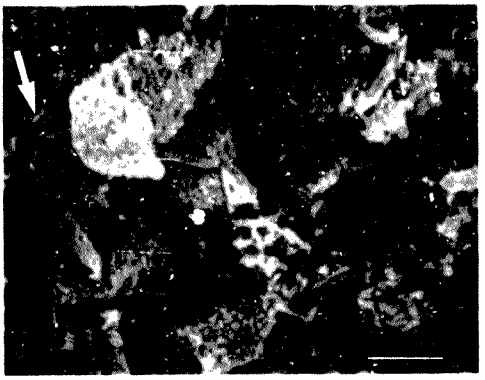

Fig. 33 Large, loosely coiled spirochaetes (arrow) with a diverse epimural microflora on the caecal epithelium of a 30 day old conventional rat. Bar=5μm



Fig. 34 Transmission electron microscopy of the caecal epithelium of a 26 day old conventional rat depicting massive colonization of the crypts by spiral bacteria. Bar=5μm

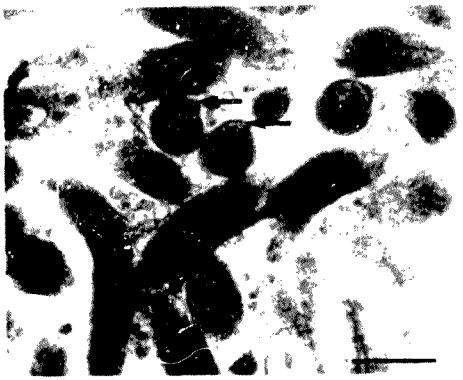

Fig. 35. Enlargement of Fig. 33 showing transverse sections of spiral bacteria packed into caecal crypts of 26 day old conventional infant. The periplasmic fibrils of the spirochaetes are evident (arrows). Bar=0.5um.

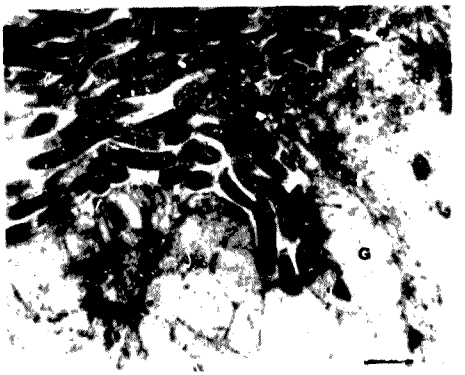

Fig. 36. Spirochaetes penetrating goblet cell (G) of crypt packed with these bacteria. The lack of microvilli is evident. Bar=1um.



Fig. 37. Spirochaetes in a less-densly colonized caecal crypt of a 26 day old conventional rat. Spirochaetes are orientated perpendicular to the epithelium. The microvillus border (MV) is intact. Bar=1µm.



Fig. 38. Enlargement of Fig. 37 depicting the close association between microvilli and spirochaetes. Periplasmic flagella can be discerned (arrows). Bar=0.2µm.

crypts with a lower bacteriological load the microvillus surface was intact (Fig. 37). In some (rare) instances, intimate associations between spirochaetes and cellular brush borders appeared to exist (Fig. 38).

By SEM a tightly coiled helical organism, probably a spirochaete, was observed on the caecal epithelium on day 20 (Fig. 39) and on the colo. epithelium the following day. Although impossible to characterize, the tight, regular coiling strongly suggests that this organism is a type C or D spirochaete.

Subsequent to day 24, SEM revealed that small loosely-coiled spiral bacteria were often associated with foci in the epithelium in which the microvillus surface had been eroded (Figs. 40 and 41). TEM of this tissue, revealed penetration of epithelial cells by two morphologically distinct spiral bacteria, spirochaetes with 2-4-2 periplasmic fibril arrangements, as well as the organisms with the loose, outer membrane (Figs. 42, 43 and 44).



Fig. 39 Helical bacteria, probably spirochaetes on microvilli (MV) of caecal epithelium of 30 day old conventional rat. The diversity of the epimural flora is evident. Bar=5μm.



Fig. 40. Loosely coiled spiral organism (arrow) associated with area of caecal epithelium of 24 day old conventional rat, in which microvilli (MV) have been eroded. Bar=5μm.

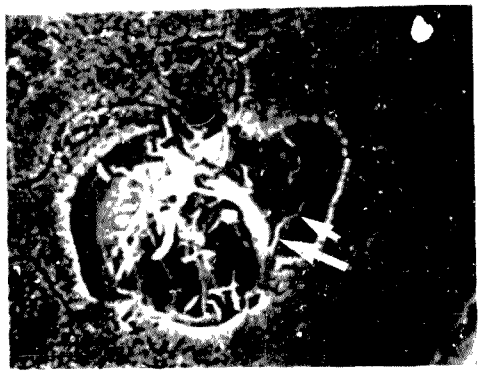

Fig. 41. Numerous loosely coiled bacte on area of caecal epithelium of 33 day old conventional rat in which microvilli (MV) have been destroyed. Two types of spiral bacteria distinguishable by different cell diameters, are present (large and small arrows). Bar=5μm.

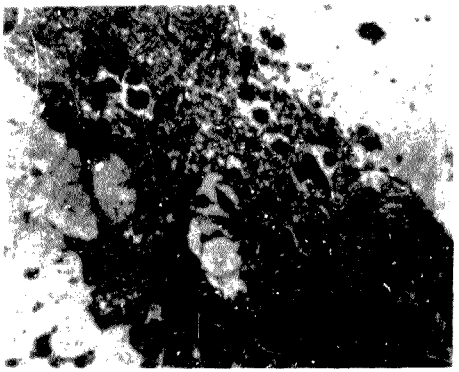

Fig. 42. Bacteria within caecal epithelial cell of 33 day old conventional rat. At this magnification the two types of bacteria could be distinguished by different cell diameters. Large arrows, organism with loose outer membrane; small arrows, spirochaete with two periplasmic fibrils. Bar 1 µm.

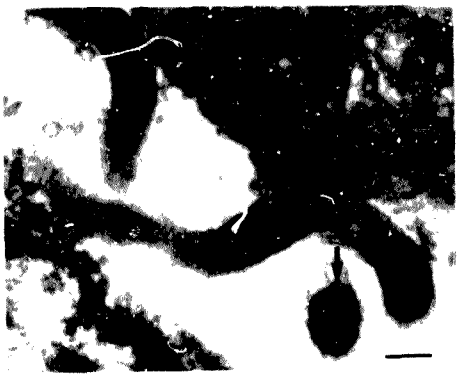

Fig. 43. Longitudinal section of spirochaetes within caecal crypts of 33 day old conventional rat. Arrows illustrate 2 periplasmic fibrils in each organism. Bar 0.2µm.

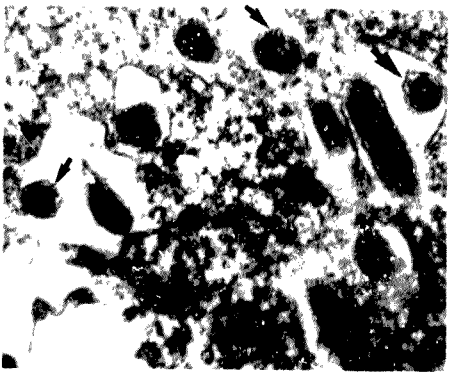

Fig. 44 Transverse section of spirochaetes within caecal epithelial cell of 33 day old conventional rat. Two (large arrow) and 4 (small arrows) periplasmic fibrils are evident, indicating that these invasive bacteria probably have a 2:4:2 periplasmic fibril arrangement. Bar=0.2μm.

2.4. Discussion

In this study it has been shown that at least four morphologically distinct spiral bacteria with ultrastructures identical to those described for "classical" spirochaetes, reside in the conventional laboratory rat GIT. In addition, bacteria possessing both periplasmic fibrils and flagella-like appendages are also consistently found in this habitat. Spirochaetes are localized primarily in the large intestine (caecum and colon) (Fig. 4), although the large "atypical"

spirochaetes may also inhabit the ileal region of the small intestine (Fig. 4e). Despite inherent sampling errors in the techniques employed, most of the bacteria were observed in all 15 of the rats examined, thus fulfilling one of the criteria necessary for establishing autochthonicity. Those organisms not observed in all animals (type D spirochaetes), may have been present at population densities below the level of detection. Samples examined by negative staining - electron microscopy (the most accurate and objective method for defining the ultrastructure of each organism) were of necessity very small, thereby adding to experimental error, whereas light microscopy, which enabled larger samples to be examined, were more subjective and misclassification of organisms particularly types C and D could easily have arisen, despite a conscious effort to avoid these experimental errors.

Enumeration of different spirochaete types in different rats, revealed some variation, which could be a function of counting inaccuracies and/or a function of natural variation within the population. Similar deviations in spiral bacterial populations have been reported in dogs (Pindak, *et al.*, 1965). Individuals with different somatic antigen compositions may harbour different flora, and this could be particularly true for those spiral bacteria with habitats in close proximity to epithelial cells. Factors such as diet, state of health and even perhaps the stage in the oestral cycle of female rats, could affect the microbial composition of the gut. Diet has been shown to have a profound effect on the composition of the rat gastrointestinal flora, although the helical bacterial population was not examined (William-Smith, 1965a). In dogs, the number and types of spiral bacteria in the GIT is subject to diurnal variations (Pindak, *et al.*, 1965) and this could be true for all animals, including rats.

In an attempt to accurately identify the precise habitats of each of the "classical" spirochaete types, fluorescent antibody tracing using antibodies raised against these spirochaetes, was attempted. Unfortunately, although the antisera were spirochaete-specific, none were strain-specific, (Fig. 5) and hence the earlier finding that these bacteria were localized in the distal regions of the gut was confirmed, but the precise location of the gastrointestinal spirochaetes was not revealed. More valid information would perhaps have been attained had the antisera been pre-adsorbed with heterologous spirochaete isolates.

The gastrointestinal spirochaetes were comparatively late colonizers of the infant rat, only becoming established when the oxygen potential of the gut would have been reduced by the preceding colonizers such as the facultative anaerobes and microaerophilic campylobacters, observed in this study to be early colonizers. Although the colonization pattern of gut bacteria in infant rats has previously been examined, no mention of spiral bacteria was made, since the authors based their study entirely on cultivable bacteria and did not employ selective techniques essential for the growth of these bacteria (Raibaud, *et al.*, 1966a; 1966b; Williams-Smith, 1965a). An investigation into the colonization sequence of mouse intestinal flora revealed that spiral bacteria were among the first to colonize the mucous blanket where they attained population densities of 10^8-10^9 bacteria/g of whole bowel within 1 week of birth (Davis and Savage, 1972; (Savage, 1970; 1972). These primary colonizers, had a single flagellum extending from each terminal end and were thus morphologically similar to the first of the helical bacteria seen colonizing the rat GIT (Davis *et al.*, 1973).

Although the sampling size of the population in this succession study was small, and therefore particularly prone to errors, the high

population densities attained on the epithelial surface by both the campylobacter-like organisms and the type A spirochaetes prior to their decline to climax adult levels was interesting (Figs. 23 and 31). It is tempting to speculate that those homeostatic factors which regulate the population size in adult animals were responsible for this decline in the population numbers. The adult rat gut flora has been shown to have a regulating effect on bacterial colonization in the neonatal rat gut (Lawrence *et al.*, 1982). Since the aim of the colonization study was to determine whether spirochaete sub-populations colonized the infant gut during normal colonization events; and this was shown to occur at similar times in two separate litters; the neonatal population size was not increased. Nevertheless, it would be particularly interesting to establish whether crypt colonization by type A spirochaetes on day 26, is a general colonization phenomenon, or was merely a localized incident; in which case the predisposing factors enabling such a colonization to occur could be investigated. The animal concerned, did not display any obvious gastrointestinal disorder. It is also interesting that in this 26 day-old individual, the colonic crypts were packed with helical bacteria with the characteristic outer membrane (Fig. 29), whereas the caecal crypts were colonized exclusively by the multifibrillar spirochaetes (Fig. 35). In contrast to the findings by Lee, (1980), this distinct localization by helical bacterial sub-populations was not observed in adult animals.

As is the case with other animals, major perturbations in the gut population occurred at weaning. Infant rats begin to consume solid food on day 16 when they also begin to nibble faecal pellets, thus seeding the gut with adult flora (Lane-Petter, 1976). This coincides almost exactly with bacterial diversification of the gut population, the increase in caecal size and the commencement of a mass penetration into the epithelial tissue by the gut flora. Mice begin to

consume solid food on day 13 (Lane-Petter, 1976) and from day 10 - day 18 profound rearrangement of the gut bacterial population occurs (Savage *et al.*, 1967). By the time weaning of the infant rats was complete (day 30), all five types of spirochaetes had reached adult climax levels. Prior to weaning, TEM of epithelial tissue revealed that the gut was lined by a dense granular material with a texture different to that of mucus, (Fig. 21). This was interpreted as being milk protein, which, by coating the epithelial cells could possibly act as an inhibitor to penetration of the epithelium by potential pathogens at a time when the infant would be unable to elicit an immune response to limit the effects of the challenge. This proteinaceous layer could act as a physical barrier by inhibiting binding of potential pathogens to epithelial cells, have an antibacterial activity by lactoferrin action and could also act as an immunological protecting agent in that it would contain maternal secretory IgA.

Massive penetration of gut epithelia by members of the normal gut microbiota is believed to cause neonatal necrotizing enterocolitis (NEC) which is a frequently fatal disease affecting preterm human infants in the first 10 days of life (Stark and Lee, 1982a). Weanling diarrhoea which is prevalent in breast-fed human infants is also believed to be caused by massive perturbation within the gut ecosystem at weaning (Stark andLee, 1982b), and may involve a penetration of the mucosal epithelium similar to that observed in this TEM study of weanling rat gut tissue (Fig. 24). Once in the lamina propria, the virgin but competent immune system of the infant would elicit a primary immune reaction against the invading bacteria and eby limit the infection. Perhaps the fatality associated with NEC is due to the incompetent immune system of the premature neonate which is unable to respond to the bacterial challenge. Although helical bacteria were among the microorganisms that penetrated the

gut epithelium, there was no evidence that they were the primary invaders. Such information could possibly be attained by a critical time-sequence study.

The lack of consistent spirochaete colonization of the stomach and small intestine, together with the sporadic appearance of low numbers of different types of spiral organisms in these areas of the adult, strongly suggests that spirochaetes are not autochthonous to these areas, but are merely transients arising from coprophagy or even perhaps translocation of oral spirochaetes. Reports concerning the colonization of the stomach epithelium by spiral bacteria possessing both periplasmic fibrils and flagella-like appendages have been made for a variety of animals (Doenges, 1951; Lockard and Boler, 1970; Pindak *et al.*, 1965; Weber and Schimitdiel, 1962) and in each case, the bacteria were associated with the fundic portion of the stomach. It is unfortunate that this region of the gastric mucosa was not processed for electron microscopy, but at the time, it was thought that the hydrochloric acid secreted by the fundic glands would not permit the colonization of spirochaetes in this habitat. Spiral bacteria capable of colonizing this habitat, would certainly warrant further study.

Sightings of spirochaetes in the distal small intestine, could have arisen by translocation of these bacteria from the caecum to the ileum via the ileo-caecal junction and this is probably true for those spirochaetes that were only occasionally seen. Nevertheless, spiral organisms with a characteristic loose outer membrane were consistently seen inhabiting the crypts of this area. The fast flow rate of lumen contents in the small intestine, necessitates the need for bacteria residing in this area to have a fast growth rate, to be firmly attached to the epithelium, or to be localized within the crypts of Lieberkuhn. The cryptal location of these bacteria would explain

why they were not observed by SEM. These bacteria are similar in both size (3-8μm x 0.6μm) and morphology to those previously described by Wendelschafer *et al.*, (1973) and are probably equivalent to the type 3 organisms described by Davis *et al.* (1972b) who saw them in the rat caecum as did Gustafsson and Maunsbach (1971). In contrast to the reports by Wendelschafer *et al.* (1973) and Phillips *et al.* (1978), these bacteria were never observed in intimate contact with either epithelial cells or goblet cells nor were they seen within epithelial cells, although they did invade the lamina propria of the infant rat. Reports by Lee (1980), that these bacteria are restricted to colonic crypts were not confirmed in this study, since they were also found in caecal and ileal crypts. In young, weanling rats however, colonic crypts were almost exclusively colonized by large numbers of these distinctive spiral organisms (Fig. 30).

Also seen occasionally on the ileal epithelium and more frequently on the large intestine epithelium, were the large spiral organsims that had both thick periplasmic fibrils and flagella-like appendages. High population densities, both on the epithelium, where they were embedded in the mucous layer (Figs. 8, 9 and 10) and in the lumen contents were attained. In cross-section, these bacteria had distinctive shell-like structures (Figs. 18 and 19), not unlike that described for members of the *Pillotina* genus which inhabits the hindgut of termites and cockroaches (Margulis *et al.*, 1981; To *et al.*, 1978). The large size and crenulations (pleats) in the outer membrane are also characteristic for this genus (Margulis *et al.*, 1981). It is possible that two species of these bacteria were represented in the rat gut, since the population was heterogeneous in size and number of periplasmic fibrils, with the smaller organisms (1.0μm in diameter) having 9 fibrils and the larger (1.5μm in diameter) having 15 fibrils (Figs. 18 and 19). Similar procaryotes have

been described by Erlandsen and Chase (1972), Phillips *et al.* (1978), Phillips and Lee (1983) and Wendelschafer *et al.* (1973), who all saw these bacteria in rat ileal crypts, where they frequently attained high population densities. No evidence was seen of crypts packed with large numbers of the aforementioned bacteria, nor their digestion by paneth cells (Erlandsen and Chase, 1972). were not observed in this study. Identical organisms were also described by Davis *et al.* (1972b), who saw them in the caeca of 6 out of the 15 rats examined, whereas in this study, these pillotina-like bacteria were present in the large bowel of all rats. In contrast to Davis *et al.* (1972b), but like Phillips and Lee (1983), these bacteria were never observed within epithelial cells or lamina propria.

TEM of adult GIT tissue revealed that spirochaetes with numerous periplasmic fibrils frequently penetrated into epithelial cells and even into the lamina propria (Fig. 13). Similar bacteria were also seen colonizing the infant gut epithelium, and in one infant attained such high population densities, that every caecal crypt was packed exclusively by these bacteria (Figs. 31 and 34). Although their numbers subsequently declined to *c.* 10^{8} organisms/g of epithelial tissue, they remained the most dominant of the "classical" spirochaetes in the rat GIT and were found in the lumen contents, in the mucous blanket and in close association with the epithelial cells. Similar bacteria have been described by Phillips *et al.* (1978) as well as by Davis *et al.* (1972b) who described them as penetrating into goblet cells, a phenomenon only seen in the infant gut epithelium in this study.

The invasion of adult epithelial cells by the aforementioned multifibrillar treponemes seemed to be a spontaneous, yet random process. Some cells were heavily infected while adjacent cells were completely free of microorganisms. This may be a function of the

cell, with age or some other factor enabling the bacteria to invade. Massive epithelial damage and penetration such as that observed in the infant rat gut, was never observed in the adult, although these treponemes were among the invasive bacteria of the infant rat. As early as 1916, gastrointestinal spirochaetes were described as having the ability to penetrate into epithelial cells (Macfie, 1916), but the mechanism by which they do this, is still obscure. It is generally assumed that spirochaetes enter cells by engulfment, but if this were the case, they would be enclosed within a membrane-bound vesicle, which was never apparent in this study. It has also been proposed that spirochaetes could have the ability to "swim" through animal cell membranes (Greenberg and Canale-Parola, 1977). Spirochaetes, within cells were generally associated in clumps (Fig. 13), and Glock *et al.* (1974), attributed this phenomenon to intracellular spirochaete division. Alternatively, several bacteria may simultaneously penetrate the cell at a particular site in the cell membrane.

The infestation of epithelial cells by spirochaetes is similar to that reported for swine dysentery (Glock and Harris, 1972; Glock *et al.*, 1974; Taylor and Blakemore, 1971) and even salmonella invasion of cells (Pasiel and Turnbull, 1985), although in these cases, degenerative changes in the infected cells occur and an inflammatory response is evoked. Perhaps in the past, members of the rat population were afflicted by a disease similar to swine dysentery, but with the passage of time, mechanisms limiting pathogenicity have been evolved. In this context, it is interesting that SPF Wistar rats as opposed to conventional Wistar rats did not harbour any of the spiral bacteria described in this study. The significance of this finding is unknown. In mice, the gut microflora in conventional animals is similar in all basic respects to that of SPF animals (Dubos *et al.*, 1965) and this includes the spiral procaryote population which is present in SPF mice (Davis and Savage, 1972a).

