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Oral spirochetes:

contribution to oral malodor

and formation of spherical bodies

by Angela De Ciccio

A thesis submitted to the Faculty of Graduate Studies and Research, McGill University, in partial fulfillment of the requirements for the degree of Master of Science.

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ABSTRACT

Spirochetes are putative periodontal pathogens because they are found in increased numbers in periodontitis. With severity of periodontal disease, there also appears to be an increase in oral malodor. This is due to a greater breakdown of tissues containing sulfurated amino acids such as cysteine, cystine, and methionine. Bacteria in the oral cavity can metabolize these amino acids to produce volatile sulfur compounds such as hydrogen sulfide and methyl mercaptan that contribute to oral malodor. Detection of volatile sulfur compounds is usually performed by gas chromatography or a portable sulfur monitor, but none of these methods provides direct evidence for the production of hydrogen sulfide from oral spirochetes. A successful method has been developed, as described in this thesis, to demonstrate that oral spirochetes are major contributors to oral malodor associated with periodontitis. This has been accomplished by modification of an established method for isolating these bacteria directly from subgingival plaques.

A morphological variation of spirochetes, called a spherical body, exists. It has been postulated that this may be a dormant form of spirochetes used as a survival strategy. A number of environmental conditions were tested to determine whether or not they could contribute to spherical body formation in the oral spirochete *Treponema denticola* ATCC 35405. Cells grown in the absence of rabbit serum, volatile fatty acids, thiamine pyrophosphate, or yeast extract showed a dramatic increase in the numbers of spherical bodies. *T. denticola* cells grown in the presence of the metabolic end-product

lactic acid or at pH 7.42 instead of 6.8 also contained more spherical bodies than the control.

RÉSUMÉ

Les spirochètes sont associés à la périodontite parce qu'ils sont retrouvés plus fréquemment durant cette maladie. Proportionnellement à la sévérité de la périodontite, il y a habituellement une augmentation de l'odeur buccale. Ceci est causé par une augmentation de la désintegration des tissus contenant des acides aminés soufrés comme la cystéine, la cystine, et la méthionine. Les bactéries de la cavité buccale peuvent métaboliser ces acides aminés pour produire des composés de soufre volatils comme l'acide sulfhydrique et le mercaptan de méthyle. Habituellement, ces composés sont détectés par chromatographie en phase gaseuse ou a l'aide d'un moniteur portatif détectant le soufre, mais ces méthodes ne donnent aucune preuve de la production d'acide sulfhydrique par les spirochètes oraux. Une méthode est décrite dans cette thèse demontrant que les spirochètes oraux contribuent à l'odeur orale associée avec la périodontite. Ceci a été accompli par modification d'une méthode établie pour l'isolation de ces bactéries directement de la plaque subgingivale.

Il existe une variation morphologique des spirochètes. Ce sont des corps sphériques pouvant être une forme latente du spirochète, utilisée comme stratégie de survie. Différentes conditions environmentales ont été testées pour voir leurs effets sur la formation des corps sphériques dans le spirochète oral *Treponema denticola* ATCC 35405. Les cellules ayant poussé dans un milieu dépourvu de sérum de lapin, d'acides gras volatils, de thiamine pyrophosphate, ou d'extrait de levure ont demontré une augmentation du nombre de corps sphériques. Les cellules de *T. denticola* qui ont poussé

dans un milieu contenant de l'acide lactique ou à pH 7.42 au lieu de 6.8 ont aussi formé plus de corps sphériques comparativement au contrôle.

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LIST OF ABBREVIATIONS

α	alpha
μ	micro
μg	microgram
μl	microlitre
μm	micrometer
μmol	micromole
ATCC	American Type Culture Collection
BANA	N-benzoyl-DL-arginine-naphthylamide
bp	base pair(s)
CFU	colony-forming unit
cm	centimeter
dATP	2'-deoxyadenosine 5'triphosphate
dCTP	2'-deoxycytidine 5'triphosphate
dGTP	2'-deoxyguanosine 5' triphosphate
dH_2O	distilled, demineralized water
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
EDTA	ethylene diamine tetra-acetic acid
g	gram
g	times the force of gravity
GC	gas chromatography
H_2S	hydrogen sulfide
kDa	kilodalton
l	litre
lb.	pound
m	milli
M	molar (mole/l)
mg	milligram
ml	millilitre
mm	millimeter
mM	millimolar
N	normal
nm	nanometer
NOS	New Oral Spirochete medium
NOS-A	New Oral Spirochete medium with 0.7% SeaPlaque agarose
NOS-GB	New Oral Spirochete medium with 0.5% gelatin and 0.5% Bacto agar
NOS-GN	New Oral Spirochete medium with 0.5% gelatin and 0.5% Noble agar
O.D.	optical density

OS	oral spirochete(s)
PBS	phosphate-buffered saline
PC	protoplasmic cylinder
PCR	polymerase chain reaction
PMN	polymorphonuclear leukocyte
PROS	pathogen-related oral spirochetes
RNA	ribonucleic acid
RNase A	ribonuclease A
rRNA	ribosomal RNA
SB	spherical body
SSC	standard saline citrate
TBE	tris borate EDTA buffer
TE	tris EDTA buffer
VSC	volatile sulfur compound
wt./vol.	weight/volume

CHAPTER I. INTRODUCTION

"I found an unbelievably great company of living animalcules aswimming more nimbly than any I had ever seen up to this time. The biggest sort (whereof there were a great plenty) bent their body into curves in going forward..." Antony van Leeuwenhoek (1632-1723) wrote this in his communication with the Royal Society (England), upon examination of a dental plaque specimen (Holt, 1978). This most likely was a description of the first microscopic observation of a spirochete.

Spirochetes are Gram-negative, helical bacteria which differ from other spiral bacteria by the presence of internal rather than external flagella. Despite the fact that spirochetes have been known to exist since the 1600's, little is known about them due to difficulty in cultivating them *in vitro* and a lack of a manipulatable genetic exchange system.

Free-living spirochetes inhabit mud (Margulis *et al.*, 1993), sewage (Johnson, 1977), high salt lakes (Greenberg and Canale-Parola, 1975), and oceans (Canale-Parola, 1978). Some spirochetes may also form part of the normal microflora of eukaryotic organisms such as *Hydra* (Canale-Parola, 1978), the crystalline style of mollusks and the gut of insects (Johnson, 1977) and mammals (Lee and Hampson, 1992, 1994; Johnson, 1977). Only very few species of spirochetes are known to be pathogenic. In 1905, Koch discovered that syphilis, one of the oldest sexually-transmitted diseases, was caused by the spirochete *Treponema pallidum*. Other diseases caused by spirochetes include diarrhea and typhlitis in birds (Swayne *et al.*, 1995), dysentery in pigs (Glock and Harris,

1972), and yaws, pinta, and relapsing fever in humans (Schmid, 1989; Steere *et al.*, 1983).

Spirochetes are also associated with diseases of the oral cavity. The oral spirochetes (OS) have been implicated in periodontitis, an inflammatory disease of the periodontium. Oral malodor is attributed in part to periodontitis. Studies implicating the role of OS in oral malodor form the main thrust of this thesis.

I. TAXONOMY OF SPIROCHETES

The spirochetes are one of the few major bacterial groups whose natural phylogenetic relationships are evident at the level of gross phenotypic characteristics (Woese, 1987). Analysis of nearly complete 16S rRNA sequences (>95%) is an accurate method for determining phylogenetic relationships (Woese, 1987). In the spirochete genera, 16s rRNA phylogenetic classifications agree well with morphology, size, helical wave amplitudes, numbers of axial fibrils, metabolic profiling, and ecology. Spirochetes contain unique signature sequences in their 16s rRNA genes which, when taken together with the cluster patterns derived from rRNA cataloguing, suggest a primordial ancestor which developed into the present genera and species through divergent evolution (Paster *et al.*, 1984, 1991).

Based on 16S rRNA sequence analysis, spirochetes are classified as a single order, *Spirochaetales*, which is further divided into the families *Spirochaetaceae* and *Leptospiraceae*. The family *Spirochaetaceae* comprises the genera *Treponema*, *Serpulina* (into which *T. hyodysenteriae* and *T. innocens* have been reclassified), *Spirochaeta*, *Borrelia*, *Brachyspira*, and *Cristispira*. The family *Leptospiraceae* encompasses the genera *Leptospira* and *Leptonema* (Paster *et al.*, 1991; Stanton *et al.*, 1991; 1992). Six other spirochete genera have recently been reported by Margulis *et al.* (1993): *Clevelandina*, *Diplocalyx*, *Hollandina*, *Mobilifilum*, *Pillotina*, and *Spirosymplokos*. However, they have not yet been fully characterized and further studies are needed in order to classify them into a particular family.

II. ECOLOGY

Most of the spirochete species are either free-living or non-pathogenic, host-associated bacteria. However, a few cause serious diseases in animals and humans. Table 1 lists the distinguishing characteristics of the different spirochete genera.

The genus *Leptospira* contains species which are free-living in soil and water, along with species which are associated with disease. Leptospirosis is caused by some strains of *L. interrogans* and is characterized by fever and hemorrhaging in humans and animals (Greenwood *et al.*, 1992; Schmid, 1989). This disease has a world-wide distribution but is most common in developing countries and warm climates, when contact with infected animal blood or urine-infected water takes place (Schmid, 1989). The leptospires enter the body through mucous membranes or abrasions with subsequent onset of illness occurring within 2 to 20 days. Symptoms of infection include fever, headaches, and myalgia. At this stage the leptospires may be found in the blood, cerebrospinal fluid, kidneys, and other organs of victims (Schmid, 1989). After a few days, the leptospires remain only in the aqueous humor and kidneys, hence they can be shed in the urine to infect other individuals. In more serious cases of disease, leptospirosis may cause jaundice, renal failure, hemorrhaging of the eyes, skin, and mucous membranes, encephalitis, meningitis, nerve palsies, and myocarditis (Schmid, 1989). Icteric disease is caused by the serovar *icterohaemorrhagiae* and is fatal in up to 10% of the cases (Greenwood *et al.*, 1992).

Table 1. General Characteristics of Spirochete Genera[1]

TRAIT	*Treponema*	*Spirochaeta*	*Borrelia*	*Brachyspira*	*Leptospira* *Leptonema*	*Serpulina*	*Cristispira*
Mol % G+C	36-54	51-66	27-32	ND[2]	37-53	26	ND
Size:							
Width (μm)	0.1-0.7	0.2-0.75	0.2-0.5	0.2	0.2	0.3-0.4	0.5-3
Length (μm)	5-20	10-500	3-30	1.7-6.0	10	7-9	30-500
Number of axial fibrils	2-32	2	7-30	4	2	18	>100
Diamino acid of peptidoglycan	ornithine	ornithine	ornithine	ND	diamino pimelic acid	ornithine	ND
Optimal growth temperature (°C)	37-39	15-40	37	ND	15-37	36-42	ND
Oxygen tolerance	obligate or facultative anaerobes	obligate or facultative anaerobes	micro-aerophiles	obligate anaerobes	aerobes	micro-aerophiles	ND
Distribution	mouth, genitals, intestines, tissues of animals and humans	natural water bodies, mud, sewage	arthropods, wild animals, humans	human intestine	surface waters, soil, humans, and animals	digestive tract of mammals	crystalline style and intestine of fresh and salt water mollusks

[1]The data contained in this table were compiled from Barbour and Fish (1993), Canale-Parola (1978), Chan *et al.* (1993), Holt *et al.* (1994), Johnson (1977), Margulis *et al.* (1993), Paster *et al.* (1991), Paster *et al.* (1984), Syed *et al.* (1993), and Schmid (1989).

[2]ND means not determined.

The only representative species of the genus *Leptonema* is *L. illini* (Holt *et al.*, 1994). This organism is found in domestic and wild animals but is non-pathogenic (Holt *et al.*, 1994; Schwan *et al.*, 1992).

The genus *Spirochaeta* comprises obligate and facultative anaerobes which are saccharolytic (Canale-Parola, 1992). These microbes may be found in aquatic environments such as fresh water marshes, oceans, ponds, swamps, lakes, and rivers, as well as in mud and sewage. They also inhabit hydrogen sulfide-containing environments. None of the *Spirochaeta* species has been reported to be pathogenic (Holt *et al.*, 1994).

The spirochete *Cristispira* has been observed in many mollusks, however, the only species presently recognized is *C. pectinis*. The cristispires are most likely commensals of marine and freshwater mollusks and inhabit the crystalline style or fluid of the digestive tract (Holt *et al.*, 1994). They are found in gastropods and non-mollusks as well. Little is known about these organisms due to the inability to cultivate them in an *in vitro* environment (Holt *et al.*, 1994).

The genus *Brachyspira* includes only one species, *B. aalborgi*. This species is pathogenic to humans as evidenced by its isolation from biopsy material of human patients with intestinal spirochetosis (Holt *et al.*, 1994).

Cells of the genus *Serpulina* have been isolated from intestinal contents and feces of swine and other mammals (Holt *et al.*, 1994). *Serpulina hyodysenteriae* is the causative agent of swine dysentery. This condition is characterized by mucohaemorrhagic diarrhea, weight loss, and lesions in the large intestine (Glock and Harris, 1972). Dysentery caused by spirochetes was thought to affect only animals. However it was recently isolated from men with acquired immune deficiency syndrome

(Miller *et al.*, 1992). The non-pathogenic *Serpulina innocens* was isolated from the intestines and feces of swine and dogs (Smibert, 1984). The newly-identified *Serpulina pilosicoli* causes porcine intestinal spirochetosis but has also been found in humans colonized by intestinal spirochetes (Trott *et al.*, 1996).

The *Borrelia* species are microaerophilic and pathogenic to both animals and humans. *Borrelia burgdorferi* is the causative agent of Lyme disease, the most common vector-borne infectious disease in temperate climates. In North America, *B. burdorferi* is carried by the ticks *Ixodes pacificus* and *I. scapularis* (Barbour and Fish, 1993). Three to thirty-two days after being bitten by a tick, a person develops symptoms including fever, headache, and, at the site of the bite, a characteristic skin rash known as *erythema chronicum migrans*. During the following three weeks, the lesions may increase in size or multiple lesions may appear (Schmid, 1989). During the secondary stage of the disease, *B. burgdorferi* disseminates, resulting in swelling, blockage, or failure of the heart, meningitis, or encephalitis (Schmid, 1989). The third stage is arthritis and occurs in 60% of cases. The arthritis may become chronic in approximately 10% of the cases. This disease is rarely fatal but causes chronic ill health if not treated (Schmid, 1989). Other species of *Borrelia* are also pathogenic. Louse-borne relapsing fever is caused by *B. recurrentis* and is spread by a human louse, whereas tick-borne relapsing fever is caused by other *Borrelia* species categorized by the agent-vector relationship. For example, *Borrelia* that infect the tick *Ornithodoros turicata* are speciated *B. turicatae* (Schmid, 1989). The symptoms of both diseases include fever, headache, and myalgia. These manifestations may be followed by or concurrent with dissemination of the spirochetes to other organs of the body. In these cases, death can occur as a consequence

of myocardial collapse. In yet other cases, a low-level spirochetemia may result because the organisms can undergo antigenic variation (Schmid, 1989).

Members of the genus *Treponema* can be part of the normal oral, intestinal, and genital microflora in humans and animals as well as causative agents of disease (Holt *et al.*, 1994). Pinta is caused by *Treponema carateum* and is limited to skin lesions. This is the least contagious of the spirochete diseases, manifests itself in people of all ages, and is now restricted to remote areas of Mexico and northern South America (Perine *et al.*, 1984). The etiologic agent of yaws is *T. pallidum* subspecies *pertenue*. Before the 1960's, it had a worldwide tropical distribution but was eradicated when single-dose penicillin treatments were employed. However, it is still common in rural areas in northern South America, the Caribbean, equatorial Africa, Micronesia, and Burma (Perine *et al*, 1984). Infection is common in children since the organism is spread by skin to skin contact. The most common spirochete disease is syphilis, caused by *T. pallidum* subspecies *pallidum*. Venereal syphilis has a worldwide distribution and is spread via sexual intercourse. The initial lesion is a painless ulcer which usually occurs on the genitalia about three weeks after infection (Schmid, 1989). The ulcer can persist for 2 to 6 weeks and the disease may develop into secondary syphilis, characterized by disseminated skin and mucous membrane lesions and systemic manifestations. If left untreated, one-third of infected individuals develop tertiary disease beginning approximately 5 years after primary infection (Schmid, 1989). This may result in hepatitis, glomerulonephritis, arteritis (often leading to strokes), impotence, bladder dysfunction, extreme pain, and dementia if dissemination to the brain takes place (Schmid, 1989). Venereal syphilis can easily be treated with penicillin if diagnosed early

but is fatal if left untreated (Schmid, 1989). Endemic syphilis is a rare disease caused by *T. pallidum* subspecies *endemicum* but is less severe than venereal syphilis. The late lesions resemble those of yaws. (Schmid, 1989). Endemic syphilis was once widespread in arid areas but is now restricted to parts of the Arabian peninsula and Africa (Csonka and Pace, 1985). It affects mostly children and first manifests itself as shallow, painless ulcers on the mucous membranes of the mouth. This disease can therefore be spread by saliva and shared eating utensils (Schmid, 1989). The only treponeme animal pathogen is *T. paraluiscuniculi*, a venereal spirochetosis of rabbits (Johnson, 1977). The oral treponemes have been implicated as etiologic agents of periodontal disease and will be further discussed in subsequent sections of the thesis.

III. GENERAL CHARACTERISTICS OF SPIROCHETES

Spirochetes are flexuous, Gram-negative helical organisms which divide by transverse binary fission (Hovind-Hougen, 1974). They differ from other prokaryotes by the presence of periplasmic flagella. A schematic representation of a spirochete cell is depicted in Figure 1. A darkfield micrograph of the OS *T. denticola* ATCC 35405 is shown in Figure 2. Spirochete structures and their functions will be outlined below.

Figure 1. **Schematic representation of a spirochete cell.**

A. Cross-section along the length of a spirochete.

B. Cross-section through the center of a spirochete cell.

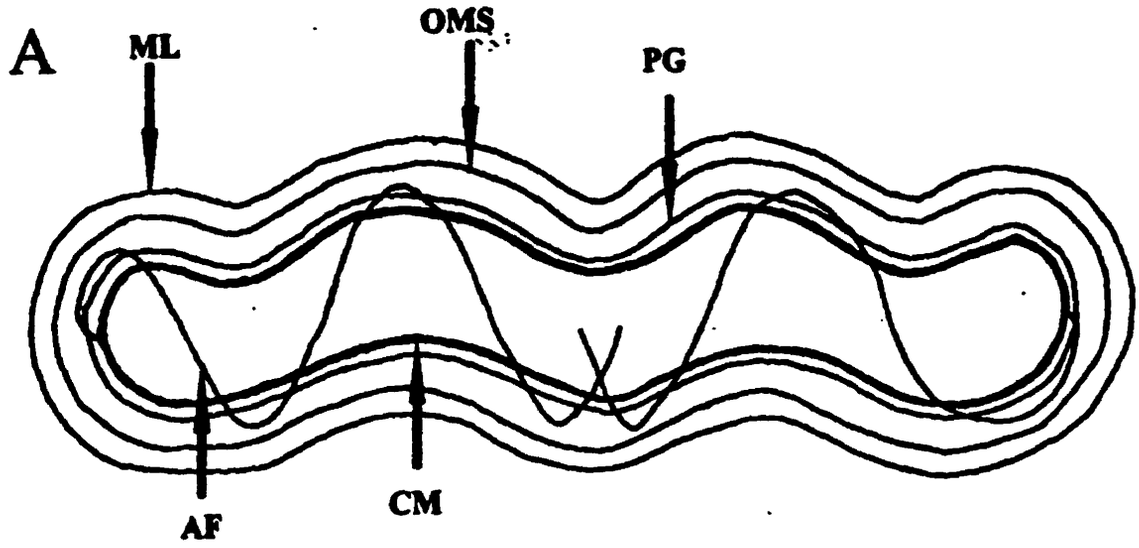
(Adapted from Holt, 1978.)

The locations of the mucoid layer (ML),

outer membrane sheath (OMS),

axial flagella (AF),

peptidoglycan layer (PG), and cytoplasmic membrane (CM) are shown with arrows.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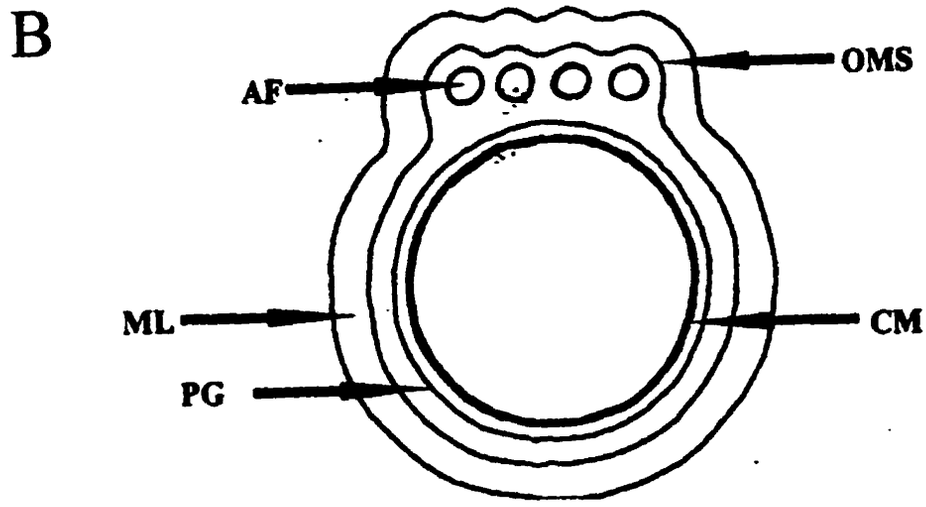
B
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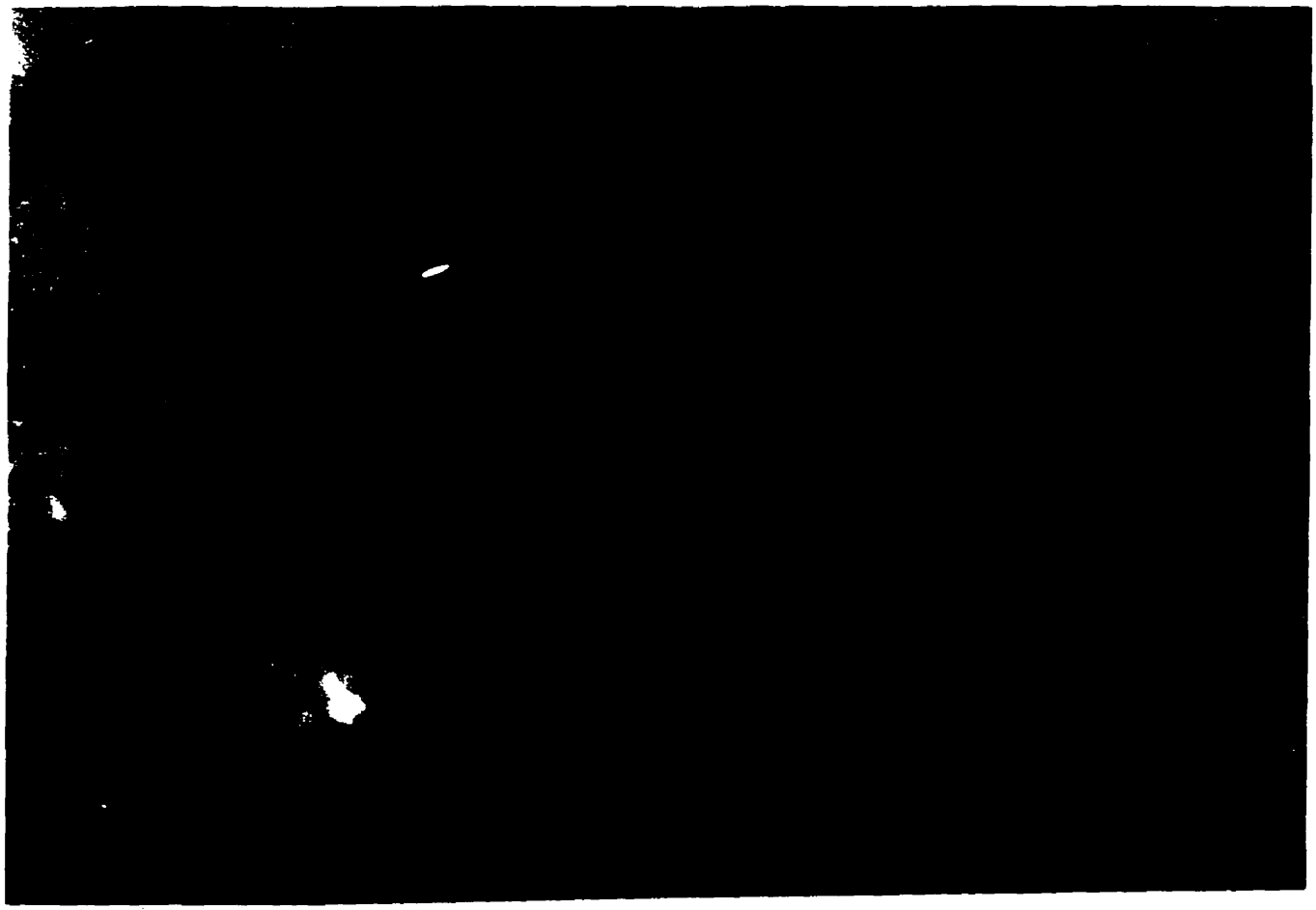
PG

Figure 2. **Darkfield photomicrograph of *Treponema denticola* ATCC 35405.**

Original magnification: 1000X



i. Mucoid Layer

The mucoid layer is the outermost component of the spirochete and is hence the first part of the cell to interact with the immune system, other bacteria in the cell's vicinity, and host cells.

