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Prevalence and Mechanisms of Antibiotic Resistance in Oral Bacteria

by

Darcie Elizabeth Roe

A dissertation in partial fulfillment
of the requirement for the degree of

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Marilyn C. Rohat PhD

Chairperson of Supervisory Committee

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Doctoral Dissertation

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University of Washington

Abstract

Prevalence and Mechanisms of Antibiotic Resistance in Oral Bacteria

by Darcie Elizabeth Roe

Chairperson of the Supervisory Committee: Professor Marilyn C. Roberts
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Periodontitis occurs in 15% of adults with teeth. Antibiotics are used as an adjunct to standard mechanical therapy for the treatment of periodontitis. As antibiotic use has increased, many bacteria have acquired resistance mechanisms. In the work presented here, the prevalence of antibiotic resistance was determined for periodontal pathogens and commensal species using minimum inhibitory concentration (MIC) assays, DNA probes, polymerase chain reaction, and mating experiments to determine the mobility determinants. The organisms studied included *Actinobacillus actinomycetemcomitans*, *Campylobacter rectus*, *Treponema denticola*, *Neisseria perflava/sicca*, *Neisseria mucosa* and *Neisseria flava*. Tetracycline resistance was found in *A. actinomycetemcomitans*, *T. denticola*, *N. perflava/sicca*, *N. mucosa* and *N. flava*. The tetracycline resistance in *A. actinomycetemcomitans* and *T. denticola* was due to the presence of the Tet B determinant. The Tet B determinant was found to be mobile in *A. actinomycetemcomitans* and appears to be associated with a conjugative plasmid. The Tet B determinant was not mobile in the *T. denticola* isolates examined and was located on the chromosome. The Tet M determinant was present in *Neisseria perflava/sicca*, and *Neisseria mucosa* but not in *Neisseria flava*.

Macrolide resistance was found in *A. actinomycetemcomitans*, *C. rectus* and commensal *Neisseria* species. These macrolide resistant species carried a variety of rRNA methylase determinants. The most common determinants found were Erm B and Erm C. The rRNA methylase determinants were located on the chromosome and were mobile in all the species examined.

This research establishes that previously described antibiotic resistance determinants originally found in other human pathogens are also present in periodontal pathogens and oral commensal species.

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DEDICATION

To my husband, Jerry

CHAPTER 1

Introduction

THE ORAL CAVITY

The world as we know it contains, ourselves (humans), plants and animals, all of which we see and notice everyday. The world also includes a microscopic population, which we cannot see. The members of this microscopic population include, fungi, protozoa, bacteria and viruses. The bacterial population represents a very large and diverse group of single celled organisms. The diversity of bacteria is clearly shown by the wide variety of environments they colonize ranging from the Antarctic frozen lakes to thermal hot springs. Another aspect of bacterial diversity is seen in their ability to adapt to form symbiotic relationships with higher life forms. Examples of this are: 1) nitrogen fixing bacteria living in plant roots which provide nitrogen, an essential nutrient, to the growing plant and 2) ruminant bacteria living in the stomach of a cow help to break down the cellulose in plant material the cow consumes. (Ryan, 1994)

Bacteria can also form symbiotic relationships with humans. Bacteria begin to colonize the human skin and mucosal surfaces moments after birth (Ryan, 1994; Socransky & Manganiello, 1971). These same bacteria may prime the human immune system by exposing it to bacterial antigens which continually trigger the immune system to be ready to fight bacterial infections. These bacteria also produce products that are useful to the host such as Vitamin K (Ryan, 1994). Many of these same bacteria help the host by forming a protective barrier through colonization, which deters the disease causing bacteria from colonizing the host (Preus, 1990; Ryan, 1994). This last

relationship is clearly evident on the tooth surface and in the gingival sulcus of the oral cavity (Slots & Taubman, 1992).

It has been estimated that 300-400 different species of bacteria colonize the oral cavity of man (Loesche, 1993; Socransky & Haffajee, 1994). Individuals normally have between 150 to 200 different bacterial species residing in the oral cavity at any given time (Socransky & Haffajee, 1994). In order to remain in the oral cavity and not be flushed away by saliva and swallowing, bacteria must adhere to each other, a tooth, oral epithelium, or the mucous membrane (Kelstrup & Theilade, 1974; Loesche, 1993; Slots & Taubman, 1992). The specific sites bacterial species colonize in the oral cavity, such as the gingival margin, are largely dependent on the characteristics of the adherence mechanisms of that specific bacteria (Socransky & Manganiello, 1971). Some bacteria are able to remain in the oral cavity by using adherence mechanisms such as; receptors or adhesins specific to the pellicle which covers the tooth, or to other bacteria attached to the tooth (Gibbons & van Houte, 1973; Listgarten, 1988; Ryan, 1994; Socransky & Manganiello, 1971). As the colonization process continues on the tooth surface above the gingival margin, the bacteria begin to form a mass called dental plaque (Listgarten, 1988). Plaque build-up most commonly occurs around the tooth irregularities and the gingival margin (Gibbons & van Houte, 1973).

In a healthy mouth, the dental plaque is dominated by Gram-positive facultative bacteria (Kelstrup & Theilade, 1974; Moore & Moore, 1994). The early colonizers are *Actinomyces viscosus*, *Actinomyces naeslundii* and *Streptococcus mitis*, *Streptococcus sanguis*, *Staphylococcus epidermidis* (Genco, 1981; Moore & Moore, 1994; Slots &

Taubman, 1992; Socransky, 1977). The *Actinomyces* spp. and *Streptococcus* spp. genera may represent up to 50% of the total flora in the healthy mouth (Kelstrup & Theilade, 1974). Without good daily oral hygiene, the dental plaque continues to grow in mass due to the cell division of the early colonizers (Kelstrup & Theilade, 1974). This is followed by the adherence of midrange colonizers. Midrange colonizers are bacterial species that cannot adhere directly to the tooth surface, but adhere to the early colonizers which are attached to the tooth (Gibbons & van Houte, 1973; Kelstrup & Theilade, 1974). These midrange colonizers include *Neisseria* spp., *Haemophilus* spp. and *Fusobacterium* ssp. (Socransky & Manganiello, 1971; Slots & Taubman, 1992). When significant accumulation of early and midrange colonizers occurs, new bacterial species labeled “late colonizers” begin to adhere to the plaque. These late colonizers, like the midrange colonizers cannot adhere to the tooth surface and therefore must adhere to the early and midrange bacteria (Gibbons & van Houte, 1973; Kelstrup & Theilade, 1974). In addition, these late colonizing species prefer an anaerobic environment. This anaerobic environment is established by the early and midrange facultative species which can grow in the presence of oxygen, but can also grow without oxygen. Therefore, the late colonizers are located in the subgingival site in the sulcus pocket, physically under the protection of the early and midrange colonizers that make up the supragingival plaque. These late colonizers include species such as; *Prevotella*, *Campylobacter* and *Veillonella* (Socransky & Manganiello, 1971; Slots & Taubman, 1992). The process of gaining mass and colonization with a more diverse bacterial species is referred to as ‘plaque

maturation'. This process continues until removed by brushing or debridement and the process starts over again (Kelstrup & Theilade, 1974).

When disease begins to occur in the gingival sulcus, a shift or change in the type of colonizing bacteria is observed (Genco, 1981). This change results in the development of gingivitis which is characterized by inflammation of the gingival tissue without loss of alveolar bone support or connective tissue (Slots and Taubman, 1992; Tanner & Stillman, 1993). As gingivitis develops, the percentage of Gram-negative organisms making up the total flora increases (Genco, 1981; Slots and Taubman, 1992; Tanner & Stillman, 1993). With gingivitis, *Actinomyces* spp. and *Streptococcus* spp. are still present in the gingival sulcus, but make-up a lower percentage of the total cultivable flora than seen in healthy gingiva (Genco, 1981; Slots & Taubman, 1992). The organisms most closely associated with gingivitis are *Fusobacterium nucleatum*, *Prevotella intermedia* and many others (Genco, 1981; Slots & Taubman, 1992).

PERIODONTITIS

When the loss of bone support and connective tissue destruction are observed the disease state is considered to be periodontitis. At this stage, 75% of the flora are Gram-negative anaerobic rods (Genco, 1981; Slots & Taubman, 1992). The most predominant bacterial species associated with periodontitis are *F. nucleatum*, *P. intermedia* and *Porphyromonas gingivalis*, making up 30 to 50% of the flora (Genco, 1981; Moore & Moore, 1994; Slots & Taubman, 1992). As bacteria accumulate on the tooth surface and the disease progresses it is characterized by, bleeding, suppuration, advanced

inflammation of the gingiva and tooth attachment loss (Slots & Taubman, 1992). There is also an apical migration of the junctional epithelium down the root surfaces, causing an increase in size of a space called the periodontal pocket located between the tooth and the epithelium (Figure 1)(Schluger *et al.*, 1990; Slots & Taubman, 1992). The periodontal pocket can become as deep as ≥ 10 mm as compared to a depth of ≤ 4 mm with healthy gingiva (Figure 1) (Schluger *et al.*, 1990; Slots & Taubman, 1992). This disease process also involves the destruction of the tissue protecting the root surface which becomes exposed to the oral cavity environment (Slots & Taubman, 1992). With advanced periodontal disease, the connective tissue, which is the attachment support for the tooth, is destroyed and supporting alveolar bone, which is the bone the tooth sits in, is resorbed. This whole process of attachment loss, creates a lack of structural support for the tooth, ultimately leading to tooth loss (Slots & Taubman, 1992). It has been estimated that 6 to 18% of adults who still have their teeth will develop some form of periodontitis, with the prevalence increasing with age (Oliver *et al.*, 1991; Slots & Taubman, 1992).

Eleven bacterial species, listed in Table 1, have been found to be closely associated with periodontitis (Dzink *et al.*, 1988; Haffajee *et al.*, 1988; Haffajee & Socransky, 1994; Moore & Moore, 1994). As more has been learned about the bacteria colonizing the oral cavity and more patients with periodontitis have been evaluated, the list of bacteria associated with the progression of periodontitis has changed over the years (Theilade, 1986; Socransky & Haffajee, 1994). Historically, many of these bacterial species have gone in and out of favor over time (Socransky & Haffajee, 1994). This is in

part due to an unclear cause and effect relationship between specific bacterial species and the disease. There is still not total agreement as to which oral bacterial species are the most important in the progression of periodontal disease. Despite this, the accepted cause of periodontitis is due to the presence of bacteria (Socransky & Haffajee, 1994). None of these organisms are found in all periodontal disease cases, but at least one or a combination of several of these presumptive pathogens are found in the subgingival microflora of most periodontal patients (Dzink *et al.*, 1988; Haffajee *et al.*, 1988). Some of these bacteria can also be found in low numbers in healthy patients and in nondiseased sites in patients with periodontitis. This suggests that these bacterial species are probable normal residents of the oral cavity rather than foreign invaders (Loesche, 1993; Moore & Moore, 1994). Another possibility is that some of the bacteria labeled as “pathogens” may be present due to the results of the disease process rather than the cause of the disease process (Socransky & Haffajee, 1993). All of these factors have made the determination of the etiology of periodontitis very difficult (Haffajee & Socransky, 1994; Socransky & Haffajee, 1993). This problem is further complicated by a lack of clear understanding of the disease process and what factors effect pathogenesis (Socransky & Haffajee, 1993). Factors such as smoking, genetics and immune response affect the composition of the flora, which in turn affects the disease progression. How and why they have such affects is poorly understood (Moore & Moore, 1994; Slots & Taubman, 1992; Socransky & Haffajee, 1993). In addition, difficulties in the “proper” classification of various forms of periodontitis and current disease status only adds to the complexity (Socransky & Haffajee, 1993). The lack of a direct correlation between

disease and a single bacterial species suggests the disease process is more complex than the presence or absence of a bacterial species colonizing a patient (Moore & Moore, 1994; Socransky & Haffajee, 1993). The progression of periodontitis is believed to be due to a number of factors that must all occur at the same time; the pathogenic bacteria must be present in high enough numbers (above the threshold of the host's resistance), a susceptible host, a susceptible host immune system, a conducive local oral environment and the absence of enough beneficial bacteria (Socransky & Haffajee, 1993; Theilade, 1986).

The classification of specific forms of periodontal disease is based on the severity of the clinical symptoms, history and biological behavior of the lesions, status of the patient's immune system and characteristics of the patient such as age (Schluger *et al.*, 1990; Tanner & Stillman, 1993). Examples of some of the different forms of periodontitis are: adult periodontitis, juvenile periodontitis, rapidly progressive periodontitis, refractory periodontitis, and early-onset periodontitis (Loesche, 1993; Schluger *et al.*, 1990; Slots & Taubman, 1992). Since the disease status with periodontitis is based on subjective clinical parameters and ranges of measured values, there is much debate as to the correct classification of disease. The definitions of the forms of periodontitis continue to change as more is learned about the disease process (Theilade, 1986).

Adult periodontitis is the most common form of the disease accounting for more than 90% of the cases of periodontitis. It is characterized by disease onset at age 35 or older (Schluger *et al.*, 1990). High levels of inflammation are not observed. The disease

can affect a few sites or all the teeth with little or no evidence of progression of active disease. The level of plaque accumulation correlates with the severity of the lesions (Schluger *et al.*, 1990). However, this correlation does not extend to the bone loss that is observed which is variable through out the bone structure. *P. gingivalis* is closely associated with the progression of this form of periodontitis and currently is thought to be the most important pathogen with this form of periodontitis (Slots & Listgarten, 1988; Slots & Rams, 1991). *P. intermedius* and *B. forsythus* are also associated with adult periodontitis but not as strongly as *P. gingivalis* (Slots & Listgarten, 1988; Slots & Taubman, 1992).

Localized juvenile periodontitis is seen in prepubescent children (Schluger *et al.*, 1990). Lesions are characteristically seen at the first molars and/or incisors. The gingival tissue appears normal and there is less plaque build-up than might be expected. This form of periodontitis is more common in females, African-Americans, and may also involve genetic factors. Many of these patients also have functional defects in their immune system (Schluger *et al.*, 1990). *Actinobacillus actinomycetemcomitans* is thought to be the primary pathogen associated with this form of periodontitis and is less commonly found in the adult forms of periodontitis (Loesche, 1993; Slots & Listgarten, 1988; Slots & Rams, 1991).

Rapidly progressive periodontitis is seen in patients between puberty and age 35. Plaque accumulation is variable and lesions are generalized, affecting most of the teeth. Patients commonly have a previous history of juvenile periodontitis. Patients in the active phase of disease have gingival tissue that is acutely inflamed. Severe bone loss is

profoundly evident in these patients and more extensive than what is observed with adult periodontitis.

Refractory periodontitis usually develops from other forms of periodontitis (Listgarten *et al.*, 1993). These are cases of destructive periodontitis which respond poorly to the conventional therapy of scaling and root planing, periodontal surgery, and regular maintenance (Fiehn, 1990; Haffajee *et al.*, 1985; Hirschfeld & Wasserman, 1978; Listgarten *et al.*, 1993). These patients were described by Page (1985) as progressing in a “rapid and unrelenting manner regardless of the type and frequency of therapy applied.” It appears that periodontal surgery and improved hygiene may postpone the loss of teeth but most therapies are unable to prevent exacerbated disease and tooth loss (Hirschfeld & Wasserman, 1978). Slots and Rams (1991) report that 10% of periodontitis patients fall into the category of refractory periodontitis. It has been suggested that therapeutic failures are due to the inability to remove or reduce the number of subgingival pathogenic organisms during therapy (Slots & Rams, 1991). It has also been suggested that some of these patients exhibit weakened immune responses with regards to periodontal infections (Slots & Rams, 1991). Antibiotics have been used as a supplement to therapy for this form of periodontal disease, in an attempt to eradicate periodontal pathogens from the periodontal pocket (Goodson, 1994; Slots & Rams, 1991). In reality, antibiotic therapy is not able to remove all bacteria from the gingival sulcus, rather, the antibiotic therapy slows down the disease process thus aiding the response to mechanical therapy in these cases.

Early onset periodontitis occurs during or soon after eruption of the primary teeth. There are two forms of early onset periodontitis, generalized and localized (Schluger *et al.*, 1990). Generalized early onset periodontitis is characterized by extreme acute inflammation and rapid destruction of the alveolar bone and gingival tissue affecting all of the teeth. Localized early onset periodontitis is characterized by little or no inflammation. Destruction occur slowly and only affects some of the teeth (Schluger *et al.*, 1990). *P. gingivalis* has been shown to be closely associated with the progression of this form of periodontal disease (Slots & Rams, 1990).