The rat multifibrillar spirochaetes not only penetrated into epithelial cells, but also appeared to traverse the basement membrane directly (Fig. 14). This suggests that the spirochaetes may be capable of direct enzymatic breakdown of the basement membrane collagen matrix, perhaps by hyaluronidase activity which is present in *T. pallidum* but not in *T. denticola* or *T. vincentii* (Fitzgerald and Gannon, 1983). Despite the presence of spirochaetes in the lamina propria, no obvious inflammatory response was initiated by the host. This could be related to the finding that extracts from a number of spirochaete species can suppress lymphocyte proliferation (Shenker *et al.*, 1984). *In vivo T. pallidum* may escape host recognition by adsorbing host proteins onto its outer membrane (Cox *et al.*, 1984): a ploy that could be utilized by the gastrointestinal spirochaetes. It has also been proposed that the outer membrane may shield the majority of treponemal proteins from surface exposure and thereby protect the pathogen from host recognition (Penn *et al.*, 1985). Alternatively, host unresponsiveness to these bacteria may be due to co-evolution of the host and the bacterium, which is a requirement for autochthonicity.

The other spirochaete subpopulations described in this study have not previously been reported as inhabiting the rat GIT, probably because previous studies have concentrated on the epimural and particularly the cryptal microflora. The low population densities attained by the spirochaete with the 1-2-1 periplasmic flagella arrangement, may explain why other workers have not reported the presence of this organism on the epithelial surface. Spirochaetes with the periplasmic fibril arrangements of 2-4-2 and 3-6-3 selectively colonized the lumen contents of the large bowel (Figs. 4b and 4c). This is in direct contrast to numerous reports where spirochaetes are considered as only being associated with the mucosal layer (Davis, 1976; Knehans and O'Del, 1980; Leach *et*

al., 1973; Savage *et al.*, 1971). Despite being associated with the epithelium and even residing in the crypts, the large multifibrillar spirochaetes as well as the pillotina-like organisms also attained high population densities in the lumen contents (Figs. 4a and 4e). This is in conflict to the findings of Phillips *et al.* (1978), who rarely saw these treponemes in the lumen. In contrast to the findings of Lee (1980), who described different spiral bacterial populations colonizing highly specific areas within the adult rat gut, no perceptible difference in the types of spiral organisms inhabiting the caecum and the proximal colonic epithelium could be discerned in this study. However, the reduction in both numbers and frequency of spiral bacteria seen in the distal colon (Fig. 4) does suggest that this area of the GIT is not the natural habitat of these bacteria, but that they are merely transients in this locality. Environmental conditions in the distal and proximal areas of the colon are so different that it is unlikely that similar autochthonous populations would be present in both areas.

Total counts of the more dominant spirochaete types in the adult revealed that a population density of 10^{8} organisms/g of lumen contents or epithelial tissue could be attained. Although these counts are subject to inherent errors, they do indicate that the spirochaete population could have a significant effect on the GIT ecosystem. Savage *et al.* (1971), estimated that the population levels of spiral bacteria on the mouse colonic epithelium can reach 10^{9} organisms/g but this was not limited to spirochaete sub-populations. In swine, small spirochaetes of similar dimensions to those in the rat GIT and having 1 or 2 periplasmic fibrils, attain densities of 10^{7}-10^{8}/g of colonic contents (Harris and Kinyon, 1974), and thus are similar to the population densities reported in this study.

In conclusion, four morphologically distinct spirochaetes with "classical" ultrastructure and possibly two types of "atypical" spirochaetes possessing both periplasmic fibrils and flagella-like appendages, were recognized as inhabiting the large bowel of the conventional rat. These bacteria all colonized the infant rat in a sequential pattern and were consistently seen in the adult animal. From published reports, at least some of these bacteria are present in rats of different colonies on a global scale, being found in the United States, Australia and now South Africa. These factors, strongly imply that spirochaetes are constituents of the rat gastrointestinal autochthonous microflora.

CHAPTER 3

ISOLATION AND CHARACTERIZATION OF RAT GASTROINTESTINAL SPIROCHAETES

3.1 INTRODUCTION

Refractiveness to *in vitro* culture, is a common feature of gastrointestinal spirochaetes, and, with the exception of swine enteric treponemes, spirochaetes from the monogastric gut have rarely been isolated and biochemically characterized. Initial success in the cultivation of these bacteria arose from concerted efforts to grow the causative agent of swine dysentery. Three species of *Treponema* have been isolated from the swine caecum, but only one of these; *T. hyodysenteriae* is pathogenic (Harris *et al.*, 1972a; 1972b). *T. innocens*, is morphologically and serologically indistinguishable from *T. hyodysenteriae* (Kinyon and Harris, 1979), and was originally thought to be a non-pathogenic variant of the swine dysentery agent; but low DNA homologies (Miao *et al.*, 1978b), unrelated haemolysins (Saheb *et al.*, 1981a), different fatty acid profiles (Matthews and Kinyon, 1984) and other criteria, have proved that this spirochaete is a separate species. Strains of *T. innocens* have also been isolated from dog caeca ("puppy strain") (Kinyon *et al.*, 1977) as well as from feral rodents (Joens and Kinyon, 1982). The other non-pathogenic swine spirochaete species is *T. succinifaciens* which in earlier literature was termed the "small spirochaete" and strain 6091 (Cwyk and Canale-Parola, 1979).

Attempts to cultivate the bacteria associated with the condition known as intestinal spirochaetosis were also, for many years, unsuccessful. Eventually, employing a high hydrogen gas phase, Hovind-Hougen *et al.* (1982), succeeded in growing these

organisms, and, on the basis of a lack of cytoplasmic microtubules, classified them as belonging to a new spirochaete genus; *Brachyspira* (gen. nov.). Treponemes have also been isolated from the caeca of opossums infected with intestinal spirochaetosis (Turek and Meyer, 1979). Neither of these isolates, have been fully characterized.

Spiral bacteria from the rodent gut have been cultivated, but most of these have ultrastructures consistent with the *Campylobacter* genus (Lee and Phillips, 1978; Roach and Tannock, 1979; Savage *et al.*, 1972). Lee and Phillips, (1978), did however isolate a "classical spirochaete" from the mouse caecum, but failed to characterize the organism. These authors were unsuccessful in cultivating spirochaetes from the rat caecum. More recently, the same authors isolated large spirochaetes possessing both periplasmic fibrils and flagella-like appendages from the rat small intestine (Phillips and Lee, 1983). These bacteria are not "classical spirochaetes" but share an ultrastructure more similar to that of members of the *Pillotina* genus. Spiral shaped bacteria from the rat large intestine, have never been cultivated (Phillips and Lee, 1984).

Proposals that spirochaetes can be classified purely on ultrastructural criteria at the electron microscope level (Hovind-Hougen, 1976) are generally considered to be inadequate, and consequently, spirochaetes from different animal hosts can only be compared once they have been subjected to exhaustive biochemical and chemical characterization. The finding that rodents, can, under laboratory conditions, transmit *T. hyodysenteriae* from one swine herd to another (Joens, 1980), emphasizes the need for a study on the natural spirochaete population in these and similar animals. It was for this reason that spirochaetes from rat GIT were isolated,

characterized and taxonomically compared with *T. hyodysenteriae* and *T. innocens*. Furthermore, speculation on the niche occupation of an organism in its natural habitat can only be made after the biochemical potential has been determined in pure culture. To this end, isolates from the rat GIT were subjected to biochemical analysis and speculations on their function in the gastrointestinal ecosystem were made.

3.2 MATERIALS AND METHODS

3.2.1 In vitro culture

Primary Isolation

Inocula were obtained from 25 conventional Wistar rats (*Rattus norvegicus*), many of which were also used for determining the position of spirochaetes in the rat GIT. Material for culturing purposes was taken prior to fixation. Samples were taken from the stomach, duodenum, ileum, caecum and proximal large colon from the first four animals, while only caecum and colon were sampled from the other rats. Animals were sacrificed by dislocation of the neck and were immediately transferred into, and dissected in, a Forma anaerobic chamber (Forma Scientific, Idaho, USA) with a gas phase of 83% nitrogen, 12% carbon dioxide and 5% hydrogen, operating at a redox potential of less than -180mV as shown by indicator dyes. In each case, a 1 in 5 dilution of lumen contents was made, using sterile anaerobic diluting fluid (ADF) (Appendix D(2)) as the diluent. Epithelial tissue (c. 4cm^2) was washed lightly in diluting fluid and either homogenized in a sterile Griffith's tube with c 1mℓ of ADF, or the mucosal surface scraped with a sterile blade, the scrapings picked up with a pasteur pipette and placed into 1mℓ of

diluting fluid. Samples for culturing were also taken from 5 SPF rats and infants used in the succession study.

Culture media

media were prepared anaerobically (Appendix D(1)) and were duced in the anaerobic cabinet for at least 48 hours prior to on. Modified RGCA and E media (Holdeman *et. al.*, 1974) (Appendix D(4) and D(5)) were routinely used for the isolation of the IT spirochaetes. These media were modified, in that 10% citrated ine or human blood was used instead of haemin and inactivated m, and after initial tests, vitamin K and cocarboxylase were omitted. Furthermore, 0,1% thioglycollate was used as a reducing agent instead of cysteine-HCl, and for primary isolation, 3% agar was used. The medium employed by Turek and Meyer (1977) as well as that used by Smibert and Claterbaugh (1972) (Appendix D(6) and D(7)) were also used on three occasions, but were not routinely used since a wide variety of morphologically distinct spirochaetes were not cultivated, and spirochaetal growth was less prolific than on the other media. RGCA medium containing 0.3% polygalacturonic acid (Alltech) instead of cellobiose, glucose and starch was also used on two occasions but did not support spirochaete growth. All cultures were incubated anaerobically in the gas cabinet at 37°C for 7 days. In addition, on two occasions both E and RGCA media were incubated under microaerophilic conditions, achieved by using a gas-pak system (BBL) without palladium catalyst.

Selective isolation techniques

Spirochaetes were selectively isolated from other intestinal bacteria by utilizing the motility properties of these prokaryotes. The "fil-

ter" method, selects for small spirochaetes, which can migrate through membrane filters whose pore size is too small for most other bacteria (Smibert, 1981). The "well" method, selects for bacteria capable of migrating through agar concentrations of more than 3%. Spirochaetes are thought to migrate through the agar in search of limiting growth factors in the medium, to which they are chemotactically attracted (Canale-Parola, 1978). In addition, intrinsic resistance by treponemes to certain antibiotics was used to enrich for spirochaetes, usually in combination with other selective techniques. The antibiotics, naladixic acid, rifampicin, and polymyxin B are all recommended by Holdeman *et al.* (1974), while the antibiotic spectinomycin is used to selectively isolate for *T. innocens* and *T. hyodysenteriae* from swine caecal contents (Songer *et al.*, 1976). Of these antibiotics, rifampicin and polymyxin B were extensively used for isolating rat gastrointestinal spirochaetes. The mechanism by which spirochaetes exhibit resistance to these antibiotics is not known, although it has been proposed that rifampicin resistance is due to low affinity of spirochaete RNA polymerase for the antibiotic (Leschine and Canale Parola, 1980). Resistance to polymyxin B could be due to the lack of LPS in treponemes, which is the target of this antibiotic (Penn *et al.*, 1985).

Filter method

Sterile filters (Millipore) of 0.22 or 0.45 µm pore size (25mm diameter) were placed directly onto the surface of isolation medium plates. A ring of sterile vaseline was piped around the edge of the filter, and an o-ring from the filter unit was placed directly onto the vaseline ring. A drop of inoculum (0.05 mℓ) was placed onto the filter.

Well method

A well which had been cut into the center of agar plates using a glass tube of approximately 5mm in diameter, was filled with inoculum. Plates were incubated in an upright position.

Antibiotics

The antibiotics polymyxin B (Wellcome); rifampicin (Ciba Geigy); naladixic acid (Boehringer) and spectinomycin (Upjohn) were incorporated into the isolation medium. The concentrations at which these antibiotics were employed, and the solvent in which they were dissolved, are indicated in Table 3.

Table 3. Concentrations and solubilities of antibiotics incorporated into culture media for isolating rat GIT spirochaetes.

Antibiotic	Solubility	Final concentration
polymyxin B	water	88 units/ml
rifampicin	ethanol	1µg/ml
naladixic acid	1N NaOH	800units/ml
spectinomycin	water	400µg/ml

Antibiotic solutions were filter sterilized and added aseptically to sterile culture media. The antibiotics were used either individually, or in combination, and with both the well and filter isolation methods. Polymyxin B and rifampicin, were particularly useful in inhibiting the growth of contaminating bacteria without affecting the spirochaete growth. Of the isolation techniques, the well method, used in conjunction with polymyxin B and/or rifampicin was the most successful for isolating rat gastrointestinal spirochaetes.

Purification of primary cultures

Spirochaete growth could be visualized as a fine haze spreading over the culture medium, usually extending 2-3 cm from the well or filter. Initially, all spirochaete growth was subcultured onto prereduced media of similar composition to that used for isolation, but once it had been ascertained that they could grow on modified RGCA medium and/or Bacto tryptose blood agar base (TSBA) (Difco) containing 5% citrated bovine or human blood, they were routinely subcultured onto one of these media, not usually containing any antibiotics. For subculturing spirochaetes, a small agar block of *c.* 1cm² was cut from an area displaying spirochaete growth and placed in an inverted position directly onto the new culture medium. Spirochaetes would then migrate away from the agar blocks. By continuously selecting for spirochaete growth, pure cultures of spirochaetes could be obtained after 3-4 weeks. Purity of cultures were assessed using the modified Gram stain (Appendix A(1)) and by examination at the electron microscope level using negative staining techniques (Appendix B(7)).

In an attempt to obtain axenic spirochaete cultures, contaminated primary cultures were streaked for isolated colonies onto agar plates containing 3% agar. In addition, roll tubes containing modified RGCA medium (Appendix D(4)) supplemented with 5% foetal calf serum (FCS) instead of blood were also prepared and inoculated with contaminated cultures scraped off agar plates and suspended in saline. Manipulations were all done in the anaerobic cabinet, while roll tubes were prepared on an Astell roll-tube apparatus (Astell, London, UK) using the Hungate anaerobic technique (Holdeman *et al.*, 1974). Cultures were incubated at 37°C for a week and colonies were picked off under a dissecting microscope and stained using the modified Gram stain (Appendix A(1)).

Maintenance of cultures

Lyophilization

Spirochaetes were harvested from RGCA maintenance medium by flooding the plates with sterile ADF (Appendix D(2)) and scraping growth off with a sterile glass spreader. The spirochaete suspension was centifuged at 2 500g for 15 min in gas-tight glass centrifuge tubes and the pellet resuspended in minimum amount of diluting fluid. Spirochaetes were added to 0.1mℓ aliquots of either heat-inactivated horse serum containing 7.5% (w/v) glucose, or skim-milk solution with 0.3% sodium ascorbate as reducing agent, both of which had been prereduced in the cabinet for 24 hours. All manipulations were done in the anaerobic cabinet. Vials were sealed, transferred to the freeze-dryer (VirTis Co. Ltd. New York, USA) and organisms briefly exposed to air while bungs were removed so that the vials could be placed in the freeze-dryer. Cultures were lyophilized overnight, and viability checked the following day and then at monthly intervals for 3 months. For reconstitution, cultures were resuspended in minimum amount of ADF and inoculated onto RGCA maintenance medium plates.

Freezing at -70°C.

Cultures were harvested as described above, and resuspended in liquid medium (Appendix D(8)) containing 20% glycerol as a cryoprotectant. Tubes were sealed with gas-tight stoppers and placed immediately into a -70°C deep-freeze. Viability was checked 7 days later and then at monthly intervals for 3 months by inoculating onto RGCA maintenance medium plates.

Agar slants

Maintenance medium agar slants were prepared, cultures inoculated onto the slants, grown at 37°C for 4-5 days, and maintained at 11°C. Cultures were subcultured every 2 months.

Liquid cultures

Spirochaete cultures are often refractive to growth in liquid culture media, and those isolated in this study were no exception. Unsuccessful attempts to obtain liquid cultures included:

- inoculation of liquid media of similar composition to that used for maintaining and isolating the organism,
- inoculation onto agar slants, superimposed with a nutrient liquid medium,
- inoculation onto agar slants containing salts, blood and rumen fluid and a liquid phase containing peptones and carbohydrates,
- gradually decreasing agar concentration.

Limited success was finally obtained using the Lemcke technique for growing *T. hyodysenteriae* in liquid culture (Lemcke *et al.*, 1979b), in which cultures are incubated in minimum amount of medium in a slanted position. The composition of the liquid growth medium and method for preparation are given in Appendix D(8). Using this technique, spirochaete isolates with numerous periplasmic fibrils could be cultivated although other isolates did not grow. Growth could be visualized as a fine, non-uniform, hazy turbidity during log phase and a white precipitate on the glass surface during stationary phase of growth. Incubation time was variable and depended on initial inoculum size, as well as the physiological state

of the inoculum culture. In order to standardize conditions, an inoculum volume of 10% of a fresh, late log-phase culture was routinely used. This gave a final concentration of *c.* 5 x 10^4 spirochaetes/mℓ of culture medium and maximum numbers of bacteria were attained after 10 days of incubation at 37°C. Growth was assessed using a Bausch and Lomb Spectronic 1001 spectrophotometer (Bausch and Lomb, Rochester, New York, USA) with a 1 cm light path and a wavelength of 640nm. Using uninoculated culture media as a blank, maximum turbidity that could be attained was *c.* 0.4 units.

Growth requirements of isolated spirochaetes

Solid media

Growth requirements of all the isolated spirochaete strains were determined by omitting rumen-fluid, blood or carbohydrates from RGCA maintenance medium. Control plates were simultaneously inoculated, and all cultures were incubated anaerobically for two weeks, after which, plates were examined and Gram stains made of all cultures.

Liquid culture

Analysis of growth requirements and optimum growth conditions of those spirochaetes that would grow in liquid culture was determined in the liquid culture medium described previously, with the exception that the medium was not supplemented with 0,5% yeast extract since it was only in this study that the advantages of this supplement became apparent.

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Variations made to the basic liquid medium (Appendix D(8)) included:

- replacement of foetal calf serum with

  a. heat inactivated (56°C for 15 min) horse serum (Gibco)

  b. heat inactivated rabbit serum, prepared in the laboratory from rabbit blood.

- addition of a vitamin solution (2ppm biotin, 2ppm folic acid, 10ppm pyridoxine hydrochloride, 5ppm riboflavin, 5ppm thiamine, 5ppm nicotinic acid, 5ppm pantothenic acid, 0.1ppm vitamin B_{12}) (Wolin *et al.*, 1963).

- addition of trace mineral solution (3000ppm $MgSO_4$, 1500ppm nitrilotriacetic acid, 1000ppm NaCl, 500ppm $MnSO_4$, 100ppm of $FeSO_4$, $CaCl_2$, $CoCl_2$, $ZnSO_4$ and 10 ppm of $CuSO_4$, Al_2SO_4, H_3BO_3 and Na_2MoO_4) (Wolin *et al.*, 1963).

- addition of a volatile fatty acid solution (0,17% acetic acid, 0,06% proprionic acid, 0,04% butyric acid and 0,01% valeric, isovaleric and isobutyric acids).

- addition of 0.5% yeast extract (Oxoid).

- change of gas phase from 100% CO_2 to 100% N_2.

- incubation at various temperatures (42°C, 30°C, and 25°C).

- addition of cocarboxylase (0.5mg/100mℓ) and vitamin K (0.1mg/100mℓ).

- cultures incubated in a vertical position.

Control cultures were included in all studies, and all cultures were examined for turbidity at 5 day intervals for a period of 30 days. Turbidity was assessed by determining absorbance at 640 nm.

To determine whether spirochaete isolates had an absolute requirement for carbohydrates, a sugar-free medium was prepared. The papaic digest of soybean meal in trypticase soy broth (Oxoid CM129) intrinsically contains glucose (Oxoid manual). Initially, a formulated trypticase soy broth containing the constituents of CM129 (Appendix D(9)) but lacking the papaic digest was used in Lemcke liquid growth medium (Appendix D(8)). This failed to support spirochaete growth and a range of peptones were added to the basic medium at a concentration similar to that found in trypticase soy broth, *viz.* 0.3%. Peptone aus fleisch (Merck); mycological peptone L40 (Oxoid); bactopeptone (Difco); pepton (indeterminate origin); bacteriological peptone L37 (Oxoid) and proteose peptone (Difco) were all added individually to the tryptone medium. Peptones were autoclaved separately and aseptically added to the basal medium. All cultures were inoculated from the same stock culture and were examined at two day intervals for the presence of spirochaetal turbidity. Only mycological peptone L40, bacteriological peptone L37 and pepton supported adequate growth, and on this basis carbohydrate-free Oxoid bacteriological peptone L37 was chosen to replace the papaic digest of soybean meal component in the formulated trypticase soy broth medium.

Growth curve

A growth curve of isolate D29 grown in Lemcke liquid growth medium (Appendix D(8)) was constructed. Evaluation of bacterial numbers

at various sampling times was problematical since spirochaetes would not grow as isolated colonies on agar plates or in roll tube cultures. For this reason, a viable count could not be ascertained, but the curve was constructed using a total count obtained with a Neubauer counting chamber. Liquid culture media were inoculated with active log phase cultures at final concentrations of 5 x 10^4/mℓ. Four, 30mℓ cultures were set up, and at 24 hour intervals, 0.3mℓ aliquots were withdrawn from each culture and combined. Total counts and optical density were determined for each sample. While doing total counts, it was noticed that as the culture approached stationary phase, the number of spiral forms of the organism decreased and a number of granular forms were seen. These variants of the organism were counted separately. The growth curve for isolate D29 was repeated three times. The morphology of the spirochaetes at different stages of the growth curve was determined using negative staining - transmission electron microscopy (Appendix B(7)).