Most of the work on mucoid layers was done on *T. pallidum*. Mucopolysaccharides were shown to accumulate in the lesions associated with syphilis, and it was proposed that the mucopolysaccharides resulted from capsule formation by *T. pallidum* (Turner and Hollander, 1957). Later, electron microscopy revealed that this layer was actually a mucoid layer (Christiansen, 1963; Fitzgerald *et al.*, 1978). Initially, it was believed that host proteins adhered to the mucoid layer, thereby preventing an immune response against *T. pallidum* (Christiansen, 1963). It was also hypothesized that the mucoid layer could hide the spirochete antigens that were on the cell surface (Fitzgerald and Johnson, 1979). More recent studies show that *T. pallidum* contains relatively few outer membrane proteins (Cox *et al.*, 1992). It is, however, possible that both the lack of outer membrane proteins and the presence of a type of protective coat might aid *T. pallidum* to evade the immune system.

Few studies deal with mucoid layers in the OS. Recently, though, Scott *et al.* (1997) confirmed the presence of a mucoid layer in *T. denticola* ATCC 35405.

ii. Outer Membrane Sheath

The outer membrane sheath (which can also be called simply the outer membrane or outer sheath) is a three-layered membrane for most spirochetes but a five-layered one for some leptospires (Johnson, 1977). The viability of spirochetes is dependent on an intact outer sheath. Studies showed that damage to the outer sheath of leptospires by antibody and complement resulted in the loss of intracellular components and cell death. Furthermore, *Borrelia* and *Treponema* cells are immobilized or killed by the antibody-complement system (Anderson and Johnson, 1968) and the use of hypotonic solutions to extend the outer sheath of treponemes results in cell death (Johnson, 1977).

The outer sheath in treponemes and leptospires contains protein, lipid, and carbohydrate. The presence of lipopolysaccharide has also been noted but may be species-specific (Hardy and Levin, 1983; Penn *et al.*, 1985; Strugnall *et al.*, 1990). Also, the outer membrane of spirochetes appears to contain lipopolysaccharide with different composition from other bacteria and may thus have a different biological activity than in other Gram-negative bacteria (Zeigler and Van Eseltine, 1975). Haapasalo *et al.* (1992) studied the protein content of the outer membrane of *T. denticola* and found a 53 kDa protein which bound to fibronectin, fibrinogen, and laminin and might therefore aid in the organism's attachment to periodontal tissue. Egli *et al.* (1993) subsequently purified the protein and showed that it was the only known porin in *T. denticola*. This porin might serve as a crude filtration device and might mediate concentration of food near the opening of the channel (Egli *et al.*, 1993).

iii. Periplasmic Flagella

Unlike other flagellated bacteria, spirochetes contain flagella beneath the outer membrane sheath, inserted subterminally at each cell end and extending toward the opposite pole of the cell. These periplasmic flagella have been designated various names including axial fibrils, axial filaments, axial flagella, endoflagella, periplasmic fibrils and axistyles. The number of periplasmic flagella per spirochete cell can range from two to hundreds (Canale-Parola, 1978; Charon *et al.*, 1992). A three-digit system is used to describe the arrangement of the axial fibrils. For example, the designation 1-2-1 means that one axial fibril is inserted at each end of the cell and in the middle, where the filaments overlap, there are two fibrils. Only the leptospires and *T. phagedenis* contain periplasmic flagella which do not overlap (Margulis *et al.*, 1993). In addition, in older cultures of *T. denticola* ATCC 33520 and *T. phagedenis* Kazan 5, axial filaments were found external to the cells, yet the organisms still exhibited motility (Ruby *et al.*, 1991).

Other than their characteristic periplasmic location, the fine structure of spirochete axial filaments appears similar to that of flagella in other organisms. That is, the axial filament consists of a filamentous portion or shaft, a hooked region near the insertion into the cell body, and an insertion apparatus made up of a number of disks. The axial fibrils are wound around a helical protoplasmic cylinder. Chemically, the filaments are more complex than the flagella in other bacteria (Holt, 1978). Studies showed that purified periplasmic flagella from a variety of spirochetes consisted almost entirely of protein and that their amino acid composition was similar to that of other bacterial flagella. Bacterial flagella usually consist of one protein, flagellin, whereas the spirochete axial fibrils

contained several different proteins (Holt, 1978). The only exceptions are *B. burgdorferi* and *Spirochaeta zuelzerae*, whose periplasmic flagella contain only one polypeptide (Charon *et al.*, 1992).

Klitorinos *et al.* (1993) showed that the oral spirochetes *T. denticola*, *T. socranskii*, and *T. vincentii*, can locomote through viscosities of up to 700 mPas, possibly due to the periplasmic location of their flagella. This could explain the presence of spirochetes in viscous host tissue and their ability to penetrate the oral mucosa. Other flagellated bacteria exhibit optimal motility at 2 to 5 mPas and their speed usually decreases with increasing viscosity, so they are not frequently isolated from tissues (Schneider and Doetsch, 1974).

iv. Protoplasmic cylinder

The protoplasmic cylinder is found directly beneath the outer membrane sheath and is made up of both a peptidoglycan layer and a cytoplasmic membrane. The peptidoglycan layer maintains the helical shape of spirochetes since treatment of cells with lysozyme or penicillin, which affect the integrity of the peptidoglycan layer, results in the loss of their spiral shape (Canale-Parola, 1978). On the other hand, purified peptidoglycan retains its helical shape (Canale-Parola, 1978). The peptidoglycan of *Treponema* and *Spirochaeta* cells contains ornithine as its diamino acid component but *Leptospira* contains diaminopimelic acid (see Table 1) (Johnson, 1977). All three genera, though, contain muramic acid, alanine, glucosamine, and glutamate in their peptidoglycan layers (Johnson, 1977).

v. Cytoplasm

The spirochete cytoplasm is like that of other bacteria, containing vesicles and inclusion bodies (Johnson, 1977). The genome of spirochetes can range in size from 900 kb for *T. pallidum* to 3100 kb for *L. interrogans* (Taylor *et al.*, 1991; Walker *et al.*, 1981). The G+C content of spirochete DNA can range from 26 to 66 mol%, as demonstrated in Table 1 (Paster *et al.*, 1991; Canale-Parola, 1978).

Extrachromosomal elements were shown to exist in spirochetes. For example, Caudry *et al.* (1995) found a 2.6-kb plasmid in both *T. denticola* ATCC 33520 and *T. denticola* e', similar to the spirochete plasmid pTD1, previously reported by Ivic *et al.* (1991). A 4.2-kb plasmid was also found in four strains of oral treponemes studied (Chan *et al.*, 1996; Caudry *et al.*, 1995). The fact that the same plasmid was found in different strains of treponemes suggests a natural mechanism of exchange of the plasmid between different organisms. Until recently, there was no known mechanism of genetic exchange for spirochetes. Li and Kuramitsu (1996) reported the construction of a specific *flg*E mutant of *T. denticola* ATCC 35405 after electroporation using an erythromycin resistance cassette inserted into an *flg*E DNA fragment. The *flg*E gene encodes the flagellar hook protein. Therefore, the resulting mutant lacked periplasmic flagella and did not display any visible motility. This method of transformation holds much promise for future work in genetic exchange for spirochetes.

The cultivable spirochetes were once thought to exhibit a resistance to rifampin, suggesting an alternative transcription machinery or other resistance mechanism (Paster *et al.*, 1984). However, Trott *et al.* (1996) described a new spirochete species, *Serpulina pilosicoli*, which was moderately susceptible to rifampin. Their studies demonstrated that

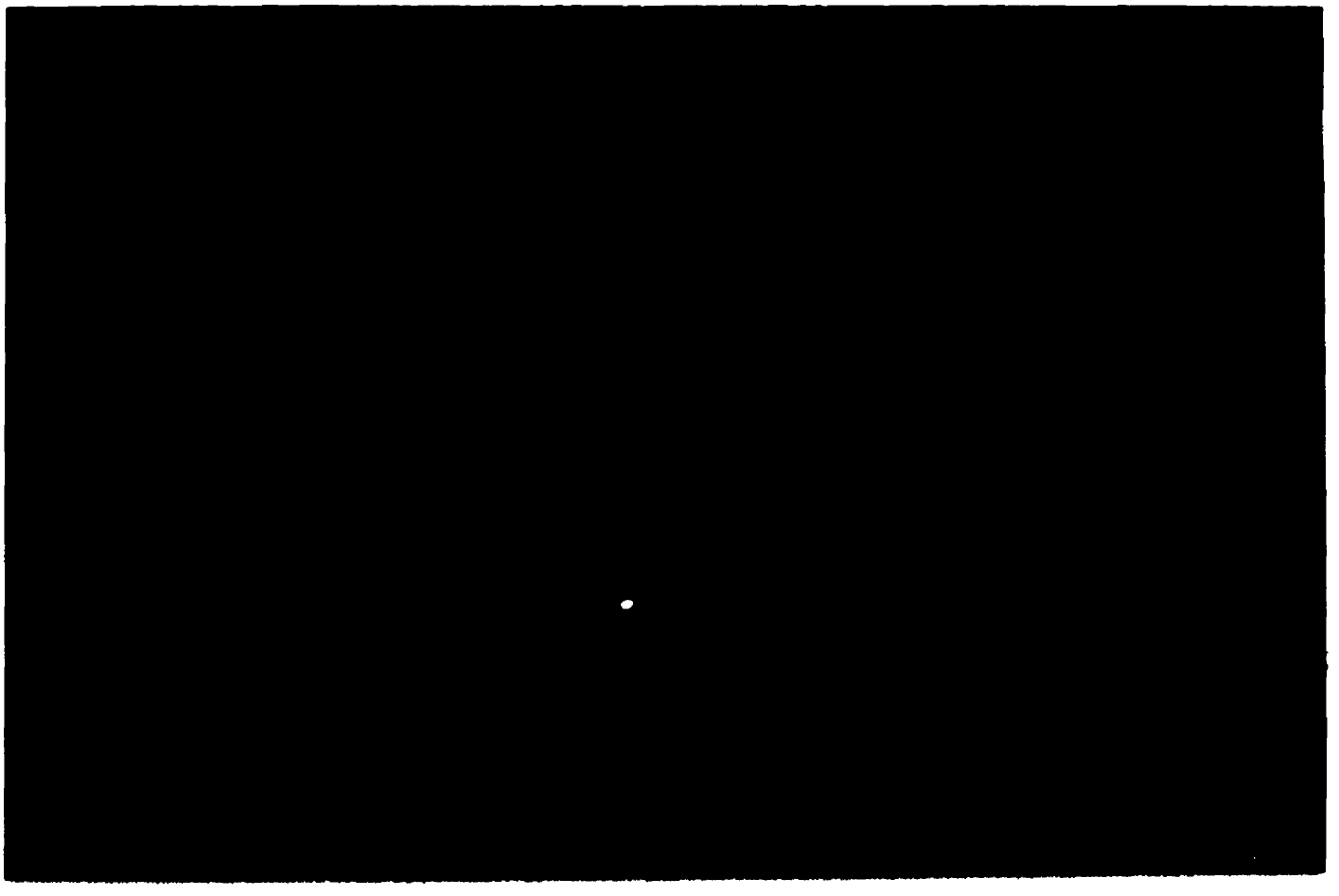
S. innocens was also moderately susceptible to rifampin at a concentration of 20 μg/ml. Similarly, experiments in our laboratory suggest that *T. denticola*, *T. vincentii*, and *T. socranskii* are inhibited by 20 μg/ml rifampin (unpublished data).

IV. SPHERICAL BODIES OF SPIROCHETES

Not all of the spirochetes in a culture exist in the typical helical form. Certain morphological variations of spirochetes, previously called "granules", were first described in 1948 (Hampp *et al.*). Reports on the spherical structures of different spirochete species were later published (Hampp and Bethesda, 1950; Czekalowski and Eaves, 1954; Listgarten *et al.*, 1963; Kawata and Inoue, 1964; Bladen and Hampp, 1964) and were thereafter termed "spherical bodies" (Umemoto and Namikawa, 1980; Umemoto *et al.*, 1982, 1984; Gebbers *et al.*, 1989). A darkfield micrograph of a spherical body formed by *T. denticola* ATCC 35405 is shown in Figure 3. Though the cause of formation of the spherical bodies is not known, the development of these quasi-multicellular bodies was elucidated (Wolf *et al.*, 1993; Wolf and Wecke, 1994). The cells begin as typical helical treponeme forms, but upon cell division, the protoplasmic cylinders separate while the outer sheath remains intact (Figure 4a, b). The two spirochetes then move in contrary directions, initiating plait formation (Figure 4c, d). As the treponemes continue to move, one side is further compressed into a club shape (Figure 4e). This movement continues until a spherical body containing many spirochetes is formed (Figure 4f, g). The spirochetes in spherical bodies lose their

Figure 3. **Darkfield photomicrograph of a spherical body formed by *T. denticola* ATCC 35405.**

Original magnification: 1000X



individual outer sheaths. Instead, many protoplasmic cylinders lie side by side on the inner surface of a common outer sheath (Wolf and Wecke, 1994). As well, the axial flagella become randomly arranged in the body rather than being organized around the protoplasmic cylinder and vesicles or central bodies of different sizes may be present.

Until recently, it was believed that spherical bodies existed only *in vitro* but not *in vivo*. Studies by Wecke *et al.* (1995) show that treponemal spherical bodies were found on expanded polytetrafluoroethylene membranes used for the treatment of deep periodontal pockets. Their results indicate that all forms of the spirochetes and spherical bodies were present on the membrane, and hence, *in vivo*.

It has been postulated that spherical bodies may be dormant forms of spirochetes used as a survival strategy. Other types of bacteria are able to form endospores when the surrounding conditions are unfavorable. This may be caused by a lack of proper environmental nutrients, an accumulation of toxic by-products, or a change in the environmental gas content. Similar to spore-forming bacteria, if conditions are improved, spherical bodies can revert to their normal helical form (Wolf and Wecke, 1994).

Figure 4. **Schematic drawing of the morphogenetic process of formation of spherical bodies of spirochetes, beginning with the normal helical form.**

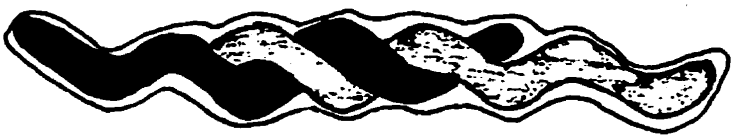
a. Typical helical form of treponemes; **b.** separation of protoplasmic cylinders (PC) of dividing treponemes, the outer sheath (S) remains intact; **c.** movement of two spirochetes in contrary directions initiates formation of a plait; **d.** plait as an intermediate form of a spherical body; **e.** further movements compress the individual treponemes to a club; **f.** final stage: the spherical body containing many spirochetes; **g.** cross section of a spherical body.

(Adapted from Wolf and Wecke, 1994.)

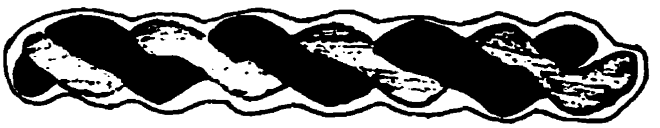
a. **Typical helical form.**

b. **Separation of protoplasmic cylinders.**

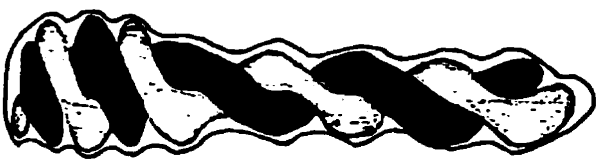
c. **Initiation of plait formation.**



d. **Plait.**



e. **Club.**



f. **Spherical body.**

g. **Cross section of a spherical body.**

V. THE ETIOLOGY OF PERIODONTAL DISEASE

i. General Characteristics of Periodontal Disease

Periodontal disease is an inflammatory response of the gingiva and connective tissue to accumulation of bacteria on the base of the teeth along the gum margin. It is characterized by destruction of the supporting tissues of the teeth and may eventually lead to tooth loss.

Chronic marginal periodontal disease, or adult periodontitis as it is also called, encompasses two separate disease entities: gingivitis and periodontitis. Gingivitis is the early stage of gum disease and is considered to be a minor inflammation of the gums that may ultimately lead to periodontitis in the susceptible subject (Robinovitch, 1994). The gums are red and inflamed and may bleed spontaneously, during brushing or flossing, or upon probing (Loesche, 1993). There is, however, no clinical bone loss or mobility of the tooth. Periodontitis is used to describe the stage of chronic marginal periodontal disease in which there is progressive loss of attachment of the tooth to its supporting structures caused by the accumulation of bacterial plaque subgingivally. The bone and fibers surrounding the tooth are damaged, forming a space between the tooth and the gingival epithelium. As this gingival crevice deepens, it forms a pocket which may eventually exceed 10 mm in depth. Bacteria invade the site and release cytotoxic and degradative products into the periodontal pocket. There is also alveolar bone loss, and mobility of the tooth, eventually leading to tooth loss if the condition is left untreated (Kleinberg and Wolff, 1986; Loesche, 1993). This disease is not considered to be reversible since the

lost alveolar bone and periodontal ligament do not regenerate with cessation of the inflammation, even though further progression may be halted. Periodontal disease activity is indicated by bleeding upon probing, pus formation, and other signs of inflammation (Loesche, 1993). Adult periodontitis is the most common form of periodontal disease. It mostly affects people aged 30 years and older (Riviere *et al.*, 1991) and is responsible for most tooth loss in people of this age group.

Other types of periodontal disease exist, categorized mainly by the age of onset of the diseases and the rapidity of their progression. Early-onset periodontal diseases occur after puberty and progress rapidly (Scheinken, 1994). For example, localized juvenile periodontitis, which affects the first molars and incisors, generalized juvenile periodontitis, which can affect many teeth, and rapidly-progressing periodontitis are all types of early-onset periodontal diseases. Acute necrotizing ulcerative gingivitis, also known as Vincent's infection or trench mouth, is distinctly different from adult periodontitis. It has an acute onset, frequently associated with periods of stress and poor oral hygiene. There is rapid ulceration of the gingiva that can quickly lead to pathologic bone resorption. Spirochetes and fusiform bacteria have been implicated in this disease. In fact, studies have shown that the spirochetes actually invade the tissues (Robinovitch, 1994).

Severe periodontal disease is highly prevalent amongst older people. In Ontario, 22% of older adults were reported to have severe periodontitis (Locker and Leake, 1992). The Piedmont 65+ Dental Study showed that 16% of whites and 46% of blacks had severe periodontal disease (Beck, 1990). Risk markers for periodontal disease can include aging, smoking, and dental hygiene.

Three hypotheses exist to explain the relationship of plaque to periodontal disease. The specific-plaque hypothesis says that specific microbes in the plaque cause the disease (Loesche, 1976). The nonspecific plaque hypothesis states that the effects of many nonspecific bacteria in the dental plaque cause periodontitis (Loesche, 1976). The last hypothesis, the exogenous hypothesis, says that the normal plaque microflora are disrupted by a newly-acquired pathogen, thereby causing the clinical manifestations of periodontal disease (Genco, 1987). To date, no specific bacterium has been shown to be the sole causative agent of periodontal disease. The oral cavity is inhabited by a number of different species of bacteria that may contribute to or cause disease. These issues will be addressed in the following section.

ii. The Role of Bacteria in Periodontitis

It has been suggested since the 19th century that bacterial overgrowth on the dento-gingival surface is the cause of periodontal disease (Harlan, 1883; Miller, 1890). This concept was reintroduced in the 1950s when it was suggested that plaque control was essential in the treatment of periodontal disease (Loesche, 1976).

A healthy gingival crevice contains few bacteria, most of which are Gram-positive, aerobic cocci. During periodontal disease, though, the microflora shifts to predominantly Gram-negative, anaerobic motile bacteria (Kleinberg and Wolff, 1986, Loesche, 1993). A study by Singletary *et al.* (1982) found that 62% of bacteria in healthy sites were cocci and 5% were motile bacteria, while in diseased sites, 18% were cocci and 44% were motile.