THERAPY FOR PERIODONTITIS

The objective behind the treatment of periodontal disease is the reduction of the number of bacteria in the gingival pocket with the long term goal of retaining as many teeth as possible (Hirschfeld & Wasserman, 1978). Conventional therapy involves the removal of bacterial plaque and calculus from the teeth (mechanical therapy) via scaling and root planing (Slots & Rams, 1990). Plaque and calculus are physically removed from the tooth by scraping the supragingival and subgingival tooth surfaces with scalers and currettes. The effect of this treatment is the removal of bacteria which lowers the total number of bacteria in the periodontal pocket (Socransky & Haffajee, 1993). Up to 90% of the bacteria in a pocket can be removed from a site by this method (Socransky & Haffajee, 1993). The removal of such a large percentage of bacteria also alters the local environment (Socransky & Haffajee, 1993). With fewer bacteria present, inflammation of the gingiva is normally reduced (Socransky & Haffajee, 1993). These changes in turn

affect the temperature of the pocket and the amount of crevicular fluid flow which ultimately affects the growth environment of the bacteria (Socransky & Haffajee, 1993). Even with all of these changes, the removal of bacteria is only temporary. Early colonizers start to recolonize the tooth surface within one to two hours after removal and most species can recolonize a site within 42 days (Socransky & Haffajee, 1993).

Educating the patient in dental hygiene is vital to improve daily hygiene habits at home in order to accomplish adequate supragingival plaque control. If the disease is in an advanced state, surgery is a common procedure (Schluger *et al.*, 1990). The aim of periodontal surgery is to reduce and eliminate if possible the periodontal pocket. Surgery allows full access to the root surface which permits complete removal of plaque and calculus build-up on the root surface. Surgery allows the recontouring of the soft and/or hard tissue to a healthy conformation. This leads to a reduction of the periodontal pocket which in turn eliminates anaerobic environment which is needed by most periodontal pathogens. Surgery also allows easier home care oral hygiene and professional oral hygiene care. To prevent the reoccurrence of disease, improved and maintained dental hygiene is crucial. This is accomplished by further educating the patients in dental hygiene and continued regularly scheduled dental cleaning, every 3-6 months (Goodson, 1994; Slots & Taubman, 1992). Approximately 80 to 90% of cases of periodontitis respond to periodontal surgery and are stable if good plaque control is maintained (Becker *et al.*, 1984; Hirschfeld & Wasserman, 1978; Knowles *et al.*, 1979; Lindhe *et al.*, 1984; Pihlstrom *et al.*, 1983; Slots and Taubman, 1992; Socransky & Haffajee, 1993).

When therapy fails in the presence of good therapy and good oral hygiene, it is often

difficult to determine what factors were the cause of the failure, again returning to the fact that periodontal disease progression is complex and is affected by many factors (Haffajee *et al.*, 1995).

Of the many forms of periodontitis, early-onset, rapidly progressing, and refractory periodontitis represent 10 to 20% of periodontitis cases and are the most difficult to treat (Slots & Taubman, 1992). When conventional mechanical treatments and surgery have failed, the dentist often uses antibiotics as an adjunct to therapy with these forms of periodontitis (Genco, 1981; Slots & Rams, 1990). The following antibiotics are used in the treatment of periodontitis; penicillin, cephalosporin, macrolides, tetracycline, clindamycin, or metronidazole (Coco & Pankey, 1989; Fiehn, 1990). Antibiotic dosage and duration are based on the clinical judgment of the dentist (Slots & Rams, 1990). In 1988, 83% of periodontists in the United States which answered a survey stated they prescribed antibiotics following periodontal surgery (Slots & Rams, 1990). Drugs of choice in order of use were: 1) tetracycline 2) doxycycline 3) penicillin 4) amoxicillin (Slots & Rams, 1990). Tetracycline is thought to be the most commonly used antibiotic for periodontal therapy in the United States. However, the current usage in 1996 and amounts of antibiotics being prescribed are unknown. There is much debate as to when antibiotics are appropriate to use. It is also under debate if microbiological examination of a site should be done and if so when, and whether the susceptibility testing of bacteria from infected sites is necessary or relevant (Fiehn, 1990; Genco, 1981; Slots & Rams, 1990).

There have been many reports describing the use and efficacy of antibiotic therapy. When evaluating these studies, one must consider the variation between studies in sample size, differences in criteria of “diseased patients,” differences in the antibiotic therapies, dosage prescribed, length of therapy, and mechanical treatments (Ram and Slots, 1990). These variabilities make interpretation and comparisons of studies very difficult. Slots and Ram (1990) also discuss some of the adverse effects of antibiotic therapy. They suggest that antibiotic therapy with an agent the periopathogen is resistant to could result in the aggravate the disease process rather than resolution of the disease (Slots & Rams, 1990). The reason for this would be the antibiotic used for therapy would inhibit the growth of all antibiotic susceptible bacteria, which would reduce the competition for antibiotic resistant periodontal pathogen which would then be able to proliferate in the presence of antibiotic and the lack of bacterial competition, allowing the periodontal pathogens to continue the disease process. It has also been reported, that patients who received multiple courses of antibiotics have dramatic changes in their flora. These changes include colonization by enteric bacteria, pseudomonads or yeast in the subgingival flora, which are not normally found in the human oral cavity of healthy individuals (Helouvo, 1986; Slots *et al.*, 1988).

ANTIBIOTICS

The first natural antibacterial agent, pyocyanase, was discovered in 1888 (Levy, 1992a). de Frudenreich found that bacteria *Bacillus pyocyaneus* (now known as *Pseudomonas aeruginosa*) released a blue pigment which had an inhibitory effect on

other bacteria. In 1889, Rudolf Emmerich and Oscar Loew performed the first susceptibility trials with pyocyanase (Levy, 1992a). In the laboratory, pyocyanase was so effective in inhibiting bacteria that many species were actually killed, including pathogenic bacterial species. However, when pyocyanase was tested for human use with *in vivo* studies, it was toxic and unstable, and was deemed unfit for human use. (Levy, 1992a)

In 1929, Alexander Fleming discovered a dish containing *Staphylococcus* ssp. that had been lysed apparently due to a substance produced by a *Penicillium* species of mold also growing on the petri dish (Fleming, 1929). The mold's product was named penicillin, and this is considered the first "true" antibiotic that could be used as a therapeutic agent in humans (Fleming, 1929; Levy, 1992a; Slots & Taubman, 1992). However, it was not in a pure enough form in high enough quantities to use clinically until the early 1940 (Chain *et al.*, 1940; Florey, 1945). In 1944, the second antibiotic streptomycin, a product of a actinomycete *Streptomyces griseus* was discovered by Schatz, Brigie and Waksman (Schatz *et al.*, 1944). These findings mark the beginning of the antibiotic era which has revolutionized therapy modalities in modern medicine (Levy, 1992a).

Tetracycline

In 1948, the first tetracycline class compound, chlorotetracycline, was isolated from *Streptomyces aureofaciens* (Duggar, 1948). Subsequently, about 1000 tetracycline derivatives have been described (Chopra *et al.*, 1992). Four fused cyclic rings is the

common chemical characteristic of all members of the tetracycline class of antibiotics. Of all the tetracycline derivatives only seven have been used clinically either with humans or animals (chlortetracycline, oxytetracycline, tetracycline, methacycline, doxycycline, minocycline)(Chopra *et al.*, 1992). Tetracyclines are active against Gram-positive, Gram-negative, aerobes and anaerobes (Chopra *et al.*, 1981; Klien & Cunha, 1995; Levy, 1992a; Speer *et al.*, 1992). Due to the activity of tetracyclines on such a wide variety of bacteria, tetracyclines were the first antimicrobial group to be described as “broad spectrum” (Chopra *et al.*, 1981; Cunha, 1985). This broad spectrum also includes activity against unique bacteria such as mycoplasmas, chlamydiae, rickettsia, as well as protozoan parasites (Chopra *et al.*, 1981; Klien & Cunha, 1995; Levy, 1992a; Speer *et al.*, 1992). In addition to being broad spectrum, tetracyclines have few side effects and are quite stable (Chopra, 1990; Chopra *et al.*, 1992; Levy, 1992b). Due to these favorable characteristics and low cost, tetracyclines are one of the most commonly used antibiotics world wide, surpassed only by penicillins (Col & O’Connor, 1987; Levy, 1992a). Currently, tetracyclines are commonly used to treat bacterial respiratory infections and urogenital tract infections although this is changing as more bacteria become resistant (Chopra *et al.*, 1981; Chopra *et al.*, 1992). Tetracycline also has the ability to concentrate in the gingival crevicular fluid at levels up to five times higher than the blood level concentrations (Table 2)(Ciancio *et al.*, 1980; Gordon *et al.*, 1981; Pascale *et al.*, 1986). It has been postulated that this concentration effect is due to the ability of tetracycline to attach to the tooth surface and still remain biologically active when released (Baker *et al.*, 1983; Gordon & Walker, 1993; Wilkesjo *et al.*, 1986). It is

this last characteristic that has made tetracycline a very effective and popular therapeutic agent for the treatment of periodontal disease (Genco, 1981; Slots & Ram, 1990; Walker *et al.*, 1983).

Tetracycline enters the bacterial cell via passive diffusion through hydrophilic pores in the outer membrane (Chopra, 1990; Chopra *et al.*, 1992). Both an energy dependent active transport and an energy independent uptake system are used for the passage of tetracycline through the bacterial cytoplasmic membrane (Chopra, 1990; Chopra *et al.*, 1992; Levy, 1992b). The energy independent uptake system represents passive diffusion (Chopra, 1990; Chopra *et al.*, 1992). The energy dependent active transport is known to occur; however, the exact mechanism is still not fully understood (Chopra, 1990; Chopra *et al.*, 1992). Tetracyclines have a selective action against bacteria because the bacterial cell actively concentrates tetracycline while mammalian cells do not (Chopra *et al.*, 1992; Chopra, 1994).

The typical tetracyclines include; tetracycline, chlortetracycline, minocycline and doxycycline (Chopra *et al.*, 1992). The mechanism of action of the 'typical tetracycline' is the interference with bacterial cell growth by inhibiting protein synthesis (Chopra, 1994). Tetracyclines accomplish this by binding to a specific site on the 30S ribosome. The presence of tetracycline on the ribosome disrupts the codon-anticodon matching between tRNA and mRNA. By blocking the binding of aminoacyl-tRNA to the acceptor site on the mRNA-ribosomal complex, the addition of amino acids to the peptide stops, which results in the blocking of translation and protein production (Chopra *et al.*, 1992). There are several theories as to how tetracycline specifically blocks translation. One

hypothesis is the interaction of tetracycline with the ribosomes, may alter the three dimensional structure of the ribosome making the bases within the 30S rRNA inaccessible to the aminoacyl-tRNA (Chopra *et al.*, 1992). A second hypothesis is the tetracycline molecule simply covers the binding site of the aminoacyl-tRNA and becomes a physical barrier between the aminoacyl-tRNA and the ribosome (Taylor & Chau, 1996). It has been shown that one molecule of tetracycline per ribosome is enough to inhibit translation and that the 7S ribosomal protein appears to form part of the binding site for tetracycline (Speer *et al.*, 1992). This interaction between the ribosome and the tetracycline molecule is reversible and does not involve any permanent changes to the ribosome (Chopra *et al.*, 1992). This type of antimicrobial mechanism is classified as bacteriostatic because of its reversibility and inhibits cell growth, but does not kill the cell (Chopra *et al.*, 1992).

The atypical tetracyclines include; chelocardin, anhydrotetracycline, 6-thiatetracycline, 4-epi-anhydrochlortetracycline and anhydrochlortetracycline none of which are currently being used (Chopra, 1994; Oliva *et al.*, 1992). These antibiotics interfere with bacterial cytoplasmic membrane permeability and are poor inhibitors of protein synthesis (Chopra, 1994; Oliva *et al.*, 1992; Speer *et al.*, 1992). Their site of action is at the cell membrane which results in cell damage which causes cell lysis and death, thereby making this subgroup of the tetracycline antibiotics bactericidal rather than bacteriostatic (Chopra, 1994; Oliva *et al.*, 1992). Atypical tetracycline antibiotics have been used in human studies and were found to have adverse side effects and; therefore, are not deemed appropriate for therapeutic use in humans (Chopra, 1994).

Glycylclines are a new group of semisynthetic tetracyclines which includes DMG-MINO and DMG-DMDOT which are derivatives of minocycline and 6-demethyl-6-deoxytetracycline respectively (Testa *et al.*, 1993). These derivatives contain N,N-dimethylglycylamido substituted at the 9 position of minocycline and 6-demethyl-6-deoxytetracycline (Testa *et al.*, 1993). The glycylclines were designed to have antimicrobial activity on tetracycline resistant bacterial isolates (Testa *et al.*, 1993). The mechanism of action of these derivatives is to affect the bacterial membrane permeability (Oliva, *et al.*, 1992). These compounds are less toxic than the atypical tetracyclines and hold promise for future therapeutic uses (Goldstein *et al.*, 1994).

Another pair of antimicrobial agents, Dactylocyclines A and B have been recently isolated from *Dactylosporangium* spp. (Tymiak *et al.*, 1992; Wells *et al.*, 1992). These are the first naturally occurring tetracycline derivatives that lack cross-resistance to previously described tetracycline resistance mechanisms but have retained a C-2 amide (Tymiak *et al.*, 1992). However, the decomposition product of dactylocyclines, dactylocyclinone, is effected by previously described tetracycline resistance mechanisms (Tymiak *et al.*, 1992).

Tetracyclines also have activities that are not antimicrobial that complicate their effects on disease process. Tetracycline can inhibit gingival collagenolytic enzyme activity and bone resorption in organ culture (Gomes *et al.*, 1984). This inhibition may reduce the gingival crevicular fluid collagenolytic activity by 70% during the first few weeks of therapy (Golub *et al.*, 1984; Golub *et al.*, 1985). It has also been speculated that tetracycline is able to suppress the functions of leukocytes (Martin *et al.*, 1974;

Yanagimura *et al.*, 1989). A reduction in leukocyte function would cause a reduction in the gingival crevicular fluid collagenase activity, lipase activity and antibody production (Martin *et al.*, 1974; Yanagimura *et al.*, 1989).

Another affect tetracycline has on the human body is the suppression of neutrophil chemotaxis (Humbert *et al.*, 1991). It is believed the migration of polymorphonuclear leukocytes to a site is part of the inflammatory response. It has been postulated that reducing the migration of neutrophils by tetracycline results in a reduction of inflammation (Humbert *et al.*, 1991). Plewig and Schopf (1975) demonstrated that both topical and systemic administration of several derivatives of tetracycline were able to reduce 80% of inflammation in all of the human patients tested.

Research done by Lantz *et al.* (1987) showed that *P. intermedia* grown at sub-MIC levels of tetracycline binds less fibrinogen than *P. intermedia* grown in the absence of tetracycline. It has been suggested that sub-MIC level of tetracycline result in a decreased expression of fibrinogen-binding sites present on the *P. intermedia*. The level of reduced expression is dependent on the concentration of tetracycline present during the cell growth (Lantz *et al.*, 1987). Tetracycline induced decreased fibrinogen-binding could also be caused by a decreased affinity to fibrinogen by the *P. intermedia* (Lantz *et al.*, 1987). This modification is suspected to affect the ability of *P. intermedia* to adhere and colonize the gingival sulcus, consequently affecting the concentration of *P. intermedia* remaining in the sulcus (Lantz *et al.*, 1987). An additional affect of tetracycline is seen on gene expression in *Actinomyces viscosus* and *P. gingivalis*. In the presence of tetracycline, the number of fimbriae dramatically decreases on the bacterial

cell outer membrane of both *A. viscosus* and *P. gingivalis* reducing the bacterium's ability to attach to the tooth surface (Peros *et al.*, 1985). This in turn could affect the ability of the bacteria to colonize the surface of the tooth. The inhibition of the expression of adherence factors, fibrinogen-binding and fimbriae, may also inhibit plaque formation (Baker *et al.*, 1983). This phenomenon has been demonstrated *in vitro*, but requires the antibiotic to have antimicrobial activity and be able to be adsorbed on to the tooth surface (Baker *et al.*, 1983).

Tetracycline and minocycline can also increase the rate of connective tissue attachment (Somerman *et al.*, 1988). These antibiotics increase the attachment of human gingival fibroblasts to polystyrene (Somerman *et al.*, 1988). For periodontal regeneration to occur fibroblasts must attach and spread at the healing sites on the root surface, which in turn leads to an increase in the regeneration of connective tissue attachment. However, the *in vitro* studies, only see the increase in attachment with antibiotic concentrations lower than 100 µg/ml per dose (Somerman *et al.*, 1988). At a dose higher than 200 µg/ml per dose, the *in vitro* studies observed a decline in activity as compared to the low dosage (Somerman *et al.*, 1988). However, all of this data is based on *in vitro* studies and more research is needed to determine if these observations also occur *in vivo*.