Culture of spirochaete granules

To determine whether the round granular forms of spirochaetes were reproductive units, a liquid culture was incubated for one month and then used to inoculate Lemcke liquid growth medium (Appendix D(8)). Phase-contrast microscopy revealed that this culture was composed predominantly of granular forms, although an occasional helical organism was seen. Cultures containing no helical forms and which had been maintained at 4°C for 3 and 6 months were also used as inocula. Inoculated cultures were incubated at 37°C for three months before being recorded as negative for growth.

3.2.2 Biochemical characterization

The difficulty of routinely culturing spirochaete isolates in liquid medium meant that the majority of biochemical tests had to be performed on agar medium. In addition, standard biochemical media (MacFaddin, 1980; Smibert and Krieg, 1981a) were modified to accommodate the anaerobic growth and fastidiousness of these organisms. Unless otherwise stated, all biochemical tests were done on prereduced Bacto tryptose blood agar base (Difco) supplemented with 10% foetal calf serum (FCS) (Republic of South Africa, State Vaccine Laboratories) and other, appropriate substrates added to the medium. All test were done in duplicate, and positive and negative controls using anaerobic bacteria or facultative anaerobic bacteria grown under anaerobic conditions were used wherever possible. *T. hyodysenteriae* (ATCC 27164) and *T. innocens* (ATCC 29796) were also routinely tested. Spirochaete strains were inoculated from maintenance medium onto the test medium and were incubated in the anaerobic gas cabinet at 37°C for 7 days. Biochemical parameters selected were those listed for the characterization of *Treponema* species in Holdeman *et al.* (1974), as well as other characteristics used for the differentiation of anaerobic bacteria.

Oxidase

Strips of filter paper were impregnated with oxidase reagent (1% tetramethyl-p-phenylenediamine dihydrochloride) (Sigma), prepared immediately before use. Test cultures were grown on test medium plates and growth was smeared directly onto the oxidase test papers. The development of a purple colour within 10 seconds indicated the presence of the enzyme. *Escherichia coli*, served as the negative control, while *Pseudomonas aeruginosa* was used as a positive control.

Catalase

Bacteria growing on test medium plates were exposed to air for 30 *min prior* to a few drops of 3% hydroden peroxide being placed directly onto the cultures. Presence of the enzyme was indicated by the formation of bubbles. *Staphylococcus epidermidis* served as a positive control, while *Streptococcus lactis* was used as a negative control.

Sugar fermentation

The method of Phillips (1976) was used to determine the fermentative capacity of isolated spirochaetes. Twenty mℓ of test medium supplemented with 5% bovine blood instead of FCS, was poured into petri dishes and the plates allowed to dry in a lamina flow hood. Stock sugar solutions containing 20% of the substrate to be tested were sterilized by autoclaving and one mℓ was added to each plate, which was then allowed to dry (final concentration of sugar, 1%). Sugars tested were arabinose (BDH);, cellobiose (Merck); dulcitol (BDH); fructose (Saarchem); galactose (BDH); glucose (Saarchem); glycogen (BDH); glycerol (Saarchem); inositol (BDH); inulin (BDH); lactose (Saarchem); maltose (Saarchem); mannitol (Merck); mannose (Merck); melibiose (BDH); raffinose (BDH); rhamnose (BDH); ribose (Saarchem); salicin (Merck); sorbitol (Saarchem); sorbose (BDH); sucrose (Saarchem); starch (acid only) (BDH) and xylose (Merck). Plates were prereduced in the anaerobic cabinet prior to inoculation. Due to the migration of spirochaetes through the agar, only one spirochaete isolate per plate was tested. Fermentation was detected by removing an agar plug from the plate using a glass tube, transferring this plug to a petri-dish and placing three drops of 0,04% bromothymol blue (BDH) onto the plug. Colour changes in the indicator due to acidity in the agar were observed within a minute. A plain blood agar plate, which had 1 mℓ of sterile distilled water added to it, served as a substrate-free

control, while an additional control of an uninoculated plate containing each sugar was also included. *E. coli* was used as a control for evaluating results over several substrates. The test was also evaluated according to recorded fermentations of both *T. hyodysenteriae* and *T. innocens*.

Hydrogen sulphide production

Test medium containing 0.05% ferric citrate (Merck) and 0.1% sodium thiosulphate (Saarchem) was used. A blackening of the medium due to formation of iron sulphide indicated a positive result *Proteus vulgaris* was used as a positive control, while *E. coli* served as a negative control.

Indole production

Filter paper was moistened with Kovacs' reagent (3g p-dimethylaminobenzaldehyde (Sigma) dissolved in 75mℓ of butanol and 25mℓ of conc. HCl added). Cultures grown on maintenance medium were smeared onto the moist filter paper. The formation of a pink colour within 3 minutes indicated a positive reaction. As a positive control *E. coli* was used, while a *Klebsiella* species served as a negative control.

Aesculin hydrolysis

Test medium containing 0.1% aesculin (Merck) and 0,05% ferric citrate was employed. A positive reaction such as that given by *Klebsiella* species was indicated by a blackening of the plates. *Salmonella typhimurium* was used as a negative control.

Gelatin hydrolysis

A modification of the method described by Syed (1976) was used. After incubation of test medium supplemented with 3% gelatin (Merck), plates were refrigerated for at least 1 hour prior to examination. A white opaque zone around growth indicated a positive reaction. *Streptococcus faecalis* served as a positive control, while *E. coli* was used as a negative control.

Urease production

A modification of Christensens' urea agar was employed. Test medium containing 0,0012% phenol red (pH 6,8) (BDH) was autoclaved and after cooling an aliquot of 20% urea stock solution which had been filter sterilized was added to the medium so that a final concentration of 2% was achieved. Positive reaction was indicated by a change in the phenol red indicator from yellow (pH 6.8) to red (pH 8.4). *P. vulgaris* was used as a positive control, while *E. coli* served as a negative control.

Nitrate Reduction

Test medium was supplemented with 0.1% KNO_3. Immediately prior to testing, equal volumes of 0,5% α naphthylamine and 0,8% sulfanilic acid in 30% acetic acid were mixed and a drop placed directly onto the microbial growth. Development of a red colour within 2 min denoted a positive reaction. *E. coli* served as a positive control while *Lactobacillus casei* was used as a negative control.

Mucin hydrolysis

Test medium was supplemented with 1% hog gastric mucin (Sigma). After incubation, plates were flooded with 1% $CaCl_2$ and allowed to stand for 30 min. The $CaCl_2$ precipitated unhydrolysed mucin, leaving clear zones in the medium were hydrolysis had occurred (Roach and Tannock, 1979). *Bacteroides vulgatus* was used as a positive control, while *E. coli* served as a negative control.

Haemolysis

Haemolysis of rabbit, horse, human and bovine blood was tested by the addition of whole citrated blood to Bacto cryptose blood agar base (Difco) to a final concentration of 10%.

Starch hydrolysis

Test medium was supplemented with 1% soluble starch (Merck) After incubation, plates were flooded with Grams iodine (Appendix A(1)). A blue colouration of the agar such as that observed with *E. coli* indicated a negative result, while unstained agar around growth as seen with *Bacillus subtilis* indicated a a positive reaction.

Growth inhibition

Bile: test medium was supplemented with 2% Bacto ox bile (Difco)
NaCl: test medium was supplemented with 5% (w/v) NaCl.
Glycine: test medium was supplemented with 1% glycine.

Meat medium

Robertsons' cooked meat medium (Oxoid) was inoculated with an agar plug cut from spirochaete growth on maintenance medium plates and incubated for 4 weeks.

Decarboxylase

Liquid growth medium (Appendix D(8)) was supplemented with 0.001% bromothymol blue (BDH), 0.0005% cresol red (BDH), 0.0005% pyridoxal (BDH) and either 1% L-arginine, L-lysine or L-ornithine (Merck). A control culture without amino acids was also included. Cultures were examined for a colour change from yellow to purple, which is indicative of a positive reaction. As a positive control (for lysine, arginine and ornithine): *Salmonella typhimurium* was used while as a negative control (for lysine, arginine and ornithine): *P. vulgaris* was used. This test could only be done on isolates which would grow in liquid medium. The high concentration of lysine inhibited spirochaete growth and an incubation period of a month was necessary before medium became turbid. The other cultures attained maximum turbidity within 10 days.

Metabolic End Products

The volatile metabolic end-products of spirochaete isolates that grew in liquid medium were determined using the method of Carlsson (1973). Spirochaetes were cultivated in Lemcke liquid growth medium (Appendix D(8)) and an aliquot of inoculated, but not incubated broth was stored at 4°C to serve as a control. The end-products of isolate D29 grown in formulated trypticase soy broth, both in the presence and absence of glucose were also determined. Once turbid, a sample of culture was passed through a cation-exchange resin (Dowex AG50W, 400 mesh hydrogen form (Sigma) which had been washed in distilled water and packed onto

glass wool in a Pasteur pipette. The eluate was collected and 2µℓ samples were analysed in a Pye Unicam CCD chromatograph (Pye, Cambridge, UK) fitted with dual flame ionization detectors. A nitrogen flow rate of 60mℓ/min was used and the columns (*c.* 1.8m x 3mm inner diameter) were packed with Chromosorb 101 80/100 mesh (Alltech) Samples were run isothermally at 220°C. Results were recorded on a chart recorder and peaks were compared with those obtained using a standard volatile fatty acid solution containing 1meq/mℓ of acetic, proprionic, butyric, isobutyric, valeric, isovaleric, lactic and succinic acids.

Pigment analysis

When grown on plates containing whole horse blood instead of bovine blood, some spirochaete isolates had a tan colouration. Since pigmentation in treponemes is rare, this pigment was further characterized. Pigment analysis was only performed on isolate G40, since this was the most pigmented of all the isolates.

To determine the solubility of the pigment, loopfulls of culture were emulsified in, acetone, petroleum ether, chloroform, water, toluene and ethanol in the wells of a spot plate. Since the pigment was readily soluble in chloroform, this solvent was used to extract the pigment for further analysis.

Spirochaetes were harvested from ten RGCA plates containing 10% horse blood, washed twice in saline and then resuspended in 5 mℓ of saline. Pigment was extracted by the addition of an equal volume of chloroform and shaking the suspension for 10 min. The chloroform layer was separated from the aqueous phase by centrifugation at 3500g for 10 min. The pigment was characterized according to the criteria listed in Fox (1976).

The absorbance spectrum was determined by monitoring the absorbance from 200-700nm using a Beckman Model 25 spectrophotometer (Beckman Scientific, Irvine, California, USA). Fluorescence was determined by examining the pigment under a ultraviolet lamp that emitted light at 366 and 254 nm. Sensitivity to dilute acids, strong acids, alkalis and iodine was determined in a spot plate by mixing 0.2 mℓ aliquots of pigment extract with equal volumes of 0.1M HCl, 10M HCl, 0.1M H_2SO_4, 10M H_2SO_4, 10M NaOH, 1M NaOH and Grams iodine. Sensitivity to oxidation was determined by bubbling oxygen (Afrox) through the extract.

API ZYM

Treponema cultures J22, J25, J28, L21, L23, M32, P16, P28, T1, T9, T15, T18, T23, *T. hyodysenteriae* and *T. innocens* were scraped off TSBA plates and suspended in sterile saline. The cultures were washed and resuspended in saline at a turbidity equal to a MacFarland tube number 5. Initial harvesting was performed in the anaerobic cabinet while other manipulations were performed under a stream of nitrogen gas. The cultures were then transferred back into the cabinet where two drops were placed in each microtube of an API ZYM gallery (API System S.A., Montalieu, Vercieu, France), which was incubated anaerobically for 4 hours at 37°C. One drop each of reagents A and B (API System S.A.) were added to each microtube, and the colour developed for 5 min, after which non-specific yellowing of the suspension was removed by exposing the gallery to sunlight for 5 min. A value ranging from 0-5 was assigned to each microtube by comparison with a colour chart provided by the manufacturer.

3.2.3 DNA Characterization

Some difficulty was experienced in isolating DNA from spirochaete isolates and many variations to both the Kirby (1957) phenol method, and Marmur (1961) method were attempted. For all these methods, DNA could be readily extracted from a control *E. coli* culture but none yielded appreciable amounts of spirochaete DNA. An assay for DNase activity by the spirochaetes, using a modified version of the toluidine blue-O-DNase test (MacFaddin, 1980), in which DNase agar (Oxoid) supplemented with 10% FCS, 0.25% glucose and 0.01% Toluidine blue-O (Merck) was used. Inoculated plates were incubated in the anaerobic cabinet for a week before being exposed to air for at least 30 min prior to interpreting the results (the low redox potential of the cabinet reduced the toluidine blue making it colourless, but upon exposure to air, reoxidation restored the blue colour). A pink colouration such as that observed when *S. marcescens* was used, indicated a positive result; whereas a blue colouration as seen when *E. coli* was used, indicated a negative control. All spirochaete cultures yielded a negative reaction, indicating that DNase was not degrading the DNA before it could be isolated.

Isolation of DNA

Highly polymerized DNA was finally isolated and purified using the method of Meyer and Schleifer (1975), which is described in detail in Appendix E(1). Spirochaetes were cultivated on maintenance medium (Appendix D(4)) and were ...sted (aerobically) by pouring saline onto the medium a... the culture off the agar surface using a glass spreader. ...ells were collected by centrifugation (8000g for 10min) in a Beckman J2-21 centrifuge with a JA-14 rotor (Beckman Scientific, Irvine, California, USA) and DNA isolated from c. 0.5ml of wet packed cells. Sodium dodecylsulphate (SDS) was used to lyse the cells and the nucleic acids were ex-

tracted with hexadecyltrimethyl ammonium bromide (Sigma) DNA was precipitated with isopropanol, collected by centrifugation and suspended in sodium citrate buffer (Appendix E(1)). The concentration of DNA was adjusted to 50µg/mℓ (Appendix E(2)) using saline citrate buffer. Purity of the DNA was assessed using the extinction coefficients at A_{258}/A_{230} and A_{258}/A_{288} (Appendix E(2)).

G+C mol% Content

Thermal denaturation as described by Marmur and Doty (1962), was used to determine the DNA base composition. The DNA concentration was adjusted to 25µg/mℓ (optical density of 0.5 at 260nm) by the addition of an equal volume of saline citrate buffer (Appendix E(1)) to the DNA stock solution. A melting profile was obtained using a Varian 2200 spectrophotometer (Varian, Cary, Australia) equipped with a Julabo thermoprogrammer (Julabo Laboratory Equipment, Seelbach, West Germany). The temperature was raised at a rate of 5°C per minute until 50°C was reached, and thereafter, at a rate of 1°C per min. The G+C mol% ratio was calculated using the formula %GC = 2,44(Tm - 4) where Tm is the midpoint of thermal denaturation of the DNA (De Ley, 1970; Marmur and Doty, 1962) (Appendix E(3)). As controls, the G+C mol% ratio of DNA extracted by the above method from *E. coli* B (NCTC 9001) and *T. hyodysenteriae* (ATTC 27164) was determined.

3.2.4 Lipid analysis

Lipid extraction

Lipids were extracted by a modification of the method of Bligh and Dyer (1959) which is described in detail in Appendix F(1). Lyophilized cells of known dry weight were suspended in distilled

water and methanol and chloroform added. Lipids were extracted at 4°C, the chloroform layer was collected, evaporated and the lipids dried to constant weight. After weighing, lipids were suspended in 3mℓ of chloroform-methanol (19:1 vol/vol) and stored under nitrogen at -20°C.

Lipid fractionation

The total lipid extracts were separated into their component lipid classes by DEAE cellulose column chromatography (Rouser *et al.*, 1969). This facilitated the identification of polar lipids analysed by thin layer chromatography, as many of the standards had overlapping mobilities, but conveniently, fractionation into acidic and non-acidic fractions separated all overlapping spots. The column was packed with DEAE cellulose (Sigma) converted to the acetate form as described in Appendix F(2). Lipids were eluted by the scheme presented in Table 14 in Appendix F(2). Fractions 2 and 3 were evaporated to dryness and resuspended in 0.5mℓ of chloroform-methanol (19:1 vol/vol), while contaminating salts in fraction 4 were removed by passing this fraction through a sephadex column (Pharmacia) (Appendix F(2)), prior to being dried and resuspended in 0.5 mℓ chloroform-methanol (19:1 vol/vol). All samples were stored under nitrogen at -20°C. Fractions 2 and 4, containing non-acidic polar lipids and acidic polar lipids respectively, were analysed using thin layer chromatography (TLC), while the components in fraction 3, which contained fatty acids, were analysed using gas-liquid chromatography (G

Thin layer chromatography

The phospholipid and glycolipid components eluted from the DEAE cellulose column were analysed using TLC. Glass plates 20 x 20 cm

were coated with a 250 µm layer of Silica Gel G (Merck) using a Desaga apparatus (Desaga, Germany) and were activated at 110°C for 60 min immediately prior to use. Lipid samples (usually 30µℓ) were chromatographed two dimensionally using chloroform-methanol-ammonium hydroxide (60:30:4 vol/vol/vol) in the first direction, and chloroform-methanol-water (65:24:4 vol/vol/vol) in the second direction. A developing distance of 15cm was used for both solvents. Spots were detected using iodine vapour and/or 0,05% aqueous Rhodamine G6 (Merck) solution sprayed onto the plate and viewed with a ultra-violet light with a wavelength of 266nm. Phospholipids were identified using the ferric chloride-sulfosalicylic acid reagent (Appendix F(3)) while glycolipids were identified using the diphenylamine reagent (Appendix F(3)). Glycerides were identified by spraying the plates with 10N KOH after they had been sprayed with Rhodamine G6 solution and viewing under ultra-violet light. Retention of fluorescence after quenching with KOH indicated the presence of a glyceride (Touchstone and Dobbins, 1983). Lipids were identified by comparison of mobilities with the standards phosphatidylserine, phosphatidylcholine, cardiolipin, phosphatidylglycerol, phosphatidylinositol, lysophosphatidylcholine, phosphatidylethanolamine, sphingmyelin and monogalactosyldiglyceride, all obtained from Sigma.

Quantitation of phospholipids

The position of phospholipid spots on TLC plates was detected using iodine vapour and their position marked. To facilitate removal of the spots, the silica gel was sprayed lightly with water. Marked spots were scraped off the glass plate using a blade, and placed into acid-cleaned test tubes. For each assay, a sample of silica gel of similar size to the test spot was cut from a clean area of the plate and included as a blank. Phosphate analysis was performed using

the method described by Kirchner (1978) (Appendix F(4)). As an internal control, recovery of phospholipid standards developed two dimensionally on TLC plates was analysed.

Sugar analysis

Total extracted lipids as well as glycolipid spots on TLC plates were analysed for their sugar moieties. Glycolipid spots were collected as described previously the lipids eluted using chloroform, the silica gel removed by centrifugation, and the chloroform evaporated leaving the glycolipid sample. Sugar moieties were identified using gas-liquid chromatography as described by Clamp (1977) (Appendix F(5)). Methyl glycosides were formed using 2M methanolic-HCl and heating the sample under nitrogen at 85°C. The solution was neutralized with silver carbonate (Ag_2CO_3) (Merck), and acetic anhydride (BDH) added. After centrifugation and tituration of the sediment with methanol, supernatant fractions were evaporated to dryness and lipid material removed with n-hexane. The residue was stored in a desiccator over silica-gel until required. Trimethylsilylimidazole (TSMI) (Supelco Inc.) in pyridine (1:2 vol/vol) was used to derivatize the samples. Three µl aliquots were chromatographed in a Pye Unicam CCD gas-liquid chromatograph (Pye, Cambridge, UK) equipped with dual flame ionization detectors and dual 1.8m x 3mm (internal diameter) glass columns packed with 10% SP2330 on 100/120 supelport (Supelco) nitrogen flow rate of 60mℓ/min was used, and a temperature program of 200°C for 1 minute followed by an increase of 16°C/minute for 6.5 minutes was employed. Peaks were identified using the control sugars D-glucose, D-galactose, D-galactosamine and D-mannose treated in the same manner.

Fatty acid analysis

Total extracted lipid material as well as samples from fraction 3 of the DEAE cellulose column were evaporated to dryness and refluxed under nitrogen with 0.5mℓ with 2M methanolic-HCl at 85°C for 3-5 hours. Samples were evaporated tc dryness under nitrogen and suspended in 0.5mℓ of chloroform-hexane (1:4 vol/vol). The fatty acid methyl-esters, were analysed by gas-liquid chromatography under similar conditions to those described for sugar analysis, except that an isothermic temperature of 250°C was used. Fatty acid profiles were identified by comparing retention times with the standards 12:0, 14:0, 16:0, 18:0, 18:1 and 18:2 all obtained from Alltech.