A periodontal pocket can serve as a protected environment where a variety of bacteria can grow. Over 350 bacterial species were found in plaque and up to 60 species were recovered from a single pocket (Wolff *et al.*, 1994; Wolff *et al.*, 1988). However, only a few of these species seem to be associated with periodontitis. *Porphyromonas gingivalis*, *Actinobacillus actinomycetemcomitans*, *Fusobacterium nucleatum*, *Bacteroides forsythus*, *Campylobacter rectus*, *Prevotella intermedia*, *Eikenella corrodens*, and the treponemes, including *T. denticola*, *T. socranskii*, *T. vincentii*, and the uncultivable pathogen-related oral spirochetes (PROS), all appear to be found more frequently from periodontally-diseased as opposed to disease-free sites (Newman, 1990; Riviere *et al.*, 1991, 1992; Wolff *et al.*, 1994). Research indicates that the pathogens thought to be associated with periodontal disease are found in both healthy and diseased gingiva and that gingival inflammation does not necessarily result in periodontitis (Riviere *et al.*, 1992; Wolff *et al.*, 1994). Therefore the host's oral environment must also play a role in the progression of disease. In most people, periodontal health is well-maintained by the body defenses, despite the high microbial load in the oral cavity. However, certain factors may facilitate or deter the progression of periodontitis. For instance, commensal bacteria are known to be protective. *Streptococcus sanguis* can inhibit the growth of *A. actinomycetemcomitans* (Taubman *et al.*, 1989) and *Streptococcus mitis* inhibits bacilli which cause diphtheria (Thomson and Shibuya, 1946). In addition, certain microbes can promote the growth of other species of bacteria. For example, *P. gingivalis* secretes a substance that stimulates the growth of *T. denticola* and the occurrence of *T. denticola* requires the presence of *P. gingivalis* (Simonson *et al.*, 1992).

Factors produced by the putative pathogens of periodontitis may also be involved with the disease. Ammonia, which is produced by bacteria during amino acid fermentation, is found in the gingival crevice, irritates the gingiva, and can inhibit collagen synthesis (Kleinberg and Wolff, 1986). Endotoxin may stimulate inflammation and bone resorption *in vitro*. As well, endotoxin causes the release of lysosomal enzymes from polymorphonuclear leukocytes (PMNs), which can intensify inflammatory reactions (Listgarten, 1987). Short chain fatty acids, including propionate, formate, isovalerate, succinate, and isobutyrate, have been detected in periodontal pockets. These fatty acids are produced by anaerobic bacteria and can diffuse out of the pocket and into the gingival tissues (Kleinberg and Wolff, 1986). The level of short chain fatty acids has been correlated with the severity of periodontal disease (Tonetti *et al.*, 1987). In pockets, they are found at concentrations of 5-30 mM, concentrations which may inhibit PMNs *in vitro* (Botta *et al.*, 1985), as well as inhibit lysozyme release by PMNs stimulated with f-met-leu-phe (Tonetti *et al.*, 1987). Short chain fatty acids have also been shown to inhibit human gingival fibroblast proliferation (Singer and Buckner, 1981) and affect actin synthesis in fibroblasts (Yamada *et al.*, 1985).

Some oral bacteria produce hyaluronidases that may degrade the hyaluronic acid that is a component of connective tissue ground substances (Tam *et al.*, 1982; Tam and Chan, 1985; Scott *et al.*, 1996). Such degradation can make it easier for toxins and bacteria to gain access to gingival tissue, thereby initiating periodontal disease. In addition, phospholipase C is present in periodontally-diseased sites but not in healthy ones (Siboo *et al.*, 1989). It can participate in the destruction of host cells and in the

release of arachidonic acid. Arachidonic acid can produce the inflammatory mediators prostaglandins and leukotrienes and may thus be a factor in periodontal disease.

Another factor that is important in periodontitis is colonization of a bacterial species to a host cell or bacterium in the oral cavity. For example, *P. gingivalis*, *P. intermedia*, *Actinomyces naeslundi*, *A. viscosus*, and *F. nucleatum* adhere to blood group glycoproteins present on epithelial cells (Lie *et al.*, 1994).

When bacteria metabolize sulfur-containing amino acids, by-products include volatile sulfur compounds (VSCs) such as hydrogen sulfide, methyl mercaptan, and dimethyl sulfide. Cysteine degradation, catalyzed by the enzyme cysteine desulfhydrase, yields hydrogen sulfide, ammonia, and pyruvate. Methionine, after deamination, can result in the formation of methyl mercaptan and α-ketobutyrate. Dimethyl sulfide may also come from methionine. In the presence of serine, methionine may also form cysteine, which can, in turn, yield hydrogen sulfide. The VSCs can disrupt the structural integrity of the sulcular epithelium, allowing other microbial products to gain access to the underlying connective tissue (Ng and Tonzetich, 1984) and causing inflammation. They can also interfere with the normal maturation of collagen, prevent protein synthesis by fibroblasts, and increase the permeability of the oral mucosa by cleaving disulfide bonds of the proteoglycans and glycoproteins in the extracellular matrix of the sulcular and junctional epithelium (Ng and Tonzetich, 1984). VSCs are also responsible for oral malodor and will be discussed later with respect to this subject.

iii. The Role Of Spirochetes in Periodontal Disease

Spirochetes are one of the major putative pathogens of periodontitis. Unfortunately, few studies deal with the role of OS in periodontal disease because of the difficulty encountered in their cultivation. That is, only a relatively small number of OS species have been isolated and cultivated when considering that 23 species were detected by polymerase chain reaction (PCR) analysis of a single sample of subgingival plaque from a patient with severe periodontitis (Choi *et al.*, 1994). To date, the only known cultivable species of OS are *T. denticola, T. socranskii, T. vincentii, T. pectinovorum, T. maltophilum,* and *T. medium* (Umemoto *et al.*, 1997; Wyss *et al.*, 1996). Table 2 illustrates the major differences in characteristics between these six species.

The most frequently-isolated cultivable OS is the small to medium-sized *T. denticola* (Chan *et al.*, 1993). This organism produces many proteolytic enzymes and cytotoxic products capable of eliciting an inflammatory response in the oral cavity (Loesche, 1993). For instance, trypsin-like enzyme (Loesche *et al.*, 1990; Makinen *et al.*, 1994), hyaluronidase (Scott *et al.*, 1996), and phospholipase C (Siboo *et al.*, 1989) are all produced by *T. denticola.* It also contains a cell-bound factor that is capable of inhibiting fibroblast proliferation (Isogai *et al.*, 1993).

T. denticola and *P. gingivalis* have been used as diagnostic and prognostic markers in periodontal disease, based on their ability to produce a trypsin-like enzyme (Loesche *et al.*, 1990). The trypsin-like enzyme in these organisms can be detected by the hydrolysis of benzoyl-DL-arginine-2-naphthylamide (BANA) in subgingival plaque.

Table 2. Taxonomic characteristics of the cultivable oral spirochetes[1]

Traits	*T. denticola*	*T. vincentii*	*T. socranskii*	*T. pectinovorum*	*T. medium*	*T. maltophilum*
Cell Length (μm)	6-16	5-16	6-15	7-15	5-16	5
Cell Diameter (μm)	0.15-0.20	0.20-0.25	0.16-0.18	0.28-0.30	0.20-0.30	0.20
Carbohydrate Fermentation	–	–	glucose, fructose, mannose, maltose, galactose, starch, sucrose, ribose, xylose	pectin, poly-galacturonic acid, glucuronic acid	glucose, fructose, maltose, mannose, galactose, sucrose, ribose, trehalose, inulin, salicin, raffinose	variable
H_2S Production	+	+	+	–	+	ND
Final pH of Glucose Broth	6.7	6.9-7.2	5.1-5.9	5.3-5.9	ND[2]	ND
Indole Production	biovar *denticola* + biovar *comondonii* –	weak	–	–	–	ND
Arrangement of Axial Fibrils	2-4-2 5-10-5	5-10-5	1-2-1	2-4-2	5-10-5 6-12-6 7-14-7	1-2-1
G+C Content of DNA (mol%)	37-38	44	51	39	51	ND

[1]The data in this table were compiled from Smibert (1991), Umemoto *et al.* (1997), Rosebury and Foley (1942), Wyss *et al.* (1996), and Simonson *et al.* (1988).

[2]ND means not determined.

Levels of BANA-hydrolyzing trypsin-like enzyme have been correlated with amount of plaque, proportions of OS, and probing depth (Loesche *et al.*, 1990).

Studies strongly implicate the uncultivable PROS as etiologic agents of periodontal disease. They were found in increased numbers in subgingival and supragingival plaque from diseased sites when compared to healthy sites (Riviere *et al.*, 1991). Monoclonal antibodies against pathogen-specific determinants on *T. pallidum* subspecies *pallidum*, the causative agent of syphilis, were used to identify spirochetes in plaque. Results indicated that spirochetes with a pathogen-specific epitope were found in plaque from ulcerative gingivitis and chronic periodontitis patients but not in the healthy controls (Riviere *et al.*, 1991). The association between PROS and *T. pallidum* subspecies *pallidum* suggests that an OS similar to *T. pallidum* is the causative agent of periodontal disease and that the disease mechanisms in syphilis might apply to periodontitis as well.

In general, OS have a variety of virulence mechanisms that could implicate them as etiologic agents of periodontal disease. They are capable of invading the sulcular epithelium and initiating gingivitis (Mikx *et al.*, 1990), thereby directly presenting their endotoxins, cytotoxic enzymes and molecules, and immunologically-active compounds to host inflammatory cells. OS are also able to inhibit polymorphonuclear leukocyte function. The polymorphonuclear leukocytes can phagocytize the OS but are unable to degrade them (Boehringer *et al.*, 1986). This is associated with the inability of the lysosomes to degranulate, suggesting that the fusion of lysosomes to phagosomes is inhibited by the OS. Other immunological studies were not able to associate OS with

periodontal disease. High titers of antibodies to OS were reported in patients with moderate periodontitis but not in patients with severe disease (Tew *et al.*, 1985). These results suggest that OS may cause immune suppression that enables them to evade the host's normal immunologic surveillance.

Loesche (1988) averaged the spirochete numbers found in various studies and obtained the following results (numbers in parentheses represent the number of studies from which the percentage of spirochetes was averaged): healthy sites, 1.6% (6); sites with gingivitis, 18% (9); localized juvenile periodontitis, 12% (3), adult periodontitis 37% (16), early-onset periodontal disease, 53% (12). Since over 350 bacterial species are present in the oral cavity, the fact that spirochetes are found in increased numbers in most periodontal diseases strongly implicates them as etiologic agents of the disease.

A more recent study of detection frequency of bacteria by an immunocytochemical assay showed that PROS were the only identified bacteria at sites of both health and disease that demonstrated a significant positive relationship with the presence of periodontitis (Riviere *et al.*, 1996). It is probable, then, that PROS play an important role in either the initiation of periodontal disease or its progression.

iv. Treatment of Periodontal Disease

Periodontal disease can usually be successfully treated by a patient's change in oral hygiene methods, along with scaling and root planing performed by a dentist (Oshrain *et al.*, 1986). If the disease is more severe, then surgical debridement may be employed. This surgery involves elevating the gingival flap, debridement and scaling of

the surface of the root, removal of granulomatous tissue from the lesion, and replacement of the flap, usually leading to clinical improvement (Egelberg, 1987).

When the disease shows no improvement by the previously-mentioned methods, then antimicrobial therapy may be suggested. Tetracyclines and debridement are used in treating localized juvenile periodontitis and rapidly-progressing periodontitis, which are associated with the bacterium *A. actinomycetemcomitans* (Gordon and Walker, 1993). For cases of adult periodontitis, conventional therapy is usually sufficient, whereas for refractory cases of periodontal disease, metronidazole, tetracycline, amoxicillin-clavulanate potassium, or metronidazole-amoxicillin are used (Gordon and Walker, 1993).

For periodontal diseases where spirochetes predominate, scaling and root planing alone are usually sufficient. Al-Joburi *et al.* (1989) showed that in patients with adult chronic periodontitis, spirochete numbers were similar in groups treated with tetracycline or spiramycin along with scaling and debridement compared with those patients treated with scaling, root planing and a placebo pill.

No one type of therapy has been shown to be good for all patients. Therefore, the potential pathogen of the periodontal disease must be determined and the treatment administered accordingly. It is also imperative for the patient to prevent further disease by maintaining proper oral hygiene practices.

VI. ORAL MALODOR

i. Etiology of Oral Malodor

Oral malodor is a general term used for foul smells emanating from the mouth. It is commonly noticed in patients and is often an important clinical sign or symptom that aids dentists in establishing a diagnosis of underlying pathology (Tessier and Kulkarni, 1991). Oral malodor can come from various sources and it is necessary to identify the source of the malodor in order to treat the patient's condition effectively. For example, ozostomia refers to a putrid smell that is detected from the mouth but derives from the upper respiratory tract whereas stomatodysodia is foul breath originating from areas in the lower respiratory tract (Williams and Wilkins, 1982). Halitosis refers to oral malodor arising as a consequence of systemic metabolic conditions (Rosenberg and Gabbay, 1987). Fetor oris, also called fetor ex ore, is an oral malodor which emanates from the mouth (Williams and Wilkins, 1987).

About 90% of mouth odors are due to fetor oris (Tonzetich, 1978). This condition is associated mostly with protein degradation by bacteria covering the oral hard and soft tissues, especially the tongue (De Boever and Loesche, 1995). Changes in the oral microflora may cause fetor oris. For example, poor oral hygiene, menstruation, and the use of antibiotics alter the microbiota in the mouth and are often associated with subsequent oral malodor (Tonzetich, 1978; McNamara *et al.*, 1972; Ogunwande, 1989).

The predominant VSCs produced in the oral cavity are hydrogen sulfide, methyl mercaptan, and, to a lesser extent, dimethyl sulfide. Other compounds produced by oral

putrefaction processes such as ammonia, amines, and organic acids may be involved, too, but may function as modifiers. Studies on VSCs have shown that oral malodor increases with worsening periodontal disease. This can be attributed to a number of factors, the most important being a shift from mostly aerobic microorganisms to predominantly anaerobic bacteria which produce large amounts of VSCs. Other factors involved in fetor oris from gum disease include a higher number of desquamating cells and greater bleeding and flow of fluid from the gingival crevice and periodontal pockets. Blood and gingival crevicular fluid provide substrates, lysed leukocytes, and growth factors that may stimulate the development of heavier bacterial loads on the tissues and might therefore cause an increased odor production.

Other factors are also needed in order for oral malodor to be produced. McNamara *et al.* (1972) showed that a pH above neutrality was also required for malodor formation. Other researchers demonstrated that the reduction of oxygen also caused an increase in oral malodor. Oxygen reduction lowers the oxidation-reduction potential, thereby promoting the growth of anaerobic bacteria, which are known to produce copious amounts of VSCs.

Oral malodor is at its worst in the morning and is often referred to as "jungle mouth". This is because salivary flow is diminished during sleep. Therefore, putrefaction can occur without interruption by salivary washings and without the dislodgment of bacteria from oral surfaces during eating. Furthermore, pH of plaque in the morning is also at its highest level, favoring malodor formation.

A number of studies prove that oral malodor and periodontal disease are related. Yaegaki and Sanada (1992) demonstrated that the amounts of VSCs were 8 times greater

in patients with periodontal disease as compared to control patients. Persson (1992) used gas chromatography (GC) to show that hydrogen sulfide was detected in 61 out of 79 periodontally-diseased pockets, and was therefore the predominant VSC. Methyl mercaptan was found in only 20% of the pockets. However, with worsening periodontal disease, there was a subsequent increase in the methyl mercaptan to hydrogen sulfide ratio. Miyazaki *et al.* (1995) assessed the role of oral health habits, smoking habits, and medical history on oral malodor. They observed a significant correlation only between the VSC value and periodontal conditions and tongue coating. Their results also suggested that oral malodor might be caused mainly by tongue coating in the younger generation. In the elderly, periodontal disease and tongue coating both play a role in oral malodor.

ii. Measurement of Oral Malodor

Oral malodor measurements are complicated by a variety of parameters including complexity of gaseous molecular species, sampling difficulties, temporal variations, choice of suitable subject populations, and a lack of agreement on reference standards (Rosenberg and McCulloch, 1992). Some of the available methods of measuring oral malodor are outlined below, along with the relative benefits and potential problems associated with each technique.

a. Subjective Measurement of Oral Malodor

Organoleptic assessment of mouth air is the most simple and commonly-used method, involving direct nasal sniffing of expelled mouth air. However, there is much variation in the appraisal of bad breath by different judges and at different times. Hunger, menstrual cycle, head position, degree of attentiveness, and pregnancy all influence organoleptic measurements. Also, olfactory sampling should be performed by taking a short, rapid sniff, which is difficult to perform in the exact same way for a long period of time.

b. Instrumental Analysis of Oral Malodor

GC with flame photometric detection has been used to analyze oral malodor (Tonzetich and Richter, 1964; Richter and Tonzetich, 1964; Tonzetich, 1971). GC studies have allowed researchers to come to the conclusion that oral malodor is associated with VSCs. GC measurement of malodor samples has several advantages compared with organoleptic measurements. Individual gases can be separated and quantitated and low concentrations of gases can be effectively measured. Some of the negative aspects of GC measurements include the high costs incurred, the need for skilled personnel, the lack of portability, and the time required for detection and measurement (Rosenberg and McCulloch, 1992).

A newer method of measuring gases associated with oral malodor, which makes use of an industrial sulfide monitor or portable sulfur monitor, is also being used. This instrument draws air out of the semi-open oral cavity at a fixed rate while the subject breathes through the nose (Rosenberg *et al.*, 1991a). Use of the monitor is advantageous compared to GC since it is less expensive, it can be operated by non-skilled personnel, it is portable, and there is a rapid turn-around time between measurements (Rosenberg and McCulloch, 1992). Unfortunately, the instrument cannot be used to distinguish between individual sulfides, measurements cannot be made in the presence of high levels of ethanol and essential oils (therefore, assays on mouthwash efficacy can only be performed once these components have dissipated), and the instrument may need periodic recalibration due to loss of sensitivity with time (Rosenberg *et al.*, 1991b).

c. Indirect Measurement of Oral Malodor

The most widely-used indirect method of assessing oral malodor is the measurement of malodor and VSCs in putrefying saliva samples (Tonzetich and Richter, 1964; Richter and Tonzetich, 1964; Tonzetich, 1971; Solis-Gafar *et al.*, 1975). Usually, saliva obtained from the mouth is almost odorless. After several hours of incubation, though, malodor is easily detected, due mostly to breakdown of matter by microorganisms (Yaegaki and Sanada, 1992). This method is disadvantageous since putrefied saliva differs from directly-sampled oral malodor. Other less-frequently

employed indirect methods measure VSCs produced from bacterial isolates and pure cultures such as *Fusobacterium* and *Bacteroides* species (Claesson *et al.*, 1990; McNamara *et al.*, 1972; Tonzetich and McBride, 1981).

iii. Effective Control of Malodors of the Mouth

Oral malodor is indicative of a problem and can be a useful diagnostic tool. Since 90% of mouth odors originate from the putrefaction of the cellular debris present in the oral cavity, then treatments that improve periodontal conditions will, in general, result in lower levels of VSCs. Therefore, good oral hygiene and periodontal surgery will eliminate the majority of oral malodors. Brushing alone is only effective in reducing VSCs levels by 25% (Tonzetich, J., 1978). However, a 75% reduction in VSCs is accomplished by cleaning the dorso-posterior part of the tongue and rinsing with a zinc-containing mouthwash (Tonzetich, J., 1978). Zinc forms insoluble non-volatile thiol derivatives, thereby reducing oral malodor. Sanguinarine, a benzophenanthridine alkaloid, has also been shown to control oral malodors. This compound contains an iminium group that reacts with thiols to prevent the formation of or to neutralize VSCs (Southard *et al.*, 1984). Other methods of controlling malodors of the mouth merely mask the problem but do not cure it.

VII. RESEARCH OBJECTIVES

We were interested in assessing the contribution of OS, taken directly from periodontally-diseased pockets, to oral malodor. Bacterial culture media have been used for the detection of many oral odor-producing bacterial species other than spirochetes, but no medium has been described for identifying hydrogen sulfide-producing OS associated with periodontitis. Conventional detection of hydrogen sulfide production by bacteria is through the incorporation of ferrous or ferric salts in the growth medium. This allows black iron sulfide salt clusters to form within hydrogen sulfide-producing colonies or along an inoculum stab line after growth (Gerdhardt *et al.*, 1994). NOS medium, previously used for the isolation of OS from periodontal pockets by Chan *et al.* (1993), was modified for the detection of hydrogen sulfide by the addition of iron salts. In addition, this medium was modified by using an alternate gelling combination of 0.5% gelatin with 0.5% Noble agar which is more economical than 0.7% SeaPlaque agarose normally used in growing OS (Chan *et al.*, 1993).

The last part of the focus of my research was to determine whether certain environmental factors could contribute to the enhanced formation of spherical bodies or to an increase in the ratio of spherical bodies to normal helical spirochete forms.

CHAPTER II. MATERIALS AND METHODS

I. STERILIZATION

Media and glassware were sterilized at 121°C, 15 lb. per square inch for 20 minutes in a pressure-steam sterilizer. Heat-unstable liquids were sterilized by passing through 0.45 μm pore size cellulose acetate membrane filters (Fisher Scientific, Fair Lawn, NJ).

II. DEMINERALIZATION AND DISTILLATION OF WATER

Single-distilled water was demineralized by passing it through a demineralizer (dH_2O) (Corning, Fair Lawn, NJ, Model Ld-2).

III. ANAEROBIC CULTIVATION CONDITIONS

The spirochetes studied were maintained in 0.3% NOS Noble agar medium and subcultured monthly. They were grown anaerobically at 35°C in an atmosphere of 85% N_2, 10% H_2, and 5% CO_2 in an anaerobic glove box (Coy Laboratory Products, Ann Arbor, MI).

IV. MEDIUM

The medium used to grow spirochetes was the "New Oral Spirochete" (NOS) medium formulated by Leschine and Canale-Parola (1980) and modified for our use with the substitution of brain heart infusion for heart infusion.

The composition of complete NOS medium was as follows (in grams per 100 ml dH_2O): Brain heart infusion, 1.25; trypticase, 1.0; yeast extract, 0.25; and glucose, 0.2, all purchased from BBL (Becton Dickinson and Company, Cockeysville. M.D.); sodium thioglycollate, 0.05; L-cysteine hydrochloride, 0.1; and L-asparagine, 0.025, all purchased from Sigma Chemical Co. (Mississauga, Ontario); Noble agar (Difco Laboratories, Detroit, MI), 0.3 for maintenance and 0.7 for use as a top layer; SeaPlaque agarose (FMC BioProducts, Rockland, ME), 0.7 for isolation. The medium was autoclaved at 121°C for 20 minutes and the following filter-sterilized supplements were then added (ml/100 ml medium): 0.2% (wt./vol.) thiamine pyrophosphate (Nutritional Biochemical Corporation, Cleveland, Ohio), 0.3; 10% sodium bicarbonate (Fisher Scientific), 2.0; normal rabbit serum (Sigma Chemical Co.), and a mixture of volatile fatty acids, 0.2, prepared by dissolving 0.5 ml of each of the following fatty acids in 100 ml 0.1N KOH: isobutyric acid (Fisher Scientific); D,L-2-methylbutyric acid; isovaleric and valeric acids (Eastman Kodak Company, Rochester, NY).

V. BACTERIAL CULTURES

The treponemes studied included *Treponema denticola* ATCC 35405, isolated and characterized in our laboratory (Cheng and Chan, 1983); *T. socranskii* subspecies *socranskii* ATCC 35536; and *T. vincentii* ATCC 35580, purchased from the American Type Culture Collection (Rockville, MD).