Tetracycline can bind to the dentin surfaces on teeth (Wikesjo *et al.*, 1986). When the environmental concentration of tetracycline drops, bound tetracycline is released from the dentin surface (Wikesjo *et al.*, 1986). The levels of biologically active tetracycline, due to release from the dentin, are above the MIC for most pathogenic bacteria and are maintained at this level for a minimum of 48 hours after the completion

of oral therapy (Baker *et al.*, 1983; Wikesjo *et al.*, 1986). This phenomenon is referred to as root conditioning, because it changes the root surface in such a manner as to favor the binding of fibronectin (Terranova *et al.*, 1986; Wikesjo *et al.*, 1986). The addition of fibronectin to the dentin stimulates the attachment and growth of fibroblasts, while suppressing the attachment and growth of epithelial cells on the dentin (Terranova *et al.*, 1986). This results in the regeneration of connective tissue attachment, which aids in the overall support of the tooth. These activities may be of therapeutic value in treating periodontal disease.

Macrolides, Lincosamides and Streptogramin B

Erythromycin, a member of the macrolide group of antibiotics, was discovered by McGuire *et al.* in 1952 as a substance produced by *Streptomyces erythreus* (McGuire *et al.*, 1952). Other naturally produced compounds have been discovered and added to the macrolide class of antibiotics and include; oleandomycin, spiramycin, joseycin, and medecamycin. Little chemical modification has been done to the original chemical structures of these macrolides. Erythromycin is the only macrolide that is approved for humans use. Spiramycin has been used in animals. The common chemical structure of the macrolide group is a large lactone ring with one or more sugars attached via a glycosidic bond (Mazzei *et al.*, 1993). Recently, new synthetic macrolides have been developed through the modification of the erythromycin lactone ring system, these derivatives have a different pattern of substitutions on the lactone ring (Mazzei *et al.*, 1993). They have improved chemical, microbiological and pharmacokinetic properties

and they include; roxithromycin, clarithromycin, flurithromycin, azithromycin, dirithromycin, miocamycin, and rokitamycin (Mazzei *et al.*, 1993). Out of all of the synthetic derivatives, clarithromycin and azithromycin are currently being used.

The lincosamides and streptogramin B antibiotics are chemically distinct, but have a mode of action and spectrum of activity similar to the macrolides (Arthur *et al.*, 1987; Leclercq & Courvalin, 1991). The macrolides, lincosamides and streptogramin B inhibit protein synthesis by binding to the 50S ribosome, which results in the inhibition of bacterial cell growth (Leclercq & Courvalin, 1991). Data suggests the binding site for these three groups of antibiotics overlap on the ribosome or functionally interact (Vazquez, 1966; Vazquez & Monro, 1967; Weisblum, 1995a; Weisblum, 1995b). These antibiotics interfere with the transpeptidation which stimulates the dissociation of peptidyl-tRNA from ribosomes during translocation (Mazzei *et al.*, 1993). However, the exact site of action for these antibiotic is not known (Eady *et al.*, 1990). Like tetracycline, the macrolides, lincosamides and streptogramin B are classified as bacteriostatic agents (Lorian, 1991).

Erythromycin has a wide spectrum of activity against both Gram-positive aerobic and anaerobic bacteria (Lorian, 1991). Erythromycin is widely used as an alternative antibiotic for penicillin allergic patients that have a Gram-positive bacterial infection (Slots & Taubman, 1992). Erythromycin is also used for the treatment of dental abscesses and oral infections, but rarely for periodontitis. This is due to the low concentration achievable $\leq 1 \mu\text{g/ml}$ in the gingival crevicular fluid following multiple doses (Table 2)(Genco, 1981; Papas & Walker, 1987; Slots & Ram, 1990; Walker *et al.*,

1993). At this level, most periodontal pathogens are not susceptible, especially *A. actinomycetemcomitans*, *F. nucleatum*, *Eikenella corrodens* and black-pigmented *Bacteroides* (*Prevotella* or *Porphyromonas* spp.) (Gordon & Walker, 1993; Papas & Walker, 1987). In contrast, clindamycin, a member of the lincosamide group, has been shown to be effective for the treatment of periodontal disease, especially with patients who are thought to have tetracycline resistant periodontal pathogens (Genco, 1981; Walker & Godson, 1990). Clindamycin penetrates into the gingival crevicular fluid at high enough concentrations to be effective against most periodontal pathogens (Table 2) (Walker *et al.*, 1981). However, with clindamycin therapy 1% to 10% of the patient develop antibiotic-associated pseudomembranous colitis (Bartlett, 1981; Bartlett *et al.*, 1978; Slots and Taubman, 1992). Streptogramin B is not currently available for use in the United States but is available in Europe (Weisblum, 1995b).

Like the tetracycline group, derivatives of the macrolide group have also been developed which can have activity on erythromycin resistant strains. The 6-O-methyl-11, 12-cyclic carbamate derivatives of erythromycin are active and can inhibit protein synthesis of ribosomes that have been dimethylated by rRNA methylase genes in MLS resistant bacteria (Goldman & Kadam, 1989). It has been shown that the 6-O-methyl-11, 12-cyclic carbamate derivative 1a binds sensitive and dimethylated ribosomes with the specificity for a single site, in a one-to-one stoichiometry (Goldman & Kadam, 1989). This is the same as is seen with erythromycin and sensitive ribosomes (Goldman & Kadam, 1989). The derivative has been shown to induce MLS resistance when bound to a sensitive ribosome, but does not induce the expression of the MLS resistance mRNA

when on a dimethylated ribosome (Goldman & Kadam, 1989). Based on competition binding assays it is evident that the 1a derivative does bind to the macrolide binding site on the ribosome (Goldman & Kadam, 1989). This derivative holds promise for future therapeutic use.

ANTIBIOTIC RESISTANCE

The discovery of antibiotics has had a dramatic effect on modern medicine (Levy, 1992a). Today, antibiotics are used to treat most bacterial infections, and have made many once life threatening diseases treatable. This rapid cure of “deadly diseases” lead the general public to think of antibiotics as the ‘miracle cure’ (Levy, 1992a). The general public has come to demand antibiotics from physicians when they are ill, even if antibiotic therapy is not appropriate for the illness. In the last 20 years, there has been a dramatic increase in the amount of antibiotics being used in medicine and in agriculture (Col & O’Conner, 1987). The bacteria have acquired resistance mechanisms to be able to survive in an environment containing antibiotics (Amabile-Cuevas *et al.*, 1995).

Bacteria have five main mechanisms of antibiotics resistance: 1) change the bacterial target of the antibiotic so the target is no longer susceptible to the antibiotic (Weisblum, 1995a), 2) change the outer membrane so it is less permeable to the antibiotic (Levy, 1992a), 3) acquire or increase productivity of an efflux pump which removes the antibiotic from within the cell keeping the antibiotic away from the target (Chopra *et al.*, 1992), 4) acquire an enzyme that can modify the antibiotic making it inactive (Speer, 1991) or 5) bypass the pathway being affected by the antibiotic (Lorian, 1991).

Tetracycline Resistance

The first tetracycline resistant strain was a *Shigella* spp. isolate from Japan, found in 1953 (Tateno, 1955). By the 1960s, 10% of the Japanese *Shigella* spp. isolates were multi-drug resistant (Akiba *et al.*, 1960). The problem of tetracycline resistance has continued to spread and increase (Levy, 1992a). After evaluating the presence of tetracycline resistance, it was later (late 1970's-80's) discovered the most common mechanism of resistance was due to acquisition of tetracycline resistance genes rather than mutations in existing chromosomally located bacterial genes (Chopra *et al.*, 1992; Levy, 1989; Roberts, 1989). There are currently eighteen different determinants which have been described, many of which are located on mobile elements and can be easily transferred between bacterial species (Table 3)(Chopra *et al.*, 1992; Roberts, 1996; Taylor & Chau, 1996). Among these determinants, there are three different mechanisms of resistance (Table 3)(Levy, 1989; Roberts, 1989; Chopra *et al.*, 1992).

The efflux mechanism accounts for eleven of the eighteen characterized tetracycline resistance determinants(Chopra *et al.*, 1992; Jones, 1992; Roberts, 1994; Roberts, 1996; Speer *et al.*, 1992). An efflux gene codes for a protein located in the bacterial cell membrane with 12 (Gram-negative) or 14 (Gram-positive) hydrophobic regions which cross the bacterial cell membrane (Chopra *et al.*, 1992; Levy, 1992b). A multimer of the efflux protein functions as a pump and exports the tetracycline out of the cell. This movement of tetracycline occurs against a concentration gradient and involves the exchange of a proton for a tetracycline-cation complex in an energy dependent

fashion (Levy, 1992b; Yamaguchi, 1990). This process reduces the intracellular concentration of tetracycline as compared to extracellular concentration of tetracycline (Jones, 1992; Roberts, 1994; Speer *et al.*, 1992). The lowering of the tetracycline concentration protects the ribosome from being exposed to tetracycline and allows protein synthesis to continue.

The efflux genes are commonly found on a variety of mobile elements. The efflux genes of Gram-negative origin (Table 3) are often found on transposons. The genes are commonly inserted into large plasmids which can come from a variety of different plasmid incompatibility groups (Jones, 1992). Resistance determinants for other antibiotics are often found on these same plasmid (Gillespie, 1986; Jones, 1992; Mendez *et al.*, 1980). The efflux genes of Gram-positive origin (Table 3) are often found on small mobilizable plasmids which have been found integrated in the chromosomes of some bacterial species and are only rarely seen in large conjugative plasmids except for *tetA(P)* (Gillespie, 1986; Projan & Novick, 1988; Schwarz *et al.*, 1992). In these small plasmids, the efflux gene may be located in a cassette, with other antibiotic resistance determinants (Projan & Novick, 1988; Schwarz *et al.*, 1992). The *tetB* gene is the most widely distributed efflux determinant and has been found in Gram-negative bacterial species (Jones, 1992; Roberts, 1994). The *tetB* gene is the only efflux mechanism that confers resistance to both tetracycline and minocycline, the rest of the efflux determinants are effective only on tetracycline and doxycycline (Chopra *et al.*, 1992).

The mechanism of ribosomal protection accounts for five of the eighteen characterized tetracycline resistance genes (Table 3). These genes code for

cytoplasmic proteins which prevent tetracycline from binding to the ribosomes (Burdett, 1991; Sanchez-Pescador, 1988). This group of protein are similar to the elongation factors Tu and G suggesting they might function as tetracycline resistant elongation factors or prevent binding of the tetracycline to the ribosome (Burdett, 1991; Sanchez-Pescador, 1988). However, the exact steps behind this protective mechanism are still not clear.

The ribosomal protection genes are commonly found on a variety of mobile elements. The *tetO* genes are usually found on conjugative plasmids (Charpentier *et al.*, 1993; Taylor & Courvalin, 1988). The *tetM*, *tetS* and *tetQ* genes are commonly associated with conjugative transposons, and *tetM* and *tetS* are also found on conjugative plasmids (Flannagan *et al.*, 1994; Li *et al.*, 1995; Salyers *et al.*, 1995). The *Bacteroides* conjugative transposon, which often carries the *tetQ* gene, is able to mediate the movement of itself, coresident plasmids and unlinked integrated elements into a recipient isolate (Li *et al.*, 1995; Salyers *et al.*, 1995). This process of mobilization is stimulated 100 to 1000 fold by the presence of tetracycline (Li *et al.*, 1995; Salyers *et al.*, 1995).

Enzyme modification accounts for only one of the eighteen characterized tetracycline resistance genes (Table 3). The Tet X determinant, in association with the erythromycin resistance Erm F determinant has only been found in *Bacteroides* spp. to date (Speer, 1991; Speer, 1992). This protein has amino acid similarities to NADPH-requiring oxidoreductase and has been shown to require both oxygen and NADPH to be functional(Speer, 1991). Therefore, the *tetX* gene should not be able to function and

cause tetracycline resistance when it is carried by anaerobic species, such as *Bacteroides* spp. (Speer, 1991).

The tetracycline resistance determinants listed above have very little effect on the atypical tetracyclines (Chopra, 1994). This is not surprising since the mechanism of action for this group of antibiotics is the bacterial membrane, not in the cytoplasm with the ribosome where these resistance mechanisms are functioning (Chopra, 1994). The new glycyliclins are classified as atypical tetracyclines and have activity in the presence of the ribosomal and efflux tetracycline resistance mechanisms (Oliva *et al.*, 1992; Testa *et al.*, 1993). These agents have good activity against *Neisseria*, *Moraxella*, *Haemophilus* and *Bacteroides* spp. and many anaerobic species (Nord *et al.*, 1993; Whittington *et al.*, 1995; Wise & Andrews, 1994). The glycyliclins have antibacterial activity against *Escherichia coli* and *S. aureus* carrying efflux mechanism or ribosomal protection (*tetM*) (Rasnussen *et al.*, 1994; Testa *et al.*, 1993). In addition, these derivative are very effective on methicillin resistant *S. aureus* and methicillin sensitive *S. aureus* and could prove to be a breakthrough for the treatment of tetracycline resistant organisms (Eliopoulos *et al.*, 1994; Goldstein *et al.*, 1994; Testa *et al.*, 1993; Weiss *et al.*, 1995; Wise & Andrews, 1994).

Macrolide resistance

Erythromycin resistant *S. aureus* was first detected in France, England, Japan and the USA shortly after its introduction in 1953 (Garrod, 1957, Haight & Finland, 1952; Jones *et al.* 1956; Weisblum, 1995a). Resistance to the macrolides occurs by the

following mechanisms: alterations in the target site, modification of the antibiotic, or alteration of the transport of the antibiotic (Arthur *et al.*, 1987; Weisblum, 1995a).

Target modification due to the methylation of the rRNA by an acquired rRNA methylase is the most common mechanism of macrolide resistance (Weisblum, 1995a). Over 30 structurally homologous rRNA methylase genes have been described which code for an adenine-specific N-methyltransferase which methylates a single adenine nucleotide (A2058) on the 23S component of the 50S ribosomal subunit (Weisblum, 1995a). These rRNA methylase genes bear the name *erm* (erythromycin ribosomal methylation) followed by a letter to distinguish them from each other (Weisblum, 1995a). The high level of amino acid homology in the protein product among these rRNA methylase genes and identical enzymatic activity is suggestive of a common ancestor for the *erm* genes. Similar genes are found in the erythromycin producing species (Arthur *et al.*, 1987). Due to the substantial DNA sequence diversity among the *erm* genes, it is thought the relatedness among these genes must be due to divergence of a common ancestor and not due to independent evolution (Arthur *et al.*, 1987). RNA protection studies and point mutations suggest the peptidyltransferase loop in domain V of the 23S rRNA is the binding site for the MLS antibiotics to the ribosome (Weisblum, 1995a). The methylation of the ribosome prevents binding of the macrolides and also includes activity against the lincosamides, and streptogramin B due to the overlapping binding sites of all three antibiotics on the ribosome (Leclercq & Courvalin, 1991). The rRNA methylase resistance determinants have no effect on the streptogramin A class of antibiotics (Leclercq & Courvalin, 1991). All of the rRNA methylase determinants can be divided

into a minimum of 8 classes of resistance determinants based on sequence homology and stringent hybridization experiments (Leclercq & Courvalin, 1991; Weisblum, 1995a). Of these determinant groups, five groups appear to be the most common, represented by the *ermA*, *ermB*, *ermC*, *ermF* and *ermQ* genes (Weisblum, 1995a).