3.2.5 Immunological characterization

Antisera were raised against selected spirochaete isolates as described in Appendices C(1)and C(2), and purified using the rivanol method (Appendix C(3)). To detect antigenic differences of the various isolates, an agglutination test and a disc-growth inhibition test were used.

Agglutination

Normal and immune antisera were diluted ten fold, and thereafter a two fold dilution series made using physiological saline as the diluent. Spirochaete isolates were harvested from maintenance medium plates, and after washing in saline, their concentration adjusted to 5 x 10^6 organisms/mℓ using a Neubauer counting chamber. An equal volume of antigen was added to each antiserum dilution, mixed and incubated at 37°C for 2 hours, followed by 4°C for 1 hour. The tubes were then examined for agglutination using a 10X hand lens and compared with control tubes containing the antigen

diluted with saline. The end point was considered as being that dilution at which no agglutination of the bacteria occurred.

Disc growth inhibition test

Sterile filter paper discs of 5mm in diameter were impregnated with each antiserum and placed onto the agar surface of RGCA maintenance medium plates which had been inoculated with 0.1mℓ of spirochaete suspension containing 5 X 10^6 organisms/mℓ. After incubation, plates were observed for zones of inhibition around discs.

RESULTS:

3.3.1 Isolation of rat intestinal spirochaetes

Despite the occasional observation of spirochaetes in areas of the GIT proximal to the large intestine, these bacteria were never cultured from these sites, and after four attempts, the stomach, duodenum and ileum were discarded as potential sources of spirochaetes. However, spirochaetes could be routinely isolated from both the lumen and epithelial tissue of the caecum and proximal colon.

Four morphologically distinct spirochaetes were axenically cultured *in vitro*. Although these were distinguishable at the light microscope level (Fig. 45) they were more readily differentiated with the electron microscope, where size and number of periplasmic fibrils were the main differentiating criteria (Figs. 46-49). Isolates that were axenically cultivated and characterized are listed in Table 4 together with the anatomical site from which they were obtained and their general morphology. Other isolates which were not obtained

in pure culture or were lost before they could be further characterized, are not listed in this table.

Of the selective methods employed for isolating spirochaetes, the "well method" was consistently found to be the best, yielding spirochaete cultures relatively free from contaminating bacteria. The "filter method" was not satisfactory, in that heavily contaminated cultures were obtained. Experience showed that axenic spirochaete cultures were rarely obtained from badly contaminated primary cultures. Contamination of plates with migratory *Clostridium* species was also problematical and the faster growth rate of the contaminant meant that spirochaete cultures were seldom obtained once contaminated with these clostridia. In some instances, extreme difficulty was experienced in obtaining spirochaete cultures free from a gram-positive, facultative coccus. Negative staining of these cultures revealed an intimate association between the coccus and the spirochaete, thereby explaining why selection methods which continuously selected for highly motile bacteria failed to eliminate the coccus contamination (Fig. 50).



Fig. 45. Modified Gram stains of the four types of spirochaetes isolated in pure culture from the rat GIT. A: spirochaetes with 7-14-7 or 8-16-8 periplasmic fibril arrangement (isolate G40). The large size and tapered ends are evident. B: Spirochaetes (isolate G33) with 2-4-2 periplasmic fibril arrangement. Many of the bacteria have rounded up to form coccoid bodies. C: Spirochaetes (isolate G39) with 3-6-3 periplasmic fibril arrangement. Many dense coccoid bodies are present. D: Spirochaetes (isolate F25) with 1-2-1 periplasmic fibril arrangement. The small size in comparison with the other spirochaete types is evident. Spirochaete granules can also be seen in this culture. (Bar=10μm).

Table 4. Spirochaete strains isolated *in vitro* in axenic culture: original isolation site, and gross morphology of each isolate

isolate	isolation site	size (μm)
isolates with numerous periplasmic flagellae (Type A)		
D29	caecum lumen	7.3-10.5 x 0.42-0.53
E9	caecum epithelium	6.5- 9.2 x 0.38-0.51
G11	caecum lumen	5.8-10.4 x 0.42-0.53
G18	caecum lumen	7.6-11.2 x 0.44-0.54
G40	colon lumen	5.2-10.3 x 0.42-0.52
G67	caecum epithelium	7.9-11.5 x 0.41-0.50
J22	caecum epithelium	8.3-10.4 x 0.40-0.50
J25	caecum epithelium	8.5-11.3 x 0.38-0.49
J28	caecum lumen	8.6-10.8 x 0.39-0.50
P16	colon lumen	8.5-11.0 x 0.38-0.49
P28	colon epithelium	8.8-11.4 x 0.38-0.45
T1	caecum epithelium	8.6-10.6 x [illegible] 0.49
T9	colon lumen	8.5-11.5
T15	colon lumen	8.0-10.5
T18	caecum lumen	8.5-11.5 x
T23	caecum lumen	8.4-11.4 x 0.42-0.50
isolates with 3-6-3 periplasmic flagella arrangement (Type C)		
G15	caecum lumen	4.2-9.8 x 0.30-0.35
G39	colon lumen	4.0-8.5 x 0.30-0.36
J3	colon lumen	4.0-8.4 x 0.32-0.35
L21	colon epithelium	4.2-8.6 x 0.34-0.36
L23	colon epithelium	4.0-8.6 x 0.31-0.35
M32	caecum epithelium	4.0-8.5 x 0.32-0.36
isolates with 2-4-2 periplasmic flagella arrangement (Type B)		
E7	caecum lumen	5.0-7.3 x 0.28-0.33
G33	colon lumen	6.0-8.2 x 0.30-0.35
J9	colon epithelium	4.8-7.2 x 0.31-0.35
M9	colon epithelium	4.5-7.0 x 0.31-0.35
isolates with 1-2-1 periplasmic flagella arrangement (Type D)		
F25	caecum epithelium	3.0-5.8 x 0.26-0.30



Fig. 46. Negative staining of spirochaete (isolate G40) with numerous periplasmic fibrils. Bar = 0.5μm



Fig. 47. Negative staining of spirochaete (isolate G33) with 2-4-2 periplasmic fibril arrangement. Bar=0.5μm.



Fig. 48. Negative staining of spirochaete (isolate G39) with 3-6-3 periplasmic fibril arrangement. Bar=0.5um.



Fig. 49. Negative staining of spirochaete (isolate F25) with 1-2-1 periplasmic fibril arrangement. The outer sheath (OS), insertion disc (ID) and periplasmic fibrils (PF) are clearly visible. Bar=0.5μm.

Spirochaete growth could be visualized as a fine haze spreading over the agar surface. In primary cultures, this haze extended about 3cm from the well in the agar. Cultures isolated from the periphery of the haze were less contaminated than those isolated closer to the point of inoculation. Gram stains of cultures from these two sites suggested that similar spirochaete populations were present in both areas of the plate. On primary isolation, two morphologically distinct spirochaete populations were frequently co-isolated, but with repeated subculturing, a pure culture of either one of the two spirochaete types (usually the large multifibrillar spirochaete) could be obtained.

Of the media used, modified RGCA and E medium were extensively used since these supported the growth of all four morphological types of treponemes isolated. The other media appeared to be more specific in the types of spirochaetes which they grew. Incubation under microaerophilic conditions failed to grow any treponemes, although pure cultures of *Campylobacter* species could be isolated using the selective procedures employed for spirochaetes. Inclusion of polygalacturonic acid as the only carbon source in RGCA medium



Fig. 50 Contaminating gram positive coccus in intimate association with spirochaetes in culture. Bar 1µm

also failed to support spirochaete growth and was discontinued after two attempts. The antibiotics rifampicin and polymyxin B were consistently found to be the most useful in inhibiting contaminating bacteria without affecting spirochaete growth and were extensively used. Spectinomycin was of limited usefulness in primary isolations, since some spirochaete isolates were inhibited by it and the growth rate of others was reduced, nevertheless, it was occasionally useful for eliminating contaminating bacteria resistant to the other antibiotics. Naladixic acid was not used since all spirochaete isolates were sensitive to it.

Spirochaete cultures were routinely maintained on maintenance medium slants at 11°C although subculturing every 6-8 weeks was essential. Cultures could also be maintained at -70°C for periods of up to 3 months. Lyophilization, was not a satisfactory method for storage, as cultures could only be reconstituted for 1 week after being freeze-dried.

Growth Requirements

Although the multifibrillar spirochaete isolates would grow for one subculture on RGCA plates containing neither rumen fluid nor serum, they were clearly growing under stress conditions as indicated by their morphology, which was long and filamentous. Upon subculture onto similar medium, they failed to grow. All isolates had an obligate serum requirement. Haemin, cocarboxylase and vitamin K were not essential co-factors for any of the spirochaete morphotypes. Rabbit, sheep, bovine and human blood all supported spirochaete growth equally well, although horse blood reduced the growth rate of the bacteria.

Table 5. Effect of various modifications to basic Lamcke liquid growth medium (not supplemented with 0.5% yeast extract) on growth of isolate D29.

modification to basic medium.	number of days for culture to attain turbidity ≥0.4 at 640nm.
control	10
no serum	no growth
+ horse serum	>30
+ rabbit serum	10
+ 0.5% yeast extract	7
+ vitamin solution	7
+ volatile fatty acid mixture	20
nitrogen gas phase	10
+ cocarboxylase + vitamin K	12
standing vertically	>30
incubated at 42°C	9
incubated at 30°C	no growth
incubated at 25°C	no growth

The analysis of growth requirements of isolate D29 in liquid medium (Table 5), also indicated a serum requirement, with FCS and rabbit serum being superior to horse serum for supporting growth. Vitamin solution and yeast extract also increased the growth rate of the organism, while the addition of volatile fatty acids appeared to inhibit growth, but this may have been due to a lowering of the pH of the medium. The importance of incubating the cultures in a slanted position was also made apparent in this study, and although the reason for this was investigated, no explanation can be made. Although a strict anaerobe, the composition of the gas phase for D29 was not important. The spirochaete grew at 37-42°C, but not at temperatures of 30°C and below.

Growth Curve

Spirochaetes in liquid culture displayed a typical logarithmic growth curve, with a generation time of 19.07 hours (Fig. 51). A common feature of all the spirochaete cultures was that as they aged, round irregular bodies or granules were produced. Negative staining of

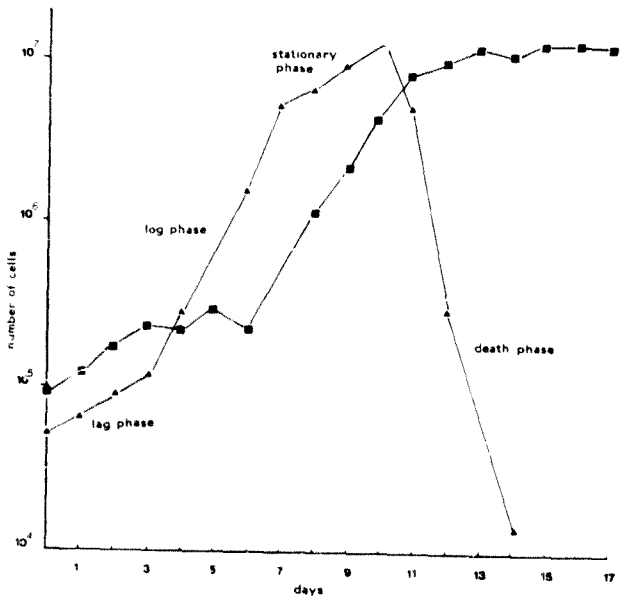


Fig. 51 Growth curve of isolate D29 cultivated in liquid medium. ■——■ coccoid bodies; ▲——▲ spiral forms.

the culture at various stages of the growth curve revealed variations in spirochaete morphology, which are summarized in Table 6. and are illustrated in Fig. 52. From negative staining it appeared as if two distinct types of granules were formed; the first being a rolled-up form of the bacterium (Fig. 52A and M) and the other a degenerative form comprising of what appeared to be sheath components together with periplasmic fibrils (Fig. 52 G and H). Studies undertaken to determine whether these granules could be reproductive forms suggested that they were not, since 3 month old



Fig. 52. Morphology of isolate 029 at various stages in the growth curve.
A. Log phase: spirochaete with one end 'rolled up'. Bar = 1µm.
B. Log phase: division by binary fission. Bar = 0.1µm.
C. Log phase: newly divided spirochaete. Bar = 1µm.
D and E. Log phase: spirochaetes intimately associated together. Bar = 1µm.
F. Log phase: spirochaetes coiled together to form a plait. Bar = 1µm.
G and H. Stationary phase: granule forming from sheath material. Bar = 1µm.
I. Stationary phase: spirochaetes attached to granules. Bar = 1µm.
J. Death phase: large aggregate of granules associated with few spirochaetes. Bar = 1µm.
K and L. Death phase: small granules with protruding periplasmic fibrils. Bar = 0.5µm.
M. Death phase: wound up spirochaete with protruding periplasmic fibrils. Bar = 1µm.

Table 6. Morphological characteristics of multifibrillar spirochaete (isolate D29) at various stages of the growth curve

days after inoculation	stage in growth curve	length (μm)	spirochaete motility	characteristic appearance
3	end lag phase	5-10	slight motility, occasional flexing	spirochaetes in single units, helix often terminating in a sphere (Fig. 51A).
6	mid log phase	10-20	highly motile	few spirochaetes with sphere attached to helix, binary fission evident (Fig. 51B,C), spirochaetes associated together forming knots or plaites (Fig. 51D,E,F)
7	mid log phase	5-20	highly motile	long and short spirochaetes present (pleomorphism)
9	stationary phase	10-20	sluggish	spheres attached to 30% of organisms (Fig. 51G,H)
11	death phase	10-20	almost non-motile	spirochaetes associated in large aggregates comprising of spiral forms and spheres (Fig. 51I,J)
13	death phase		non-motile	spirochaetes loose helical configuration forming long flexuous rods, spheres dissociate from aggregates forming numerous small bodies (Fig. 51K,L,M)

cultures which were composed entirely of granules, failed to grow when introduced into fresh medium, although month old cultures occasionally produced a viable culture. If the granular forms were reproductive units of the organism, all cultures should have grown. Furthermore, the long incubation period (up to 30 days), before turbidity of the medium became apparent, strongly suggests that growth arose from few viable forms. Phase contrast microscopy of inocula used from month old cultures, revealed the presence of few helical bacteria (<0.01%) which could have been responsible for the growth occurring in these cultures.

3.3.2 Biochemical characterization

All isolates failed to grow on modified RGCA plates containing 1% glycine, 5% NaCl or 2% oxgall. Litmus milk and Robertsons' cooked meat medium also failed to support their growth, and their reactions in these milieaux could not be determined. Biochemically,

spirochaete isolates were all negative for oxidase, catalase, urease, indole production, H_2S production, nitrate reduction and starch hydrolysis. Strain D29, the only isolate tested for decarboxylase activity, was also negative for this characteristic. All isolates were positive for aesculin hydrolysis, weak haemolysis of bovine, rabbit and human blood, and gelatine hydrolysis (weak reaction). Mucin digestion using the $CaCl_2$ precipitation test was also weakly positive. The biochemical profiles of the multifibrillar spirochaete isolate T9 is presented in Table 7, together with the reactions obtained for *T. hyodysenteriae* and *T. innocens*

The range of fermentable carbohydrates was only determined for the multifibrillar isolates. Inositol, xylose, lactose, mannose and inulin exhibited weak positive reactions while other sugars were not fermented. That glucose is not fermented, is also inferred from the finding that isolate D29 produces the same metabolic end-products when grown in the presence or absence of glucose. All multifibrillar isolates produced major amounts of acetic acid and trace amounts of butyric acid as metabolic end products (Fig. 53). The growth medium intrinsically contained small amounts of acetic and proprionic acids (Fig. 53) and the addition of rifampicin, introduced alcohol into the medium.

The yellow pigment produced by multifibrillar spirochaete isolates when grown on modified RGCA containing 10% horse blood was identified as being a carotenoid on the basis that it was:

- readily soluble in chloroform, weakly soluble in acetone and insoluble in alcohol and water,

- reacted with iodine to form a red colour,

Table 7. Biochemical profiles of spirochaete T9 isolated from rat caecum, *T. innocens* (ATCC 29796) and *T. hyodysenteriae* (ATCC 27164).

Biochemical test	Isolate T9[a]	*T. innocens* (ATCC 29796)	*T. hyodysenteriae* (ATTC 27164)
oxidase	-	-	-
catalase	-	-	-
sugar fermentation			
arabinose	-	-	-
cellobiose	-	-	-
dulcitol	-	-	-
fructose	-	+	-
galactose	v[b]	-	+
glucose	-	-	-
glycogen	-	-	-
glycerol	-	-	-
inositol	w	-	-
inulin	w	-	-
lactose	+	-	+
maltose	-	-	-
mannitol	-	-	-
mannose	+	-	-
melibiose	-	-	-
raffinose	-	NT	-
rhamnose	-	-	-
ribose	-	-	-
salicin	-	-	-
sorbitol	-	-	-
sorbose	-	-	-
starch (pH)	-	-	-
sucrose	-	-	-
xylose	+	-	-
H_2S production	-	-	-
indole production	-	-	+
aesculin hydrolysis	+	+	+
gelatin digestion	w[c]	-	+
urease	-	-	-
nitrate reduction	-	-	-
mucin ($CaCl_2$)	w	NT	NT
starch hydrolysis	-	-	-
β haemolysis	w	w	+
1% bile growth	-	-	-
5% NaCl growth	-	NT	NT
1% glycine growth	-	-	-
decarboxylase (lysine, ornithine arginine)	-	NT	NT
NH_4 production	-	NT	NT

[a] identical reactions for isolates D29, G18, G40, G67, J22, P16, P28, T1, T15, T18, T23.
[b] positive for isolate T9, negative for other isolates.
[c] strong reaction in isolate G40.
abbreviations: +, positive for reaction; -, negative for reaction; w, weak positive reaction; NT, not tested.

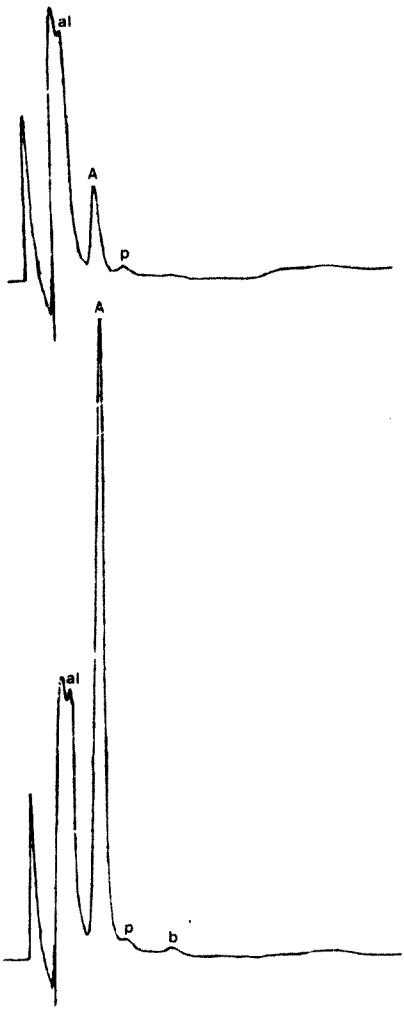


Fig. 53 Metabolic end-products produced by isolate D29 growing in Lemcke liquid growth medium, both in the presence and absence of glucose. Top: control medium, inoculated but not incubated. Bottom: test medium. Symbols: A, acetic acid; p, proprionic acid; b, butyric acid; al, alcohol.

- non-fluorescent at both 366nm and 254nm,
- had absorbance peaks at 445-450nm and 320-330nm when chloroform was used as a solvent,
- did not react with dilute acids, strong acids or alkalis.

API-ZYM characterization revealed that all treponeme isolates had the enzymes alkaline phosphatase (EC 3.1.3.1), acid phosphatase (EC 3.1.3.2), C4 esterase (EC 3.1.1.8) and β galactosidase (EC 3.2.1.23). The multifibrillar isolates also hydrolysed the longer chain fatty acid caprylate but only weakly hydrolysed the C14 (myristate) fatty acid. In addition, multifibrillar isolates had strong β glucosidase (EC 3.2.1.21) activity but had no activity for the more complex glycosidases. A total lack of proteolytic and amino acid degradation activities was also evident for the multifibrillar spirochaetes. The isolates with the 3-6-3 periplasmic fibril arrangement however were distinguishable by the presence of leucine arylamidase and phosphoamidase activity (EC 3.9.1.1). A table recording the enzymic activity of each of the tested isolates, together with that obtained for *T. innocens* *T. hyodysenteriae* is presented in Table 8.

3.3.3 DNA characterization

The G+C mol ratio of the multifibrillar spirochaete isolates was 30.7±0.5% (n=4). DNA extracted from *E. coli* B (NCTC 9001) which was used as an internal control, had a G+Cmol ratio of 50.9%.

Table 8. Enzymatic activities of isolated treponemes as indicated by the API ZYM test.