VI. DETERMINATION OF PROPER IRON SALT CONCENTRATION

In order to determine an appropriate concentration of iron salts to be used in contemplated experiments, several salts were evaluated for their ability to demonstrate blackening of media upon hydrogen sulfide production. Five-percent stock solutions of ferric ammonium citrate (ICN Biomedicals Inc., Aurora, Ohio), ferric chloride (J.T. Baker Chemical Co., Phillipsburg, NJ), ferric oxalate (John Matthey, Ward Hill, MA), ferric sulfate (Fisher Scientific), ferrous sulfate (Fisher Scientific), and ferrous ammonium sulfate (B.D.H. Laboratory Chemicals Group, Poole, England) were prepared and filter-sterilized. These solutions were added to molten NOS containing 0.7% SeaPlaque agarose (from now on referred to as NOS-A) in separate 25 cm^2 polystyrene flasks at final concentrations of 0.5%, 0.05%, and 0.005%. Once solidified, the media were then stabbed with a sterile Pasteur pipette containing approximately one ml of a log-phase *T. denticola* culture and incubated anaerobically. The results were recorded after 10 days.

VII. TOXICITY OF IRON SALTS

A comparison of the previously-mentioned iron salts at 0.05% final concentration was undertaken to determine their toxicity on spirochetes. Ten-fold serial dilutions of *T. denticola* were prepared in NOS broth. One ml of each dilution was inoculated into separate 25 cm^2 polystyrene flasks containing NOS-A alone as a control and NOS-A with 0.05% of one of the salts. Toxicity was evaluated by comparing the recovery of colony forming units (CFUs) in the control flasks (without salts) with the CFUs of flasks containing one of the salts.

VIII. COMPARISON OF CFU RECOVERY OF *T. denticola* and *T. vincentii* USING DIFFERENT SOLIDIFYING AGENTS

Serial ten-fold dilutions of *T. denticola* and *T. vincentii* were performed in fluid NOS. One ml of each dilution was inoculated into separate flasks containing either molten NOS-A, NOS with 0.5% gelatin and 0.5% Noble agar (NOS-GN), or NOS with 0.5% gelatin and 0.5% Bacto agar (NOS-GB). The media were allowed to solidify at room temperature, were overlayed with 5ml molten NOS containing 0.7% Noble agar, and were incubated in an anaerobic glove box for 10 days.

IX. SUBGINGIVAL PLAQUE SAMPLE COLLECTION

Subgingival plaque samples were obtained by inserting 2 sterile paper points into periodontal pockets greater than 6 mm in depth for approximately 10 to 15 seconds.

Male and female patients of all ages were selected for the study, provided they had not received antibiotic therapy three months preceding sample collection. The paper points were placed in 2 ml of reduced transport medium in a 5ml screw-cap glass vial at chairside and taken to the laboratory to be processed.

Reduced Transport Medium

Reduced transport medium was prepared as follows: boiled 0.025% resazurin solution (Allied Chemical Corporation, Rochester, NY), 0.4 ml; salts solution (see below), 4 ml; dH_2O, 100.0 ml. Cooled and added hemin solution (see below), 1.0 ml; Vitamin K1 solution (see below), 0.02 ml; and L-cysteine hydrochloride (Sigma Chemical Co.), 0.05 g. Two ml of the medium were dispensed into vials, autoclaved, and placed in the anaerobic glove box for about 24 hours prior to use for sampling.

Salts Solution

0.02 g anhydrous $CaCl_2$ (Fisher Scientific) and 0.02 g anhydrous $MgSO_4$ (Fisher Scientific) were mixed in 30 ml dH_2O until dissolved. 50 ml more dH_2O were added as well as 0.1 g K_2PO_4 (Fisher Scientific), 0.1 g KH_2PO_4 (J.T. Baker Chemical Co.), 0.2 g NaCl (Fisher Scientific), and 1.0 g $NaHCO_3$ (American Chemical Co., Montreal, Canada). All salts were dissolved by mixing and a further 20 ml dH_2O were added.

Hemin Solution

50 mg hemin (Sigma Chemical Co.) were dissolved in 1 ml 1 N NaOH (B.D.H. Laboratory Chemicals Group) and made up to 100 ml with dH_2O.

Vitamin K1 Solution

0.15 g vitamin K1 (Sigma Chemical Co.) was dissolved in 30 ml 95% ethanol.

X. PROCESSING OF SUBGINGIVAL PLAQUE SAMPLES

i. Comparison of Spirochete Viable Counts Using Different Solidifying Agents

The recovery of spirochete CFUs from subgingival plaque samples in NOS-A was compared to that obtained in NOS-GN and NOS-GB media.

Reduced transport medium containing the subgingival plaque sample on paper points was vortexed vigorously for about 15 seconds. A 5 µl aliquot was removed for darkfield observation and the remainder of the sample was placed in the anaerobic glove box for approximately 2 hours..

The sample was then serially diluted ten-fold in supplemented NOS broth and one ml of the dilution was added to 29 ml molten NOS-A medium containing either 2 µg/ml rifampin alone or 1 µg/ml rifampin with 100 µg/ml phosphomycin. The procedure was repeated with NOS-GN medium. The media were allowed to solidify at room temperature and 5 ml NOS containing 0.7% Noble agar were used as an overlay on the medium of each flask. Once the top layer had solidified, the flasks were placed in the anaerobic glove box and the samples incubated for 4 weeks.

ii. Enumeration of Hydrogen Sulfide-producing Spirochetes from Periodontal Pockets

In order to enumerate hydrogen sulfide-producing spirochetes from periodontal pockets, the procedure outlined above was repeated with 0.05% ferrous ammonium sulfate incorporated into the media. The subgingival plaque samples were diluted in fluid NOS and these dilutions were added to control flasks containing NOS-GN medium along with another set of flasks containing NOS-GN with 0.05% ferrous ammonium sulfate. The control flasks served to demonstrate whether or not ferrous ammonium sulfate was inhibiting any of the subgingival spirochetes.

XI. VERIFICATION OF SPIROCHETAL CFUs

After growth in the anaerobic glove box, each colony in flasks containing 20 to 200 CFUs was picked by stabbing it with a sterile Pasteur pipette. A portion of the CFU was resuspended in 1 ml of fluid NOS medium and an aliquot was examined for the presence of spirochetes by darkfield microscopy. Alternatively, the top of the flask was cut off by melting the polystyrene with a hot wire. The slab of solid NOS medium with spirochete CFUs was removed into a sterile dish and the colonies examined as before.

XII. PURIFICATION OF NEWLY-ISOLATED OS

The CFUs containing spirochetes did not usually consist solely of spirochetes. Hence, subsequent purification of these colonies was required. The method chosen was

repetitive high dilution. A portion of the spirochete colony was transferred into 10 ml fluid NOS medium in a screw-capped test-tube and vortexed. Serial ten-fold dilutions of the spirochete CFU in fluid NOS were performed and 1 ml of each dilution transferred to separate tissue flasks containing 29 ml NOS-GN medium.

After growth, the purity of the isolated colonies was examined by darkfield microscopy as before. This method was repeated until each individual CFU consisted only of spirochetes. Pure isolated spirochete colonies were maintained in NOS medium containing 0.3% Noble agar and transferred monthly.

XIII. SPHERICAL BODIES

i. Effect of Different Growth Temperatures and Presence of Oxygen on Spherical Body Formation

Environmental factors such as different incubation temperatures and the presence of oxygen during growth were studied to see their effects on the formation of spherical bodies. Four glass test-tubes, each containing 10ml of NOS broth were inoculated with 250 µl of a log-phase *T. denticola* culture. The control culture was grown anaerobically at 35°C, while the others were grown aerobically at 35°C, at room temperature, or at 45°C. A 5 µl aliquot of the culture was removed, placed onto a clean glass microscope slide, and a coverslip was placed over the drop. The cells were allowed to settle for approximately 10 minutes, were examined by darkfield microscopy, and the numbers of

free spirochetes and spherical bodies in four fields were enumerated. Counting was repeated in a similar manner on days 3, 7, and 15.

ii. Effect of Omission of NOS Basal Medium Components on Spherical Body Formation and Cell Growth

The effects of omitting one of the NOS basal medium components - brain heart infusion, trypticase, yeast extract, sodium thioglycollate, asparagine, cysteine hydrochloride, or glucose - on spherical body formation were tested. Complete NOS broth was prepared as a control, along with NOS without one of each of the ingredients. 5 ml of the media were dispensed into each of three glass test-tubes. Two test-tubes were inoculated with a log-phase *T. denticola* culture and the third was used as a blank for optical density measurements. The number of free spirochetes and spherical bodies per field (averaged from 4 fields) was counted as before and repeated on days 3, 7, and 14. Optical density measurements of the cultures at 620 nm were recorded with a Spectronic 20 spectrophotometer (Milton Roy Company, USA) on days 0, 3, 7, and 14.

iii. Effect of Omission of Supplement Components on Spherical Body Formation and Cell Growth

The effect of omitting supplement components from NOS medium on the formation of spherical bodies in *T. denticola* was studied. Fluid NOS was prepared and complete supplements were added to a set of three test-tubes as a control. Either sodium

bicarbonate, rabbit serum, volatile fatty acid mixture, or thiamine pyrophosphate was omitted from the other sets. Cell counts and optical density measurements were repeated as before.

iv. Effects of Metabolic End-Products on Spherical Body Formation and Cell Growth

Some of the end-products of *T. denticola* metabolism as reported by Hespell and Canale-Parola (1971), were tested to determine their effect on spherical body formation. The authors determined the number of moles of different acids formed during metabolism of *T. denticola*. Two test-tubes containing 5 ml supplemented fluid NOS medium were inoculated with log-phase cultures of *T. denticola* and a third was used as a blank for optical density measurements. Other test-tubes containing either 26500 μmol/l acetic acid, 272 μmol/l pyruvic acid, 2720 μmol/l lactic acid, 2108 μmol/l formic acid, or 5990 μmol/l formic acid were similarly inoculated. Cell counts and optical density measurements were repeated as before.

v. Effect of Rifampin and Changes in pH of NOS Medium on Spherical Body Formation

Rifampin, at a final concentration of 2 μg/ml, was added to complete NOS medium containing supplements in order to determine whether or not this antibiotic could

affect the ratio of spherical bodies to normal helical spirochetes. Furthermore, cells of *T. denticola* were inoculated in NOS medium whose pH has been changed to 7.42 by the addition of 5 M NaOH. The pH of NOS medium is normally 6.8, but following growth of a culture to stationary phase, the pH of the medium becomes 7.42. Therefore, we were interested in determining whether the pH of the growth medium somehow affected the formation of spherical bodies. The experiment was performed as previously described.

XIV. DNA-DNA HYBRIDIZATION OF UNKNOWN SPIROCHETE ISOLATES

i. DNA Isolation

Unknown spirochete isolates A24 and A38 as well as *T. denticola* ATCC 35405 and *T. socranskii* subspecies *socranskii* ATCC 35536 were inoculated into 100 ml fluid NOS and grown to optical density readings, at 620 nm, of 0.5. The DNAs were extracted using Qiagen Genomic DNA Buffer Set (Qiagen Inc., Chatsworth, CA) according to the following manufacturer's protocol. The spirochete cultures were spun for 10 minutes at 5000 *g*. The supernatant was discarded and the pellet resuspended by vortexing in 3.5 ml Buffer B1. The suspension was transferred to a new test-tube and 80 μl of a 100 mg/ml solution of lysozyme (Sigma Chemical Co., Saint Louis, MI) and 100 μl of a 20 mg/ml Qiagen protease solution were added. The suspension was incubated at 37°C for 30 minutes. 1.2 ml of Buffer B2 was added to the test-tube, the contents were mixed thoroughly by inverting and the tube was incubated at 55°C for 30 minutes. Qiagen tip t-

100 was equilibrated with 4 ml Buffer QBT. The sample was vortexed for 10 seconds and poured into tip t-100. Once the sample had flowed through the tip, the tip t-100 was washed with 5.5 ml Buffer QC. Finally, the DNA was eluted with 5 ml of Buffer QF and precipitated by adding 0.7 volumes (3.5 ml) of isopropanol. The sample was dispensed into microcentrifuge tubes and spun for 15 minutes at 12000 *g*. The isopropanol was decanted and 1 ml ice-cold 70% ethanol (Fisher Scientific) was added to wash the pellet. The solution was once again spun for 5 minutes and the ethanol decanted. The DNA was air-dried for 10 minutes. The pellet was resuspended in 30 μl Tris-EDTA buffer, pH 8.0 (TE buffer) and dissolved in a 55°C water-bath for 1 hour.

ii. Determination of DNA Concentration

The DNA was diluted in TE buffer to achieve an optical density reading at 260 nm of 0.1 to 2.0. The DNA concentration was calculated as follows: optical density at 260 nm of 1.0 = 50μg/ml.

iii. Labeling of DNA Probes

T.denticola and *T. socranskii* DNAs were labeled using a Random Primers DNA Labeling System Kit from Gibco BRL. The DNA concentration was adjusted to 0.4 mg/ml in TE buffer. 1 µg of DNA was dissolved in 5 µl of dH_2O in a microcentrifuge tube by heating for 10 minutes in a boiling water bath and then immediately cooling on

ice. The following were added, on ice: 2 µl dCTP solution, 2 µl dATP solution, 2 µl dGTP solution, 15 µl Random Primers Buffer Mixture all from Gibco BRL, 5 µl of a 10 µCi/µl solution of (methyl-^{3}H)thymidine-5'-triphosphate, ammonium salt (Amersham), and 23 µl dH_2O. The solution was mixed briefly and 1 µl Klenow fragment (Gibco BRL) was added. The solution was mixed gently, centrifuged briefly, and incubated for 3 hours at 25°C. 5 µl Stop Buffer (Gibco BRL) were added, the solution was mixed, and a 2 µl aliquot of the mixture was added to 498 µl dH_2O. A 5 µl aliquot of this last dilution was spotted on a glass fiber filter disk. The filter was washed three times with 50 ml ice-cold 10% (w/v) trichloroacetic acid containing 1% (w/v) sodium pyrophosphate and once with 50 ml 95% ethanol at room temperature. The filter was dried at room temperature for 15 minutes and the precipitable radioactivity was determined by liquid scintillation counting.

iv. Separation of Labeled Probe from Unincorporated Nucleotides

The labeled probe was separated from unincorporated nucleotides by chromatography on a 0.9×15 cm Sephadex G-50 Nick column (Pharmacia Biotech.) The column was rinsed with 3 ml equilibration buffer (10 mM Tris-HCl, pH7.5, containing 1 mM EDTA) and left until the buffer had flowed through. The column was equilibrated with 3 ml of the same buffer and the sample was added to the column. 400 µl of the buffer was added to the column and then the purified sample was eluted with another 400 µl of the equilibration buffer. Approximately 2 ml of the sample was collected in fractions into microcentrifuge tubes and the radioactivity in a 5 µl aliquot from each tube

was counted. All eluted samples with the first high counts were pooled into one microcentrifuge tube and the labeled DNA was precipitated with 2 volumes 100% ethanol and 0.1 volume of 3 M sodium acetate. The labeled DNA was stored at -20°C overnight and then centrifuged for 15 minutes at 12000 *g*. The supernatant was decanted into another tube and the pellet was resuspended in 30 µl TE buffer. The radioactivity in 1 µl of both the pellet and the supernatant was counted on two filters. The radioactivity in the pellet was adjusted to 3×10^6 counts per minute with TE buffer.

v. Preparation of Salmon Sperm DNA

Salmon sperm DNA (Type III sodium salt, Sigma) was dissolved in dH_2O at a concentration of 10 mg/ml. NaCl was then added and adjusted to a final concentration of 0.1 M. The solution was extracted once with phenol and once with a phenol-chloroform mixture. The aqueous phase was recovered and the DNA sheared by passing it 12 times rapidly through a 17-gauge hypodermic needle. The salmon sperm DNA was precipitated with 2 volumes ice-cold 100% ethanol and recovered by centrifugation at 12000 *g* for 5 minutes. The pellet was redissolved in dH_2O at a concentration of 10 mg/ml and the optical density reading at 260 nm was determined. The DNA concentration was adjusted to 0.4 mg/ml and 1.2 mg/ml in separate microcentrifuge tubes. The solution was boiled for 10 minutes and stored at -20°C in small aliquots.

vi. Reassociation of Labeled and Unlabeled DNA

The DNA preparations were thawed and allowed to warm to room temperature. The following were added, in a 2 ml screw-capped vial: 10 µl labeled DNA, 50 µl 13.2×SSC-1 mM HEPES buffer (pH7.0), and 50 µl of 0.4 mg/ml unlabelled DNA (A24 or A38). The reaction mixture was denatured by boiling for 10 minutes. The salmon sperm DNA (0.4 mg/ml) was denatured by boiling for 10 minutes and placing on ice. The denatured salmon sperm DNA was placed into 2 control vials to measure the amount of self-renaturation of the labeled fragments. All the vials were incubated at 60°C for 20 to 24 hours. 1 ml acetate-zinc buffer (0.05M sodium acetate, 0.3M NaCl, 0.5mM $ZnCl_2$, pH 4.6) and 50 µl denatured salmon sperm DNA (0.4 mg/ml) were added to each vial, along with 250 units of S1 nuclease. The contents were mixed by vortexing and the tubes were incubated at 25°C in a water bath for 1 hour. 50 µl sheared salmon sperm DNA (1.2 mg/ml) and 2 ml ice-cold 10% tricholoracetic acid were added to each tube, the contents were mixed and the tubes were incubated at 4°C for 1 hour. A 5 µl aliquot of each mixture was spotted on separate filters and the radioactivity was determined by liquid scintillation counting. The percent similarity was calculated as follows: the counts per minute of the heterologous S1-resistant DNA divided by the counts per minute of the homologous S1-resistant DNA fragments multiplied by 100.

CHAPTER III. RESULTS

I. DETERMINATION OF OPTIMAL IRON SALT CONCENTRATION FOR DETECTION OF HYDROGEN SULFIDE

As summarized in Table 3, the optimal concentration of iron salts for the detection of hydrogen sulfide production by *T. denticola* is 0.05%. At a final iron salt concentration of 0.005%, no blackening of the media was observed. At 0.5% concentration, only a thin black line of growth was observed with ferrous ammonium sulfate and ferrous sulfate but no growth was observed with the other iron salt additives. Therefore, a final concentration of 0.5% of the tested iron salts was determined to be inhibitory to spirochete growth.

II. TOXICITY OF IRON SALTS

Table 4a and Figure 5 give the CFU recovery of *T. denticola* with various iron salts incorporated into NOS-A medium at a concentration of 0.05%. With 41.7% growth compared to the control flasks, ferrous ammonium sulfate was the least inhibitory of all the salts tested. It was therefore chosen as the iron salt for the detection of hydrogen sulfide production for all future experiments. The CFU recovery of *T. vincentii* in the presence of 0.05% ferrous ammonium sulfate was also compared to the CFU recovery in NOS-A medium. The results, as shown in Table 4b, indicate that approximately eight

Table 3. Growth of *Treponema denticola* ATCC 35405 stabs in NOS-A containing iron salts in differing concentrations

Iron Salt	Concentration of Iron Salt		
	0.5%	**0.05%**	**0.005%**
Ferrous Ammonium Sulfate	Thin black line	Black along stab	White growth
Ferric Sulfate	No growth	Black along stab	White growth
Ferric Chloride	No growth	Black along stab	White growth
Ferrous Sulfate	Thin black line (but media turbid)	Black along stab	White growth
Ferric Ammonium Citrate	No growth	Black along stab	White growth
Ferric Oxalate	No growth	Thin black line	White growth

Figure 5. **Recovery of *T. denticola* in the presence of iron salts.**

Comparative CFU recovery of a 10^{-5} dilution of *T. denticola* ATCC 35405 in the presence of various iron salts used to detect hydrogen sulfide production. Left to right: control NOS-A medium (numerous white colonies); with ferrous ammonium sulfate added (highest number of black colonies; too numerous to be discerned as discrete colonies at this dilution); with ferrous sulfate added (black colonies); with ferric ammonium citrate added (very few black colonies).

Table 4a. Comparison of recovery of CFUs[1] of *T. denticola* in NOS-A and NOS-A containing 0.05% of various iron salts

Dilution Factor	Control	Ferrous Ammon. Sulfate	Ferric Sulfate	Ferric Chloride	Ferrous Sulfate	Ferric Ammon. Citrate	Ferric Oxalate
10^{-3}	TNTC[2]	TNTC	6	4	TNTC	TNTC	8
10^{-4}	TNTC	TNTC	1	0	TNTC	49	1
10^{-5}	211	88	0	0	37	7	0
10^{-6}	40	14	0	0	4	0	0

[1]Counts (cells/ml) represent averages of CFUs obtained from two flasks.

[2]TNTC means too numerous to count.

Table 4b. Comparison of recovery of CFUs[1] of *T. vincentii* in NOS-A and NOS-A containing 0.05% ferrous ammonium sulfate

Dilution Factor	Control	Ferrous Ammonium Sulfate
10^{-3}	TNTC[2]	TNTC
10^{-4}	TNTC	TNTC
10^{-5}	TNTC	TNTC
10^{-6}	TNTC	45, 43 (avg. 44)
10^{-7}	31, 33 (avg. 32)	2, 8 (avg. 5)
10^{-8}	3, 1 (avg. 2)	0

[1]Counts (cells/ml) represent number of CFUs obtained.

[2]TNTC means too numerous to count.

times fewer CFUs were obtained in flasks containing NOS-A and 0.05% ferrous ammonium sulfate compared with control flasks containing only NOS-A. Although ferrous ammonium sulfate was inhibitory to *T. denticola* and *T. vincentii* in pure culture, there did not appear to be reduced CFU counts of spirochetes in its presence when subgingival plaque samples were assayed (see Table 5). An example of a flask containing both hydrogen sulfide-producing and non-hydrogen sulfide producing spirochetes isolated from periodontal pockets is shown in Figure 6.

III. CFU RECOVERY USING DIFFERENT SOLIDIFYING AGENTS

Table 6a and Figure 7 illustrate the CFU recovery of *T. denticola* using three different solidifying agents in NOS medium. The highest CFU recovery, 5.2×10^9 cells/ml was obtained in NOS-GN medium. NOS-GB and NOS-A media gave CFU recoveries of 2.1×10^9 cells/ml and 9.2×10^8 cells/ml, respectively. Besides giving higher colony counts, the gelatin-containing media had colonies that grew up faster than those in NOS-A medium. For example, colonies of *T. denticola* were visible after 4 to 5 days of growth in NOS-GN medium whereas they were only apparent in NOS-A medium after 7 days.

Table 6b and Figure 8 give the CFU recovery of *T. vincentii* in the same three media, NOS-A, NOS-GB, and NOS-GN. The three media gave approximately equal viable counts of 3.0×10^8 cells/ml in NOS-A, 2.5×10^8 cells/ml in NOS-GB, and 3.2×10^8 cells/ml in NOS-GN medium.