The rRNA methylase determinants have been shown to be inducible in some organisms and expressed constitutively in others (Leclercq & Courvalin, 1991). When the rRNA methylase is expressed constitutively, the isolate is resistant to macrolides, lincosamide, and streptogramin B antibiotics all of the time (Leclercq & Courvalin, 1991). When the rRNA methylase gene is inducible, the isolate does not express the rRNA methylase and appears to be susceptible to the MLS antibiotics until it is exposed to a 14-membered (erythromycin, roxithromycin) or 15-membered (azithromycin) macrolides. This exposure trigger the translation of rRNA methylase gene from the mRNA (Leclercq & Courvalin, 1991). If an inducible isolate is exposed to a 16-membered macrolide (spiramycin, josamucin, micamycin and midecamycin), lincosamide, or streptogramin B antibiotic, the isolate will usually appear susceptible to these antibiotics (Leclercq & Courvalin, 1991). This is due to the fact that the 14 and 15-membered macrolides are usually but not exclusively the only effective inducers of rRNA methylase synthesis of the MLS antibiotics (Leclercq & Courvalin, 1991). The sequence upstream of the structural gene determines if the *erm* gene will be expressed constitutively or in an inducibly and is not affected by the class of rRNA methylase present (Leclercq & Courvalin, 1991). The inducibility of a rRNA methylase gene is due to a translational attenuation mechanism (Leclercq & Courvalin, 1991). Upstream of the

structural gene for the rRNA methylase is an open reading frame which encodes a fourteen amino acid control peptide (Leclercq & Courvalin, 1991). Both this peptide and the structural gene are transcribed as a single unit (Leclercq & Courvalin, 1991). This single unit of the mRNA has three different conformations (Figure 2). It has two ribosomal binding sites (Figure 2, SD-1 & SD-2) that can be recognized by the ribosome; however, a few base pairs separates them from the initiation codon (Leclercq & Courvalin, 1991; Weisblum, 1995b). Inverted repeating sequences form a stem-loop structure which sequesters the second open reading frame and the initiation codon for the rRNA structure gene making it inaccessible to the ribosomes (Figure 2A)(Leclercq & Courvalin, 1991). This process of sequestering occurs when erythromycin is not present and the control peptide is the only protein that is transcribed from the mRNA (Leclercq & Courvalin, 1991). When an inducing antibiotic is present, it binds to the ribosome and interferes with translation. This interference stalls the ribosome and allows a conformational rearrangement in the mRNA to occur causing the stem-loop structure to unfold. This rearrangement allows the second open reading frame and initiation sequence to be recognized by the ribosome and the structural rRNA methylase can then be transcribed (Figure 2B)(Leclercq & Courvalin, 1991; Weisblum, 1995b). The enzyme then begins to methylate the bacterial ribosomes. When a ribosome has been methylated, it no longer stalls in the presence of the MLS antibiotic and the original mRNA returns to the inactive conformation when being transcribed (Figure 2C). When the antibiotic is removed, the mRNA can refold and change from conformation 2B with one stem-loop structure to conformation 2C with two stem-loop structures, which are depicted in

Figure 2. This conformational change is the energy favored refolding, where as the change from 2B to 2A requires the addition of energy (Weisblum, 1995b). This occurs even when erythromycin is still present in the cell. This process is the negative feedback feature that allows the down regulation of expression when the methylase is no longer needed (Leclercq & Courvalin, 1991; Weisblum, 1995b). This feed back mechanism will respond to removal of the enzyme and saturation of methylated ribosomes (Weisblum, 1995b).

By plating inducible strains on noninducing macrolides, lincosamides or streptogramin B antibiotic plates, mutations can be selected which changes in the regulation of the rRNA methylase gene from inducible to constitutive. This occurs at a frequency of 10^{-7} to 10^{-8} (Leclercq & Courvalin, 1991; Weisblum, 1995b). The mutant may exhibit point mutations that decrease the stability of the stem-loop structure, but this is not commonly seen in nature. The mutant may have a deletion of a few amino acids of leader peptide or the isolate may have a duplication of repeated sequences (Hahn *et al.*, 1982). The last two changes in the rRNA are the ones that are commonly seen in clinical isolates (Weisblum, 1995b). Any mutation in region 1, 3, or 4 (Figure 2) would result in a constitutive expression of the *erm* gene. Mutations in region 2 would leave regions 3 and 4 still in a stem-loop conformation and the expression would remain inducible (Weisblum, 1995b). A deletion of the leader peptide (59 nucleotides), which would include a deletion in region 1, would be an alternative mechanism to the point mutation which would also to allow constitutive expression of the *erm* gene (Weisblum,

1995b). When *erm* genes are found in clinical isolates, whether inducible or constitutive, none of the MLS antibiotics should be used for therapy (Weisblum, 1995b).

EFFECTS OF ANTIMICROBIAL THERAPY ON THE ORAL FLORA

In the last 20 years, many studies have shown the clinical outcome of antibiotic therapy for the treatment of periodontal disease. Several reviews have summarized these studies (Genco, 1981; Gordon & Walker, 1993; Slots & Rams, 1990; Walker *et al.*, 1993). The use of antibiotics as an adjunct to mechanical therapy is only appropriate with cases such as refractory periodontitis, where the patient has a history of not responding to mechanical therapy over a long period of time (Genco, 1981; Gordon & Walker, 1993, Slots *et al.*, 1979; Slots & Rams, 1990; Walker *et al.*, 1993). It is generally accepted that antibiotic therapy changes the bacterial species composition in the oral flora and the percentage of the total flora that a particular species represents. However, these changes are dependent on the antibiotics being used, the dosage used and the duration of used. The general trend that is observed after the completion of tetracycline therapy is an overall reduction by 10 to 100-fold in the total number of bacteria present and specifically a 3 to 4 fold reductions in the numbers of anaerobes, Gram-negative, motile rods and spirochetes present (Slots *et al.*, 1979). Repopulation of these organisms to the level of baseline is slow, taking 3 to 6 months depending on the patient (Slots *et al.*, 1979). There is also an increase in the proportions of *Actinomyces* spp. and *Streptococcus* spp. in the subgingival plaque after antibiotic therapy (Slots *et al.*, 1979).

Walker and Gordon (1990) found before treatment with clindamycin the oral flora was made up of 50% spirochetes and motile rods, 40% gram-negative anaerobic rods, and 20% *P. intermedius* and *P. gingivalis*. One year post-therapy, the oral flora was 15% spirochetes and motile rods, 20% gram-negative anaerobic rods, and less than 2% *P. intermedius* and *P. gingivalis*. However, most of the antibiotic therapy studies do not evaluate the susceptibility of the pathogens to the antimicrobial agent being administered. There is even less consideration given to the changes that occur due to antibiotic therapy with regards to changes in the susceptibility of the oral bacteria.

ANTIBIOTIC SUSCEPTIBILITY OF ORAL BACTERIA

The National Committee for Clinical Laboratory Standards (NCCLS) and the Centers for Disease Control and Prevention have established guidelines for minimum inhibitory concentration (MIC) testing of many clinical isolates seen in general medicine. However, these guidelines have not been determined for most oral bacterial species, especially periodontal pathogens (Baker *et al.*, 1985; NCCLS, 1993). As a consequence there are no well established guidelines as to what the appropriate MIC values are that represent the breakpoints for antibiotic resistance in periodontally associated bacteria. The concentration of antibiotics that are achievable in the gingival crevicular fluid should have an effect on what level of antibiotic is regarded as resistant when working with oral bacteria (Table 2). If an organism is susceptible to higher levels of an antibiotic than is achievable in the periodontal pocket, the organism should be regarded as resistant. Table 2 lists the antibiotic dosage used for treatment of periodontal disease and the

concentration of antibiotic that is obtained from this therapy in the blood and the gingival crevicular fluid. A few papers have tried to evaluate the susceptibility of the oral flora after antibiotic therapy, some of which do take gingival crevicular fluid concentrations into account (Baker *et al.*, 1985; Fiehn & Westergaard, 1990; Slots & Rams, 1990).

Fiehn and Westergaard (1990) evaluated the susceptibility of oral bacteria to doxycycline. Two groups of patients were examined, healthy patients and patients with periodontal disease which were treated with doxycycline. The healthy group was followed for six months and 2-6.6% of the subgingival plaque bacteria and 3-12.4% of the tonsil bacteria were found to be doxycycline resistant. The authors used the ability to grow in the presence of 10 µg/ml doxycycline as resistant. The patients with periodontal disease were placed on 1000 mg per day of doxycycline for three weeks. This level of systemic therapy corresponds to 6-10 µg/ml of doxycycline in the gingival crevicular fluid which makes the level of 10 µg/ml as a reasonable breakpoint for resistance. The baseline level of doxycycline resistance in the therapeutic group was 3.6% for the tonsils and 0.9% for the subgingival plaque bacteria. One week post-therapy, a significant increase in the percentage of Gram-positive cocci making up the total cultivable flora was noted. At 52 weeks post-therapy, the percentage of doxycycline resistant bacteria had increased 10 times in the tonsil bacteria and 20 times in the gingival sulcus bacteria as compared to the baseline. These data agree with other previously published studies (Kornman & Karl, 1982; Williams *et al.*, 1979).

Another study evaluated the long term effects of low dose tetracycline therapy, using 250 mg/ day, over a 2 to 7 years (Kornman & Karl, 1982). The authors found that

at the end of two years of therapy, on average 77% of the cultivable flora from subgingival plaque samples was resistant to 1 µg/ml of tetracycline (Kornman & Karl, 1982). However, it has yet to be determined if 1 µg/ml of tetracycline is an appropriate concentration to indicate tetracycline resistance in these bacterial species. Once therapy was discontinued, the tetracycline resistance persisted in these patients. The percentage of cultivable organisms that were resistant to tetracycline varied between 20% to 50% depending on the patient, with the average being 26% at 6 month post-therapy. These percentages are dramatically different as compared to the 7.1% resistant subgingival flora detected in the control group that received no antibiotic therapy. It was also reported that half of the patients treated with the low dose tetracycline therapy still had periodontal pathogens present in their subgingival flora. This finding represents an important side effect of long term therapy.

Listgarten *et al.* (1993) evaluated the subgingival plaque sampled from refractory periodontitis patients for resistance to penicillin, tetracycline, or metronidazole. The authors used the ability to grow in the presence of 1 µg/ml of the appropriate antibiotic as indication of resistance. The authors found variable resistance in the periodontal pathogens they isolated. They also found many of the resistant pathogens were resistant to all three antibiotics (Listgarden *et al.*, 1993). Based on this finding, the authors recommend microbial testing before using antibiotic therapy. However, it needs to be determined if 1 µg/ml is the appropriate level for determination of antibiotic resistance with these three antibiotics. Considering the fact that many of these antibiotics are able

to reach levels in the gingival crevicular fluid above 1 µg/ml as shown in Table 2, this level may not be appropriate for indicating antibiotic resistance.

Baker *et al.* (1985) evaluated the susceptibility of clinical isolates from the oral cavity to 18 different antibiotics. The species that were included in this study were *A. actinomycetemcomitans*, *P. intermedius*, *Bacteroides melaninogenicus*, *Porphyromonas gingivalis*, *Bacteroides oralis*, *Campylobacter* spp., *Capnocytophaga* spp., *Eikenella corrodens*, *F. nucleatum*, *Haemophilus aphrophilus*, *Veillonella parvula*, Anaerobic vibrios, *Actinomyces israelii*, *A. naeslundii*, *A. viscosus*, *Lactobacillus casei*, *Streptococcus mitor*, *Streptococcus mutans*, and *Streptococcus salivarius*. The authors used the NCCLS established breakpoints for aerobic and anaerobic bacteria to attempt to determine sensitivity the oral bacterial species to each antibiotic. For the antibiotics that were not covered by NCCLS, breakpoints were chosen that were 2 to 4 fold less than the highest attainable antibiotic level. All of these values were also compared to the level of antibiotic attainable in the gingival crevicular fluid (Table 2). The assays were done by the agar dilution method according to NCCLS recommendations (NCCLS. 1993). The process by which susceptibility was determined in this study most closely follows the procedures done for MIC testing done in the medical community (NCCLS, 1993).

The authors found the highest percentages of susceptible strains to the following antibiotics: tetracycline (89%), minocycline (82%), and carbenicillin (92%) (Baker *et al.*, 1985). The lowest percentage of susceptible strains was seen with penicillin (65%) and chloramphenicol (65%)(Baker *et al.*, 1985). None of the Gram-negative species were classified as susceptible to vancomycin (MIC value of ≤ 4 µg/ml indicating susceptible).

Most of the strains evaluated were classified as resistant to spiramycin (MIC values of > 6.7 µg/ml indicating resistance). The anaerobic bacteria evaluated were resistant to the aminoglycoside group of antibiotics (resistance defined as MIC of ≥ 32 µg/ml for kanamycin, ≥ 10 µg/ml for neomycin and ≥ 15 µg/ml for streptomycin)(Baker *et al.*, 1985). Therefore, the authors conclude the aminoglycoside would not be appropriate for used with periodontal disease (Baker *et al.*, 1985). However, this study did not look for the presence of antibiotic resistance determinants in the resistant isolates, so it is not known what any of the mechanism of resistance were in any of these species examined.

Another study that also used MIC assays as recommended by NCCLS was done by Williams *et al.* (1979). This study compared the effects of two different therapy regiments; one was two weeks of high dose (1000 mg/day) tetracycline therapy and the other was one week high dose (1000 ml/day) tetracycline therapy followed by low dose (250 mg/day) for an extended period of time. After two weeks of tetracycline therapy the predominant flora was *Streptococcus* and *Actinomyces* spp. From three of these patients, the *Streptococcus* spp. tested had MIC of ≥ 16 µg/ml to tetracycline and the fourth patient had *Streptococcus intermedius* with an MIC of 8 µg/ml to tetracycline (Williams *et al.*, 1979). None of the *Actinomyces* spp. or *Rothia* spp. that were evaluated were classified as resistant, having MIC values between 1-2 µg/ml to tetracycline. A *Veillonella parvula*, a Gram-negative species, from one of the patients had an MIC of 32 µg/ml to tetracycline. All of the other Gram-negative species evaluated were susceptible to tetracycline concentrations at ≤ 1 µg/ml.

With the patients that were on the long term tetracycline therapy, it was observed that the oral flora became more variable after the therapy had been completed. *Rothia dentocariosa* and *Neisseria sicca* were isolated in high percentages of the total flora from two of the long term therapy patients (Williams *et al.*, 1979). The *N. sicca* isolates were shown to have MIC values of 32 µg/ml to tetracycline. Two of the other patients had *F. nucleatum* comprising a high percentage of the total flora. In contrast to the high dosage patients, these patients continued to have periodontal pathogens present in their oral flora at the end of therapy. This findings agree with the results of the Kornman & Karl (1982) study. Most of the *Streptococcus* and *Actinomyces* spp. evaluated from the long term therapy patients had MIC values of ≥ 16 µg/ml for tetracycline. The authors concluded patients prescribed low dosage therapy for the longest time period (121 days) had the highest number of Gram-negative species present (Williams *et al.*, 1979). The authors also noted that tetracycline resistance was observed in 5 out of 6 of the Gram-negative bacillus species studied (Williams *et al.*, 1979).

A study done by Walker *et al.* (1983) approached the idea of bacterial susceptibility in a unique way using a new technique. The authors believed their technique was simple, easily interpreted and could be done in any laboratory with basic anaerobic microbiological equipment. Rather than evaluating the susceptibility of certain bacteria species isolated from the periodontal pocket, they evaluated the susceptibility of the entire subgingival plaque sample. The authors wanted to determine which antimicrobial agents would inhibit the largest percentage to the total bacteria in a diseased periodontal site. The logic behind this type of testing comes from the fact that

no bacterial species or group of bacterial species have not been clearly established as the etiologic agents of any of the specific forms of periodontal disease. The authors believed suppression of the majority of the bacteria with an antibiotic will also have the highest probability of also inhibiting the etiologic agent that is causing the disease. The authors stated this type of testing would not be appropriate once the etiological agent is known. The following antibiotics plus some of their derivatives were tested; penicillin, tetracycline, erythromycin, clindamycin, and metronidazole. The concentration of antibiotic used was based on the concentrations achievable in the gingival crevicular fluid, if known, or the blood concentrations which are achieved following the normal recommended oral dosage. This comparison was determined by plating the subgingival plaque sample on media containing no antibiotic and media containing one antibiotic. The results indicated that penicillin was the only antibiotic that reproducibly inhibited 90% of the plaque sample in all the patients evaluated. However, there are many patients who are penicillin allergic and β -lactamase production, which causes resistance to penicillin, has been reported in many oral bacteria (Walker *et al.*, 1983). Tetracycline was also shown to be very effective at inhibiting most plaque samples (>90%). However, this level of inhibition was not seen in patients that had been previously treated with tetracycline, many of which had received prolonged tetracycline therapy. Erythromycin was shown to be ineffective at inhibiting the plaque bacteria at the levels achievable in the gingival crevicular fluid. Clindamycin was effective in inhibiting 90% of the bacteria from most of the patients evaluated; however, caution should be used with this antibiotic since it is associated with antibiotic induced pseudomembranous colitis. The authors

concluded that even though antibiotics are currently being used to treat some forms of periodontal disease, one antibiotic will not be effective on all patients. Therefore, they proposed antibiotic susceptibility testing using the above method to evaluate the sensitivity of the subgingival plaque bacteria before any antibiotic is prescribed for the patient. However, this method has not been accepted and the use of whole species MIC are still not an approved method in the 1990's.