Treponema strains	Enzymes																			
	1 control	2 alkaline phosphatase	3 C4 esterase	4 C8 esterase lipase	5 C14 lipase	6 leucine arylamidase	7 valine arylamidase	8 cystine arylamidase	9 trypsin	10 chymotrypsin	11 acid phosphatase	12 phosphoamidase	13 α galactosidase	14 β galactosidase	15 βglucuronidase	16 α glucosidase	17 β glucosidase	18 N-acetyl-β-glucosaminidase	19 α mannosidase	20 α fucosidase
Strains with numerous periplasmic fibrils																				
J22	-[1]	1	3	2	1[2]	-	-	-	-	-	1	-	-	4	-	-	3	-	-	-
J25	-	1	4	2	-	-	-	-	-	-	2	-	1[2]	4	-	-	3	-	-	-
J28	-	1[2]	4	3	-	-	-	-	-	-	2	-	-	4	-	-	5	-	-	-
P16	-	1	4	2	1	-	-	-	-	-	1[2]	-	-	3	-	-	2	-	-	-
P28	-	1	4	2	1	-	-	-	-	-	1[2]	-	-	4	-	-	3	-	-	-
T1	-	1[2]	3	3	1[2]	-	-	-	-	-	1	-	-	5	-	-	5	-	-	-
T9	-	1[2]	3	3	-	-	-	-	-	-	1	-	-	4	-	-	4	-	-	-
T15	-	1[2]	2	2	1[2]	-	-	-	-	-	2	-	1[2]	5	-	-	5	-	-	-
T18	-	1[2]	4	3	1[2]	-	-	-	-	-	2	-	1[2]	5	-	-	5	-	-	-
T23	-	-	2	1	-	-	-	-	-	-	2	-	1[2]	5	-	-	3	-	-	-
strains with 3 6-3 periplasmic fibril arrangement																				
L21	-	1	3	-	-	2	-	-	-	-	3	1	-	4	-	-	-	-	-	-
L23	-	1	2	1[2]	-	2	-	1[2]	-	-	2	1	-	5	-	1[2]	1[2]	-	-	-
M32	-	1	3	1[2]	-	2	-	1[2]	-	-	3	1	-	4	-	-	-	1[2]	-	-
type *Treponema* cultures																				
T. hyodysenteriae ATCC 27164	-	2	1	1[2]	-	-	-	-	-	-	1	-	-	5	-	5	5	-	-	-
T. innocens ATCC 29796	-	2	2	1	-	-	-	-	-	-	3	-	5	5	-	2	3	-	-	-

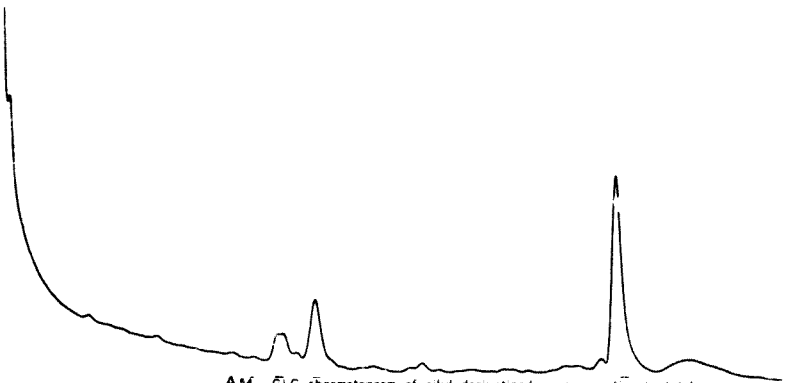
[1] numerical values assigned to each microtube when compared with colour intensity chart; - negative reaction, 1-5 represents the number of nmoles of liberated substrate; 1 = 5 nmoles; 2 = 10nmoles; 3 = 20 nmoles; 4 = 30nmoles; 5 ≥ 40nmoles.

[2] reaction very weak and could be interpreted as either 1 or -, (a colour reaction observed, but of less intensity than number 1 of colour intensity chart).

3.3.4 Lipid analysis

Approximately 15% of the cellular dry mass of isolated spirochaetes was in the form of extractable lipid material. The polar lipid component of the multifibrillar isolates was comprised of both glycolipids and phospholipids. Although neutral lipids, mainly sterols, were probably present; non-polar solvents which would separate these were not employed in this study. Neutral lipids would have migrated with both the solvent fronts. Of the phospholipids, spots with mobilities similar to phosphatidylglycerol (PG), phosphatidylserine (PS), cardiolipin (C) and phosphatidylcholine (PC) were identified. Phosphate analysis revealed that PG was the predominant phospholipid, making up almost 40% of the lipid component. Twenty-two % of the phospholipids were in the form of PS, 18% in the form of PC and 9% in the form of C. The spot identified as PC, did not react with iodine vapour and was visible only when plates were sprayed with rhodamine G6. This indicates that the fatty acids in this phospholipid, were completely saturated. The other polar lipids all stained with iodine, as did all the standard solutions used for comparing mobilities.

Also present in the polar lipid fraction were three glycolipids, the predominant one being monogalactosyldiglyceride (MGDG), while the others, which eluted with the acidic lipid fraction, were minor components and remained unidentified. These lipids were only detected when large amounts of lipid (≥50µℓ) were spotted onto TLC plates. Analysis of the sugar composition of the total cellular lipid, revealed that galactose was the only sugar present, although trace amounts of other sugar components could be detected (Fig. 54). Analysis of sugar moieties in the 3 glycolipids separated by TLC, revealed that both MGDG and one of the unidentified minor glycolipid components contained only galactose anomers (Fig. 55); whereas the other unidentified glycolipid was complex and contained galactose



A54 GLC chromatogram of silyl-derivatized sugar moieties in total lipid of isolate P28. All three peaks were identified as galactose

Fig. 54 GLC chromatogram of silyl-derivatized sugar moieties in total cellular lipid of isolate P28. All three peaks were identified as galactose anomers. A: position of glucose peaks; B: position of galactose peaks; C: position of galactosamine peaks.

and galactosamine together with trace amounts of glucose (Fig. 55). That this complex glycolipid was a minor lipid constituent, is emphasized by the fact that galactosamine was only detected in trace amounts in sugar analysis of the total lipid fraction. Analysis of individual glycolipids was only performed on isolate P28, although isolates P16, P28, T9 and T18 were analysed for sugars present in their total lipid fraction and in all these, galactose was the only sugar detected.

The cellular fatty acid composition of the various isolates grown on TSBA maintenance medium supplemented with bovine blood is represented in Table 9, together with the fatty acid composition of reference strains of *T. innocens* and *T. hyodysenteriae*. A typical profile is presented in Fig. 56. The profile obtained from the free fatty acids eluted from the DEAE cellulose column (fraction 3), consisted almost exclusively of 12:0 although minor amounts of longer chain fatty acids were present. The major fatty acid components

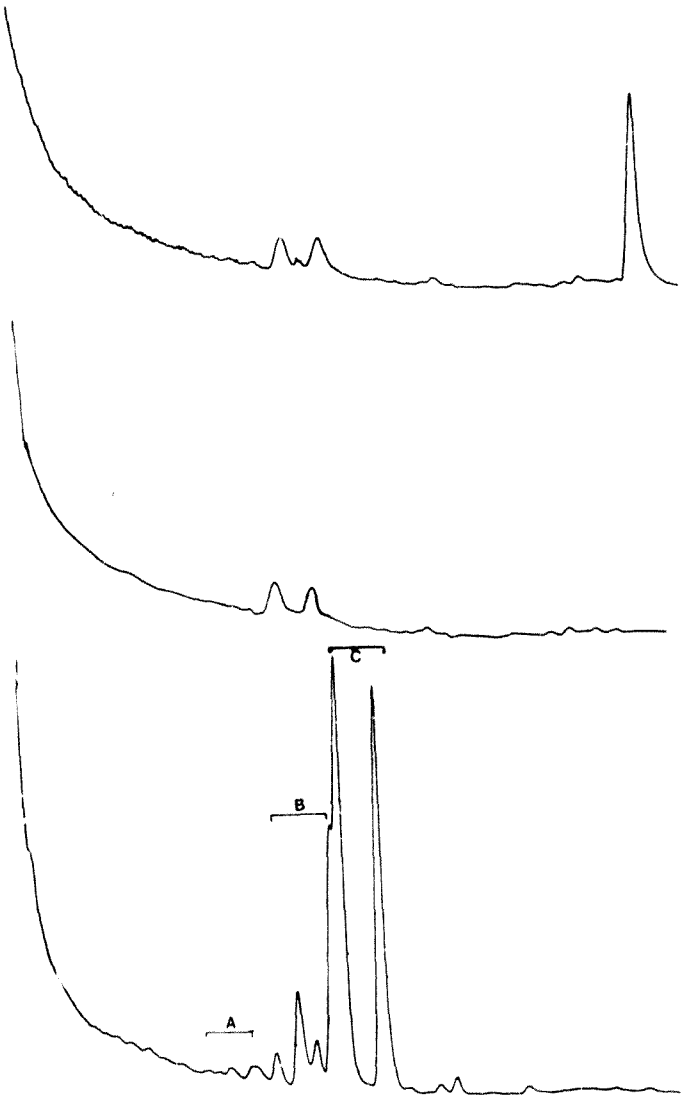


Fig. 55 GLC chromatogram of silyl derivatized sugars of individual glycolipids of isolate P28 top: MGDG containing only galactose; middle: unidentified glycolipid containing only galactose; bottom: unidentified glycolipid containing trace glucose anomers (A); galactose anomers (B); and galactosamine anomers (C).

of total cellular lipid of the multifibrillar spirochaete isolates were C16:0 and C18:1 which together contributed 70% of fatty acids. Dodecanoic acid (12:0), 14:0, 15:0, 17:0 and 18:0 and an unidentified fatty acid believed to be alkenyl 14:0 were also present, but in smaller quantities. Quantitative fatty acid data obtained for isolates with 3-6-3 and 2-4-2 periplasmic fibril arrangements is also presented in Table 9. The isolates M9 and J9, both of which have a 2-4-2 periplasmic fibril arrangement, contain significantly larger amounts of the fatty acid believed to be alkenyl 15:0 while the 3-6-3 periplasmic fibril isolates also have a distinct fatty acid profile, and

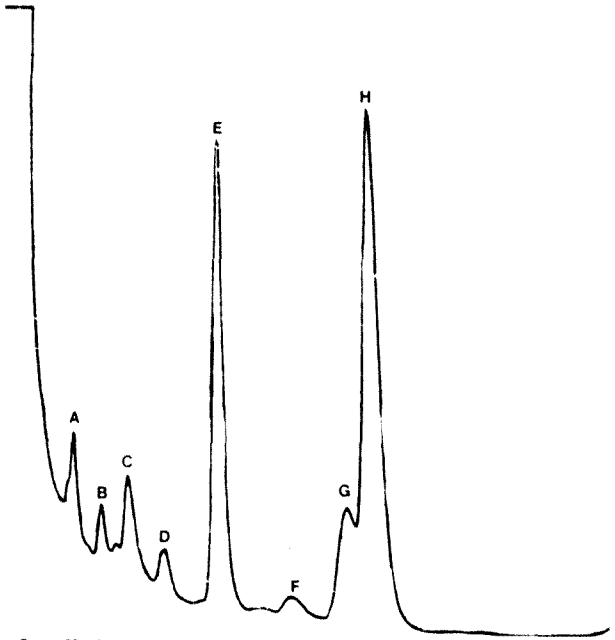


Fig. 56 GLC fatty acid profile of total cellular lipid extracted from P28. A: 12:0 n-dodecanoic (lauric acid); B: unidentified peak, probably alkenyl 14:0; C: 14:0 n-tetradecanoic (myristic) acid; D: 15:0 n-pentadecanoic acid; E: 16:0 n-hexadecanoic (palmitic) acid; F: 17:0 n-heptadecanoic acid; G: 18:0 n-octadecanoic (stearic) acid; H: 18:1 octadecenoic acid.

Table 9. Fatty acid composition of morphologically distinct spirochaetes isolated from the rat gut

Treponema isolates	Fatty acid (% of total fatty acid)[a]									
	10:0[b]	12:0	alkenyl[c] 14:0	alkenyl[c] 15:0	14:0	15:0	16:0	17:0	18:0	18:1
isolates with numerous periplasmic fibrils										
P28	-	7	3	T[d]	6	3	34	2	7	38
P16	-	-	-	T	6	3	39	2	8	41
T18	-	4	2	T	8	1	39	1	6	38
isolates with 3-6-3 periplasmic fibril arrangement										
J3	8	-	-	1	17	-	33	T	4	37
L21	6	-	-	T	14	-	29.5	T	12.5	36
isolates with 2-4-2 periplasmic fibril arrangement										
M9	1	-	-	9	3	-	37	1	5	43
J9	2.5	-	-	10	7	-	35	T	5	40
type *Treponema* cultures										
T. hyodysenteriae (ATTC 27164)	-	3	10	1	15	1	52	-	2	2
T. innocens (ATTC 29796)	-	3	2	3	2	25	57	-	2	6

[a] Numbers to the left of the colon refer to the number of C atoms in the chain; numbers to the right refer to the number of double bonds. 12:0, n-dodecanoic = lauric acid; 14:0, n-tetradecanoic = myristic acid; 15:0, n-pentadecanoic acid; 16:0, n-hexadecanoic acid = palmitic acid; 17:0, n-heptadecanoic acid; 18:0, n-octadecanoic = stearic acid; 18:1, octadecenoic acid.

[b] Unidentified peak, probably 10:0 decanoic acid

[c] Though not positively identified by the use of standards, these peaks are tentatively classified as being alkenyl fatty acids on the basis of results obtained for *T. hyodysenteriae* and *T. innocens* and comparison of these with those obtained by Matthews and Kinyon, 1984.

[d] T: trace amount <1% detected

contain larger amounts of 14:0. In all cases however, 16:0 and 18:1 were the predominant fatty acids.

3.3.5 Immunological characterization

Antisera raised against morphologically distinct isolates showed a lack of specificity and reacted with many heterologous isolates in the agglutination test (Table 10) although more specificity was displayed in the disc-growth inhibition test (Table 11). The titres of the various antisera against homologous antisera are presented in

Table 10. Agglutination of spirochaete isolates using different antisera

spirochaete isolate	heat inactivated antiserum (1 in 10 dilution)					
	saline	normal	D29	G15	G39	G33
isolates with numerous periplasmic flagella						
D29		-	***	**	-	*
G18	-	-	**	**	-	*
G40	-	-	**	***	-	**
G67	-	-	-	-	*	-
P16	-	-	**	**	-	-
P28	-	-	***	**	*	-
T1	-	-	**	**	*	*
isolates with 3-6-3 periplasmic flagella arrangement						
G15	-	-	*	***	-	**
G39	-	-	-	-	*	***
isolate with 2-4-2 periplasmic flagella arrangement						
G33	-		*	***	-	**
type *Treponema* cultures						
T. hyodysenteriae	-	-	-	**	**	*
T. innocens	-	-	**	**	-	*

***, indicates strong agglutination; **, indicates moderate agglutination; *, weak agglutination; -, no agglutination

Table 11. Disc growth inhibition test of spirochaete isolates using different antisera

spirochaete isolate	heat inactivated serum				
	normal	D29	G15	G39	G33
isolates with numerous periplasmic flagella					
D29	-	+	-	-	-
G18	-	+	-	-	-
G40	-	+	+	-	+
G67	-	-	-	+	-
P16	-	+	-	-	-
P18	-	+	-	-	+
T1	-	+	-	-	-
isolates with 3-6-3 periplasmic flagella arrangement					
G15	-	-	+	+	+
G39	-	-	+	+	-
isolate with 2-4-2 periplasmic flagella arrangement					
G33	-	-	-	-	+
Type *Treponema* cultures					
T. hyodysenteriae	-	-	-	-	-
T. innocens	-	-	-	-	-

-, indicates no inhibition of growth; +, indicates inhibition of growth: no growth within 5mm of disc.

Table 13 (Appendix C(5)). For estimating cross-reactivity between isolates using the agglutination test, a 1 in 10 dilution of the antisera was used (Table 10).

3.4 DISCUSSION

Using selective isolation techniques, four morphologically distinct spirochaetes were isolated from the rat GIT (Figs. 46-49). These techniques have been employed for obtaining *Spirochaetales* from various environments, and are based primarily on the motility properties of members of this order which can migrate through

3% agar; a concentration that is inhibitory for other prokaryotes. The extreme difficulty experienced with contamination was therefore rather surprising and even more so when it was realised that non-motile cocci were the most persistent contaminants. The electron microscopic observations of these bacteria intimately associated with the spirochaetes (Fig. 50), led to the hypothesis that the spirochaetes were themselves, aiding the persistence of the contaminats. Similar findings have been reported for oral treponemes by Socransky *et al.* (1978) and To *et al.* (1978b), who, using ingeneous experiments, showed that spirochaetes can transport other prokaryotes both in liquid and on agar medium. Such transportation would be an obvious advantage for those spirochaetes which have symbiotic relationships with other bacteria, such as *T. vincentii* and *T. denticola* both of which require thiamine pyrophosphate (TPP) for growth (Austin and Smibert, 1982). Similar transportation, may also occur *in vivo*, since To *et al.* (1980), have shown that rods and coccoid bacteria ride on spirochaetes in the hindgut of the sonoran desert termite *Pterotermes occidentis*.

The difficulty encountered with persistent contamination, combined with the slow growth rate of the spirochaetes, resulted in many isolates never being obtained in pure culture. The contamination problem was not aided by the growth characteristics of the spirochaetes, which usually spread over the entire plate. In pioneer work of the cultivation of oral treponemes, similar problems were reported,

> "Inability to obtain true colonies of spirochaetes is perhaps the most serious obstacle to the effective study of these organisms. The conditions of their separation from bacteria and their subsequent cultivation are such that there can be

no guarantee of true purity..... This difficulty is characteristic of all cultures based on Noguchi technique, but as noted previously no adequate alternative to this technique is available." (Rosebury *et al.*, 1951).

Occasionally, spirochaete cultures which had been passaged a number of times would sporadically produce smooth, round, translucent microcolonies of 0.5mm in diameter. The factors inducing this form of growth could not be identified. In an effort to routinely obtain colonial growth, variations to the basic culture media, as well as roll-tube culture techniques were tried, but without success. Smibert (1981) indicated that for oral spirochaetes "getting colonies of treponemes may be difficult and requires patience," an attribute seemingly lacking in this author. If the theory of Canale-Parola (1978), that spirochaetes meander across agar plates in search of limiting growth factors is correct, then presumably, identification of these factors and increasing their concentration would induce colonial formation. The lack of colony production is perhaps indicative that the optimal fulfilment of nutritive requirements has not been achieved, and that the cultures are probably not growing at their maximum rate.

The difficulty experienced in growing spirochaetes in liquid culture is an extraordinary characteristic of these fastidious organisms, which has been reported for both free-living and host associated forms (Hovind-Hougen *et al.*, 1982; Lemcke *et al.*, 1979; Phillips and Lee, 1983; Rosebury *et al.*, 1951). The limited success eventually achieved, was related to the incubation of culture flasks in a slanted position. The reason for this is not obvious, since the organisms did not appear to adhere to the glass nor to require increased gas diffusion into the medium since shaking of the culture in an orbital shaker, did not affect the growth rate. In liquid me-

dium, 5% glucose, but not 5% galactose, inhibits the growth of *T. phagedenis* biotype Reiter although the Kazan 5 biotype is not affected (Thomas *et al.*, 1976).

The formation of coccoid bodies in older cultures, is a peculiar characteristic shared by spirochaetes and other spiral bacteria. The morphological study of spirochaetes at various stages of the growth curve, confirmed the finding that these forms are produced primarily at the onset of stationary phase, although a small increase in number was observed in lag phase (Fig. 51). This could represent the release of "preformed" granules into the culture medium. Whether these rounded forms are reproductive units or not is still controversial. In 1981, Kameneva *et al.* described cystic, filterable, reproductive forms of a marine spirochaete, and more recently, Al-Qudah *et al.* (1983) proposed a life cycle for the Reiter treponeme, in which spirochaetes "emerge" from cystic forms. Several investigators have claimed to have isolated spirochaetes from aged cultures containing only round forms (Hampp, 1946), whereas others claim that these forms are degenerative spirochaetes, incapable of reproduction. Evidence presented in this study, suggests that 100% granular cultures are non-viable, although two granular forms were seen, one of which could be a cystic form. This variety seemed to be a "rolled up" spirochaete (Fig. 52M), which could perhaps have the capacity to "uncurl" and reproduce when introduced into suitable medium. The other granular type, was waste material produced terminally from external sheath components (Fig. 52G and H). Organisms were capable of producing copious granular material, which initially remained associated with the bacteria, but was subsequently released as free granules. In their study, Al-Quadah *et al.* (1983) also described two forms of "cysts", one of which developed spontaneously and was non-viable, whereas the

other was induced by specific, unfavourable conditions and could "germinate" to release viable spirochaetes.