Figure 6. **Recovery of OS from periodontal pockets in NOS-GN medium containing 0.05% ferrous ammonium sulfate.**

CFU recovery of a 10^{-4} dilution of a sample from a periodontal pocket. Hydrogen sulfide-producing (black) and non-hydrogen sulfide producing (white) colonies are visible.

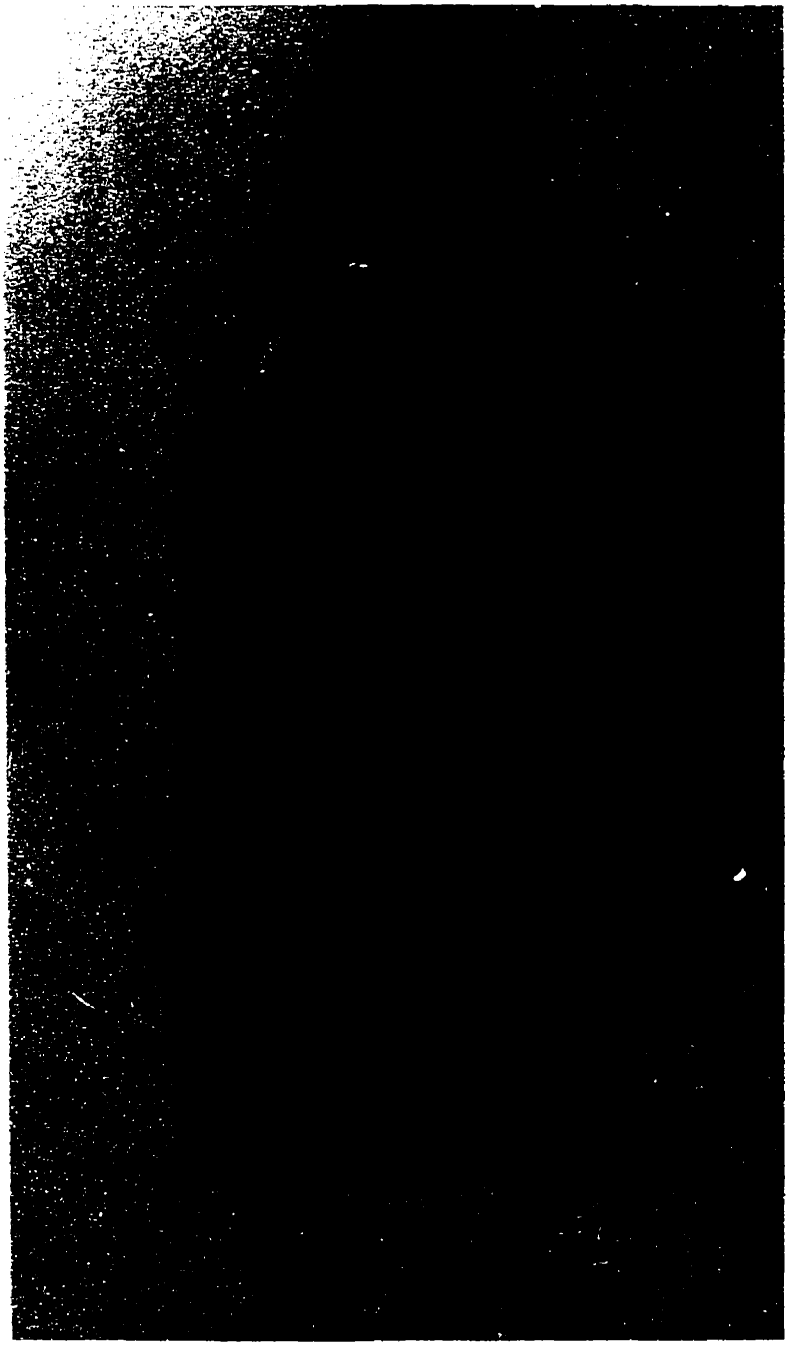


Table 5. CFU recovery from subgingival plaques and enumeration of hydrogen sulfide-producing spirochetes[1]

Sample Number	NOS-GN Control	NOS-GN + 0.05% Ferrous Ammonium Sulfate	
1	2.5×10^5	2.0×10^5 Black[2]	
2	1.7×10^5	1.8×10^5 Black	
3	1.9×10^5	1.6×10^5 Black	
4	3.2×10^4	2.0×10^4 Black	
5	2.7×10^4	2.2×10^4 Black	1.5×10^3 White
6	1.4×10^5	1.5×10^5 Black	
7	1.7×10^4	1.6×10^4 Black	5.0×10^2 White
8	1.5×10^5	9.5×10^4 Black	
9	2.0×10^4	2.0×10^4 Black	1.0×10^3 White
10	6.5×10^5	3.4×10^5 Black	
11	2.5×10^5	2.7×10^5 Black	1.5×10^5 White
12	5.0×10^4	7.5×10^4 Black	

[1]Counts (cells/ml) represent averages of CFUs obtained from two flasks.

[2]Spirochetes that form black colonies are hydrogen sulfide producers; spirochetes that form white colonies are non-hydrogen sulfide producers.

Figure 7. **CFU recovery of *T. denticola* in NOS-A, NOS-GB, and NOS-GN media.**

Recovery of CFUs of a 10^{-8} dilution of *T. denticola* in NOS-A (left), NOS-GB (center), and NOS-GN media. The highest recovery of CFUs was obtained in NOS-GN medium, while the lowest was in NOS-A medium.

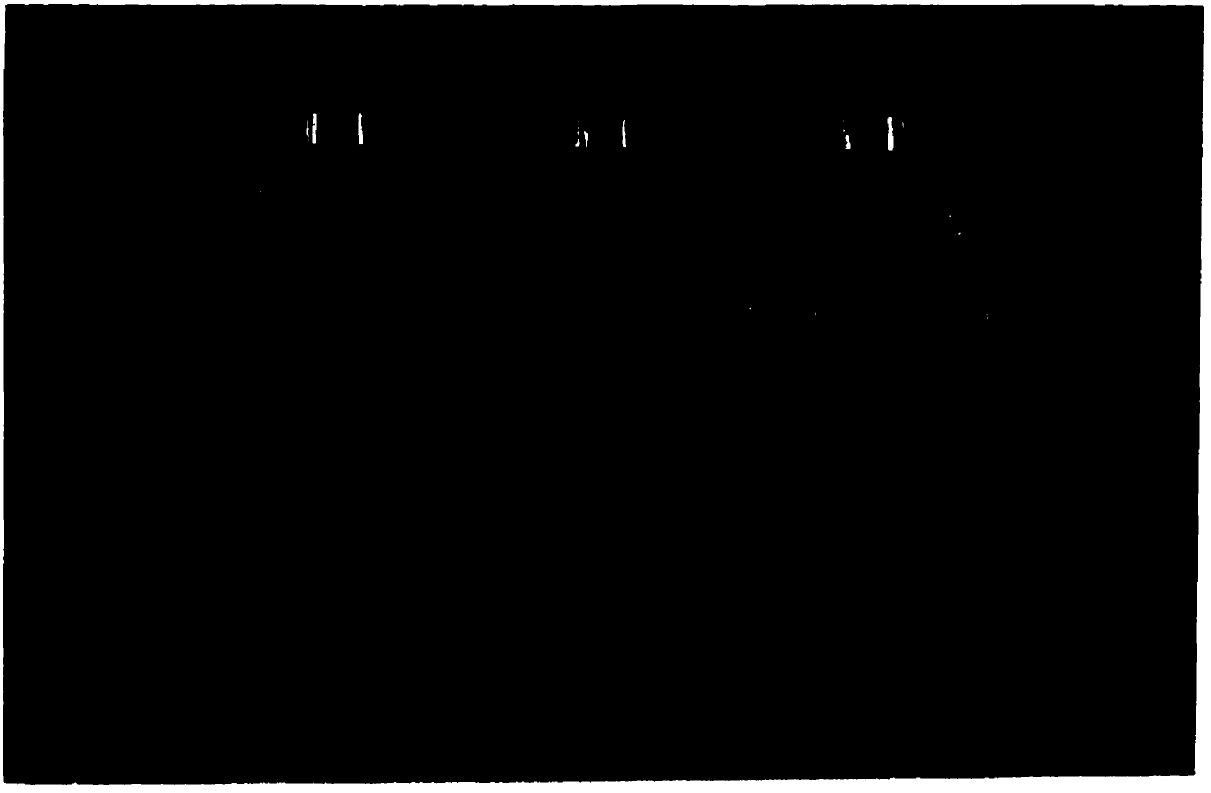


Figure 8. **CFU recovery of *T. vincentii* in NOS-A, NOS-GN, and NOS-GB media.**

Growth of 10^{-7} dilution of *T. vincentii* in NOS-A (left), NOS-GN (center), and NOS-GB (right) media. Similar recoveries were obtained in NOS-A and NOS-GN media, but almost no CFUs grew at this dilution in NOS-GB medium.

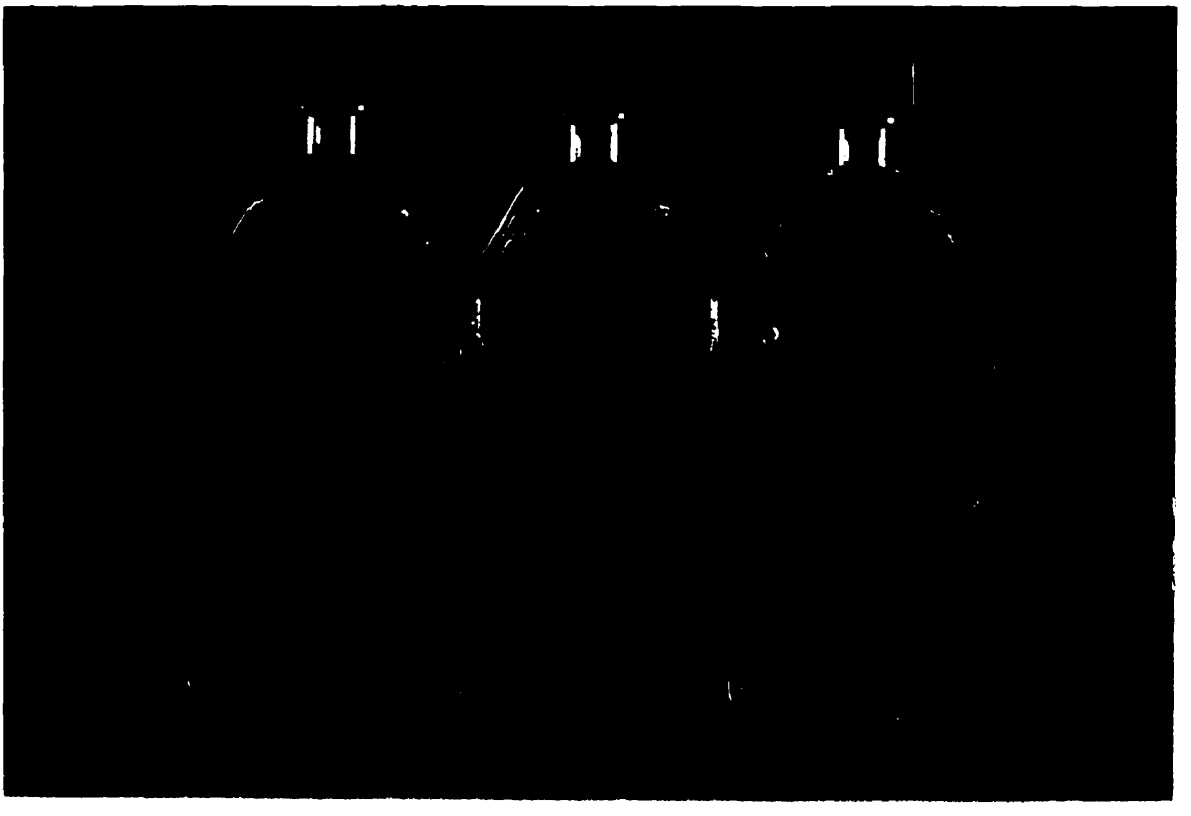


Table 6a. CFU recovery of *T. denticola* in NOS-A, NOS-GB, and NOS-GN media

T. denticola dilution	NOS-A	NOS-GB	NOS-GN
10^{-4}	TNTC*	TNTC	TNTC
10^{-5}	TNTC	TNTC	TNTC
10^{-6}	TNTC	TNTC	TNTC
10^{-7}	91,93 (avg. 92)	205,215 (avg. 210)	TNTC
10^{-8}	14,10 (avg. 12)	20,30 (avg. 25)	47,57 (avg. 52)
10^{-9}	2,2 (avg. 2)	4,6 (avg. 5)	9,11 (avg.10)

*TNTC means too numerous to count

Table 6b. CFU recovery of *T. vincentii* in NOS-A, NOS-GB, and NOS-GN media

T. vincentii dilution	NOS-A	NOS-GB	NOS-GN
10^{-4}	TNTC*	TNTC	TNTC
10^{-5}	TNTC	TNTC	TNTC
10^{-6}	TNTC	TNTC	TNTC
10^{-7}	32,28 (avg. 30)	24,26 (avg. 25)	31,33 (avg. 32)
10^{-8}	4,8 (avg. 6)	4,6 (avg. 5)	4,2 (avg. 3)
10^{-9}	0	0	0

*TNTC means too numerous to count.

Since NOS-GB medium gave lower CFU recoveries of *T. denticola* than did NOS-GN medium, only NOS-GN medium was further tested for recovery of spirochetes from periodontal pockets.

IV. SPIROCHETE RECOVERY FROM PERIODONTAL POCKETS

The results illustrated in Table 7 indicate that NOS-GN medium is superior to NOS-A with respect to CFU recovery from subgingival plaques (see Figure 9). As well, no significant difference in spirochete CFU recovery was observed when a combination of 1 μg/ml rifampin and 100 μg/ml phosphomycin was used to inhibit bacteria other than spirochetes in place of 2 μg/ml rifampin alone. However, the rifampin-phosphomycin mixture is favored because it inhibits a greater number of nonspirochetal bacteria than does 2 μg/ml rifampin (see Figure 10). Many bacteria appear to be resistant to 2 μg/ml rifampin, making accurate enumeration of spirochete CFUs difficult. Higher concentrations of the antibiotic rifampin were tested on both stock spirochete cultures and subgingival plaque samples, but the spirochete viable counts obtained were lower than in the control flasks (data not shown).

Figure 9. **Comparison of recovery of OS from periodontal pockets in NOS-A and NOS-GN media.**

CFU recovery of a 10^{-3} dilution of a sample isolated from periodontal pockets. Left: no growth in NOS-A medium containing 1 μg/ml rifampin and 100 μg/ml phosphomycin; Right: growth of spirochete CFUs in NOS-GN medium containing 1 μg/ml rifampin and 100 μg/ml phosphomycin.

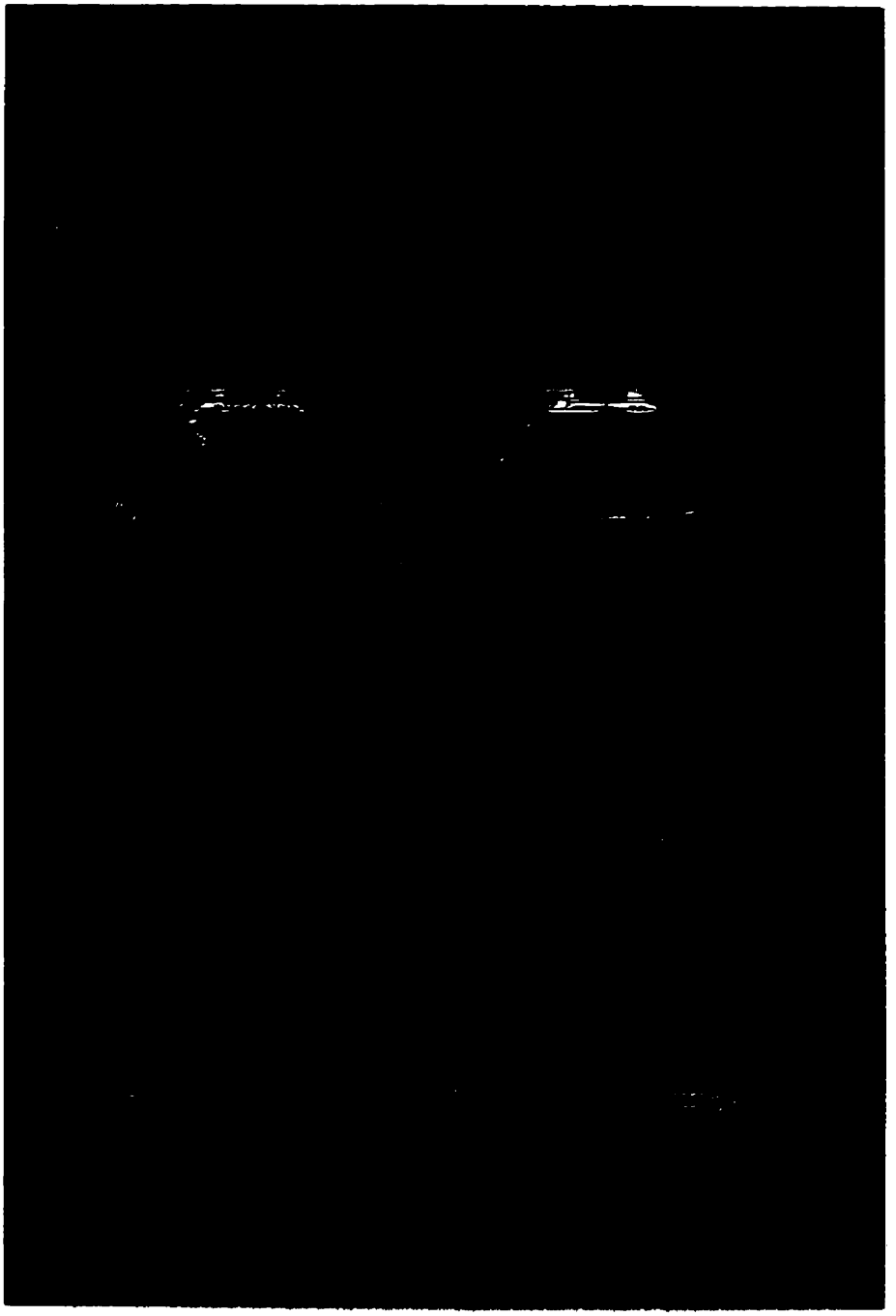


Figure 10. **Recovery of OS from subgingival plaques in NOS-GN medium containing different antibiotics.**

Growth of a 10^{-3} dilution of a subgingival plaque sample. Left: CFU recovery in NOS-GN medium containing 2 μg/ml rifampin. Note that the majority of the isolated CFUs are not OS indicating that many bacteria in the oral cavity are resistant to this concentration of rifampin. Accurate enumeration of the spirochete colonies in this medium is therefore difficult. Right: Recovery of CFUs of OS in NOS-GN medium containing 1 μg/ml rifampin and 100 μg/ml phosphomycin. The majority of the colonies are OS.

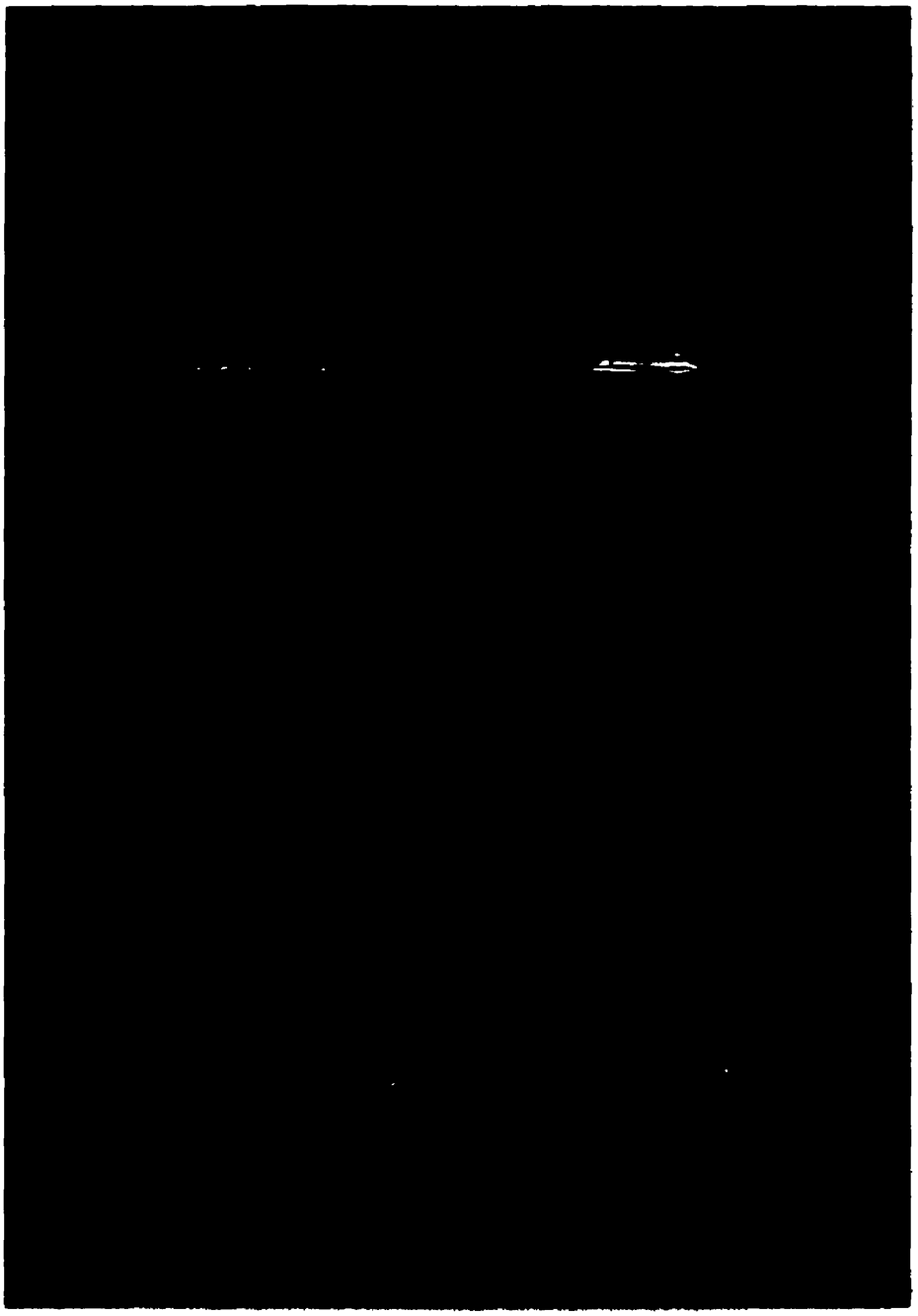


Table 7. Viable counts of OS from subgingival plaque samples inoculated into NOS-A, NOS-GN, and NOS-GB media[1]

Sample number	NOS-A + 2 μg/ml rifampin	NOS-A + 1 μg/ml rifampin + 100 μg/ml phosphomycin	NOS-GN + 2 μg/ml rifampin	NOS-GN + 1 μg/ml rifampin + 100 μg/ml phosphomycin
1	1.1×10^{4}	1.3×10^{4}	1.9×10^{4}	2.7×10^{4}
2	6.5×10^{4}	8.0×10^{4}	1.8×10^{5}	1.4×10^{5}
3	1.1×10^{4}	1.0×10^{4}	1.3×10^{4}	1.7×10^{4}
4	5.0×10^{3}	6.0×10	2.1×10^{4}	2.0×10^{4}
5	2.5×10^{3}	0	4.0×10^{4}	2.0×10^{4}
6	2.1×10^{5}	1.8×10^{5}	6.5×10^{5}	1.6×10^{5}
7	1.6×10^{4}	2.7×10^{4}	2.3×10^{5}	3.1×10^{5}
8	8.0×10^{4}	ND[2]	2.5×10^{5}	ND
9	2.0×10^{5}	ND	7.0×10^{5}	ND
10	2.0×10^{4}	ND	2.1×10^{5}	ND
11	3.0×10^{4}	ND	5.0×10^{4}	ND

[1]Counts (cells/ml) represent averages of CFUs obtained from two flasks.