MECHANISMS THAT CAUSE RESISTANCE IN ORAL BACTERIA

The previous sections describe some of studies that have evaluated the effect of antibiotics on the oral flora. Some of these papers have made attempts to evaluate the presence of resistance in the oral bacteria. However, there are only a few papers which have characterized the mechanisms that are responsible for the resistance being seen in oral species of bacteria and/or periodontal pathogens.

Work done by Hawley *et al.* (1980) described the effects of tetracycline on the streptococcal flora of patients with periodontal disease. Their findings indicated that tetracycline therapy selected for a highly resistant population of *Streptococcus* spp.. In some cases this population was not maintained after completion of therapy. It was also noted that selection with a single antibiotic did not lead to the isolation of *Streptococcus* spp. that were resistant to multiple antibiotics. The tetracycline resistance found in these isolates was not associated with a plasmid. However, the authors did not make attempts to transfer the resistance or determine if the resistance was associated with a conjugative transposon. When some of the isolates were later characterized further, it was found that

most of the tetracycline resistant isolates carried the Tet M determinant (Burdett *et al.*, 1982; Hartley *et al.*, 1984). This study also demonstrated the transfer of the Tet M determinant from a donor *Streptococcus sanguis* isolate to *Enterococcus faecalis*, *Streptococcus sanguis*, and *Streptococcus mutans* in the absence of detectable plasmids (Hartley *et al.*, 1984). The transfer of the Tet M determinant occurred at a frequency ranging from 1.0×10^{-4} to 3.0×10^{-7} / recipient depending on the recipient used (Hartley *et al.*, 1984).

The Tet M determinant has also been reported in the oral species *F. nucleatum*, *Neisseria perflava*-*N. sicca*, *Streptococcus* spp., *Peptostreptococcus anaerobius*, *Veillonella* spp. and facultative Streptococci (Roberts & Moncla, 1988). These bacterial species were isolated from six patients with periodontal disease. They were selected by plating bacterial plaque samples on blood agar plates supplemented with 10 µg/ml of tetracycline. This study did not evaluate the mobility of the Tet M determinant in these species. The authors found no plasmids in these species and reported the Tet M determinant to be located in the chromosome (Roberts & Moncla, 1988). In a second study, the authors show the Tet M determinant was associated with a functional conjugative transposon in *F. nucleatum* (Roberts & Lanciardi, 1990). The Tet M was transferable from a tetracycline resistant *F. nucleatum* to a tetracycline susceptible *F. nucleatum*, *Peptostreptococcus anaerobius* and *E. faecalis*. The frequency of transfer ranged from 10^{-1} to 10^{-8} /recipient. This was the first report of a Gram-negative rod carrying a functional conjugative chromosomal Tet M determinant, and is the third report of mobile conjugative tetracycline resistance in anaerobic species with *Clostridium*

difficile with *tetM* (Hachler *et al.*, 1987) and *Bacteroides* spp. with *tetQ* being the first two (Shoemaker *et al.*, 1989).

The Tet Q determinant has been found in *P. intermedia*, and *P. denticola* where it is located in the chromosome (Guiney *et al.*, 1989; Shoemaker *et al.*, 1989). The authors also showed that the Tet Q determinant was transferable from both species; however, the frequency of transfer was ten fold higher in *P. intermedia*. The transfer of penicillin resistance was determined to be linked to the transfer of tetracycline resistance in both species. The Tet Q determinant in *P. denticola* was transferable to both nonpigmented oral *B. buccae* and to intestinal *B. fragilis* isolates (Guiney *et al.*, 1990; Shoemaker *et al.*, 1989).

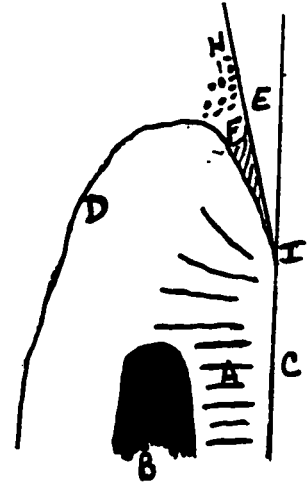
These studies have established periodontal pathogens do carry the same antibiotic resistance genes as other human pathogens. However, the frequency of these determinants, the host range of these determinants, and the rate of transfer are now beginning to be established. In addition, the effects of these antibiotic resistance genes on therapy and the affect the presence of these determinants will have on other bacterial species colonizing the host has not been evaluated.

AIM OF RESEARCH

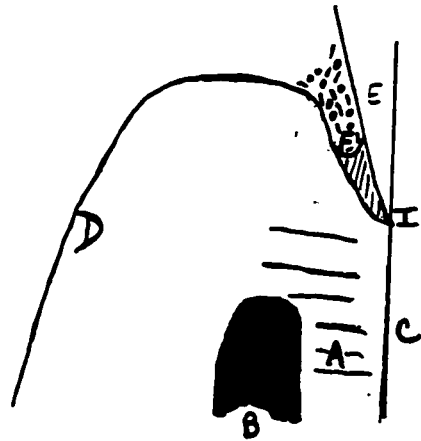
Antibiotics are used as part of the treatment for cases of periodontitis that do not respond to standard mechanical therapy. Little work has been done to evaluate the effects of antibiotic therapy on the periodontal pathogens or the commensal oral species. The purpose of the research for this dissertation was to evaluate if previously

characterized antibiotic resistance determinants were present in any of the bacteria associated with periodontal disease or the commensal species that comprise the normal oral flora. The prevalence of tetracycline and erythromycin resistance was determined using MIC assay, DNA probes and PCR. The mobility of these antibiotic resistance determinants was also evaluated using conjugal mating experiments.

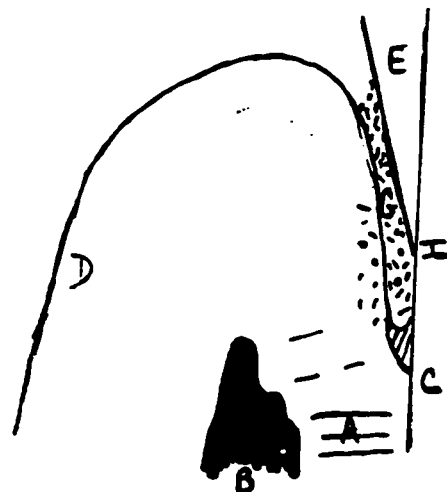
A. Healthy Gingiva



B. Gingivitis



C. Periodontitis



Key:

- A. Periodontal Ligament
- B. Alveolar Bone
- C. Cementum
- D. Oral Epithelium
- E. Tooth Enamel
- F. Gingival Sulcus
- G. Periodontal Pocket
- H. Dental Plaque
- I. Junctional Epithelium

Figure 1. Comparison of gingival health to gingivitis and periodontitis

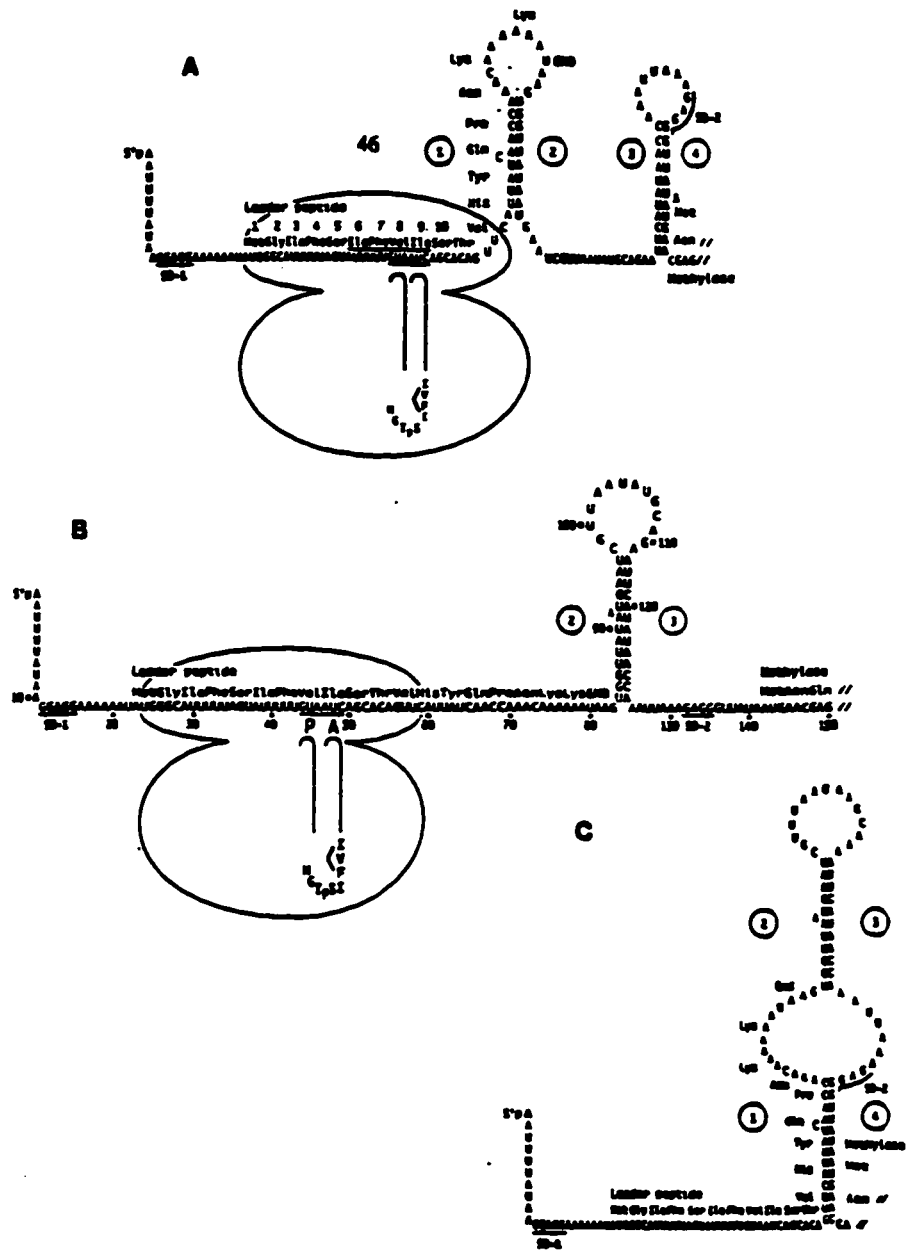


Figure 2. Conformational transitions of mRNA during induction by erythromycin (Weisblum, 1995b)

The numbers 1,2,3,4 represent segments of the mRNA to help clarify the segment locations in the three figures.

SD = Ribosomal binding sites

Table 1. Culturable bacteria associated with periodontal disease

Species	Oxygen Sensitivity
<i>Actinobacillus actinomycetemcomitans</i>	Facultative ^{a)}
<i>Bacteroides forsythus</i>	Anaerobe
<i>Campylobacter rectus</i>	Anaerobe / Microaerophilic ^{b)}
<i>Capnocytophaga</i> spp.	Microaerophilic ^{c)}
<i>Eikenella corrodens</i>	Microaerophilic
<i>Fusobacterium nucleatum</i>	Anaerobe
<i>Peptostreptococcus micros</i> ^{d)}	Anaerobe
<i>Porphyromonas gingivalis</i>	Anaerobe
<i>Prevotella intermedius</i>	Anaerobe
<i>Selanomonas</i> spp.	Anaerobe
<i>Treponema denticola</i>	Anaerobe

a) can grow in the presence or absence of oxygen, but prefers CO₂

b) only a few isolates have been shown to grow in the presence of low levels of oxygen, therefore most isolates are still considered anaerobes

c) all isolates can grow in the presence of low levels of oxygen

d) Gram-positive bacteria, all other bacteria in this table are Gram-negative

Table 2. Antibiotic Levels in bodily fluids

Antibiotic	Dosage	Serum levels (µg/ml)	Serum Breakpoint (µg/ml)	Gingival crevicular fluid levels (µg/ml)	
Penicillin V	500 mg every 6 hrs	3-20	16		Genco, 1981
Ampicillin	500 mg single dose	2-4			Slots & Rams, 1990
Chloramphenicol		3-12	8		Genco, 1981
Clindamycin	150 mg every 6 hrs	5-26	8	2	Genco, 1981; Walker <i>et al.</i> , 1981
	300 mg single dose		1.9	2	
Erythromycin	250 mg every 6 hrs	<1-10	4	1	Genco, 1981; Slots & Rams, 1990
Metronidazole	500 mg every 6 hrs	4-10	8	8	Genco, 1981; Slots & Rams, 1990; Olsvik & Tenover, 1993
	250 mg single dose		6.0	3.6	
Tetracycline	250 mg every 6 hrs	1-5	4	4-8	Genco, 1981; Walker <i>et al.</i> , 1991; Slots & Rams, 1990; Olsvik & Tenover, 1993
	500 mg single dose		1.5	5-12	
Minocycline	100 mg every 12 hrs	0.7-4.5	4	8-16	Genco, 1981; Slots & Rams, 1990; Olsvik & Tenover, 1993
	100 mg single dose		1	5.3	

The antibiotic breakpoint is defined as the concentration of antibiotic that can be achieved with optimal therapy

Table 3. Tetracycline Resistance Determinants

Determinant	Mechanism	Origin
A	Efflux	Gram-negative
B	Efflux	Gram-negative
C	Efflux	Gram-negative
D	Efflux	Gram-negative
E	Efflux	Gram-negative
G	Efflux	Gram-negative
H	Efflux	Gram-negative
I	Efflux	Gram-negative
K	Efflux	Gram-positive
L	Efflux	Gram-positive
A(P)	Efflux	Gram-positive
M	Ribosomal Protection	Unknown
O	Ribosomal Protection	Unknown
S	Ribosomal Protection	Unknown
B(P)	Ribosomal Protection	Unknown

Table 3. continued

Q	Ribosomal Protection	Unknown
U	Ribosomal Protection	Unknown
X	Enzymatic inactivation	Unknown

CHAPTER 2

Tetracycline resistance in *Actinobacillus actinomycetemcomitans*

BACKGROUND

Localized juvenile periodontitis, is closely associated with the presence of *Actinobacillus actinomycetemcomitans* (Slots *et al.*, 1980; Zambon, 1985). The disease affects people, 12 to 20 years of age, and leads to exfoliation of the permanent incisors and first molars (Slots *et al.*, 1980; Zambon, 1985). Tetracyclines are currently the antibiotics of choice, as an adjunct to mechanical debridement, for the treatment of localized juvenile periodontitis (Genco, 1981). With few exceptions, tetracycline resistance is due to the acquisition of new genes (Marshall *et al.*, 1984; Morse *et al.*, 1986). These genes code for either a protein which pumps tetracycline out of the cell, or a protein which protects the ribosome from the action of tetracycline (Roberts, 1989). These genes are usually associated with transposons and/or conjugative elements (Roberts, 1989).

A. actinomycetemcomitans has been reported as being susceptible to tetracycline (Goodson & Tanner, 1992; O'Connor *et al.*, 1990; Pavicoc *et al.*, 1992; Slots *et al.*, 1982). However, no standard minimum inhibitory concentration (MIC) conditions have been established for *A. actinomycetemcomitans*. As a result, laboratories have used a variety of media, air conditions and incubation times making it difficult to compare results from different studies (Jousimies-Somers *et al.*, 1988; O'Connor *et al.*, 1990; Pajukanta *et al.*, 1992; Pavicoc *et al.*, 1992; Slots *et al.*, 1982). In addition, no value which indicates resistance has been established, nor do we know whether *A.*

actinomycescomitans carries acquired antibiotic resistance genes, as do other species such as the taxonomically related *Haemophilus* species (Marshall *et al.*, 1984; Roberts, 1989).

The aim of this project was to compare different media, atmospheric conditions and incubation times for determination of MIC values, and to determine if tetracycline resistance determinants are present in *A. actinomycescomitans* isolates.

MATERIALS AND METHODS

Bacterial isolates. We examined 18 clinical isolates of *A. actinomycescomitans* from 16 patients with periodontitis. Unfortunately, no patient histories regarding disease progression or therapy treatments were available. Isolates were collected in Seattle between December 1985 and February 1987. Four ATCC strains (33384, 43717, 43718, 43719) of *A. actinomycescomitans* from the American Type Culture Collection, (Rockville, MD) were also included in the study.