The generation time of 19 hours for isolate D29, is slow when compared to other bacteria including spirochaetes. *T. hyodysenteriae* has a doubling time of 5.2 hours (Lemcke *et al.*, 1979b), *T. succinifaciens* of 3.5 hours (Cwyk and Canale-Parola, 1979), rumen spirochaetes of 60-85 minutes (Ziolecki, 1979), while the oral treponemes have generation times of 12-20 hours and in this respect are similar to the rat isolates (Hardy *et al.* 1964). If this doubling time is indicative of the *in vivo* division rate, then the rat gastrointestinal spirochaetes would have to reside in protected environments to maintain themselves against the GIT flow rate. The active motility of these bacteria would also enable them to maintain themselves in the GIT. *In vitro*, dividing spirochaetes were exceptionally motile.

It is surprising that none of the spirochaete-types isolated from the rat intestine had a requirement for short chain fatty acids while all had an obligate requirement for long chain fatty-acids as supplied in the serum component. Several of the oral and gastrointestinal spirochaetes including *T.innocens, T.hyodysenteriae, T. vincentii, T. denticola, T. refringens, T. phagedenis* require serum whereas most rumen treponemes and a few oral and gastrointestinal species including *T. succinifaciens, T. bryantii, T. pectinovorum* and *T. macrodentium* require short chain fatty acids as supplied in rumen fluid. The preference for foetal calf, rabbit or human sera as opposed to horse serum, is probably related to differences in fatty acid compositions of the sera. Similar preferences have been reported for *T. hyodysenteriae*. These findings, again reflect the fastidious nutritional needs of host-associated spirochaetes.

The ability to hydrolyse the carboxyl ester from both long and short chain fatty acids was tested using the API-ZYM system. The isolates with numerous periplasmic fibrils, all exhibited strong hydrolysis of the short chain fatty acid butyrate, somewhat less hydrolysis of caprylate and only very weak hydrolysis of the C14 fatty acid (myristate) (Table 7). This is in strong contrast to the results obtained for both *T. hyodysenteriae* and *T. innocens*, which exhibited weak activity for butyrate and caprylate and no activity for the C14 fatty acid. Similarly, the spirochaete with the 3-6-3 periplasmic fibril arrangement, showed relatively strong activity for butyrate, negligable activity for caprylate and no C14 lipase activity (Table 7). With their obligate serum requirements, it is surprising that the isolated spirochaetes could not utilize the long chain fatty acids to a greater extent. Similar results were reported for *T. denticola*, which also has an obligate serum-requirement, but negligible or no C14 lipase although it does exhibit a strong C8 esterase-lipase reaction (Laughon *et al.*, 1982). *T vincentii* which is also a serum requiring treponeme, does however utilize the C14 fatty acid but fails to hydrolyse the carboxly group from butyrate (Laughon *et al.*, 1982).

The yellow pigmentation observed in multifibrillar spirochaete isolates and particularly G40, grown on horse-blood medium, was unexpected, since only two spirochaete species, both belonging to the *Spirochaeta* genus, are pigmented. *Spirochaeta aurantia* and *Spirochaeta* , strain RS1 produce the carotenoid pigment hydroxytorulene (Davis, 1965) when grown aerobically, but not anaerobically. The pigment isolated from G40 was identified as being a carotene, since it conformed to all the criteria for this class of pigments (Fox, 1976). Surprisingly, the absorbance spectrum of G40 pigment did not conform to that of hydroxytorulene or any other carotenoid (Davis, 1965). Since the spirochaetes were only

pigmented when grown on horse-blood media and not other other blood media, it is possible that the colour could have arisen from absorption of β-carotenes which are present in horse blood (Fox, 1976). The unique absorption spectrum of the pigment, could be due to modification made to the β-carotene molecule.

The ability to haemolyse rabbit, bovine, and human blood cells, is an attribute of intestinal spirochaetes in common with those isolated from dogs (Turek and Meyer, 1977), opossums (Turek and Meyer, 1978) and pigs (Lemcke and Burrows, 1982; Saheb *et al.*, 1981a; 1981b). The isolated spirochaetes, were all weakly haemolytic and in this respect are similar to *T. innocens*. Although some intestinal spirochaetes have an obligate requirement for haeme (Smibert and Claterbaugh, 1972), the isolated strains did not require this co-factor.

As with other intestinal spirochaetes, the isolated strains had particular nutrient requirements, as shown by the importance of peptone composition used to supplement the medium. Similar fastidiousness has been reported for *T. hyodysenteriae*, which will grow in trypticase soy broth, but not in a basal medium containing peptone and yeast extract (Kinyon and Harris, 1974). In liquid culture, the addition of yeast extract had a beneficial effect on the growth rate of the isolated bacteria. Since a vitamin solution had a similar effect, it is tempting to suggest that the yeast extract fulfilled a vitamin requirement of the spirochaetes. It is possible however that the yeast extract was used as a nitrogen source, since this is known to occur with certain rumen spirochaetes (Ziolecki *et al.*, 1975).

The specific peptone requirements, as well as growth in the absence of carbohydrates, both suggest that proteinaceous material can serve

as carbon and energy sources. The means by which this is accomplished, remains unexplained, since most isolates were only weakly proteolytic, and furthermore, amino acids did not appear to be utilized. None of the stains produced indole from tryptophan nor decarboxylated selected amino acids. The simple metabolic end-products of acetic acid and trace amounts of butyric acid, further suggest that amino acids are not degraded, since this usually results in the production of a variety of volatile fatty acids, notably isobutyric and isovaleric acids. Nevertheless, some *Clostridium* species do ferment amino acids with the production of only acetic acid (Barker, 1981). Furthermore, ammonia, which is also indicative of amino acid degradation, could not be detected in the culture medium. The inability to produce H_2S, also suggests that sulphur amino acids are not metabolized. Inhibition of growth, provoked by the addition of high concentrations of lysine suggests that this amino acid may have triggered a feedback system that inhibited the synthesis of essential amino acids, peptone degradation or transport systems by which utilizable amino acids enter the cell.

Proteolytic activity as indicated by gelatin digestion is an attribute shared by many *Treponema* species including *T. hyodysenteriae* but excluding *T. innocens* (Smibert, 1984a). Of the biochemical tests performed on the isolated spirochaetes, gelatin hydrolysis was one of the few tests in which variation among isolates was observed, with G40 producing a strong reaction, while other isolates of similar morphology, displaying only weak hydrolysis (Table 7). Unfortunately G40 was not characterized according to the API-ZYM system, but the other isolates were all consistently negative for the enzymic assays detecting trypsin and chymotrypsin activities (Table 8). The complete lack of proteolytic or amino acid degradation activities by the multifibrillar isolates (indicated by API-ZYM), is consistent with that found for other gastrointestinal spirochaetes (Hunter and

Wood, 1979). In contrast, many oral treponemes are active trypsin and chymotrypsin producers and some also produce enzymes capable of degrading amino acids particularly leucine (Laughon *et al.*, 1982). Although the treponeme isolates with the 3-9-3 periplasmic fibril configuration also lacked trypsin and chymotrypsin activities, they did have the ability to hydrolyse leucine and possibly even cysteine (Table 8). Furthermore, they also exhibited phosphoamidase activity; a rare attribute of treponemes which has only been reported in a poultry isolate and one strain of *T. hyodysenteriae* (Hunter and Wood, 1979).

The ability to hydrolyse mucin as indicated by $CaCl_2$ precipitation of undigested mucin, may also occur by proteolytic attack. None of the spirochaete isolates possessed the enzymes α-fucosidase, α-galactosidase, or N-acetyl-β-glucosaminidase, suggesting that they do not hydrolyse the sugar moieties from the mucin molecule. Nevertheless all spirochaete isolates hydrolysed aesculin, indicating that they can hydrolyse some glycosidic bonds (MacFaddin, 1980). Aesculin hydrolysis is a common attribute of treponemes, shared not only by *T. hyodysenteriae* and *T. innocens*, but also by the oral spirochaetes *T. denticola*, *T. refringens* and *T. minutum* (Smibert, 1984a).

API-ZYM assays indicate that the multifibrillar treponemes isolated from the rat gut are similar to *T. hyodysenteriae* and *T. innocens* in that they are prolific producers of β-galactosidase and β-glucosidase. Both *T. hyodysenteriae* and *T. innocens* are also producers of α-glucosidase but this enzyme was not detected in the rat isolates. The presence of α galactosidase activity is used to differentiate *T. innocens* from *T. hyodysenteriae* (Joens and Kinyon, 1982), and in this respect, the isolated spirochaetes are like *T. hyodysenteriae* in that they produced this enzyme.

The range of carbohydrates fermented by the multifibrillar spirochaetes is narrow, with only galactose, inositol, inulin, lactose, mannose and xylose being weakly fermented (Table 7). The enzymes β-galactosidase and β-glucosidase would be involved in the liberation of simple sugars from these complex molecules. The fermentation test employed in this study, gave negative results for glucose fermentation for spirochaete isolates, *T. innocens* and *T. hyodysenteriae*. This is in contrast to the published descriptions of these reference strains which are considered to be glucose fermenters (Smibert, 1984a). In PYG broth, glucose fermentation by *T. hyodysenteriae* and *T. innocens* is considered positive if a pH reduction of 0.25 units occurs. This could be beyond the sensitivity of the agar test employed, although using a similar test, Lemcke and Burrows (1981) recorded a positive reaction for glucose fermentation for both *T. hyodysenteriae* and *T. innocens*. A further discrepancy was observed in the fermentation reaction for lactose by *T. hyodysenteriae*, which in this study was positive although Smibert (1984a) records this as being negative. Lemcke and Burrows (1981) also recorded a positive lactose fermentation reaction for *T. hyodysenteriae*. Conventional biochemical tests, including sugar fermentation reactions are not reliable in differentiating *T. hyodysenteriae* from *T. innocens*, and conflicting results are obtained in different laboratories (Matthews and Kinyon, 1984). Kinyon and Harris (1979) consider indole production and fructose fermentation as being of value for differentiating these two species, while Lemcke and Burrows (1981), dispute these findings.

The inability to hydrolyse starch is a characteristic the spirochaete isolates shared with most of the carbohydrate-fermenting species of *Treponema* including both *T. hyodysenteriae* and *T. innocens* (Smibert, 1984a). None of the spirochaete isolates produced indole, and in this respect are like many strains of *T. innocens* but unlike *T.*

hyodysenteriae which is usually positive for this characteristic (Kinyon and Harris, 1979). Although not usually used for characterizing treponemes, the three that have been analysed for urease and nitrate reduction (*T. phagedenis*, *T. hyodysenteriae* and *T. innocens*) are, like the rat isolates; negative for these characteristics. H_2S production is also one of the characteristics differentiating the non-carbohydrate-fermenting treponemes from the carbohydrate fermenting species. In not producing H_2S, the isolated spirochaetes again conform to the latter group in which both *T. hyodysenteriae* and *T. innocens* have been placed (Smibert, 1984a).

The isolated multifibrillar spirochaetes produced acetic acid and trace amounts of butyric acid as metabolic end products. These volatile fatty acids are also produced by *T. hyodysenteriae*, *T. innocens* and *T. vincentii*, although the swine species produce more buryric acid than the rat isolates, while *T. vincentii* produces major amounts of both acetic and butyric acids (Lemcke and Burrows, 1981; Holdeman *et al.*, 1974). The similarity in metabolic end products strongly suggests that these spirochaete species share a common metabolic pathway which is distinct from *T bryantii* and *T. succinifaciens*, both of which produce acetic, formic and succinic acids (Stanton, 1984). The acetic and proprionic acids intrinsically in the medium, were probably introduced in the Trypticase soy broth (Carlsson, 1973).

Using conventional biochemical tests, the isolated spirochaetes appeared to be relatively inert, although the API-ZYM micromethod for detecting the presence of particular enzymes was more revealing in the biochemical attributes of these organisms. Unlike other API systems, the API-ZYM test monitors enzymes produced by cells on any chosen medium, and hence does not rely on the multiplication

of bacteria in the microtubes; a distinct advantage for fastidious organisms such as treponemes. The API-ZYM reaction has been used extensively to characterize both intestinal spirochaetes (Hovind-Hougen, *et al.* 1982; Hunter and Wood, 1979; Joens and Kinyon, 1982) and treponemes of oral origin (Laughon *et al.*, 1982). The API-ZYM profiles obtained in this study for *T. innocens* and *T. hyodysenteriae* are remarkably comparable to those obtained by Hunter and Wood (1979).

The G+Cmol% value of 30.7 obtained for the multifibrillar spirochaete isolates, makes these organisms distinct from any other cultivated *Treponema* species. Both *T. hyodysenteriae* and *T. innocens* have a G+C mol% ratio of 25-26, while other oral, rumen and intestinal strains (*T. succinifaciens*) all have G+C ratios between 36 and 42%. A control culture of *E coli* B (NCTC 9001), gave a G+C mol% ratio of 50.9 which is exactly that reported by Delley (1970). Some difficulty was experienced in isolating spirochaete DNA, although the reason for this is obscure, since methods used for isolation of DNA from other *Treponema* species (T. B. Stanton, personal communication) were not successful on these isolates. No DNase activity could be detected in the spirochaete cultures, and all methods worked well for the isolation of *E. coli* B DNA. Furthermore, spirochaete cultures lysed readily with sodium dodecylsulphate. One possible explanation is that due to difficulties in culturing large numbers of spirochaetes, too few organisms were used, yielding a low concentration of DNA which would be particularly susceptible to degradation. Difficulty in preparing DNA from spirochaetes precluded the logical step of measuring the degree of DNA homology between rat spirochaete isolates and *T. hyodysenteriae* and *T. innocens*

As with other cultivable treponemes, approximately 15% of the cellular dry weight was composed of extractable lipid material. The multifibrillar spirochaete isolates P16, P28, T9 and T28, all had similar polar lipid profiles and similar fatty acid compositions. These isolates all contained the glycolipid monogalactosyl diglyceride (MGDG) and the phospholipids phosphatidylglycerol (PG), cardiolipid (C), phosphatidylserine (PS) and phosphatidylcholine (PC). In this study no attempt was made to determine the proportions of glycolipids and phospholipids in the polar lipid fraction, but spot size and density suggested that MGDG was a major lipid component. MGDG is a common component of all cultivable treponemes (Livermore and Johnson, 1974), although *T. pallidum* is devoid of this glycolipid (Matthews *et al.* 1979). The phospholipids PG and C are common lipid components among spirochaetes while the presence of PC is one of the recommended criteria for differentiating *Spirochaeta* species from *Treponema* species, which in all instances have this phospholipid (Livermore and Johnson, 1974). The presence of PS is more unusual, and has been detected previously in only *T. refringens*, *S. stenostrepta* and *T. pallidum* (Livermore and Johnson, 1974; Matthews *et al.* , 1979). The phospholipid profile of the multifibrillar isolates, is distinct from the morphologically similar treponemes *T. innocens* and *T. hyodysenteriae*, both of which do not contain PS, but do contain sphingomyelin and lysophosphatidylcholine as minor components (Matthews *et al.*, 1980a). With the exception of PS, which formed 22% of the phospholipid component, the proportions of PG, PC and C in the rat isolates were comparable to those found in *T. innocens* (Matthews *et al.*, 1980b). The C phospholipid component of treponemes is particularly important, since it is against this phospholipid that Wassermann antibodies are formed during the early stages of syphilis (Matthews, *et al.*, 1980a). The generalized presence of this component could explain the large degree of immunological

cross-reactivity between different *Treponema* species. Since it is not a taxonomic criterion, no effort to identify sterols in the lipid material of the rat isolates was made, although these neutral lipids would be expected to be present since the isolates were cultivated on serum-containing medium. Cholesterol is a major constituent of lipids of *T. pallidum*, while *T. phagedenis* contains this lipid when grown on serum-containing medium, but not when grown on media lacking cholesterol (Matthews *et al.*, 1979). Approximately 33% of total lipids of *T. innocens* is in the form of neutral lipids derived from serum containing medium.

In addition to MGDG, the multifibrillar spirochaete isolates also possessed two unidentified glycolipids. Neither of these two lipids is believed to be acylMGDG, since they eluted from the DEAE cellulose column with the acidic lipid fraction and were less mobile than MGDG on TLC plates. In an identical system, acylMGDG was shown to have a greater mobility than MGDG (Matthews *et al.*, 1980b). One of the unidentified glycolipids was exceptionally unusual in that it contained the sugar galactosamine in addition to galactose. This sugar has not previously been reported in any spirochaete in which the sugar moieties of the lipid component have been analysed. That this lipid was a minor constituent of the glycolipid fraction is emphasized by sugar analysis on the total lipid fraction, where only galactose was detected. Galactose is normally the only sugar present in spirochaete glycolipids, although some *T. innocens* strains also contain glucose (Matthews and Kinyon, 1984).

The fatty acid profile of total extracted lipid revealed that the three morphologically distinct spirochaetes isolated from the rat gut, could be differentiated according to their fatty-acid composition. The multifibrillar spirochaetes were characterized by the presence of 12:0 and 15:0 fatty acids, the isolates with the 3-6-3 periplasmic fibril

arrangement all had comparatively large amounts of 14:0 fatty acids, and the isolates with 2-4-2 periplasmic fibril configuration were characterized by the presence of what is thought to be alkenyl 15:0 fatty acids. The fatty acid profiles of all three morphologically distinct variants, are distinct from all other treponemes including *T. hyodysenteriae* and *T. innocens*, which in this study gave similar results to those reported for these organisms by Matthews and Kinyon, (1984). These strains do not contain significant amounts of fatty acids with carbon chain length of more than 16 carbon molecules, whereas all the isolated treponemes possessed large amounts of 18:1 fatty acid, and in this respect are similar to *T. pallidum*, and some oral treponemes particularly *T. zuelzerae* (Cohen *et al.*, 1970), and *T. phagedenis* (Livermore and Johnson, 1974). These organisms however also have significant amounts of 18:2 fatty acid, which was not detected in the rat isolates (Cohen *et al.*, 1970). In having large amounts of 14:0 fatty acids, the isolates with the 3-6-3 periplasmic fibril arrangement are similar to *T. microdentium*. Unfortunately, rumen *Treponema* species have not been subjected to fatty acid analysis, and thus can not be compared with the rat isolates.

Serological differentiation of the isolated spirochaetes indicated that the raised antisera lacked specificity. Similar non-specificity is problematical in positive diagnosis of both *T. hyodysenteriae* and *T. pallidum* infections. Direct and indirect fluorescent antibody tests as well as agglutination tests, are regarded as inadequate for diagnostic purposes, as sera must be preadsorbed with non-pathogenic treponemes (Beebe and Nouri, 1984; Burrows and Lemcke, 1981; Joens, 1979; Saunders and Hunter, 1974). Non-specificity of antisera has also been reported for oral treponemes (Jacob *et al.*, 1980; Jacob and Nauman , 1981; 1982). *T. pallidum* and *T. phagedenis* biotype Reiter share at least eight common antigens

(Hanff *et al.*, 1982) and monoclonal antibodies react with both species (Saunders and Folds, 1983). Nevertheless, species-specific *Treponema* antigens are present (Hanff *et al.*, 1982) and adsorption of the raised antisera with heterologous isolates, may have yielded more specific sera. The disc-growth inhibition test has been used to distinguish between *T. hyodysenteriae* and *T. innocens*, (Lemcke and Burrows, 1979a) and in this study also proved to be an improvement over tube agglutination (Tables 10 and 11). It is possible that spirochaete isolates incorporated antigenic serum components from the culture media into their outer membrane. In the disc-growth inhibition test, these non-specific antibodies may have been adsorbed in the medium thus permitting growth of heterologous strains, whereas specific binding with homologous isolates prevented spirochaete replication.

Although four morphologically distinct spirochaetes were isolated from the rat GIT, only one of these has been sufficiently characterized to enable a taxonomic comparison with other *Treponema* species. Morphologically this organism resembles *T. hyodysenteriae* and *T. innocens* in that it is motile by means of 7 or 8 periplasmic flagella originating at each pole, although in size, this isolate is slightly larger than either of the two swine species (Kinyon and Harris, 1978; Smibert, 1984a). On morphological criteria, the rat isolates can be differentiated from other intestinal *Treponema* species, including *T. succinifaciens*, which has a 2-4-2 periplasmic fibril arrangement (Cwyk and Canale-Parola, 1979), and *Brachyspira aalborgi* which is only 0.2µm wide and has four periplasmic flagella at each pole (Hovind-Hougen *et al.*, 1982). Morphologically, the rat isolates are different from all other recognized *Treponema* species. All are significantly smaller in cell diameter, and, with the exception of *T. phagedenis*, all have four or less periplasmic flagella originating from each pole (Smibert, 1984a). They are also morphologically and

physiologically distinct from *T. socranskii* (sp. nov.) (Smibert *et al.*, 1984b), *T. saccharophilum* (sp. nov.) (Paster and Canale-Parola, 1985) and *T. pectinovorum* (sp. nov.) (Smibert and Burmeister, 1983), all of which have been described since the 1984 edition of Bergey's Manual of Systematic Bacteriology was published (Canale-Parola, 1984).

Biochemically, the rat multifibrillar isolates are similar to *T. phagedenis*, *T. hyodysenteriae* and *T. innocens*, all of which belong to the carbohydrate-fermenting group of *Treponema* in the classification system of Smibert, 1984a. Nevertheless, their unique fatty-acid and lipid compositions, unusual API-ZYM reaction pattern and DNA G+C mol% ratio of 30.7 makes them distinct from these *Treponema* species. It is proposed that the rat multifibrillar isolates represent a new species, with the proposed name of *Treponema caeci* and isolate T9 (ATCC 43151) as the type strain.