[2]ND means not determined.

V. DESCRIPTION OF NEW SPIROCHETE ISOLATES

To date, three new oral spirochetes have been isolated and purified by the method described.

Colony A24 is grey, cottony, round, and measures 2 mm in diameter. By darkfield microscopy, the spirochetes appear to be small to medium-sized.

Colony A38 is large, measuring 4 cm in diameter, cream-coloured with a dense center and hazy periphery. The spirochetes are medium to large and may contain external flagella in older cultures.

Colony A39 is very diffuse (almost transparent) and measures 5 mm in diameter. The spirochetes are tightly-coiled and some are extremely long, as can be seen in Figure 11.

Other oral spirochete isolates are in the process of being purified and all will be further characterized in our laboratory at a later time.

VI. STUDIES ON SPHERICAL BODY FORMATION

As demonstrated in Figure 12, the number of spherical bodies increases with age of the spirochete culture. That is, when a culture of treponemes is inoculated into fresh medium, there is an initial drop in the ratio of spherical bodies to normal helical forms. As the culture ages, there is then an increase in this ratio.

The presence of oxygen in the environment during growth of *T. denticola* does not appear to affect the ratio of spherical bodies to free spirochetes compared to control

Figure 11. **Darkfield photomicrograph of spirochete A39, isolated from periodontal pockets.**

Original magnification: 1000X

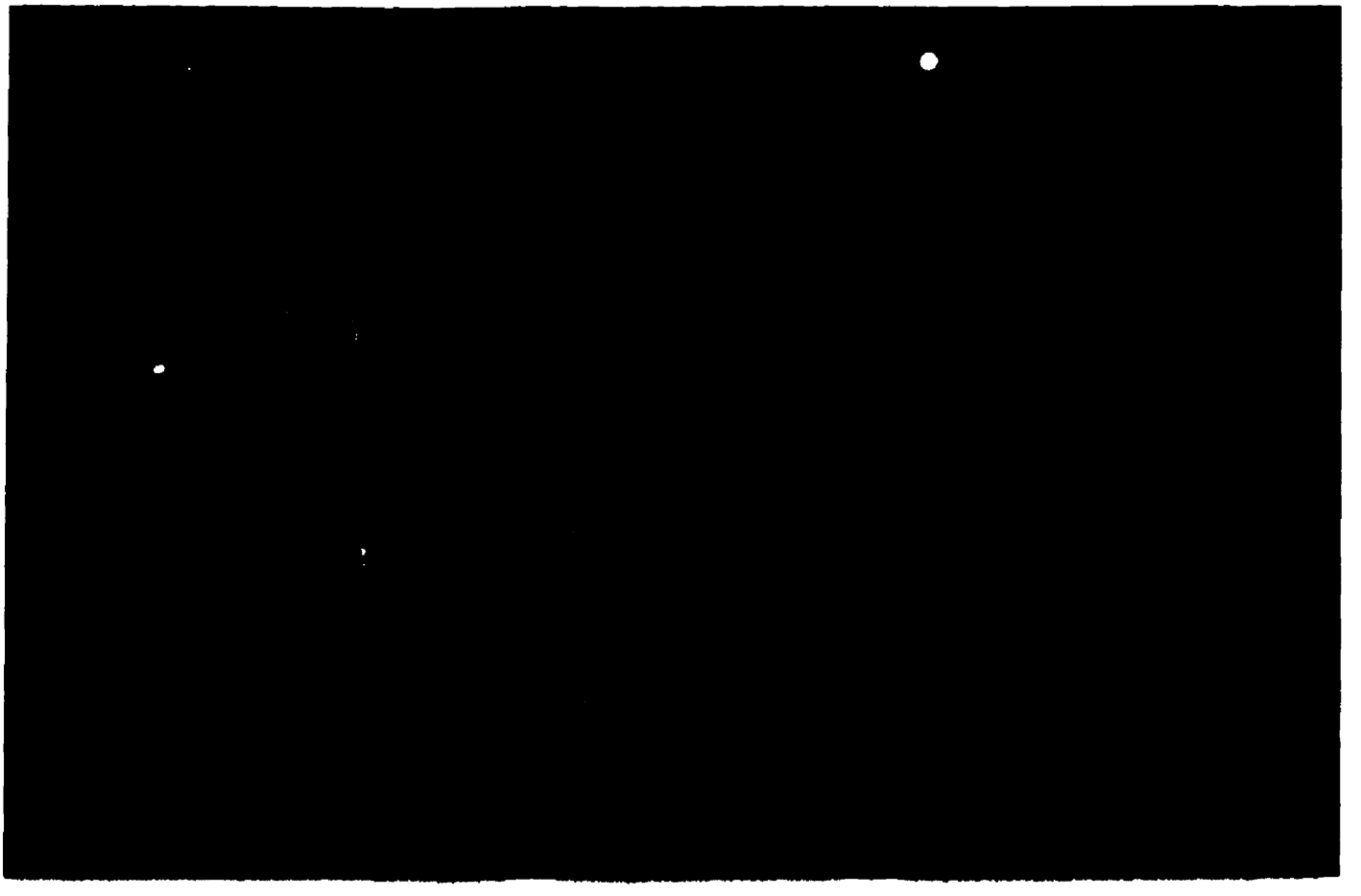
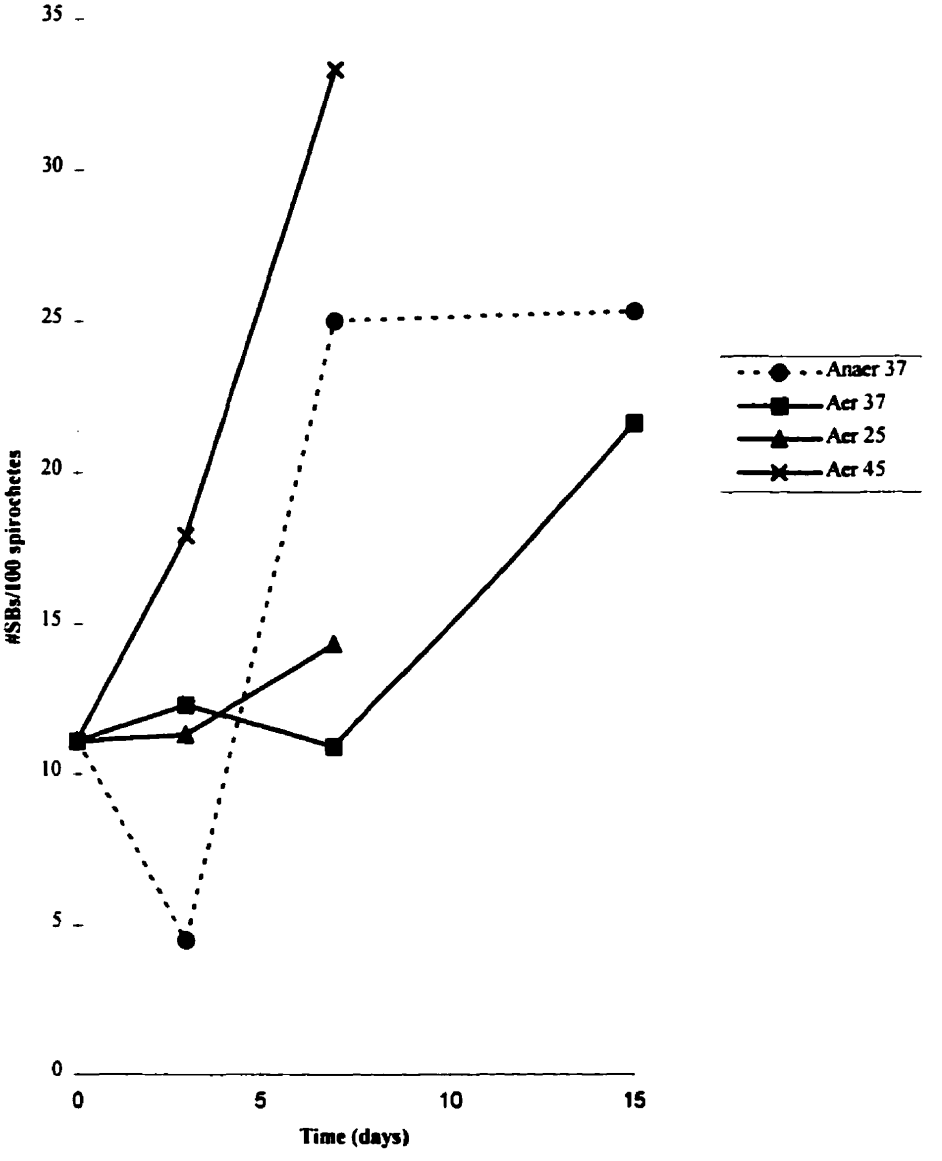


Figure 12. **Comparison of spherical body to free spirochete ratio in different growth conditions.**

A log-phase culture of *T. denticola* ATCC 35405 was inoculated into complete NOS medium and different sets of tubes were incubated in the following ways: anaerobically at 37°C, as a control (● Anaer. 37); aerobically at 37°C (■ Aer. 37); aerobically at room temperature (▲ Aer. 25); or aerobically at 45°C (× Aer. 45). All results are the averages of counts of the numbers of spherical bodies per 100 free spirochetes in four fields, as seen in the culture on days 0 (day of inoculation), 3, 7, and 15.

Note: Counting was not performed after day 7 for tubes incubated aerobically at room temperature and 45°C. Since the numbers of free spirochetes remained constant from the day of inoculation, it was determined that the cells were unable to reproduce aerobically at these temperatures.


35
30
25
20
15
10
5
0
#SBs/100 spirochetes
0
5
10
15
Time (days)
Anaer 37
Aer 37
Aer 25
Aer 45

cells grown anaerobically. However, other factors affect this ratio. For example, in Figure 13, it is clear that the absence of the NOS basal medium components yeast extract and brain heart infusion caused the greatest increase in the ratio of spherical bodies to normal helical spirochete forms. Similarly, as shown in Figure 15, when the supplement components rabbit serum, volatile fatty acids, and thiamine pyrophosphate were omitted from the growth medium, the ratio of spherical bodies to free spirochetes increased.

Figure 20 illustrates the proportion of spherical bodies to free spirochetes upon addition of end-products of *T. denticola* metabolism to NOS medium. Only the presence of lactic acid appeared to greatly increase this ratio. In this case, for every 100 free spirochetes, there were 70 spherical bodies.

The presence of the antibiotic rifampin at a concentration of 2 μg/ml in NOS medium, does not affect the spherical body to free spirochete ratio (Figure 18). However, when *T. denticola* cells are grown at a pH of 7.42, rather than at 6.8, the ratio of spherical bodies to normal helical spirochete forms is approximately doubled.

Therefore, a number of environmental and nutritional factors seem to affect the formation of spherical bodies. Figures 14, 16, 19, and 21 give the optical density measurements of *T. denticola* ATCC 35405 cultures grown under similar conditions as for spherical body studies. The optical density measurements on the last day of growth of the cultures were then compared with the ratio of spherical bodies to normal helical spirochetes. The results are shown, in the form of a scatter plot, in Figures 17 and 22. In general, the ratio of spherical bodies to free spirochetes is inversely proportional to the optical density reading. That is, in a treponeme culture that is growing well, the

Figure 13. **Effect of omission of NOS basal medium components on the spherical body to free spirochete ratio in *T. denticola*.**

A log-phase culture of *T. denticola* ATCC 35405 was inoculated into the following sets of media: NOS medium with complete supplements, used as a control (● Control); NOS medium without brain heart infusion (■ No BHI); NOS medium without cysteine (◆ No CYST); NOS medium without trypticase (▲ No TRY); NOS medium without yeast extract (× No YE); NOS without asparagine (+ No ASP); NOS without sodium thioglycollate (○ No THIO); or NOS without glucose (□ No GLUC). All results are the averages of counts of the number of spherical bodies per 100 free spirochetes in four fields, as seen in the culture on days 0 (day of inoculation), 3, 7, and 14.

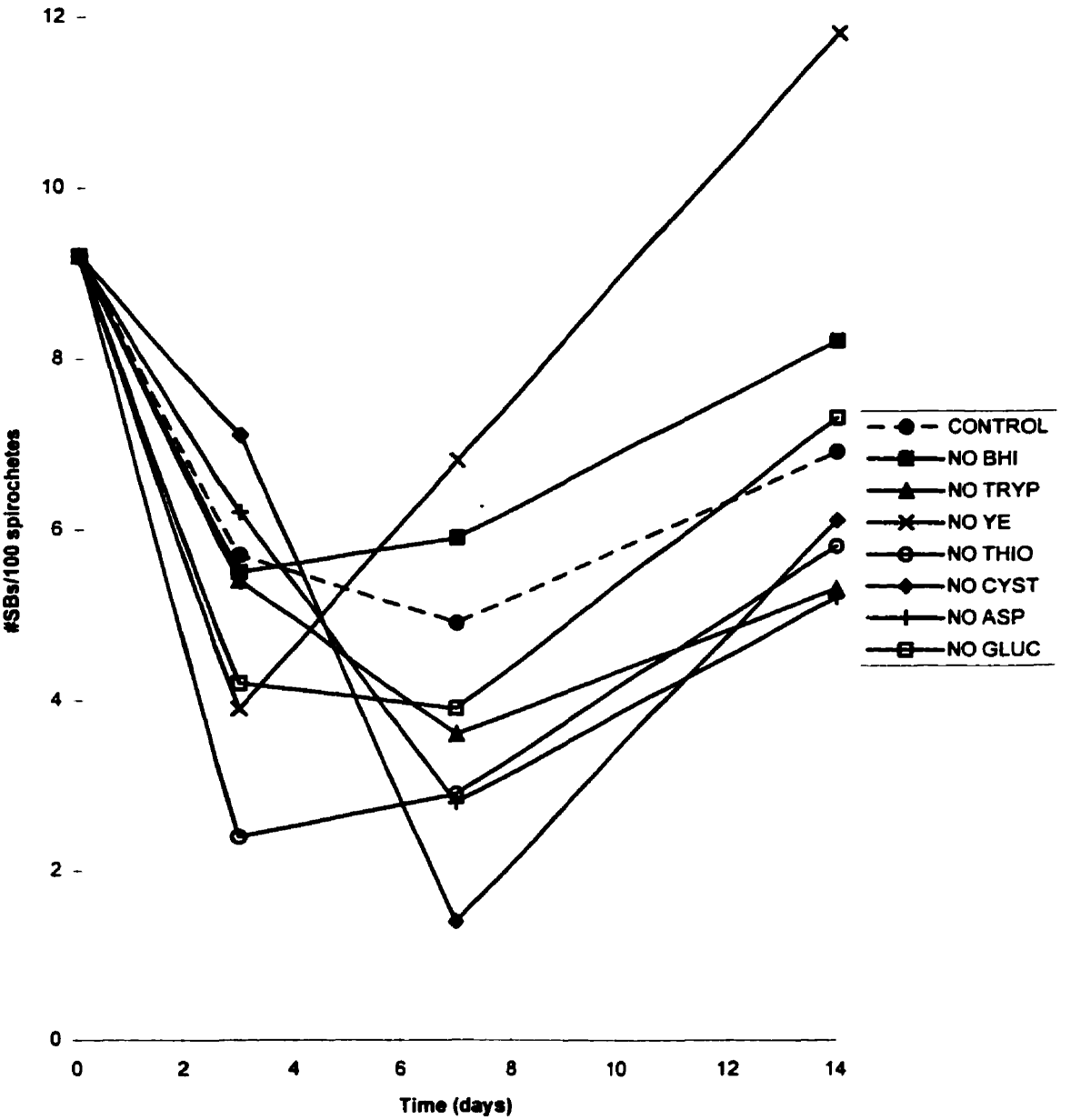

12
10
8
6
4
2
0
#SBs/100 spirochetes
0
2
4
6
8
10
12
14
Time (days)
CONTROL
NO BHI
NO TRYP
NO YE
NO THIO
NO CYST
NO ASP
NO GLUC

Figure 14. **Optical density measurements *T. denticola* grown in NOS medium without certain basal medium components.**

A log-phase culture of *T. denticola* ATCC 35405 was inoculated into the following sets of media: NOS medium with complete supplements, used as a control (● Control); NOS medium without brain heart infusion (■ No BHI); NOS medium without cysteine (◆ No CYST); NOS medium without trypticase (▲ No TRY); NOS medium without yeast extract (× No YE); NOS without asparagine (+ No ASP); NOS without sodium thioglycollate (○ No THIO); or NOS without glucose (□ No GLUC). Optical density measurements were recorded, at 620 nm, on days 0 (day of inoculation), 3, 7, and 14.

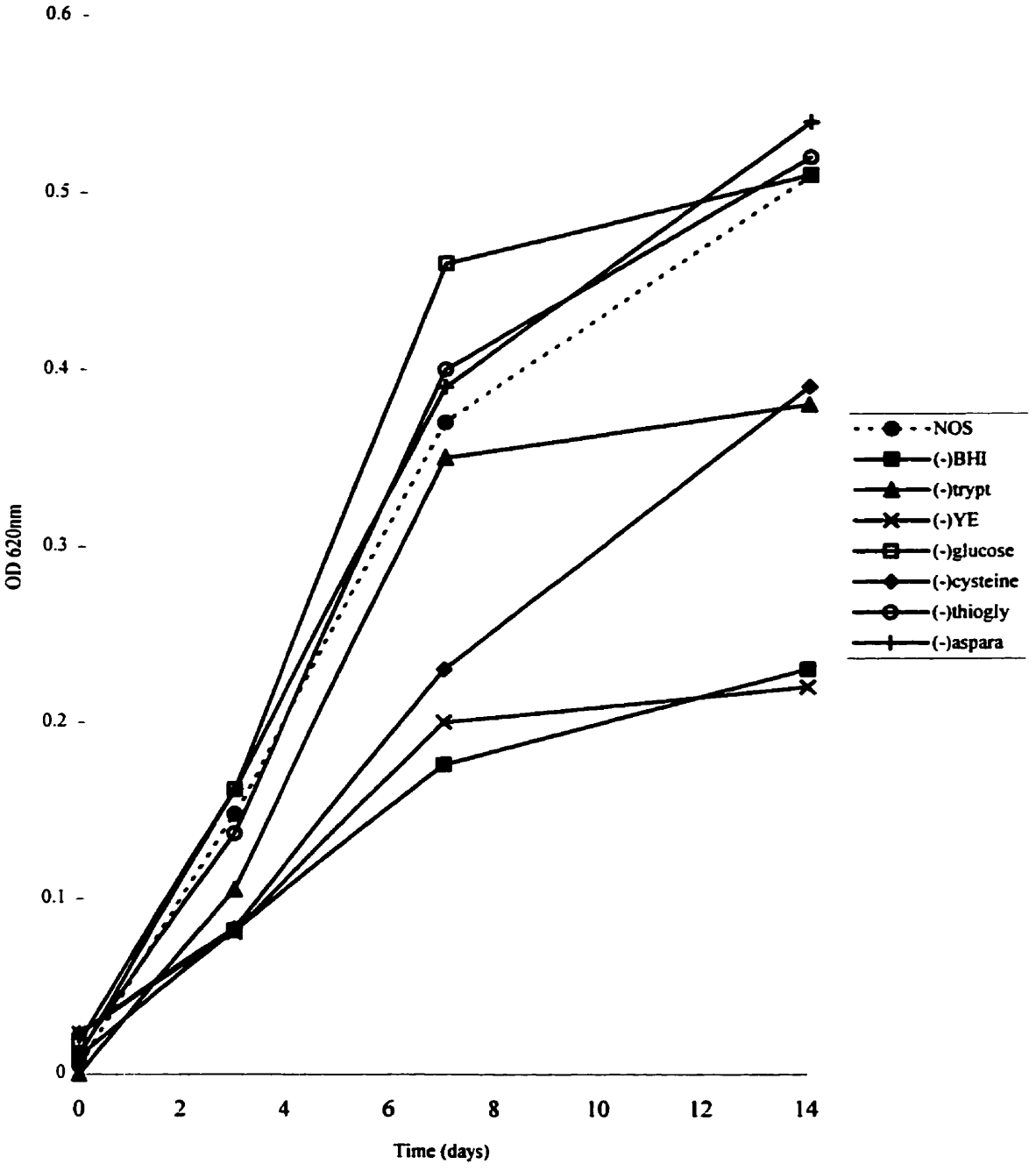

0.6
0.5
0.4
0.3
0.2
0.1
0
OD 620nm
0
2
4
6
8
10
12
14
Time (days)
NOS
(-)BHI
(-)trypt
(-)YE
(-)glucose
(-)cysteine
(-)thiogly
(-)aspara

Figure 15. **Effect of omission of NOS supplements on the spherical body to free spirochete ratio in *T. denticola*.**

A log-phase culture of *T. denticola* ATCC 35405 was inoculated into the following sets of media: NOS medium with complete supplements, used as a control (● Control); NOS medium without rabbit serum (■ No RS); NOS medium without sodium bicarbonate (◆ No Bicarb.); NOS medium without thiamine pyrophosphate (Δ No TP); or NOS medium without volatile fatty acid mixture (× No VFA). All results are the averages of the number of spherical bodies per 100 free spirochetes in four fields, as seen in the culture on days 0 (day of inoculation), 3, 7, and 14.