Media. Brain Heart Infusion (BHI) agar or BHI broth (Difco Laboratories, Detroit MI), 20% (w/v) infusion from calf brains, 20% (w/v) infusion from beef hearts, 1% (w/v) proteose peptone, 0.2% (w/v) bacto dextrose, 0.5% (w/v) sodium chloride, 0.25% (w/v) disodium phosphate and 1.5% (w/v) bacto agar, both supplemented with 10 µg of hemin, 10 µg of L-histidine and 2 µg of nicotinamide adenine dinucleotide per ml (Roberts & Smith, 1980) were used for routine growth of isolates. The following media were used for MIC determinations: *Haemophilus* Test Medium (HTM)(Doern, 1992), Modified

Enriched Trypticase Soy Agar (METSAs)(Slots, 1982; Syed, 1980), Chocolate Mueller Hinton agar (CMH)(NCCLS, 1990), Brain Heart Infusion agar (BHI, supplemented as above), and Wilkins-Chalgren agar (WC)(Jousimies-Somer *et al.*, 1988). All media were purchased from Difco Laboratories, Detroit MI. BHI has not previously been reported as a MIC medium for *A. actinomycetemcomitans*, but is currently being used at CDC for this purpose (F. Tenover, personal Communication, CDC, Atlanta, GA).

Minimum Inhibitory Concentration. The agar dilution method was used for all MIC determinations (NCCLS, 1990). The inoculum used was 1×10^4 organisms per spot using a Steers replicator (Steers *et al.*, 1959). Concentrations of tetracycline used were 0.125 to 64 $\mu\text{g/ml}$. Triplicate plates were incubated under ambient air, 5% CO_2 and anaerobic conditions (85% N_2 , 10% H_2 , 5% CO_2) and read at 24, 48 and 72 h. All MIC assays were repeated a minimum of three times and read independently by two to three different people. *Haemophilus influenzae* ATTC 49247, *Escherichia coli* ATTC 25922, a tetracycline resistant (Tc^r) *Haemophilus aphrophilus* clinical isolate, *H. influenzae* Rd 8, and *H. influenzae* Rd 8 with plasmid pMR387 which contains the Tet B determinant (Roberts & Smith, 1980) were used as controls.

Mating Experiments. The characterized *H. influenzae* Rd 8 strain, which is chromosomally resistant to rifampin and streptomycin (Roberts & Smith, 1980), and a tetracycline susceptible (Tc^s) *tetB* negative *A. actinomycetemcomitans* (9501) were used

as recipients. The 9501 isolate was selected for chromosomal resistance to rifampin (10 µg/ml) and reselected for streptomycin (50 µg/ml) resistance as previously described for other species (Roberts & Lanciardi, 1990). The donors used were susceptible to rifampin and streptomycin. Donor and recipient were grown separately to a density of 10^8 per ml in broth, equal volumes were mixed and plated directly onto agar plates and incubated overnight in 36.5°C in 5% CO₂ (Roberts & Lanciardi, 1990).

Transconjugants were selected on media supplemented with 10 µg rifampin and 4 µg/ml of tetracycline then screened for streptomycin resistance (50 µg/ml) (Roberts & Smith, 1980). The *H. influenzae* transconjugants were confirmed by DNA hybridization.

DNA Isolation. DNA from *A. actinomycetemcomitans* was prepared using the previously described protocols for *H. influenzae* (Roberts & Smith, 1980) with the following modifications: prior to phenol-chloroform extraction, Triton X-100 (1%) was substituted for sodium dodecyl sulfate (SDS), and incubated 55°C, 2 hrs, followed by 1 µg of Proteinase K (Sigma Chemical Co., St Louis, MO) incubated 37°C, 2 hrs.

Hybridization. Isolates were screened with ten different Tet determinants (Table 4) under stringent conditions as previously described which requires > 80% identity for a positive assay (Roberts *et al.*, 1991). Tet determinants were labeled with two ³²P-labeled nucleotide triphosphates and two unlabeled nucleotides by nick translation to screen the isolates, as previously described (Roberts *et al.*, 1991). Dot blots and Southern

blots were prehybridized for ≥ 2 hrs at 42°C with 50% (v/v) formamide, 0.1% (w/v) polyvinylprollidone, 0.1% (w/v) albumin, 0.1% (v/w) Ficoll, 0.1% (w/v) SDS, 0.05% M-monobasic sodium phosphate (pH 7.4) and 400 μ g per ml calf thymus DNA.

Hybridization in prehybridization solution containing, 32 P-labeled plasmid DNA, 0.76 M-NaCl, and 200 μ g per ml boiled calf thymus DNA occurred overnight. Filters were washed three times for 30 min at 52°C in 0.1% SDS, 0.015 M-NaCl and 0.0015 M-sodium citrate (0.2 X SSC) followed by three min washes at 52°C in 0.015 M-NaCl and 0.0015 M-sodium citrate. Filters were dried and exposed to x-ray film. Under these highly stringent conditions, none of the probes cross-hybridized. Positive and negative controls were used with each experiment.

Hybridization with the Tet B Oligonucleotide. Isolates were confirmed as carrying the Tet B determinant with an oligonucleotide specific for Tet B (5'CAG TGC TGT TGT TGT CAT TAA 3')(Kariuki *et al.*, 1992). The oligonucleotide was labeled in the 5'-end with γ 32 P using T4 Kinase (Kariuki *et al.*, 1992). Dot blots and Southern blots were prehybridized for ≥ 2 hrs at 37°C, then hybridized overnight at 37°C (Kariuki *et al.*, 1992). Filters were rinsed twice with 2 X SSC at room temperature, followed by a wash for one hr, at 57°C in 2 X SSC. The final wash was 45 min, at 57°C in 2 X SSC, 0.1% SDS. Filters were dried and exposed to X-ray film.

Polymerase Chain Reaction. PCR amplification used 10 ng of cloned, Tet B plasmid DNA (pRT11) as a positive control and 20-40 ng of genomic DNA as template for the *A. actinomycetemcomitans* isolates. PCR primers were 5'CAG TGC TGT TGT TGT CAT TAA 3' (Kariuki *et al.*, 1992) and 5'GCT TGG AAT ACT GAG TGT AA 3'. The reaction had a 100 µl volume and contained: 2 units of Taq polymerase (Perkin Elmer-Cetus, Norwalk, CT), 200 mM dNTP, 1 X PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM MgCl₂, 0.001% w/v gelatin) and 100 ng of each oligonucleotide. Using a Perkin Elmer-Cetus DNA thermal cycler, the reaction was incubated at 94°C for three min, then 35 cycles consisting of denaturation at 96°C for 20 sec, annealing at 52°C for 30 sec, and elongation at 72°C for 2 min. Cycle 36 was the same but the elongation step was for 5 min. Positive and negative controls were used with each run. The amplified products were dried and resuspended in 1/10 volume of sterile water and the entire sample was run on a 1.5% agarose gel. The DNA was visualized by ethidium-bromide staining and a Southern blot was prepared. The primers generated a 572 bp fragment and was confirmed by hybridization with a non-radiolabeled Tet B probe.

Non-radiolabeled hybridization. Plasmid pRT11 was labeled with the nonradioactive labeling system GeniusTM 3 kit. The GeniusTM 3 labeling system randomly primes with DIG-11-dUTP which is detected with an anti-DIG-alkaline phosphatase chemiluminescent substrate (Boehringer Mannheim Biochemica, Indianapolis IN). The labeling mixture contained 1 - 5 µg boiled DNA, 2 µl hexanucleotide mixture, 2 µl dNTP

labeling mixture, 1 μ l Klenow enzyme and 9 μ l dH₂O. The mixture was incubated at 37°C overnight, and the reaction was stopped by adding 2 μ l of 0.2 M EDTA pH 8.0. Blot were prehybridized for >2 hrs at 68°C in hybridization buffer (5 X SSC, 0.1% N-lauroylsarcosine NaCl, 0.02% SDS and 2% blocking reagent). The buffer was removed and replaced with new hybridization buffer containing the DNA probe diluted 1/500 and incubated overnight at 68°C. The probe was removed and two 5 min washes were done at room temperature in 2 X SSC and 0.1% SDS. This was followed by two more 15 min washes, in 0.1 X SSC and 0.1% SDS, at 68° C. Filters were washed for one min in buffer A (150 mM NaCl, 100 mM Tris-HCL, pH 7.5), then washed in buffer B (4% blocking reagent in buffer A) at room temperature for \geq 3 hrs. The next wash was in a-digoxigenin alkaline phosphate conjugate (diluted 1:5000 in buffer B) for 30 min at room temperature. The filters were washed twice in buffer A for min at room temperature. The last wash was for one min in 100 mM Tris-HCl, 100 mM NaCl, 50 mM MgCl, pH 9.5. Lumiphos was added at a rate of 500 ml/100 cm² filter. Filters were incubated overnight at room temperature, and then exposed to x-ray film for 30 min and developed.

RESULTS

Tetracycline Susceptibility Testing. Five different media under CO₂, ambient air and anaerobic environmental conditions were compared for use in MIC determinations. Growth at 24 hrs was not adequate for interpretation of *A. actinomycetemcomitans* although it was for *H. influenzae*, *E. coli*, and *H. aphrophilus* controls. Therefore, 48 and

72 hrs were the incubation times evaluated for *A. actinomycetemcomitans*. Ambient air conditions did not support growth of all of our *A. actinomycetemcomitans* isolates on any of the media used and was eliminated from further studies. Haemophilus Test Media and Modified Enriched Trypticase Soy Agar did not support the growth of all of our isolates under any of the air conditions and were also removed from our study. CMH, BHI, and WC supported growth of all of our isolates and controls under 5% CO₂ and anaerobic conditions (10% H₂, 85% N₂, and 5% CO₂) and were used for further evaluations. This was done in accordance to the NCCLS standards which state that fastidious organisms may be grown in the presence of 5% CO₂, if necessary to achieve adequate growth. It has been established that media incubated in the presence of CO₂ may have an altered pH (Sabath *et al.*, 1968; NCCLS, 1993). When the pH of the media used for the MIC assays was tested the values ranged from 6.7 to 7.0. The largest change in pH from the initial reading compared to the reading at 72 hrs in the presence of CO₂ observed was 0.3 (Table 5).

The MIC values are given in Table 6. Depending on the time of incubation, media, and atmospheric conditions used, *H. influenzae* ATTC 49247 isolate had MIC values ranging from 16 to 32 µg/ml. The *E. coli* ATTC 25922 isolate had an MIC of 2 µg/ml, with all conditions tested, while the tetracycline resistant *H. aphrophilus*, tetracycline sensitive *H. influenzae* Rd 8 and the R387 with plasmid pMR387 (ampicillin, chloramphenicol, and tetracycline resistant (*tetB*)) (Roberts and Smith, 1980) had MIC values ranging from 8 to 32 µg/ml, 1 to 2 µg/ml, and ≥ 32 µg/ml, respectively.

MIC assays of *A. actinomycetemcomitans* isolates ranged from 0.125 to 8 µg of tetracycline depending on the media and atmospheric conditions used (Table 6). Four isolates and one ATTC strain had the same MIC values with all three media. Six isolates and two ATTC strains varied by one dilution with the three different media. Eight isolates and one ATTC strain varied by more than one dilution on the three different media (Table 6). Overall, CMH consistently had lower MIC values than either WC or BHI, when there was variation (Table 6). WC and BHI had similar MIC values with the exception of isolate 3013. The MIC value was at most one dilution higher at 72 hrs as compared to 48 hrs incubation. BHI in CO₂ at 48 hrs gave the most reproducible results over several experiments, varying only ± 1 dilution between experiments. MIC values with the BHI media at 48 hrs for *A. actinomycetemcomitans* were compared to their ability to grow as streaked cultures on the same BHI media at 24 hrs. Fourteen of the eighteen (78%) isolates and all four (100%) of the ATTC strains had MIC values that were concordant within ± 1 dilution with their ability to grow when streaked on the same tetracycline supplemented plates (Table 6).

Identification and transfer of tetracycline resistance genes. Isolates were screened with ten different Tet determinants (Table 4). The Tet B determinant hybridized with 15 (83%) of the isolates and 3 (75%) of the ATCC strains of *A. actinomycetemcomitans* (Table 6), while the other Tet determinants did not hybridize. In some preparations, plasmid bands were evident and hybridized with the Tet B probe (Figure 3, Lane 1). In the majority of preparations there were no obvious plasmids and hybridization appeared

to be associated with the chromosome (Figure 3, Lane 2). The presence of the Tet B determinant was confirmed by hybridization with the Tet B oligonucleotide probe and by PCR. We have not been able to isolate intact plasmids from any of the *A. actinomycetemcomitans* isolates used in this study.

Four isolates and two ATTC strains of *A. actinomycetemcomitans* were used as donors in conjugation with the a tetracycline susceptible *A. actinomycetemcomitans* recipient (Table 6). The Tet B determinant transferred at frequencies of 3.5×10^{-5} to 2.5×10^{-6} / recipient. Four *tetB* positive isolates and one *tetB* positive ATTC strain of *A. actinomycetemcomitans* were mated with *H. influenzae* Rd 8. Matings gave a frequency of transconjugants ranging from 1×10^{-8} to 6×10^{-9} / recipient. All transconjugants were selected on media supplemented with 10 µg rifampin and 4 µg of tetracycline and hybridized with the Tet B probes under various detection methods. The transconjugants produced appropriate PCR products which hybridized to the Tet B determinants. The MIC values of the *A. actinomycetemcomitans* transconjugants was ≥ 4 µg/ml for tetracycline and the *H. influenzae* transconjugants has MIC values of ≥ 8 µg/ml for tetracycline. Some DNA preparations of the *A. actinomycetemcomitans* transconjugants showed faint bands above the chromosome, which hybridized with the Tet B probe (Data not shown).

CHAPTER DISCUSSION

Of the media used, BHI incubated in CO₂ and read at 48 hrs gave the most reproducible MIC results, with only a variation of ± 1 dilution between experiments.

This is the first published report using BHI for MIC determinations of *A.*

actinomycetemcomitans. The other media showed more variability between experiments for *A. actinomycetemcomitans* and some of the control isolates. However, the variability of the MIC values for control isolates was always within the range reported by National Committee on Clinical Laboratory Standards (NCCLS, 1990). Of all the media evaluated BHI media in CO₂ was the most optimal of all the MIC conditions examined and is recommended for future MIC work done with *A. actinomycetemcomitans*.

The *Haemophilus* spp. minimum inhibitory concentration interpretive standards for tetracycline are as follows; ≤ 2 μg are susceptible, 4 μg are intermediate, ≥ 8 $\mu\text{g/ml}$ are resistant (NCCLS, 1993). When the Tet B determinant is present in *Haemophilus* spp. these isolates have an MIC values of ≥ 8 $\mu\text{g/ml}$ for tetracycline (Marshall *et al.*, 1984; NCCLS, 1993). Using the criteria established by NCCLS for *Haemophilus* spp. with the *A. actinomycetemcomitans* isolates in this study, one ATCC strain would be classified as resistant, seven isolates and two ATCC strains would be intermediate and eleven isolates and one ATCC strain would be susceptible. When the NCCLS standards for MIC values are compared to the presence of the Tet B determinants, nine isolates that would be classified as susceptible carried the Tet B determinant and one isolate with an intermediate MIC values did not carry the Tet B determinant. These discrepancies can be explained in several different ways for example, the media and conditions used for

MIC determination may still not be optimal for *A. actinomycetemcomitans*. This discrepancy might be due to the difference of expression of the *tetB* gene in different host backgrounds. The later is similar to what has been described by Tompkins *et al.* (1980) where they observed different levels of expression of antibiotic resistance determinants depending on the host background among a variety of Gram-negative species. The *tetB* gene, while functional, may be poorly expressed in *A. actinomycetemcomitans*. Further studies are needed to evaluate the expression and function of the Tet B determinant in *A. actinomycetemcomitans* before it can be decided how to interpret the MIC values for tetracycline in this species. In addition, more isolates need to be evaluated to better determine the susceptibility of *A. actinomycetemcomitans* to tetracycline when tetracycline resistance determinants are not present. At the present time, one can simply report the mere presence or absence of the Tet B determinant in these isolates.