Description

Treponema caeci sp.nov. (Cowley and Hill) ('cae ci M.L. adj. of the caecum)

Helical cells measuring 0.45 x 8-12µm, exhibiting regular, loose coils. Motile by means of seven or eight periplasmic flagella inserted at each end of the protoplasmic cylinder, overlapping in the central region of the cell. An outer sheath surrounds both periplasmic fibrils and protoplasmic cylinder. Gram-negative. Growth at 42 and 37°C but not 30°C. Obligate anaerobe. Requires serum for growth. Positive for aesculin hydrolysis, weakly hydrolyses gelatin, and weakly haemolytic. Negative for oxidase, catalase, nitrate reduction, starch hydrolysis, indole production, H_2S production, and urease. Negative for ornithine, lysine, and arginine decarboxylase.

Does not grow on agar medium containing 1% bile, 5% NaCl nor 1% glycine. Ferments lactose, mannose, and xylose, weakly ferments inulin and inositol. Variable galactose fermentation. Does not ferment arabinose, cellobiose, dulcitol, fructose, glucose, glycogen, glycerol, maltose, mannitol, melibiose, raffinose, rhamnose, ribose, salicin, sorbitol, sorbose, starch (pH) and sucrose. Produces acetic acid and trace amounts of butyric acid. Guanine + cytosine content of DNA is 30.7mol% (Tm). Indigenous to rat caecum. Type strain: T9; deposited with the American Type Culture Collection under the number ATCC 43151.

CHAPTER 4

DISCUSSION AND CONCLUSIONS

Spirochaetes have been seen in the gastrointestinal contents of various animals since the turn of the century, but have never been proven unequivocably to be autochthonous, although speculations on their autocnthonous status have been made (Savage, 1983). In this study, it was shown that the "classical" spirochaete population in the conventional rat GIT is heterogeneous, and that at least four distinct types are present. These spirochaetes are ultrastructurally biochemically and physiologically distinguishable (Figs. 46-79; Tables 8 and 9). In addition large, helical bacteria possessing both periplasmic fibrils and flagella-like appendages were recognized as components of the gastrointestinal flora of the distal small intestine, caecum and colon. These microorganisms are probably also members of the *Spirochaetales* order.

Of the four types of "classical" spirochaetes isolated from the rat GIT, three fulfilled all requirements for autochthonicity: grew anaerobically, were present in all adults, colonized the gut of the infant animal and maintained stable populations in particular areas of the tract namely, the large intestine. The treponemes with the 1-2-1 periplasmic fibril arrangement are also believed to be autochthonous to the rat GIT, even though they were only seen in 14 of the 15 rats examined (Fig. 4d). The large "atypical" spirochaetes also fulfilled all requirements for autochthonicity except that no statement on oxygen growth requirements can be made since they were never cultivated *in vitro*.

Of the four spirochaete sub-populations differentiated in the rat gut, only one of these, the multifibrillar organisms, resided in the

crypts of the large intestine. This organism was not restricted to the cryptal habitat, and was frequently seen intermingling with other epimural inhabitants on the mucosa as well as in lumen contents. In one weanling rat, bacteria with this morphology attained exceptionally high population densities and were present in large numbers in every caecal crypt (Figs. 32 and 34). Any detrimental effect that this colonisation may have had on the host, was not apparent. It is not known whether this massive colonization is a general phenomenon of weanling rats, but if it is, then homeostatic factors in the gut could have reduced numbers to that of the climax community, since the infant sacrificed the following day did not display a similar colonization pattern. It is also possible that these mulifibrillar spirochaetes have a low inherent pathogenic potential, since they were frequently seen within epithelial cells and even in the lamina propria (Fig. 13). The apparent lack of an immune response towards the spirochaetes strongly suggests that they were tolerated and probably not even recognized as foreign invaders; possibly by the sharing of host antigens or masking of spirochaete antigens by the external sheath. Morphologically, these spirochaetes are similar to the intestinal pathogen *T. hyodysenteriae* (as well as *T. innocens*) and perhaps these organisms are all phylogenically related. The mechanism by which these bacteria penetrate through the cell membrane is obscure, since isolates of similar morphology lacked both trypsin and chymotrypsin activities as shown by the API-ZYM reaction (Table 8). Trypsin is thought to be a virulence determinant of bacteria involved in peridontal disease, although large oral spirochaetes also penetrate through cell membranes in the absence of trypsin activity (Laughon *et al.*, 1982). The isolated spirochaetes also lacked N-acetyl-β-glucosaminidase activity which is another enzyme involved in the breakdown of tissue-ground substance or hyaluronic acid.

Earlier reports that spirochaetes are preferentially localized at the epithelial surface, were not confirmed in this study. All four spirochaete types were seen in lumen contents, while the spirochaetes with the 2-4-2 and 3-6-3 *periplasmic flagella* arrangements even appeared to prefer this environment (Figs. 4b an 4c). Attempts to accurately identify specific habitats of different spirochaete types were unsuccessful, and merely emphasized the broad distribution of the spirochaetes in the distal GIT.

From biochemical tests, few speculations on the niches that spirochaetes occupy in the gut can be made. The lack of growth on isolation medium containing polygalacturonic acid, strongly suggests that rat gastrointestinal spirochaetes do not degrade pectin. The caecum is an area of the gut where the intestinal flow is reduced and bacterial degradation of plant polymers is known to occur, and as such it is surprising that pectinolytic treponemes were not isolated. Since several pectinolytic treponemes from the rumen and oral cavity have been isolated (Smibert and Burmeister, 1983; Weber and Canale-Parola, 1984; Ziolecki *et al.*, 1975; Ziolecki, 1979), spirochaetes with this attribute are likely to reside in the caecum and more persistent efforts to isolate these organisms should be made. The isolation of pectinolytic spirochaetes was not actively pursued in this study.

The ability to hydrolyse mucin as suggested by the $CaCl_2$ test, would be a distinct ecological advantage to intestinal spirochaetes, particularly those localized deep within crypts where a high concentration of mucin would be present. Although the mechanism of hydrolysis is unknown, it would appear as if sugar moieties are not hydrolysed from the mucin molecule since none of the isolates displayed enzymic activities for the sugar-linkages normally associated with mucin: α-galactosidase, α-glucosidase, α-fucosidase, and N

acetyl-β-glucosaminidase (Table 8). The positive reactions for aesculin hydrolysis, β galactosidase and glucosidase however does indicate that some glycosidic activity is present. It is more likely however, that mucin is subjected to proteolytic attack, thus giving a positive result with $CaCl_2$ precipitation. Spirochaete isolate G40 displayed pronounced proteolytic activity on gelatin although other isolates were less proteolytic. Despite having proteolytic activity, the multifibrillar spirochaete isolates did not appear to utilize the released amino acids, since they were negative for decarboxylase reactions, NH_4 production and H_2S production (Table 7). The multifibrillar spirochaete isolates did not have any of the arylamidase enzymes assayed for in the API-ZYM reaction (Table 8) although the spirochaete isolates with the 3-6-3 periplasmic fibril arrangement did display the ability to utilize leucine and possibly cysteine. In the gut environment these amino acids could be scavenged from other actively proteolytic bacteria.

That the isolated spirochaetes are highly adapted to their *in vivo* habitat, is shown by the physical conditions necessary for their culture. Temperatures similar to those encountered in the GIT (ie. 37-40°C) were required for growth. *In vivo* these bacteria would be actively motile in the viscous mucus, which would be comparable to their movement through agar medium. Furthermore, the spirochaetes required anaerobic conditions for replication, although the large multifibrillar isolates (but not the other isolates) could tolerate brief exposures to atmospheric oxygen. Tolerance to oxygen could be an important factor for bacteria in close contact with the epithelium, since oxygen supplied to the cells, may diffuse through the cellular membrane (Clarke, 1977). The mechanism by which spirochaetes overcome oxygen toxicity, is unknown, since neither catalase nor oxidase activity, could be detected (Table 7). In the *in vivo* situation the organisms may utilize host enzymes for

protection against toxic oxygen radicals. Similar reports have been made for both *T. pallidum* and *T. hyodysenteriae*, both of which utilize host superoxide dismutase and catalase to overcome oxygen toxicity (Austin *et al.*, 1981; Fitzgerald *et al.*, 1976; Steiner *et al.*, 1983).

It is surprising that the four morphologically distinct spirochaetes all had an obligate serum requirement and none required short chain fatty-acids, which would be present in lumen contents. Many *Treponema* species can synthesize long chain fatty acids from short chain fatty acids, and one would expect spirochaetes isolated from the lumen contents to have this ability. In the gastrointestinal ecosystem the requirement for long chain fatty acids could be supplied by mucous secretions, of which 40% in the rat is in the form of lipids (Slomiany and Slomiany, 1984). This lipid component consists of neutral lipids (free fatty acids, cholesterol, mono-, di- and triglycerides) and phospholipids (phosphatidylcoline, sphingomyelin, lysophosphatidylcholine and phosphatidylethanolamine) (Slomiany and Slomiany, 1984). Perhaps spirochaetes isolated from lumen contents derive their long chain fatty-acids from mucous secretions in more proximal regions of the GIT. Most fatty acids from dietary lipids are absorbed in the small intestine although some unsaturated fatty acids do reach the lower gut (Prins, 1977). Although they did not require short chain fatty acids for growth, the multifibrillar spirochaete isolates possessed esterase enzymes which could degrade these compounds, but surprisingly lacked lipase enzymes which would degrade longer chain fatty acids (Table 8).

The tan colouration of the multifibrillar isolates grown on plates containing horse blood but not other sera, and the identification of this pigment as a carotenoid, suggests that the spirochaetes may

have absorbed the β-carotenes which are intrinsically present at high concentrations in horse blood (Fox, 1976). In the rat intestine wall, carotene is converted to vitamin A, (Fox, 1976), and it is tempting to speculate that the spirochaetes residing within the crypts and on the mucosal epithelium are involved in this process, perhaps by merely concentrating β-carotene at the mucosal wall and making the pigment available for host conversion. Symbiotic relationships between intestinal prokaryotes and eukaryotes, in which the bacteria supply vitamins for the host are known to exist. Interestingly, isolate G40, which was the most pigmented of all the isolates, originated from the caecal epithelium.

Despite the *in vitro* isolation of four morphologically distinct treponemes, only one of these was extensively characterized. It is unfortunate that complete characterization of all isolates was not achieved in this study, but difficulty in maintaining viable cultures for extended periods of time was experienced. Biochemical parameters normally used to differentiate anaerobic bacteria were not useful for characterizing the isolated spirochaetes. Nevertheless, differences in G+C mol% ratios, fatty acid profiles, lipid compositions and unique API-ZYM profiles, all suggest that the rat multifibrillar isolates are distinct from other *Treponema* species including the morphologically similar strains of *T. innocens* and *T. hyodysenteriae*. A representative of the large multifibrillar spirochaete isolated from the rat gut (isolate T9) has been submitted to the American Type Culture Collection (ATTC) and the number 43151 has been allocated to this isolate. A manuscript in which this organism is given the proposed name of *Treponema caeci* is presently being prepared.

APPENDIX A

LIGHT MICROSCOPIC STAINING OF SPIROCHAETES

1. Modified Gram stain

Reagents

Ziehl-Neelsen carbol fuchsin

basic fuchsin (BDH)	0.5g
phenol crystals	2.5g
ethanol	5.0mℓ
distilled water	50mℓ

Fuchsin was dissolved in the phenol by heating and ethanol was added. After mixing, the water was added and stain was filtered.

Gram's iodine

	1.0g	
iodine, resublimed	2.0g	1.0g
potassium iodide	100mℓ	2.0g
distilled water		100mℓ

Potassium iodide was dissolved in small amount of water, iodine was added and when dissolved the volume made up to 100mℓ

Acetone-alcohol

30mℓ acetone, 70mℓ ethanol.

Crystal violet

0.5% crystal violet (BDH) in distilled water.

Method

1. Samp re heat-fixed, and flooded with crystal violet for 1 min.
2. Film was washed with water, and flooded with Gram's iodine for 1 min.
3. After washing in water, the film was decolourised briefly with acetone-alcohol and counter-stained with carbol-fuchsin for 3-4 min.
4. The film was washed with water, dried, and examined under an oil-objective lens (X1000).

Spirochaetes and gram-negative bacteria stained red; gram-positive bacteria, magenta in colour.

LIGHT MICROSCOPIC STAINING OF SPIROCHAETES

2. Fontana Stain

Reagents

Formol-acetic acid fixative

formaldehyde (40%)	10mℓ
glacial acetic acid	1mℓ
distilled water	100mℓ

Phenol-tannic acid mordant

phenol crystals	1.0g
tannic acid	5.0g
distilled water	100mℓ

Silver solution

10% ammonia was added to 5% aqueous solution of silver nitrate until the precipitate just redissolved.

Method

1. Heat-fixed film was flooded with formol-acetic acid fixative repeatedly for 1-2 min.
2. The film was washed with absolute ethanol and then flooded with ethanol for 3 min.
3. The film was dried, phenol-tannic acid mordant applied, and heated for 30 sec after steam had formed.
4. After washing with distilled water and drying, silver solution was added for 10 seconds, drained, fresh silver solution placed on the film and the slide heated until the film was a light golden brown in colour.
5. Film was washed in distilled water, dried and examined under oil objective lens (X1000).

Spirochaetes stained brownish-black; background stained yellow.

APPENDIX B

ELECTRON MICROSCOPY

1. Negative staining

Reagents

Phosphotungstic acid

A 2.5% aqueous solution of phosphotungstic acid was made, the pH adjusted to 6.8 using NaOH and filtered through a 0.45µm filter.

Method

Bacteria were placed onto a formvar coated, carbon reinforced grid and allowed to adsorb for 2-3 minutes. Excess culture was drawn off using filter paper, a drop of phosphotungstic acid was placed onto the grid and excess was immediately drawn off.

2. Phosphate buffer

Stock solutions of 0.2M K_2HPO_4 and KH_2PO_4 were prepared, and 72mℓ of KH_2PO_4 was added to 28mℓ of KH_2PO_4 to give a buffer of pH 7.2. This was diluted 1 in 2 to give a 0.1M solution.

3. Epon-Araldite Resin

Araldite CY212	3,7g
Epon 812	5,1g
DDSA	10,0g

The above components were mixed thoroughly and 0.47g DMP-30 was added and mixed.

APPENDIX B (continued)

ELECTRON MICROSCOPY

4. Tissue Processing

1. Glutaraldehyde-fixed tissue was washed in phosphate buffer for 10-15 min. at 4°C and post-fixed in 1% OsO_4 in buffer for 1 hour.

2. Tissue was washed twice in buffer for 15 min. in each wash and then dehydrated in alcohol using a 50%, 70%, 90% and 2 x 100% series for 30 min. in each solution.

3. Tissue was placed in propylene oxide for 20 min; 50:50 propylene-oxide:resin for 20 min. and then in resin for 3-4 hours.

4. Tissue was embedded in resin and cured for 24 hours at 70°C

5. Staining of thick sections

Reagents:

One % toluidine blue (BDH) dissolved in 1% disodium-tetraborate

Method:

Sections were flooded with stain, heated on hot plate until steam formed, and washed with water.

APPENDIX B (continued)

ELECTRON MICROSCOPY

6. Staining of thin sections

Reagents

Uranyl acetate

A supersaturated solution of uranyl acetate in 70% ethanol, was made, and the deposit removed by centrifugation. The solution was prepared within 2 hours of being used.

Lead citrate

sodium citrate	1.76g
lead nitrate	1.33g

Lead nitrate and sodium citrate were dissolved in 30mℓ of distilled water. The lead nitrate was converted to lead citrate giving the solution a milky appearance. Eight mℓ of freshly prepared 1N NaOH was added to the solution which become colourless, and the volume was made up to 50mℓ with distilled water. The solution was stored at 4°C for 3-4 months and was filtered through a 0.45µm filter immediately before use.

Method

1. Drops of freshly prepared uranyl-acetate were placed in a petridish, the grids placed in an inverted position onto the drops and stained for 10-15 min.
2. Grids were washed by dipping 10X into each of three beakers filled with filtered distilled water and dried on filter paper.
3. Grids were stained with lead-citrate for 10-15min, by placing them in an inverted position onto lead-citrate drops, in a petridish containing NaOH pellets.
4. Grids were washed and dried as described above.

APPENDIX C

FLUORESCENT ANTIBODY TRACING

1. Preparation of antigens

1. Spirochaetes were harvested from RGCA maintenance medium (Appendix D(4)) by flooding agar surface with saline and scraping bacteria off the plates using a glass spreader.

2. Bacteria were washed twice in saline (2000g for 15 min.).

3. The concentration was adjusted to 5 x 10^8 spirochaetes/mℓ using a Neubauer counting chamber to enumerate the number of spirochaetes present.

4. The bacteria were fixed in 5% formalin (final concentration) at 4°C for 24 hours, washed and resuspended in equal volume of saline.

2. Immunisation routine and pilot bleeding

1. One mℓ of antigen solution was injected into marginal ear vein of a New Zealand white rabbit at weekly intervals for a total of three weeks.

2. Five days after final injection, 10mℓ of blood was collected from each rabbit and allowed to clot at 37°C for 1 hour.

3. Serum was collected by centrifugaton (1000g for 10 min) and inactivated at 56°C for 30min.

4. A two-fold dilution series to 1 in 1024 was made and equal volumes of appropriate antigen was added to each dilution.

5. Tubes were incubated at 37°C for 2 hours followed by 4°C for 1 hour, and examined for agglutination using a 10x hand lense.

6. If the antibody titre was ≥1 in 512, (which was always the case), rabbits were anaesthetized with pentabarbitone and exsanguinated by cardiac puncture. Serum components were collected from the blood.

FLUORESCENT ANTIBODY TRACING

3. Immunoglobulin purification (Walker *et al.*, 1971).

1. pH of serum was adjusted to 8.5.
2. Serum was diluted with saline by adding a volume equal to half the original volume of serum.
3. While being stirred, a volume of Rivanol (2 ethoxy 6,9 diamino-acridine lactate (Sigma), equal to 3.5 times that of the origianl volume was added.
4. Serum was centrifuged at 15 000g for 10 min and the yellow pellet discarded.
5. Rivanol in the serum was precipitated by adding saturated KBr to the supernatant at a volume equal to half the original serum volume and centrifuging at 30 000g for 20 min.
6. To the supernatant, an equal volume of saturated ammonium sulphate was slowly added.
7. Immunoglobulins were precipitated at 4°C for 30 min.
8. Precipitate was collected by centrifugation (15 000g for 10 min) and redissolved in a volume of saline (pH 7.8) equal to the original volume of serum.
9. Ammonium sulphate precipitation was repeated and the final precipitate dissolved in the smallest possible amount of saline and dialysed against distilled water at 4°C for 3 days.
10. Serum was filter sterilized and stored at 4°C.

4. Protein concentration

Reagents

Biuret reagent

$CuSO_4.5H_2O$	1.5g
$KNaC_4H_4O_6.4H_2O$	6.0g
distilled water	500mℓ

Above reagents were dissolved in the water, 10% (wt/vol) NaOH was added and the volume made up to 1ℓ.

Method

1. Two mℓ of Biuret reagent was added to 0.5mℓ of globulin preparation and incubated at room temperature for 30min.
2. Optical density at 540nm was read and protein concentration was determined by referring to a reference calibration curve constructed from dilutions of bovine serum albumin (Fig. 57).

Table 12. Globulin concentration of purified antisera raised against various spirochaete isolates.

antigen	globulin concentration (mg/mℓ)
D29 (heat fixed)	3.8
D29 (formalin fixed)	4.0
G33	4.4
G15	4.0
G39	2.2
control (normal)	5.8

APPENDIX C (continued)

FLUORESCENT ANTIBODY TRACING

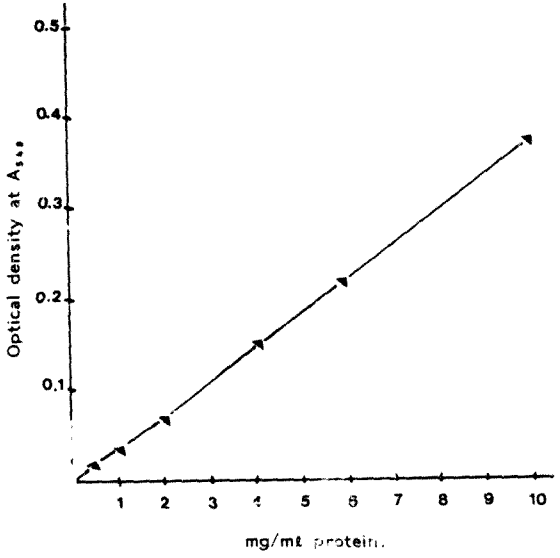


Fig. 57 Standard curve of bovine serum albumin as determined using the Biuret reagent.

APPENDIX C (continued)

FLUORESCENT ANTIBODY TRACING

5. Antibody titration of purified globulin

1. A two-fold dilution series of immune and control antisera from 1 in 2 to 1 in 8192 was prepared using saline as the diluent.