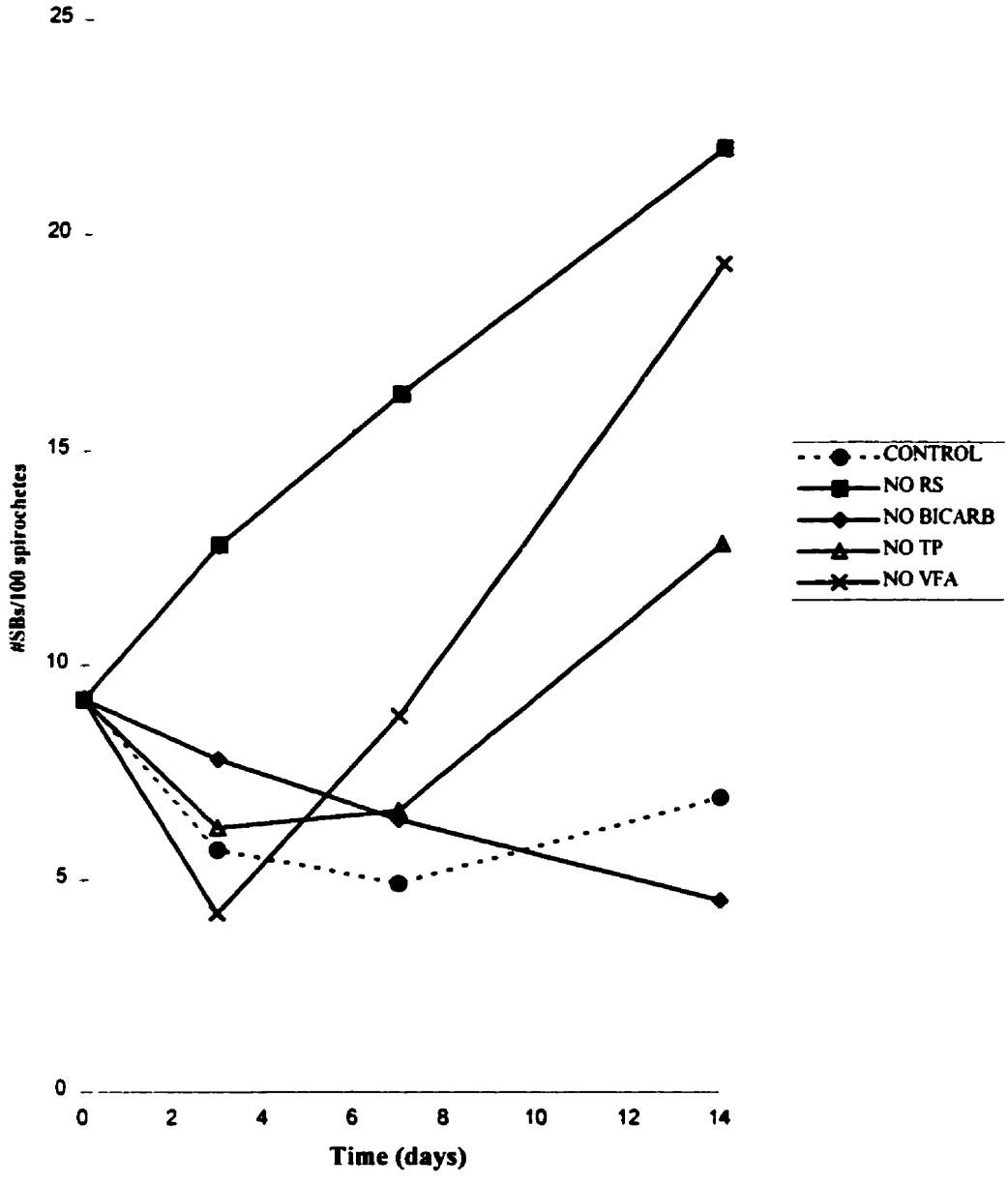

25
20
15
10
5
0
#SBs/100 spirochetes
0
2
4
6
8
10
12
14
Time (days)
CONTROL
NO RS
NO BICARB
NO TP
NO VFA

Figure 16. **Optical density measurements of *T. denticola* grown in NOS medium with supplement components omitted.**

A log-phase culture of *T. denticola* ATCC 35405 was inoculated into the following sets of media: NOS medium with complete supplements, used as a control (● Control); NOS medium without rabbit serum (■ No RS); NOS medium without sodium bicarbonate (◆ No Bicarb.); NOS medium without thiamine pyrophosphate (Δ No TP); or NOS medium without volatile fatty acid mixture (× No VFA). Optical density measurements, at 620 nm, were recorded on days 0 (day of inoculation), 3, 7, and 14.

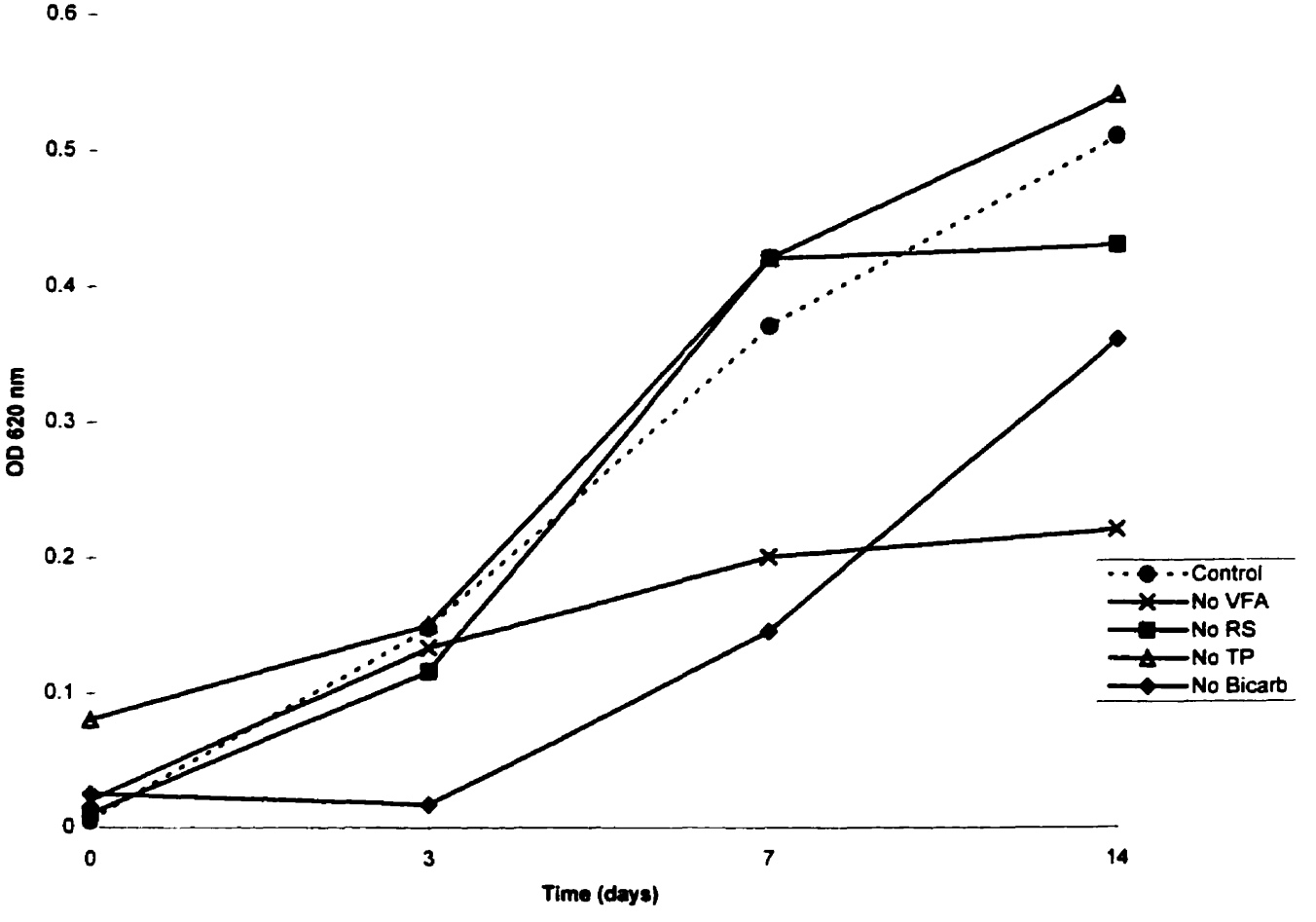

0.6
0.5
0.4
0.3
0.2
0.1
0
OD 620 nm
0
3
7
14
Time (days)
Control
No VFA
No RS
No TP
No Bicarb

Figure 17. **Scatter plot of ratio of spherical bodies to helical spirochetes vs. optical density measurements of *T. denticola* grown in NOS medium without NOS components and supplements.**

A log-phase culture of *T. denticola* ATCC 35405 was inoculated into different sets of test-tubes containing NOS medium (control), and NOS medium without one of the following components: brain heart infusion, yeast extract, trypticase, cysteine, sodium thioglycollate, asparagine, glucose, rabbit serum, sodium bicarbonate, thiamine pyrophosphate, or volatile fatty acids. The numbers of spherical bodies per field (averaged from 4 fields) were determined on day 14 of growth (stationary phase). Optical density measurements, at 620 nm, were also recorded on day 14.

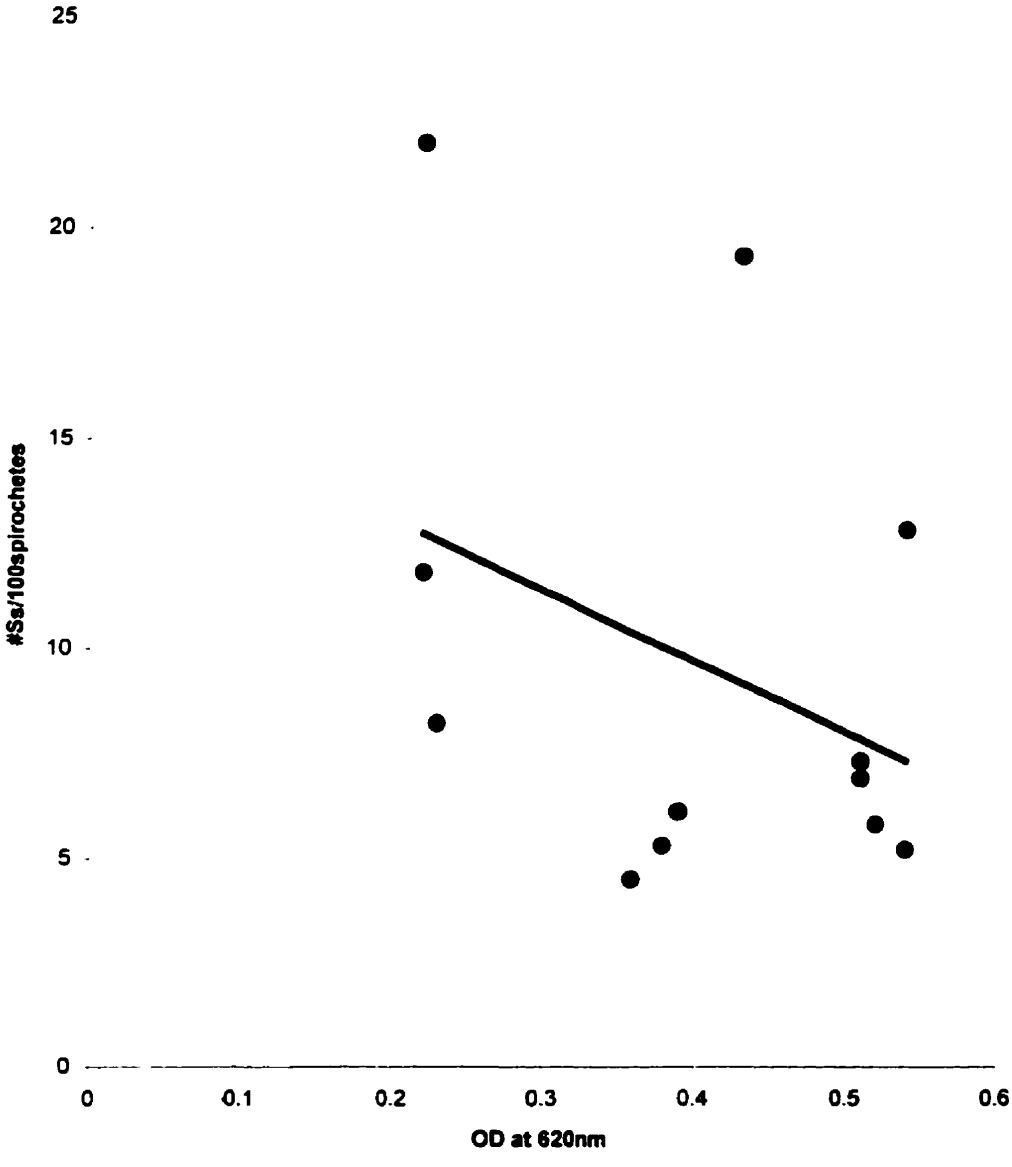

25
20
15
10
5
0
#Ss/100spirochetes
0
0.1
0.2
0.3
0.4
0.5
0.6
OD at 620nm

Figure 18. **Effect of rifampin and alteration of the pH of NOS medium on the ratio of spherical bodies to free spirochetes in *T. denticola*.**

A log-phase culture of *T. denticola* ATCC 35405 was inoculated into the following sets of media: NOS medium with supplements (used as a control), complete NOS medium containing 2 μg/ml rifampin, and complete NOS medium whose pH had been adjusted to 7.42 by the addition of 5M NaOH. Results were recorded as the number of spherical bodies per 100 free spirochetes, averaged from four fields. Counts were recorded on day 21 of growth of the culture.

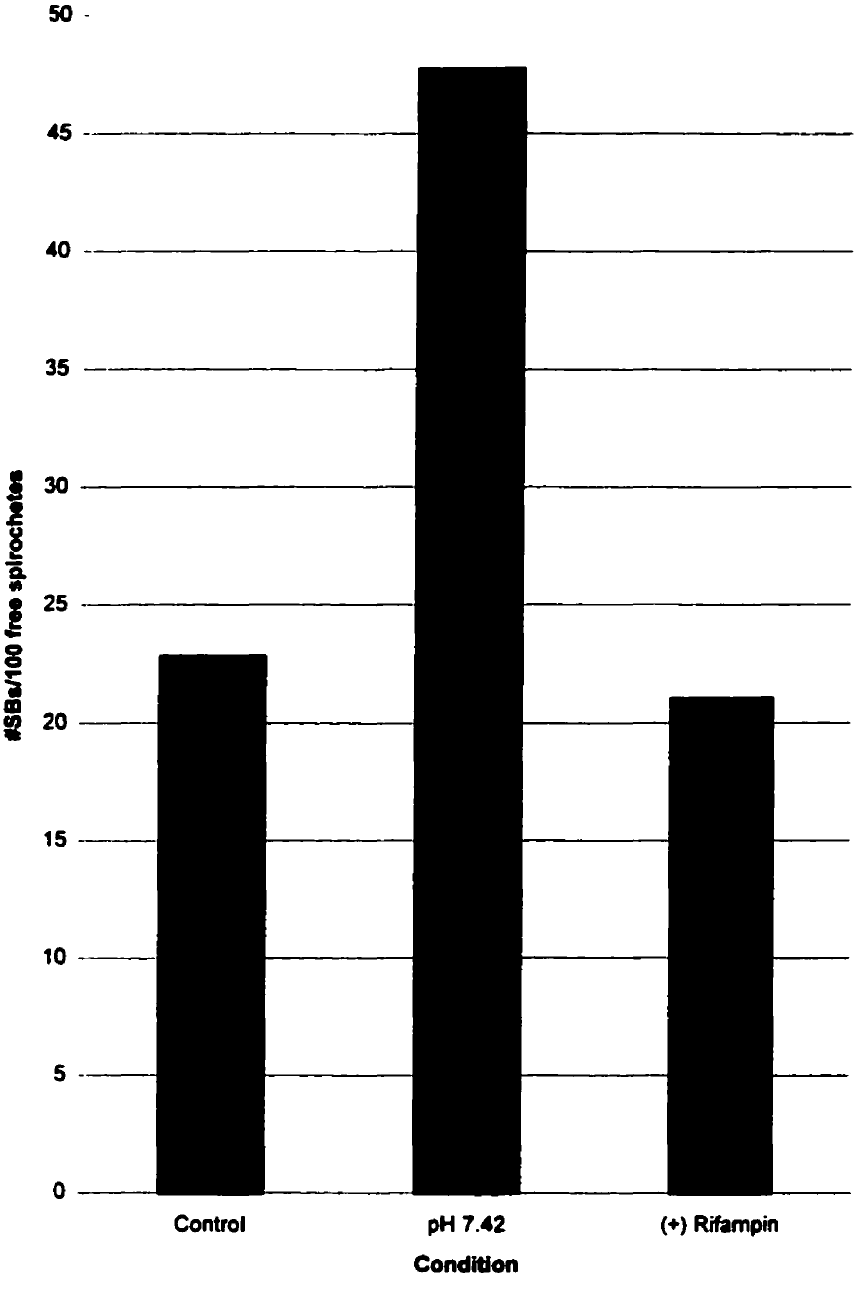

50
45
40
35
30
25
20
15
10
5
0
#SBs/100 free spirochetes
Control
pH 7.42
(+) Rifampin
Condition

Figure 19. **Optical density measurements of *T. denticola* grown in NOS medium containing rifampin and with altered pH.**

A log-phase culture of *T. denticola* ATCC 35405 was inoculated into the following sets of media: NOS medium with supplements (used as a control) (● Control), complete NOS medium containing 2μg/ml rifampin (■ Rifampin), and complete NOS medium whose pH had been adjusted to 7.42 by the addition of 5M NaOH (× pH 7.42). Optical density measurements were recorded, at 620 nm, on days 0 (day of inoculation), 2, 6, 14, and 21.

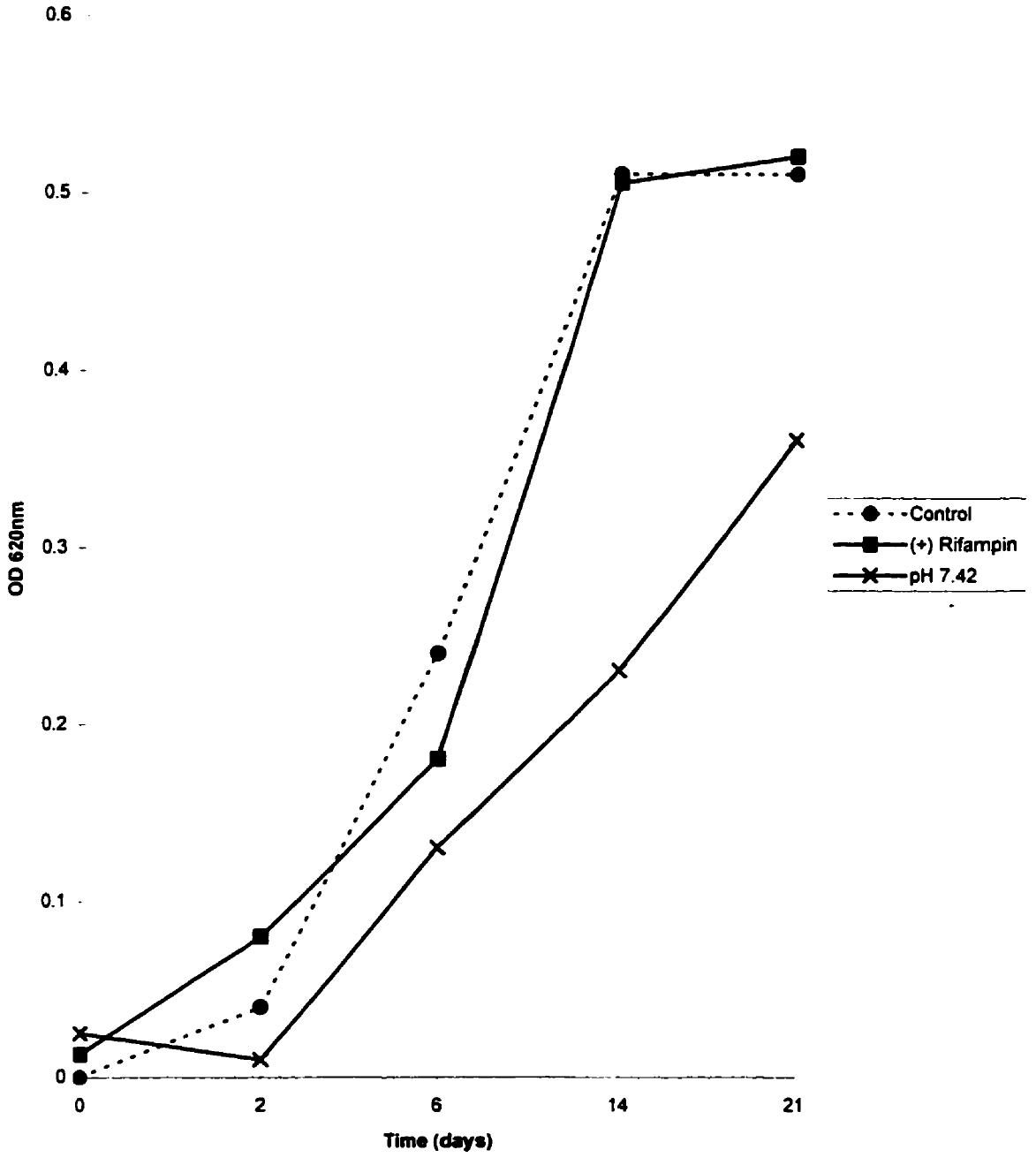

0.6
0.5
0.4
0.3
0.2
0.1
0
OD 620nm
0
2
6
14
21
Time (days)
Control
(+) Rifampin
pH 7.42

Figure 20. **Effect of the addition of end-products of *T. denticola* metabolism on the ratio of spherical bodies to free spirochetes.**

A log-phase culture of *T. denticola* ATCC 35405 was inoculated into complete NOS medium. and NOS medium containing one of the following metabolic end-products: 26500 μmol/l acetic acid , 272 μmol/l pyruvic acid, 2720 μmol/l lactic acid, 2108 μmol/l formic acid, or 5990 μmol/l succinic acid. The ratio of spherical bodies to free spirochetes on day 21 of growth of the culture was determined by taking the average of counts obtained from four fields.