The majority of Tet B determinants in *Haemophilus* spp. and other Gram-negative bacteria are associated with conjugative plasmids (Roberts, 1989). In the seven isolates and two ATCC strains of *A. actinomycetemcomitans* tested in mating experiments, all nine transferred the Tet B determinant and conferred Tc^r phenotype to the transconjugants. The transfer frequencies in these experiments are similar to frequencies previously reported for conjugative plasmids between *H. influenzae* donors and recipients (Roberts & Smith, 1980). This implies that these *A. actinomycetemcomitans* isolates carried a movable, functional Tet B determinant, which can be selected for by conjugation. Our data suggest that the Tet B determinant is located on conjugative plasmids. Plasmids have been previously reported in *A. actinomycetemcomitans*

(LeBlanc *et al.*, 1993; Olsvik & Preus, 1989). However, the two studies differ in the size, frequency of detection, and number of plasmids reported. Unfortunately antibiotic resistance was not evaluated in either study. Roberts & Smith have previously shown, with *H. influenzae*, that one and even two DNA extraction protocols did not identify all the plasmids present (1980). Plasmids in *H. influenzae* isolates with no visible plasmid, by one or two DNA protocols, could be identified because of conjugation of their plasmids to recipients (Roberts & Smith, 1980). This is what we have found with Tet B determinants in *A. actinomycetemcomitans*.

Conjugative plasmids carrying the Tet B determinant in *Haemophilus* spp. were first isolated in the 1970's (Marshall *et al.*, 1984; Roberts, 1989). As defined by NCCLS, these Tet B carrying *Haemophilus* spp. have MIC values of ≥ 8 $\mu\text{g/ml}$ for tetracycline (NCCLS, 1993). More recently, Roberts *et al.* have characterized two clones of Tc^r, Tet B determinant carrying *Moraxella catarrhalis* (1991). The presence of the Tet B determinant in *A. actinomycetemcomitans* extends the Tet B determinant host range further. In our study, 83% of the isolates carried the Tet B determinant, suggesting that the Tet B determinant carriage might be high in *A. actinomycetemcomitans* isolated from Seattle during 1985-1987. These isolates gave different pulse-field gel electrophoresis patterns and thus do not represent a single clone (Leng & Roberts, 1994). The ATCC strains 43717, and 43719 were isolated at State University of New York at Buffalo and first described in 1983 (Zambon *et al.*, 1983). The ATCC 43718 was isolated by Socransky at the Forsyth Dental Center in Boston, Massachusetts and was first described in 1983 (Zambon *et al.*, 1983). Three of the ATCC strains (43717, 43718, 43719)

hybridized with the Tet B determinant, suggesting that the Tet B determinant has been in the species at least since 1984 and is not unique to Seattle patients.

All isolates and ATCC strains of *A. actinomycetemcomitans* used as donors for mating experiments carried rRNA methylase genes which code for resistant to erythromycin (Em^r) (Roe *et al.*, 1994). Four isolates and one ATCC strain of *A. actinomycetemcomitans* (Em^rTc^r) were mated with the *H. influenzae* recipient Rd 8. When the Em^r phenotype was selected with *A. actinomycetemcomitans* donors 3013, 2608, 7601, 50% of the resulting transconjugants had both the Tet B determinant and an *Erm* determinant, while in the other 50% of the transconjugants only the *Erm* determinant was transferred (Roe *et al.*, 1996). In the other two donors, no Tc^rEm^r transconjugants were isolated. This suggests that either the Tet B and the various rRNA methylase determinants are physically linked in some isolates of *A. actinomycetemcomitans*, are able to cotransfer, or the Tet B and the various rRNA methylase determinants transfer independently but at similar frequencies.

It is currently being discussed how to address the fact that standard MIC methods may not detect inducible antibiotic resistance (Roberts, 1989; Roberts & Smith, 1980), and nonexpressing gene can be convert to an expressing gene by mutation (Roberts, 1989). Whether the use of DNA probes for the detection of antibiotic resistant genes, as we have done in this paper, should replace the conventional phenotypic assays has been suggested (Tenover, 1992). However, going to a completely DNA based detection method for antibiotic resistance would have the limitation of not being able to detect the

introduction of novel antibiotic resistance determinants into a bacterial population. No decision with regards to these issues has been resolved; however, it is clear that more work on antibiotic resistance genes in *A. actinomycetemcomitans* is needed especially since related genera such as *Haemophilus* carry a number of different antibiotic resistance genes (Roberts, 1989).

Table 4. Tetracycline DNA Tet probes used

Determinant	Source	Fragment	Vector
Gram-positive			
Tet K	pAT102	870-bp <i>HincII</i>	pUC8
Tet L	pVB11.	3.7-kb <i>HincII</i>	pVH2124
Tet M	pJI3	1.8-kb <i>KpnI-HindIII</i>	pACYC177
Tet M	pJI3	5 kb <i>HincII</i>	pACYC177
Tet O	pUOA4	1.8 kb <i>HincII</i>	pUC8
Tet P	pJIR39	800-bp <i>EcoRI-PstI</i>	pUC18
Gram-negative			
Tet A	pSL18	750-bp <i>SmaI</i>	pUC18
Tet B	pRT11	1.27 kb <i>HincII</i>	pRT11
Tet C	pBR322	929-bp <i>BstNI</i>	pBR322
Tet D	pSL106	3.05 kb <i>HindII-PstI</i>	pACYC177
Tet E	pSL04	2.5 kb <i>ClaI-PvuI</i>	pACYC177

Table 5. pH value after incubation under different atmospheric conditions

Atmosphere Conditions	Media	Time (hrs)			
		0	24	48	72
Ambient					
	BHI	7.0	6.9	6.9	6.7
	CMH	7.1	7.0	6.9	6.8
	WC	6.8	6.8	6.8	6.7
5% CO₂					
	BHI	7.0	6.9	6.8	6.8
	CMH	7.1	6.8	6.8	6.9
	WC	6.8	6.9	6.8	6.8
Anaerobic					
	BHI	7.0	7.0	6.9	6.9
	CMH	7.1	7.0	7.0	7.0
	WC	6.8	6.9	6.9	6.8

Table 6. The Presence of the Tet B determinant in *A. actinomycetemcomitans*

Isolate	MIC						Hybridization	
	CO ₂			Ama ^a	Ana ^b	Growth ^d	Tet B	Transconjugant ^e
	BHI ^c	WC	CMH	BHI	BHI		Produced	
	48 h	48 h	48 h	48 h	48h	24 h		
1011	1	1	0.5	2	0.5	4	+	A
1014	2	2	0.125	2	4	4	+	A
1528	2	2	2	4	0.5	4	+	
2102	1	0.5	0.125	2	1	4	+	H
2201	1	2	2	2	1	2	+	
2205	1	1	0.5	2	1	1	+	
2608	1	1	0.5	2	0.5	2	+	A
3013	2	0.125	0.125	2	1	4	+	A H
4001	1	0.5	0.125	2	0.5	2	+	H
5004	1	NG ^f	1	2	1	1	-	
5601	2	2	2	1	0.5	1	-	
5901	2	2	2	2	2	2	+	
6001	1	1	1	2	0.5	2	+	
7601	1	1	0.5	2	1	4	+	H
7902	1	2	0.5	2	0.5	4	+	

Table 6. Continued

8202	2	1	1	2	0.5	2	+		
9002	1	1	0.5	2	1	2	+		
9501	2	0.5	0.5	2	0.5	1	-		
ATTC 43717	2	2	2	2	1	4	+		
ATCC 43718	2	2	1	4	0.5	4	+	A	H
ATTC 43719	8	8	4	4	2	4	+	A	
ATTC 33384	1	2	0.5	2	1	1	-		
ATCC 49247 ^g	32	32	16	32	32		-		
Rd 8 ^g	2	2	1	2	2		-		
R387 ^g	>32	>32	>32	32	>32		+		
ATTC 25922 ^h	2	2	2	2	2		-		

MIC for CO₂ conditions presented for three media done at the same time.

^a Am = Ambient

^b Ana = Anaerobic

^c BHI: Brain Heart Infusion Agar; WC: Wilkins-Chalgren agar; CHM: Chocolate Mueller Hinton agar, all values are in µg/ml

^d ability to grow on Brain Heart Infusion Agar supplemented with 1, 2, or 4 µg/ml

Tetracycline in CO₂

^e A: *Actinobacillus actinomycetemcomitans*; H: *Haemophilus influenzae*, Rd 8

^f NG = No Growth

Table 6. Continued

g *Haemophilus influenzae* isolate

h *E. coli*

Isolates with the same first two numbers came from the same patient with the exception of the ATTC strains

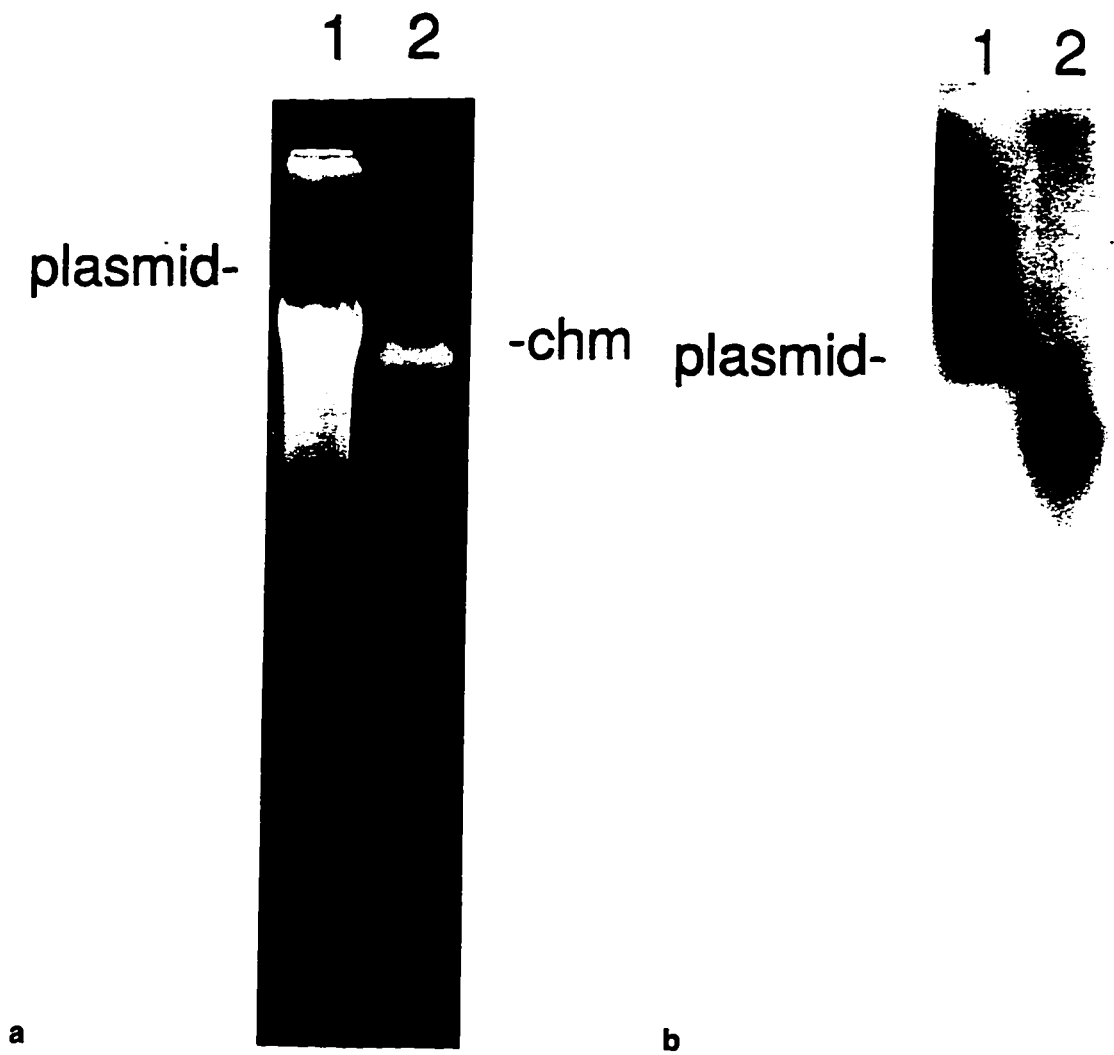


Figure 3.

A. Gel electrophoresis of two different Tet B positive *Actinobacillus actinomycetemcomitans*

B. Hybridization with radiolabeled Tet B probe

CHAPTER 3

Erythromycin resistance in *Actinobacillus actinomycetemcomitans*

BACKGROUND

Actinobacillus actinomycetemcomitans is a gram negative microaerophilic bacteria and is a member of the family *Pasturellaceae*. *Actinobacillus actinomycetemcomitans* is strongly associated with localized juvenile periodontitis, which affects persons aged 12 to 20 years (Slots, Reynold & Genco, 1980; Zambon, 1985).

Erythromycin is commonly used in dentistry as an alternative to β -lactam antibiotics for the treatment of dental abscesses, but is rarely used for the treatment of periodontitis (Genco, 1981). In contrast, clindamycin is used for the treatment of adult and refractory periodontitis (Genco, 1981). Erythromycin and clindamycin are chemically distinct, but both inhibit bacterial protein synthesis by binding to the 50S ribosomal subunit (Eady *et al.*, 1990). Resistance to erythromycin and clindamycin are usually due to the methylation of a single adenine nucleotide in the 23S component of the 50S ribosomal subunit. This change prevents erythromycin, clindamycin and streptogramin B from binding to the ribosome and is often referred to as macrolide-lincosamide-streptogramin B (MLS) resistance (Eady *et al.*, 1990; Leclercq & Courvalin, 1991; Weisblum, 1995).

Most isolates of *A. actinomycetemcomitans* have been reported to be erythromycin resistant (Erm^r) with MIC values $\geq 8 \mu\text{g/ml}$ for erythromycin (Genco, 1981; Baker *et al.*, 1985; Pavicic *et al.*, 1992; Slots & Ram, 1990). The aim of this study was to characterize resistance of *A. actinomycetemcomitans* to erythromycin, using molecular techniques. The potential for transfer of the genes responsible for erythromycin resistance was also determined.

MATERIALS AND METHODS

Bacterial isolates. We examined 21 clinical isolates and four ATCC strains of *A. actinomycetemcomitans*. The clinical isolates were collected from 16 patients in Seattle with periodontitis between 1985 and 1987. Pulsed-field gel electrophoresis indicated that these isolates did not represent a single clone (Leng & Roberts, 1994). The ATCC strains represent serotypes a (43717), b (43718), and c (33384, 43719).

Media. Brain Heart Infusion (BHI) agar or BHI broth (Difco Laboratories, Detroit, MI) supplemented with 10 µg/ml of heamin, 10 µg/ml of L-histidine and 2 µg/ml of nicotinamide adenine dinucleotide were used to culture *A. actinomycetemcomitans* (Roe *et al.*, 1995a). Brain Heart Infusion agar was used for MIC determination (Roe *et al.*, 1995a).

Minimum Inhibitory Concentration determination. MIC values were determined by agar dilution. Bacteria were suspended in 0.84% NaCl to a 0.5 McFarland standard and diluted 1:10. This dilution (10^4 cfu/spot) was inoculated by a Steers replicator according to the National Committee on Clinical Laboratory Standards (1993). Erythromycin concentrations ranged from 0.5 - 64 µg/ml. Plates were incubated at 36.5°C in air with 5% CO₂ and read at 48 h (Roe *et al.*, 1995a).

Haemophilus influenzae transconjugants were tested on Chocolate Mueller Hinton medium (Difco Laboratories, Detroit, MI) and *Enterococcus faecalis* transconjugants were tested on Blood Mueller Hinton medium (Difco Laboratories, Detroit, MI) using standard procedures (NCCLS, 1993). Plates were incubated at 36.5°C in air and read at 24 h (NCCLS, 1993).

Mating experiments. *E. faecalis* JH2-2 and *H. influenzae* Rd 8 were chosen as recipients for conjugal transfer experiments. JH2-2 has previously been selected for chromosomal resistance to fusidic acid 25 µg/ml and rifampin 25 µg/ml (Roberts *et al.*, 1992). Rd 8 has been previously selected for chromosomal resistance to fusidic acid 25 µg/ml, rifampicin 10 µg/ml, and streptomycin 250 µg/ml (Roberts *et al.*, 1980). The donor *A. actinomycetemcomitans* isolates were susceptible to rifampin and streptomycin, resistant to fusidic acid and carried the Tet B determinant and varying numbers of different rRNA methylase genes (Table 7)(Roe *et al.*, 1995a). Donor and recipient isolates were grown separately in BHI broth to approximately 10^{10} cfu/ml. Equal volumes of broth were mixed, centrifuged at 39,000 X g, resuspended, plated on BHI agar plates, and incubated overnight at 36.5°C in air with 5% CO₂, as previously described (Roberts *et al.*, 1980; Roberts *et al.*, 1992). JH2-2 transconjugants were selected on media containing rifampin 10 µg/ml and erythromycin 10 µg/ml.