2. An equal volume of appropriate antigen was added, mixed and incubated at 37°C for 2 hours and then 1 hour at 4°C.

3. Tubes were examined for agglutination using a 10X hand lens and compared with control tubes containing the antigen diluted in saline. The end-point was considered as being that dilution at which no agglutination of the bacteria occurred

Table 13. Antibody titres of purified globulins against various spirochaete strains.

antigen	antibody titre
D29 (heat fixed)	1:512
D29 (formalin fixed)	1:2048
G33 (formalin fixed)	1:1024
G15 (formalin fixed)	1:4096
G39 (formalin fixed)	1:512
control (normal serum)	1:8

APPENDIX C (continued)

FLUORESCENT ANTIBODY TRACING

6. Checkerboard titration for determination of antiserum and fluorescein labelled goat-antirabbit serum dilutions for immunofluorescence

To determine the optimum concentrations of both the antiserum and fluorescein isothiocyanate labelled goat-antirabbit serum (Boerhinger Manheim) a checkerboard titration of these two reagents was performed as illustrated in Fig. 58. The relative amount of fluorescence, estimated optically for each dilution is also indicated in this figure.

Staining procedure

1. One drop of appropriate antigen was placed into clear area of a teflon coated slide prepared by placing moist filter paper discs onto the slide prior to spraying with tefion aerosol.
2. Antigens were air dried and fixed in cold methanol for 10 min.
3. A drop of appropriately diluted immunoglobulin was placed on the antigen test spot.
4. Slide was incubated in a moist chamber at 37°C for 30 min. and rinsed three times in PBS (pH 7.2) for 10 min in each wash
5. Diluted goat-antirabbit conjugate was added to the appropriate reaction area, slide incubated at 37°C in a moist chamber for 30 min. and washed as before.
6. Slide was mounted in glycerol mounting medium (9 part glycerol : 1 part phosphate buffer (pH 8.5), viewed and scored for fluorescence.
7. As a result of this titration, all subsequent immunofluorescence work was performed using undiluted fluorescein labelled goat-anti rabbit serum, while globulin solutions G15 and D29 were diluted 1 in 10 prior to use, and G33 and G39 antisera were not further diluted.

APPENDIX C (continued)

FLUORESCENT ANTIBODY TRACING

antiserum to D29 (formalin fixed): dilution

		neat	1:5	1:10	1:20	1:40	1:80
fluorescein	neat	**	***	***	**	*	-
goat-antirabbit	1:5	**	**	**	*	-	-
dilution	1:10	*	*	*	-	-	-

antiserum to G33: dilution

		neat	1:5	1:10	1:20	1:40	1:80
fluorescein	neat	***	**	*	*	*	-
goat-antirabbit	1:5	**	*	*	*	-	-
dilution	1:10	*	*	*	-	-	-

antiserum to G15: dilution

		neat	1:5	1:10	1:20	1:40	1:80
fluorescein	neat	**	**	***	**	*	-
goat-antirabbit	1:5	*	**	**	*	-	-
dilution	1:10	-	-	*	*	-	-

antiserum to G39: dilution

		neat	1:5	1:10	1:20	1:40	1:80
fluorescein	neat	***	**	*	*	-	-
goat-antirabbit	1:5	*	*	-	-	-	-
dilution	1:10	*	-	-	-	-	-

Fig. 58 Checkerboard titration of globulin dilutions and fluorescein goat-antirabbit serum dilutions. Abbreviations: -, no fluorescence; *, slight fluorescence; **, moderate fluorescence; ***, strong fluorescence.

CULTURE MEDIA

1. Preparation of pre-reduced culture media

1. *Nutrients, resazurin (Eh indicator) and salts solutions were made in* the required volume of distilled water and boiled until the indicator became colourless.

2. The solution was cooled under a stream of 12% oxygen-free carbon dioxide in nitrogen (Afrox Ltd.).

3. Autoclavable sugars, rumen fluid and thioglycollate were added and anaerobic gas bubbled through until the pH stabilized. The pHwas then adjusted to 6.5 with 1N NaOH.

4. The prereduced medium was dispensed into gassed, air-tight, screw-capped containers, containing preweighed agar at a concentration of 1.5%.

5. Media were autoclaved for 20 min at 121°C and stored in the dark at room temperature for a maximum of three weeks.

6. Antibiotics were filtered through a 0.45µm filter unit (Millipore) and added aseptically to the molten media in the anaerobic cabinet. Bovine blood or foetal calf serum (RSA state vaccine laboratories) were added aseptically to the cooled molten medium at a concentration of 10%. All manipulations and the dispensing of media into petridishes were done in the anaerobic gas cabinet.

2 Anaerobic diluting fluid (after Holdeman *et al.*, 1974).

gelatin	0.2g
distilled water	50.0mℓ
salts solution	50.0mℓ
resazurin	0.4mℓ

Salt Solution

$CaCl_2$ (anhydrous)	0.05g
$MgSO_4$ (anhydrous)	0.2g
K_2HPO_4	1.0g
KH_2PO_4	1.0g
$NaHCO_3$	10.0g
NaCl	2.0g
distilled water	1000mℓ

$CaCl_2$ and $MgSO_4$ was dissolved in 300mℓ of distilled water. 500mℓ of distilled water was added, and remaining salts were added and dissolved. Volume was made up to 1000mℓ with remaining water. Diluting fluid was prepared anaerobically as described in Appendix D(1).

3. Production of Rumen fluid

Rumen fluid was collected from the local abbatoir and transported to the laboratory. Solid contents were removed by squeezing the rumen fluid through two layers of cheese-cloth and the filtrate collected in bottles flushed with oxygen-free 12% CO_2 in nitrogen. The rumen fluid was centrifuged at 5000g for 30 min. and the supernatant was dispensed into gassed air-tight bottles and stored at 4°C.

4. Modified RGCA Medium (after Holdeman *et al.*, 1974.)

glucose (Saarchem)	0.25g
cellobiose (BDH)	0.25g
soluble starch (BDH)	0.5g
$(NH_4)_2SO_4$	1.0g
resazurin (BDH)	4.0mℓ
distilled water	200mℓ
salts solution (Appendix D(2))	500mℓ
rumen fluid	300mℓ
thioglycollate (BDH)	1.0g
agar(Biolab)	15.0g
basal (PY) broth	200mℓ

Media was prepared as described in Appendix D(1), and was supplemented with 10 citrated bovine blood.

Basal Medium

peptone (Oxoid)	0.5g
trypticase (Oxoid)	0.5g
yeast extract (Difco)	1.0g
distilled water	100mℓ

CULTURE MEDIA

5. Modified E Medium (after Holdeman *et al.*, 1974).

glucose (Saarchem)	0.5g
maltose (BDH)	0.5g
soluble starch (BDH)	0.5g
peptone (Oxoid)	0.5g
yeast extract (Difco)	0.5g
$(NH_4)_2SO_4$	0.5g
resazurin solution (BDH)	4.0mℓ
rumen fluid	300mℓ
salts solution (Appendix D(2))	500mℓ
distilled water	200mℓ
thioglycollate (BDH)	1.0g

Media was prepared as described in Appendix D(1), and was supplemented with 10% bovine blood.

5. Smiberts' Medium (after Smibert and Claterbaugh, 1972)

peptone (Oxoid)	0.5
yeast extract (Difco)	0.
glucose (Saarchem)	1.
soluble starch (BDH)	0.5
$(NH_4)_2SO_4$	0.5g
thioglycollate (BDH)	1.0g
resazurin (BDH)	4.0mℓ
rumen fluid	280mℓ
distilled water	220mℓ
salt solution	500mℓ
agar	20.0g
bovine blood	100mℓ

Salt solution

K_2HPO_4	1,0g
KH_2PO_4	1.0g
$NaHCO_3$	10.0g
NaCl	2.0g
$CoCl_2$	0,0034g
$NaMoO_4$	0.0034g
$MnSO_4$	0.0034g
$CaCl_2$	0.2g
$MgSO_4$	0.2g
distilled water	1000mℓ

CULTURE MEDIA

6. Turks' Medium (Turek and Meyer, 1977).

brain heart infusion broth (Oxoid)	12.0g
trypticase soy broth (Oxoid)	8.0g
yeast extract (Difco)	4.0g
thioglycollate broth (Oxoid)	4.0g
thioglycollate (BDH)	0.13g
$NaHCO_3$	0.4g
agar (Biolab)	20.0g
distilled water	1000mℓ
bovine blood	100mℓ
rumen fluid	300mℓ

7. Lemcke Liquid Growth Medium (Lemcke *et al.*, 1979b).

trypticase soy broth (Oxoid)	100mℓ
foetal calf serum (RSA state vaccine laboratory	10mℓ
resazurin (BDH)	0.4mℓ
glucose	0.5g
$NaHCO_3$	0.4g
cysteine-HCl (BDH)	0.05g

Glucose and $NaHCO_3$ were sterilized separately and added immediately prior to inoculation together with serum. Two mℓ aliquots were dispensed into 150 X 10mm test tubes, or 15mℓ aliquots into 100mℓ medical flat bottles. After inoculation, culture vessels were incubated in a slanted position. such as that used in tissue culture.

8. Tryptone Soy Broth (Oxoid) CM129

pancreatic digest casein (Oxoid L42)	17.0g
papaic digest of soybean meal (Oxoid L44)	3.0g
NaCl	5.0g
K_2HPO_4	2.5g
glucose	2.5g
distilled water	1000mℓ

APPENDIX E

DNA ANALYSIS

1. **DNA extraction** (Meyer and Schleifer (1975))

solutions:

Saline-EDTA
0.15M NaCl made in 0.1M EDTA (BDH) and pH adjusted to 8.0.

SSC Buffer
0.15M NaCl in 0.015M trisodium citrate (dilute 1 in 10 for 0.1X SSC)

Method

1. 0.5-1.0g of wet packed cells were suspended in 0.4mℓ of saline-EDTA solution
2. 65µg of proteinase K (Sigma) was added to the suspension.
3. 0.4mℓ of 6% (w/v) sodium dodecylsulphate was added and the cells lysed at 60°C for 10min.
4. Using saline-EDTA the suspension was made up to 5mℓ and 0.3g of NaCl was added and dissolved (final NaCl concentration, 1M).
5. 2mℓ of 4% cetyltrimethylammonium bromide (CTAB) (Sigma) in 1M NaCl was added.
6. Nucleic acids were precipatiated by the addition of 2mℓ of isopropanol (AR grade).
7. 9mℓ of chloroform-isoamyl alcohol (24:1 vol/vol) was added and the mixture shaken vigoursly.
8. After cooling to 0°C, it was centrifuged for 30 min at 12000g in a Beckman
9. The upper phase containing the nucleic acids was collected, and 2mℓ of CTAB was added.
10. DNA was precipitated by the addition of 1 volume of water and the precipitate was collected by centrifugation at 12000g for 15 min.
11. The tubes were drained, and the pellet was slowly dissolved in 2mℓ of 1M NaCl.
12. CTAB was removed by the addition of 1 volume of chloroform and collection of the upper (aqueous) phase containing the nucleic acids.
13. Sodium-DNA salts were precipitated by the addition of 0.6 volumes isopropanol and were collected by centrifugation of 12000g for 15 min.
14. The supernatant was drained from the tube, and 1mℓ of SSC was layered onto the pellet.
15. Once the pellet had become translucent (ca. 20 min), the buffer was drained from the tube and the DNA dissolved in 1mℓ of 0.1X SSC.
16. When dissolved, 1 mℓ of 10X SSC was added.

17. The concentration of DNA was adjusted to a concentration of 50μg/mℓ (optical density of 0.8 at 260nm) using SSC solution; divided into 1mℓ volomes, and stored at -20°C.

APPENDIX E (continued)

DNA ANALYSIS

2 Important DNA coefficients

$$\mu g/m\ell \text{ DNA} = A_{258} \times \frac{0.0048}{0.021}$$

Pure DNA: $\frac{A_{258}}{A_{230}} = 1.4$

$\frac{A_{258}}{A_{280}} = 1.8 - 2.0$

3. G+C mol% ratio

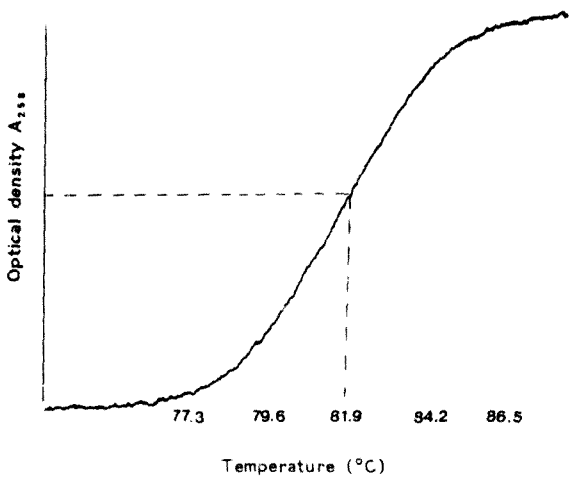


Fig. 59 Tm profile of DNA extracted form isolate T2.

Tm = the temperature corresponding to half the increase in the relative absorbance.

G+C mol% = 2.44(Tm-39.4)

G+C mol% = 2.44(81.9-69.4)

G+C mol% = 30.5

APPENDIX F

LIPID ANALYSIS

1. Lipid extraction (Bligh and Dyer, 1959).

1. Lyophilised cells were resuspended in 1.5mℓ distilled water, and 3.75mℓ methanol and 1.87mℓ chloroform were added in sequence.

2. Suspension was blended on a vortex mixer, extracted at 4° overnight and centrifuged at 2000g on a Sorval bench centrifuge for 15min.

3. Supernatant liquids were collected, cell pellet re-extracted briefly with 7mℓ of chloroform-methanol-water (2:1:0.8 vol/vol/vol) and supernatant combined with first extract.

4. A biphasic system was formed by addition of 5.6mℓ of chloroform, 1mℓ of 7.5% (wt/vol) KCl and 4.6mℓ of distilled water to the extract.

5. Phases were separated by centrifugation (2000g for 10 min.), chloroform phase removed, evaporated to dryness, and dried to constant weight over silica gel in a desiccator flushed with nitrogen.

APPENDIX F (continued)

LIPID ANALYSIS

2. Lipid fractionation

1. DEAE cellulose (Sigma) was washed with: a) 3 volumes of 1N HCl and acid removed by washing with distilled water; b) 3 volumes of 0,1N KOH and excess base removed with distilled water. Washing cycle was repeated twice.

2. DEAE was converted to the acetate form by passing 3 bed volumes of glacial acetic acid through the bed, and removing excess acid by washing with 3 bed volumes of methanol.

3. Packing was air dried, and then dried to a constant weight in a desiccator, over silica gel

4. A 1 cm chromatography tube was packed to a height of 10 cm with 1.2g of DEAE cellulose (acetate form) and washed with 6 bed volumes of methanol, 3 bed volumes of chloroform-methanol (1:1 vol/vol) and 3 bed volumes of chloroform.

5. Lipids were eluted according to the scheme presented in Table 14.

Table 14. Elution scheme used for separation of total spirochaete lipids into lipid classes (modified from Rouser *et al.*, 1969)

elution solvent	elution volumes	lipids eluted[a]	lipid class	fraction
chloroform	6	sterols	neutral lipids	1
chloroform-methanol (9:1 vol/vol)	6	MGDG, DGDG, LPC, LC	non-acidic, polar	2
chloroform-methanol (7:3 vol/vol)	4	Sph, PE	non-acidic, polar	2
chloroform-methanol (1:1 vol/vol)	6	LPE, OPE	non-acidic, polar	2
methanol	4	salts		discard
chloroform-glacial acetic acid 3:1 vol/vol)	6	FFA, pigments	fatty acids	3
glacial acetic acid	6	PS	acidic lipids	4
methanol	4	-		discard
chloroform-methanol (4:1 vol/vol) - 10mM potassium-acetate *20mL/L ammonia	6	PA, PI, PG, DPG	acidic lipids	4
methanol	6	trace lipids, salts		discard

[a]only selected lipids recorded, others not present in treponemes are also eluted (Rouser, *et al.*, 1969). Abbreviations: DGDG, diglycosyl diglycerides; DPG, diphosphatidylglycerol (cardiolipin); FFA, free fatty acids; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; MGDG, monoglycosyl diglyceride; OPE, oxidation products of phosphatidylethanolamine; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidyl serine; Sph, sphingomyelin.

6. Contaminating salts in fraction 4 were removed by passing this fraction through a sep dex column, prepared by mixing 10.0g of Sephadex G-25 urse (Pharmacia) with 50mℓ of methanol-water (1:1 vo), degassing under reduced pressure for 3 hours, and packing a column (1cm x 10cm) with the gel slurry. Column was wa d with 50 mℓ of chloroform-methanol (19:1 vol/vol) followed by 50mℓ of methanol-water (1:1 vol/vol).

7. Lipid sample was applied in chloroform-methanol (19:1 vol/vol) saturated with water (c. 5mℓ of water / ℓ of solvent) and eluted with same solvent.

8. Column was washed with methanol-water (1:1 vol/vol) prior to re-use.

APPENDIX F (continued)

LIPID ANALYSIS

Phosphate determination (Kirchner, J.G. 1978, pp647)

Reagents

70% perchloric acid (BDH)
12% perchloric acid
2,5% ammonium molybdate (Merck)
10% ascorbic acid (Saarchem)
Freshly prepared phosphate standards containing 0,1-1,0µg phosphate as Na_2PO_4

Method

1. Spots on TLC plates were removed with a blade, placed in a test tube and 0.5mℓ of 70% perchloric acid was added to the silica-gel spots in a test tube, and evaporated to dryness.
2. Residue was mixed with 2.5mℓ of 12% perchloric acid and heated in boiling water bath for 10 min.
3. 0.3 mℓ of ammonium molybdate and 0.3 mℓ of ascorbic acid was added, tubes shaken and incubated at 37°C for 2 hours.
4. Silica gel deposite removed by centifugation and absorbance of supernatant read at 820nm.

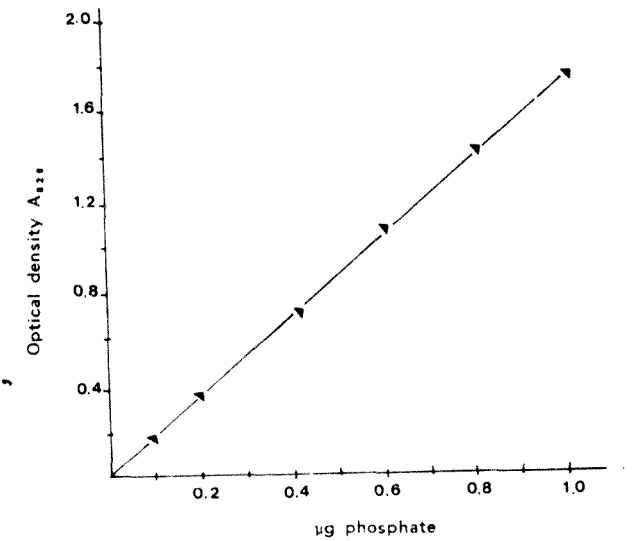


Fig. 60 Optical density (A_{820}) for various concentrations of phosphate, determined using the method of Kirchner (1978).

3. Detection of lipids on TLC plates

Ferric chloride-Sulfosalicylic acid for phospholipids (*Kirchner, 1978*)

Reagent:

sulfosalicylic acid	7.0g
$FeCl_3 6H_2O$	0.1g
distilled water	25mℓ
ethanol	75mℓ

Reagents were dissolved in water prior to the addition of the alcohol.

Test.

When sprayed onto TLC plates, phosphate groups in lipids show up *immediately as white spots on a purple background.*

Diphenylamine reagent for glycolipids

Reagent:

10% diphenylamine (Merck) in ethanol	20mℓ
conc HCl	100mℓ
glacial acetic acid	80mℓ

Test

Plate was sprayed lightly with reagent, covered with another clean glass plate and heated for 30-40 min. at 110°C. Glycolipids formed blue spots

LIPID ANALYSIS

5. Glycolipid analysis (Clamp, 1977).

1. Glycosides were methylated by the addition of 0.5mℓ of 2N methanolic-HCl to each sample and heating under nitrogen at 85°C for 5 hours. (The methanolic-HCl prepared by bubbling HCl gas generated from a mixture of $CaCl_2$ and concentrated H_2SO_4 into AR grade methanol; molarity determined by titration with 2N NaOH).

2. Ag_2CO_3 (BDH) was used to neutralize the solution, and 0.1mℓ of acetic anhydride (BDH) was added and left overnight.

3. After centrifugation and tituration of sediment with 0.5mℓ of methanol, combined supernatant fractions were evaporated to dryness.

4. Lipids were removed by addition of n-hexane (BDH) to the dried sample shaking for 5 min., centrifuging and discarding the supernatant. Residue was dried and stored in a desiccator over silica-gel until required.

5. Samples were derivatized by the addition of 0.05mℓ of *trimethylsilylimidazole* (TSMI) (*Supelco Inc*) *in pyridine* (BDH) (1:2 vol/vol) for 30 min. and chromatographed.

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