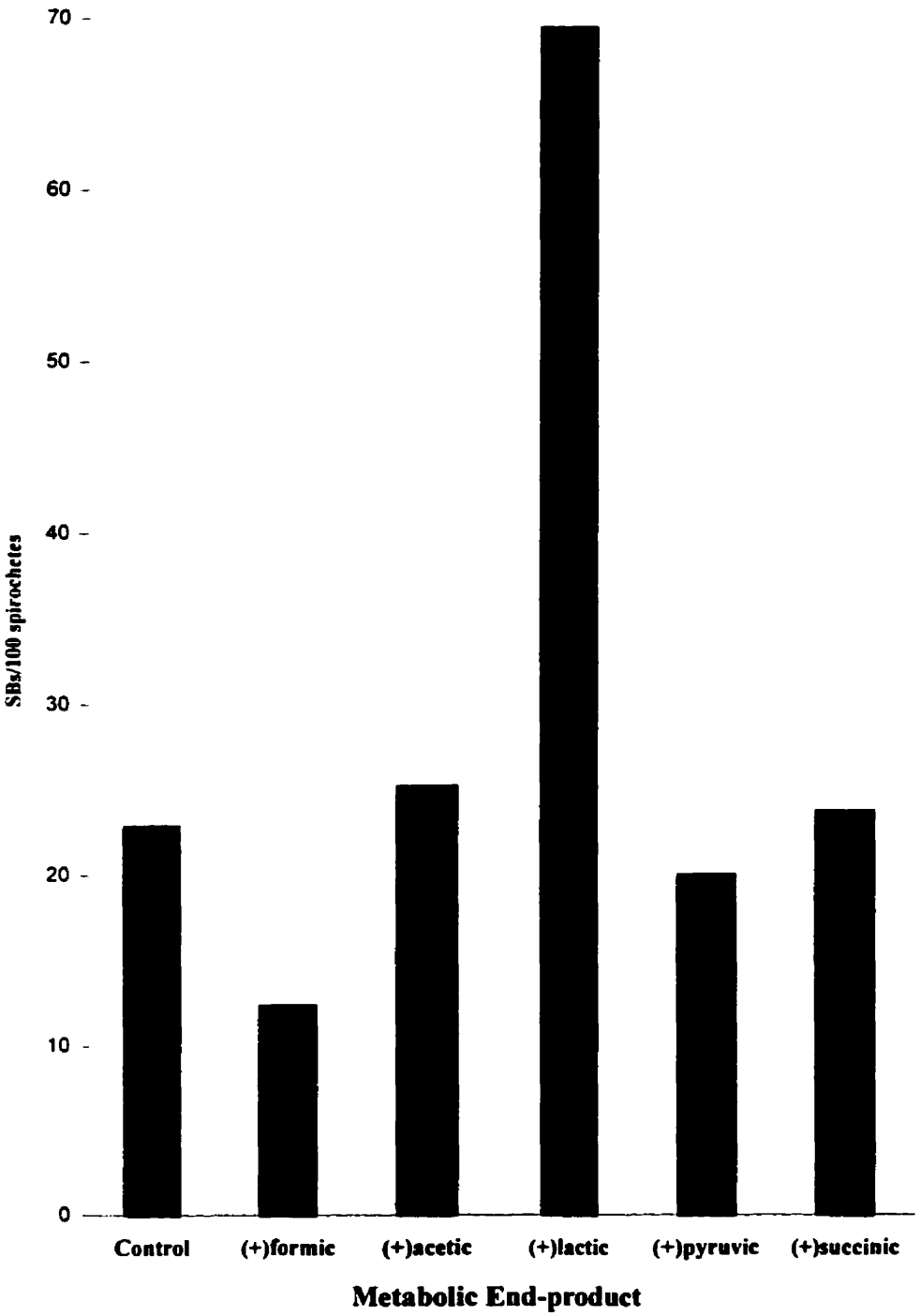

70
60
50
40
30
20
10
0
SBs/100 spirochetes
Control
(+)formic
(+)acetic
(+)lactic
(+)pyruvic
(+)succinic
Metabolic End-product

Figure 21. **Optical density measurements of *T. denticola* grown in NOS medium with metabolic end-products.**

A log-phase culture of *T. denticola* ATCC 35405 was inoculated into complete NOS medium, and NOS medium containing one of the following metabolic end-products: 26500 μmol/l acetic acid, 272 μmol/l pyruvic acid, 2720 μmol/l lactic acid, 2108 μmol/l formic acid, or 5990 μmol/l succinic acid. Optical density measurements were recorded, at 620 nm, on days 0 (day of inoculation), 2, 6, 10, and 21 of growth of the culture.

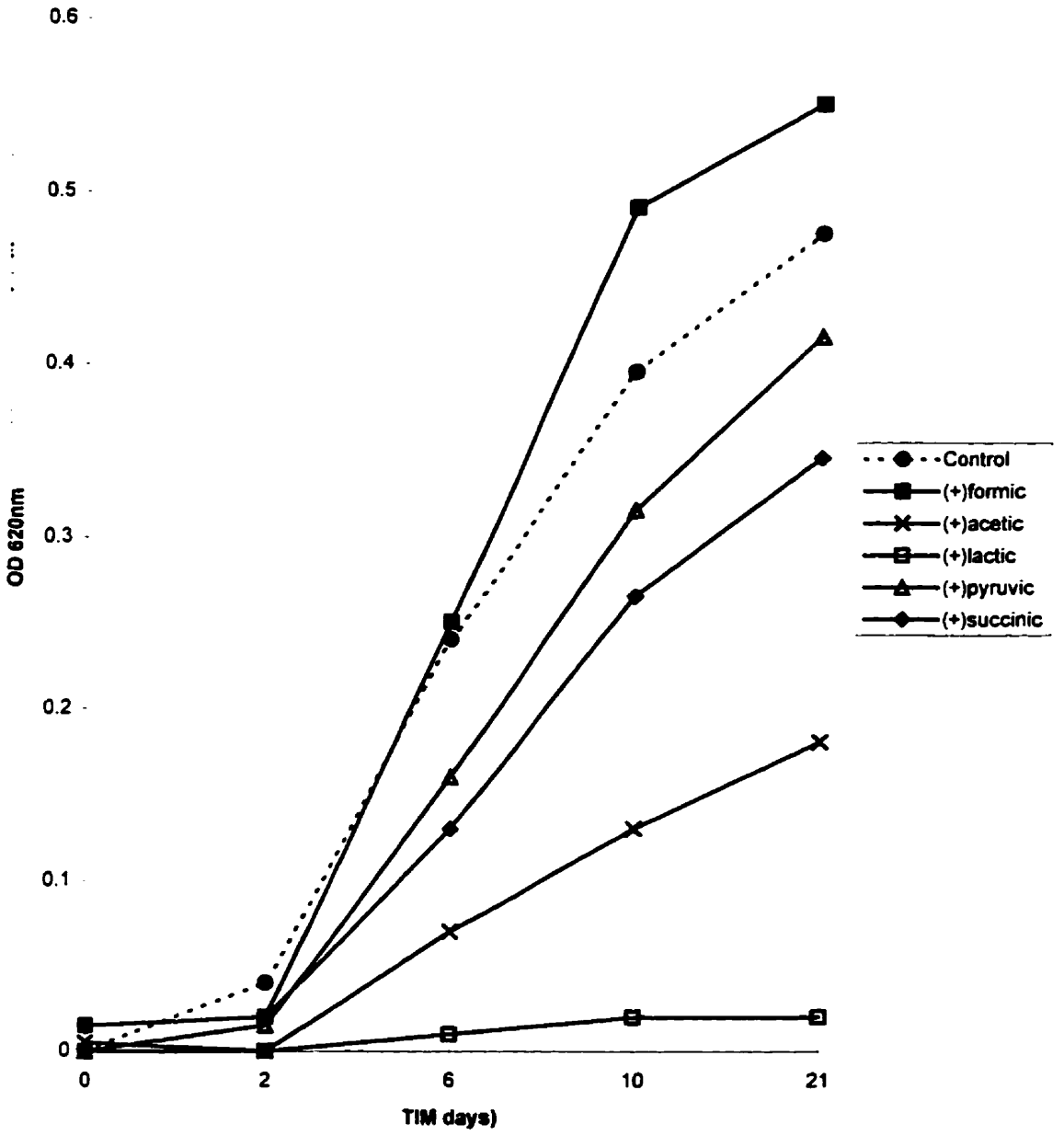
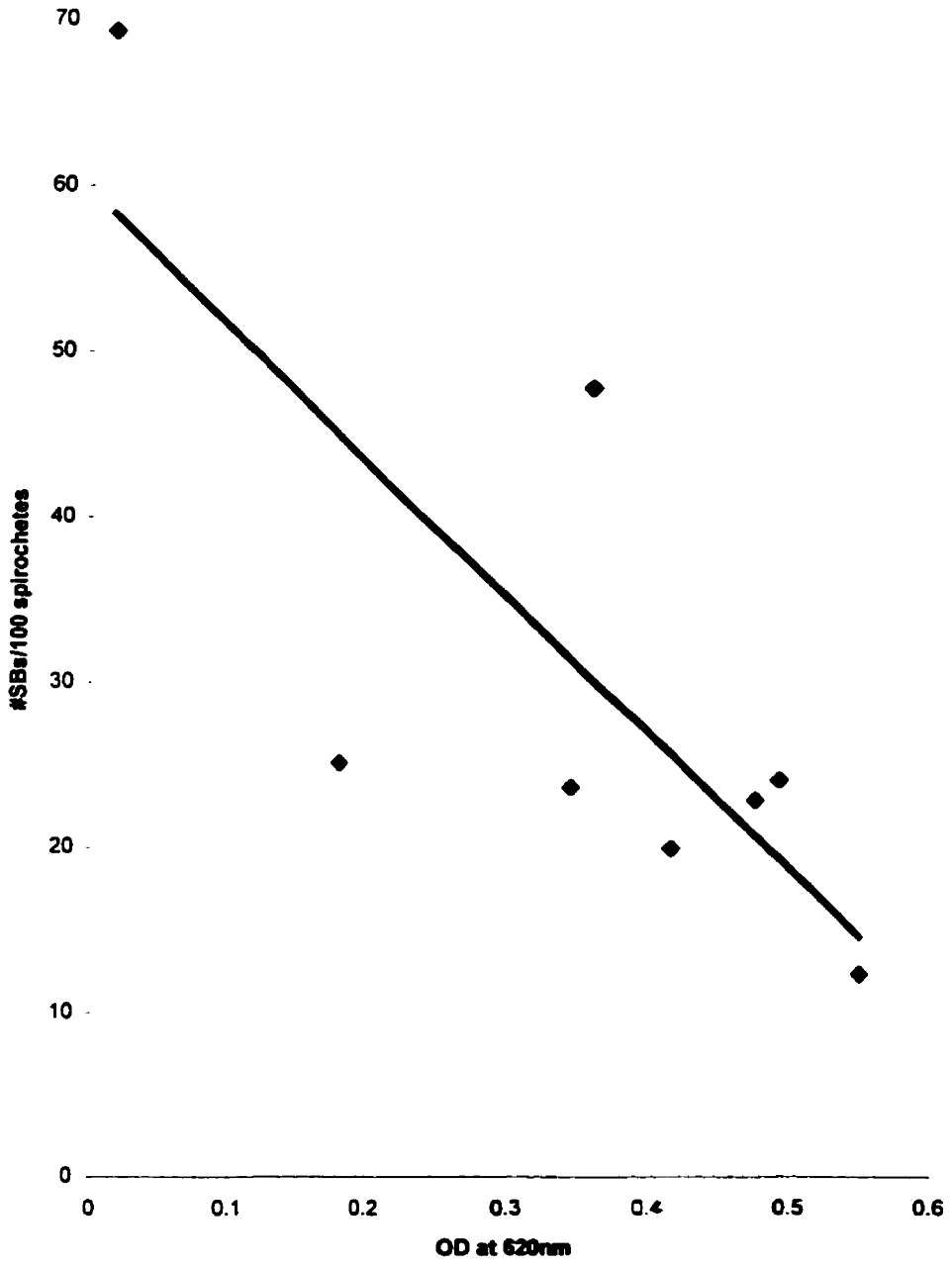

0.6
0.5
0.4
0.3
0.2
0.1
0
OD 620nm
0
2
6
10
21
TIM days)
Control
(+)formic
(+)acetic
(+)lactic
(+)pyruvic
(+)succinic

Figure 22. **Scatter plot of ratio of spherical bodies to free spirochetes vs. optical density measurements of *T. denticola* grown in NOS medium with additives.**

A log-phase culture of *T. denticola* ATCC 35405 was inoculated into different sets of test-tubes containing NOS medium (control), and NOS medium with one of the following additives: 26500 μmol/l acetic acid, 272 μmol/l pyruvic acid, 2720 μmol/l lactic acid, 2108 μmol/l formic acid, 5990 μmol/l succinic acid, 2 μg/ml rifampin, or 5 M NaOH (to bring the pH of the medium from 6.8 to 7.42). The number of spherical bodies per 100 free spirochetes (averaged from 4 fields) was determined on day 21 of growth (stationary phase). Optical density measurements, at 620 nm, were also recorded on day 21.


70
60
50
40
30
20
10
0
#SBs/100 spirochetes
0
0.1
0.2
0.3
0.4
0.5
0.6
OD at 620nm

proportion of spherical bodies to free spirochetes is low, but in a culture that is not growing well (for example, in NOS medium with lactic acid), this ratio will be relatively high.

VII. DNA HOMOLOGY STUDIES

Based on the results of DNA-DNA hybridization experiments, both spirochete isolates A24 and A38 are strains of *T. denticola*. Table 8 gives the percent homology of the isolates with the organisms *T. denticola* ATCC 35405 and *T. socranskii* subspecies *socranskii* ATCC 35536. As shown A24 is 93.4% homologous with *T. denticola* but only 42.3% homologous with *T. socranskii*. A38 is 82.5% homologous with *T. denticola* and 43.8% homologous with *T. socranskii*. A39 will be categorized, by using this method, at a later date.

Table 8. Percent homology of spirochete isolates A24 and A38 with *T. denticola* ATCC 35405 and *T. socranskii* subspecies *socranskii* ATCC 35536, as determined by DNA-DNA homology.

	Known spirochete	
Isolate	*T. denticola* ATCC 35405	*T. socranskii* subspecies *socranskii* ATCC 35536
A24	93.4%	42.3%
A38	82.5%	43.8%

CHAPTER IV. DISCUSSION

Hydrogen-sulfide production by OS

A practical and convenient method has been developed for the detection and enumeration of hydrogen sulfide-producing OS from deep periodontal pockets by a modification of an established technique for recovery of spirochetes (Chan *et al.*, 1993). Ferrous ammonium sulfate was chosen as the iron salt for detecting hydrogen sulfide since it was the least toxic of several iron salts tested for a pure culture of *T. denticola* ATCC 35405 (Figure 5 and Table 4a). Ferric ammonium citrate, which is the iron salt used for testing hydrogen sulfide production by other bacterial species, could not be used for the OS since CFU recovery was low compared to the control flasks. The OS viable counts might be lower because citrate has a chelating or sequestering property on many trace metals and might thus deprive the spirochetes of nutrients required for their growth. It is important to note that experiments with paper point samples from periodontal pockets show that ferrous ammonium sulfate may not be toxic for all OS. In some samples, CFU recovery in the presence of ferrous ammonium sulfate was higher than in the control flasks (Table 5). The OS in this instance may use the iron as a nutritional source.

As shown in Table 5, the majority of OS isolated from periodontal pockets were hydrogen sulfide producers. Therefore, OS may be considered major contributors to fetor oris from periodontal disease. These results confirm similar findings by other researchers using methods such as GC and portable sulfide monitoring to study oral malodor

(Tonzetich and McBride, 1981; Solis-Gafar *et al.*, 1975). Future experiments should deal with the correlation between the number of hydrogen sulfide-producing spirochetes and severity of periodontal disease, as determined by pocket depth. Other studies could focus on the relationship between oral malodor measurements, as determined by gas chromatography, a portable sulfide monitor, or organoleptic assessments, and the number of black spirochete CFUs.

Solidifying agents

The higher viable counts of OS in NOS-GN medium compared with NOS-A may be due to degradation of small gelatin peptides that are produced when gelatin is prepared from collagen. OS produce a proline-specific endopeptidase (Mäkinen *et al.*, 1994) which may cleave gelatin peptides and the products may serve as a nutrient source for the spirochetes. However, large molecules of gelatin in the medium are not hydrolyzed by OS nor such gelatin-hydrolyzing species as *Bacillus subtilis* and *Staphylococcus aureus*. This was shown to be true in our laboratory since NOS-GN medium remained solid even after growth of these organisms (unpublished results). At liquefaction, large molecules of agar polysaccharides exist in their primary structure. As the temperature drops to the gelation point of the agar, the polysaccharides begin to form double helices (Arnott *et al.*, 1974; Rees, 1972) and trap large molecules of gelatin within the helices. The gelatin within the double helix probably destabilizes the hydrogen bonding between D-galactose and 3,6-anhydro-L-galactose and thus reduces the gel point of Noble agar. Moreover, the large molecules of gelatin within the helices of the agar are protected against the

gelatinase (Grenier *et al.*, 1990; Uitto *et al.*, 1988) produced by both OS and other bacteria in the subgingival plaque.

The initial purpose of finding a replacement for SeaPlaque agarose was to bypass the high cost of the agarose. NOS-GN medium can serve as an inexpensive replacement for NOS-A for obtaining viable counts of OS since the GN-gelling mixture costs 0.88$ (Canadian funds) per 100ml NOS medium whereas 0.7% SeaPlaque agarose is 2.45$ for the same amount. Therefore, NOS-GN medium is superior to NOS-agarose medium in both performance and cost.

Antibiotics for selection of OS

Many of the bacteria in the oral cavity appear to be resistant to rifampin. We found that a mixture of 1 μg/ml rifampin and 100 μg/ml phosphomycin effectively inhibited most of the nonspirochetal subgingival bacteria. Rifampin is especially effective against gram-positive organisms and mycobacteria. It inhibits protein synthesis by inactivating the DNA-dependent RNA polymerase. Spirochetes are thought to contain a DNA-dependent RNA polymerase that rifampin is unable to act upon, making them resistant to the actions of this antibiotic. However, unpublished data from our laboratory show that OS are inhibited by 20 μg/ml rifampin. Other researchers similarly report that rifampin at concentrations greater than 1 μg/ml is inhibitory to *T. denticola* (Wyss *et al.*, 1996). In addition, resistance to rifampin develops rapidly both *in vitro* and *in vivo* in bacteria other than spirochetes. This resistance is caused by a mutation that leads to a change in the structure of the β subunit of RNA polymerase (Joklik *et al.*, 1988).

Therefore, accurate enumeration of OS from subgingival plaques in NOS-GN medium containing 2 μg/ml rifampin is difficult due to the growth of many bacteria present in the periodontal pocket (Table 7, Figure 9). In order to maximize the recovery of OS and minimize the growth of other bacteria, an alternative to rifampin was sought. Phosphomycin inhibits the first stage of peptidoglycan biosynthesis. Since it targets a different part of the cell than does rifampin, a combination of the two antibiotics inhibits a greater number of bacteria than rifampin alone. The efficacy of the rifampin-phosphosphomycin mixture in selecting for spirochetes is shown in Figure 10.

Spherical bodies

Spirochetes do not always exist as normal helical forms. Studies described in this thesis indicate that a number of environmental factors may induce formation of these spherical bodies. For example, *T. denticola* cultures grown in the abscence of rabbit serum, volatile fatty acids, thiamine pyrophosphate, or yeast extract had a higher ratio of spherical bodies to free spirochetes than those grown in complete NOS medium with supplements (Figure 15). In addition, this proportion was increased in spirochete cultures that contained the metabolic end-product lactic acid (Figure 20) and in those that grew in NOS medium whose pH had been altered to 7.42 (from 6.8) by the addition of 5 M NaOH (Figure 18). It was also observed that the ratio of spherical bodies to normal helical spirochetes was approximately inversely proportional to the optical density readings. That is, in healthy cultures, there were relatively few spherical bodies, but in cultures that were not growing well, there were many. Therefore, it appears that the

formation of spherical bodies by spirochetes may be a response to adverse growing conditions, similar to the formation of endospores by other bacteria.

DNA-DNA homology

The unknown OS isolates A24 and A38 were determined to be strains of *T. denticola* (Table 8). This treponeme is the most frequently recovered spirochete from periodontal pockets (Chan *et al.*, 1993). Further studies including GC analysis and plasmid screening may be performed in order to more fully characterize these organisms.

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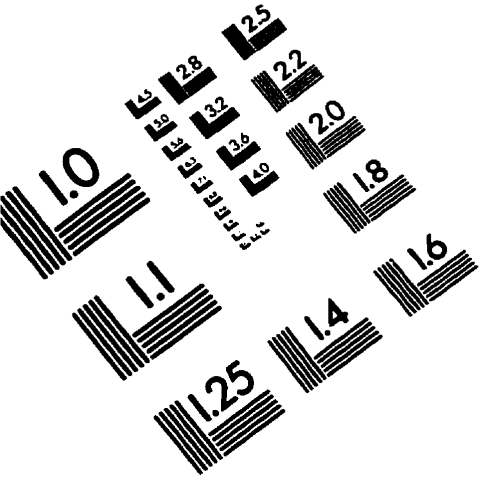
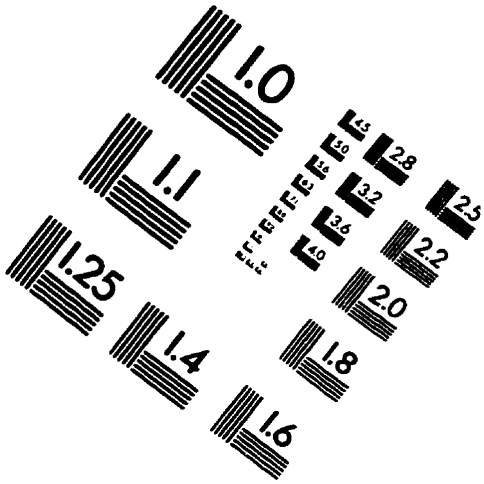
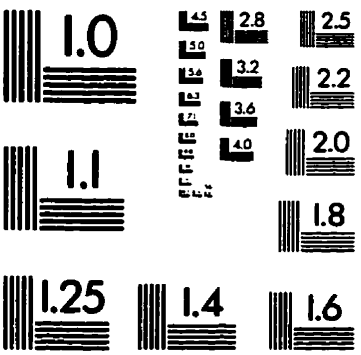
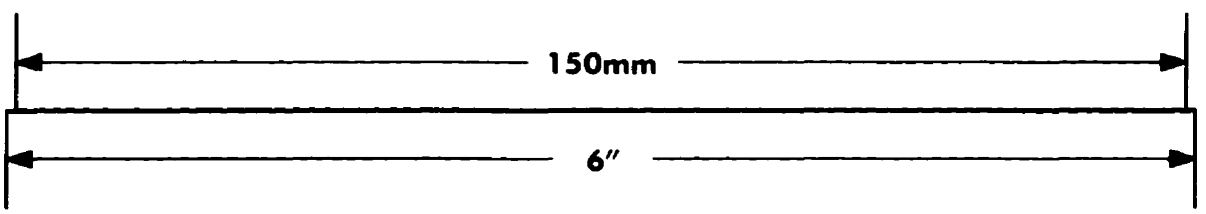
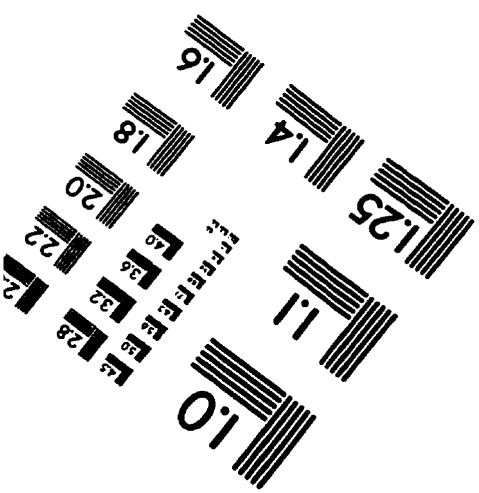
IMAGE EVALUATION TEST TARGET (QA-3)

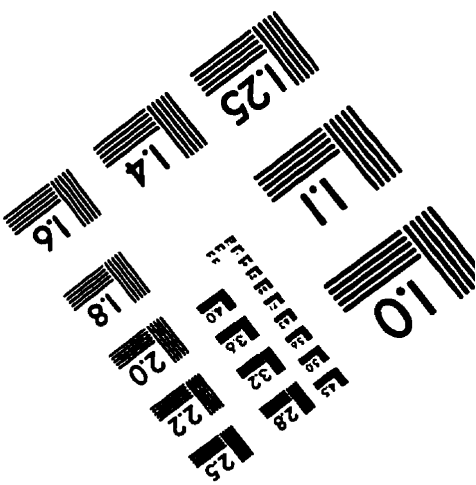












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