Transconjugants were confirmed by growth on media containing streptomycin 250 µg/ml, and by hybridization of a southern blot with an *E. faecalis* specific chromosomal DNA probe (Roberts & Lanciardi, 1990; Roberts & Brown 1994). Rd 8 transconjugants were selected on media containing streptomycin 250 µg/ml and erythromycin 10 µg/ml and then transferred to media with rifampicin 10 µg/ml. The transconjugants were confirmed as *H. influenzae* by growth requirements and colony morphology.

DNA hybridization. DNA was extracted from *A. actinomycetemcomitans*, *H. influenzae* and *E. faecalis* as previously described (LeBlanc & Lee, 1979; Roe *et al.*, 1995a). Uncut whole-cell DNA was visualized on a 0.7% agarose gel stained with ethidium bromide

and viewed under long-wave UV light. We used rRNA methylase gene probes that are representatives of the five most commonly found classes: *ermA*, *ermB*, *ermC*, *ermF* and *ermQ* (Weisblum, 1995a). Fragment DNA (Table 7) probes were labeled using the nonradioactive labeling system GeniusTM 3 kit. The GeniusTM 3 labeling system randomly primes with DIG-11-dUTP which is detected with an anti-DIG-alkaline phosphatase chemiluminescent substrate (Boehringer Mannheim Biochemica, Indianapolis IN). Hybridization and detection followed the manufacturer's instructions and are described by Roe *et al.* (1995a). Hybridization was with Southern blots of uncut whole-cell DNA under stringent conditions. Positive and negative controls were included in each Southern blot. We found that neither the susceptible JH2-2 or the Rd 8 DNA hybridized with any of the five Erm probes. None of the five Erm fragments cross-reacted with each other under the high stringency conditions used for hybridization. Each isolate and each transconjugant examined were tested at least twice on different Southern blots. Results for *ermA*, *ermB* and *ermC* were confirmed by PCR assay as previously described (Roberts & Brown, 1994).

RESULTS

Erythromycin Susceptibility Testing. The MIC assays of erythromycin for the twenty-one isolates and four ATTC strains of *A. actinomycetemcomitans* are shown in Table 7. The MIC values ranged from 2 to 32 µg/ml for erythromycin. According to NCCLS criteria (NCCLS, 1990), none of the isolates were susceptible to erythromycin, seven isolates and one ATTC strain was intermediate and the remaining 14 isolates and three ATTC strains were resistant MIC ≥ 8 µg/ml. The incubation of all MIC plates was in the presence of 5% CO₂. The NCCLS standards state that if adequate growth is not achievable for a fastidious organisms incubated in ambient air, the organisms may be

grown in the presence of 5% CO₂. Incubation in the presence of CO₂ may alter the pH of the media which in turn may affect the activity of some antibiotics (NCCLS, 1993). This phenomenon has been reported with erythromycin, where its activity changes remarkably within the pH range of 6.0 to 8.2 (Sabeth et al., 1968). To control for possible differences in the pH of the media under different air conditions the pH of the media used for the MIC assays was tested at several time points and the values ranged from 6.7 to 7.0. The largest change in pH from the initial reading compared to the reading at 72 hrs in the presence of CO₂ observed was 0.3 (Table 8). There did not appear to be any significant difference in the amount of pH change under incubation in the three different air conditions.

Identification of *erm* genes. Southern blot hybridization results are summarized in Table 9: *ermB* was found in 14 isolates (67%) and four ATCC strains (100%), *ermC* in 12 isolates (57%) and four ATCC strains (100%), *ermF* in 10 isolates (48%) and four ATCC strains (100%), *ermQ* in six isolates (25%) and *ermA* in two isolates (10%) and two ATCC strains (50%). Five isolates (20%) hybridized with one *Erm* gene, eight isolates (32%) hybridized with two *Erm* genes, five isolates (25%) and two ATCC strains (50%) hybridized with three, and two isolates (10%) and two ATCC strains (50%) hybridized with four *Erm* genes (Table 9). One isolate (5004) did not hybridize with any of the five genes, however, it had an MIC of 8 µg/ml. We found no correlation between the number of rRNA methylase genes found in an *A. actinomycetemcomitans* isolate and the MIC of erythromycin for that isolate (data not shown).

Transfer of rRNA methylase genes. Seven isolates and three ATCC strains of *A. actinomycetemcomitans* were tested for their ability to transfer the Erm^r phenotype.

Frequencies ranged from 10^{-6} to 10^{-8} /recipient with *E. faecalis*, and from 10^{-7} to 10^{-8} /recipient with *H. influenzae*. The *H. influenzae* transconjugants had MIC assays of erythromycin between 32 - 64 $\mu\text{g/ml}$, compared with MIC 4 $\mu\text{g/ml}$ for the donor strain. The *E. faecalis* transconjugants had MIC assays of erythromycin between 32 - 256 $\mu\text{g/ml}$ compared with 1 $\mu\text{g/ml}$ for the donor strain (data not shown). The Erm C determinant was transferred in six isolates and two ATCC strains of the ten donors examined. The Erm B determinant transferred from three isolates and three ATCC strains of the seven donors. The Erm F determinant transferred from one isolate and one ATCC strain of the six donors and the Erm Q determinant transferred from one of the three donors. The Erm A determinant was not transferable from either of the two ATCC strains used as donors which were examined. Matings were done at least twice and only a portion of the transconjugants were characterized; these are described in Table 10. With the recipient JH2-2, *ermB*, *ermC* or both *ermB* and *ermC* were transferred. With the recipient Rd 8, all erythromycin resistance determinants were transferable with different combinations of determinants with the exception of *ermA*. The Tet B determinant was also transferable to the Rd 8 recipient but not JH2-2.

CHAPTER DISCUSSION

This study demonstrates that previously described rRNA methylase determinants which code for erythromycin resistance have been found in *A. actinomycetemcomitans*. The rRNA methylase genes can be transferred to both closely related *H. influenzae* (Gram-negative) and unrelated *E. faecalis* (Gram-positive) recipients. Transfer of Erm^r determinants from *Campylobacter rectus* and *Actinobacillus pleuropneumoniae* donors to *E. faecalis* JH2-2 recipient has also been described (Roe *et al.*, 1995a; Wasteson *et al.*, 1996). We found the Erm B determinant was the most common in *A.*

actinomycetemcomitans, followed by Erm C, Erm F, Erm Q and Erm A determinants. Of the seven isolates and three ATCC strains of *A. actinomycetemcomitans* donors tested, all could transfer the Erm^r phenotype. The Erm B, Erm C, Erm F, and Erm Q determinants were transferable from *A. actinomycetemcomitans* donors to the *H. influenzae* recipient in various combinations (Table 10). This data indicates that the rRNA methylase genes can be selected for in *A. actinomycetemcomitans* and are functional since the transconjugants had high MIC values to erythromycin. The Erm A determinant was not transferable in the two ATCC strains used as donors to either recipient (Table 10). This suggests that the Erm A determinant is not mobile from *A. actinomycetemcomitans* or has a frequency of transfer much lower than the other determinants. More isolates would have to be evaluated to determine the mobility or non-mobility of the Erm A determinant in *A. actinomycetemcomitans*. The Erm C and Erm B determinants were the most commonly transferred from the *A. actinomycetemcomitans* donors. The Erm F and Erm Q determinants were also transferable, but at a low frequency compared with Erm C and Erm B determinants. When evaluating the transfer of determinants from *A. actinomycetemcomitans* to *E. faecalis*, we observed only Erm B and/or Erm C determinants in the transconjugants, regardless of other determinants in the donor.

Finding erythromycin resistance determinants in *A. actinomycetemcomitans* is not unexpected since several people have previously reported MIC values of erythromycin \geq 8 μ g/ml for this species (Genco, 1981; Baker *et al.*, 1985; Pavicic *et al.*, 1992; Slots & Ram, 1990). Recently, we have found rRNA methylase determinants in *A. pleuropneumoniae* (Wasteson *et al.*, 1996). With *A. pleuropneumoniae*, Erm C was found in 74% of the isolates and Erm A was found in 31% of the isolates examined. These isolates were able to transfer the Erm C determinant or both the Erm C and Erm A determinants to the *E. faecalis* JH2-2 recipient at frequencies ranging from 10^7 - 10^9 /

recipient (Wasteson *et al.*, 1996). This differs from *A. actinomycetemcomitans* which could not transfer the Erm A determinant in similar experiments. In addition, unlike *A. actinomycetemcomitans*, *A. pleuropneumoniae* did not carry the Erm B determinant.

The transfer of conjugative transposons from Gram-negative to Gram-positive species has been described previously. The conjugative *tetM* transposons, which like the erythromycin resistance determinants are of Gram-positive origin, are found in both Gram-positive and Gram-negative bacteria (Bertram, Stratz & Durre, 1991; Mabilat & Courvalin, 1988; Roberts, 1994; Roberts & Lanciardi, 1990).

There was no correlation between the MIC value and the number of erythromycin resistance determinants present in individual *A. actinomycetemcomitans* isolates. None of the isolates evaluated would be classified as susceptibility according to the standards established by NCCLS (1993). Isolate 5004 did not hybridize with any of the five Erm probes used even though the MIC for this isolate was 8 µg/ml. This isolate could carry a new or different rRNA methylase determinant or could have a different mechanism of resistance. To better evaluate the effect of the presence of the rRNA methylase determinants in *A. actinomycetemcomitans* isolates that do not carry rRNA methylase genes should be evaluated for their susceptibility to erythromycin. With this information it might be possible to determine an appropriate breakpoint value.

Erythromycin resistance determinants have been found on both conjugative plasmids and transposons, with transposons being common in Gram-positive cocci (Leclercq & Courvalin, 1991). We did not find the Erm determinants to be associated with any detectable plasmids in the donors or erythromycin resistant transconjugants. It seems highly probable that many of these erythromycin resistance determinants are on transposable elements in the *A. actinomycetemcomitans* isolates studied.

Two isolate and one ATTC strain of the *A. actinomycetemcomitans* donors were able to transfer the *tetB* gene along with the erythromycin determinants to *H. influenzae* recipient when only erythromycin was selected. The Tet B determinant was found in 33-40% of the *H. influenzae* transconjugants tested (Table 10). The Tet B determinants in these *A. actinomycetemcomitans* strains have been shown to be associated with conjugative plasmids (Roe *et al.*, 1995a). In contrast, the Tet B determinant, which has only been found in Gram-negative bacteria (Roberts, 1994) was not found in the *E. faecalis* transconjugants. Whether the presence of the rRNA methylase determinants will influence the disease process of *A. actinomycetemcomitans* associated periodontal disease treated with the lincosamide group of antibiotics such as clindamycin needs to be examined.

Table 7. Erythromycin DNA probes used

Determinant	Plasmid	Vector	Fragment	Reference
Erm A	pEM9592	pBR328	0.7-kb <i>SspI</i>	(Murphy & Lofdahl, 1984)
Erm B	pJIR229	pUC18	0.8-kb <i>PstI-EcoRI</i>	(Berryman & Rood, 1989)
Erm C	pBR328:33RV	pBR328	0.9-kb <i>HpaI</i>	(Rinckel & Savage, 1990)
Erm FS	pFD292	pUC19	0.75-kb <i>PvuII</i>	(Smith, 1987)
Erm Q	pJIR745	pUC18	1.0-kb <i>EcoRI-HindIII</i>	(Rood & Cole, 1991)

Table 8. pH value after incubation under different air conditions

Air Condition	Media	Time			
		0	24	48	72
Ambient					
	BHI	7.0	6.9	6.9	6.7
	CMH	7.1	7.0	6.9	6.8
	WC	6.8	6.8	6.8	6.7
5% CO₂					
	BHI	7.0	6.9	6.8	6.8
	CMH	7.1	6.8	6.8	6.9
	WC	6.8	6.9	6.8	6.8
Anaerobic					
	BHI	7.0	7.0	6.9	6.9
	CMH	7.1	7.0	7.0	7.0
	WC	6.8	6.9	6.9	6.8

Table 9. Comparison of MIC and gene detection for erythromycin in *A. actinomycetemcomitans*

Isolate Number	MIC ($\mu\text{g/ml}$)		Tet B ^a probe	Hybridization with Erm ^r determinant	Species of Transconjugants	Phenotype Transferred ^c
	CO ₂	Am ^b				
1011	16	16	+	B,C,F	<i>H. influenzae</i>	Erm ^r
1014	8	8	+	A,B,C,F		
1528	4	4	+	F		
2102	8	16	+	B,C,F,Q	<i>H. influenzae</i>	Erm ^r , Tet ^r
2201	4	16	+	A,B,Q		
2205	8	16	+	B,F		
2602	8	16	-	Q		
2608	8	16	+	C,F		
4001	2	16	+	C,Q	<i>H. influenzae</i>	Erm ^r
5002	8	16	-	B,C		
5004	8	16	-	None		
5601	16	16	-	B,C,F		
5901	32	16	+	B		

Table 9. Continued

6001	32	16	+	B,Q		
7601	4	16	+	C,F	<i>H. influenzae</i>	Erm ^r , Tet ^r
7902	4	8	+	B,C		
7908	16	16	-	B	<i>E. faecalis</i>	Erm ^r
7920	4	8	-	B,C,F		
8202	8	16	+	B,C,Q	<i>E. faecalis</i>	Erm ^r
9002	16	16	+	C	<i>E. faecalis</i>	Erm ^r
9501	4	2	-	B,F		
ATTC 43717	4	16	+	B,C,F	<i>H. influenzae</i>	Erm ^r
ATTC 43718	16	16	+	A,B,C,F	<i>H. influenzae</i>	Erm ^r , Tet ^r
ATTC 43719	8	16	+	A,B,C,F	<i>E. faecalis</i>	Erm ^r
ATTC 33384	16	16	-	B,C,F		

^a The Tet B data was taken from Roe *et al.*, 1995

^b Am = Ambient

^c Tet^r was found in the Rd8 recipients, Tet^r: tetracycline resistance; Erm^r: erythromycin resistance

Table 10. Distribution of Erm^r and Tet^r determinants in representative transconjugants from different experiments

Recipient	Donor	Donor Erm ^r Determinants	Number of Transconjugants Tested	Determinants in Transconjugants	
				Erm	Tet
JH2-2					
	7908	B	1	B	
	8202	B,C,Q	6	B,C	
	9002	C	4	C	
	ATCC 43719	A,B,C,F	6	B	
Rd8					
	1011	B,C,F	2	B	
	2102	B,C,F,Q	2	F	
			1	F	B
			1	B,F	B
			1	C,F,Q	
			1	B,C,F,Q	

Table 10. Continued

4001	C,Q	3	Q	
		4	C,Q	
7601	C,F	2	C	
		2	C	B
ATCC 43717	B,C,F	1	C	
		4	F	
		2	B,C,F	
ATCC 43718	A,B,C,F	2	B	
		1	C	
		2	B,C	B

CHAPTER 4

Macrolide resistance in *Campylobacter rectus*

BACKGROUND

Campylobacter (Wolinella) rectus is a Gram-negative bacteria with a monopolar flagellum. Traditionally, *C. rectus* has been considered to be an anaerobic bacterium. However, a recent study found that the ATCC 33238 isolate was able to grow in the presence of 2% CO₂ suggesting that *C. rectus* may be a microaerophilic bacterium rather than a strict anaerobe (Han *et al.*, 1991). *C. rectus* is a presumptive periodontal pathogen and is associated with adult and rapidly progressive periodontitis. Antibiotics are sometimes used as an adjunct to standard therapy. Erythromycin is used for the treatment of dental abscess and clindamycin is used as an adjunct to therapy for treatment of severe periodontitis.

There are a number of characterized mechanisms which confer resistance to erythromycin, of which rRNA methylases are the most common. The rRNA methylase determinants code for enzymes which methylate a single adenine in the 23S component of the 50S ribosomal subunit. This modification results in the inability of macrolides, lincosamides, and streptogramin B to bind to the modified ribosome (Weisblum, 1995a).

The aim of this project was to determine whether previously described rRNA methylase determinants were present in *C. rectus* isolates from periodontal patients using DNA probes. The mobility of these determinants was also evaluated using conjugal matings.

MATERIAL AND METHODS

Bacterial Isolates and Media. Eighteen *C. rectus* isolates from periodontal patients collected in Seattle, WA from 1986 through 1993 and the type strain ATCC 32238 from

the American Type Culture Collection, (Rockville, MD) were examined. Isolates were maintained on formate/fumerate supplemented Brain Heart Infusion (Difco Laboratories, Detroit, MI) as previously described (Tanner, 1987). Growth conditions were at 36.5°C , for 72 hrs. in an anaerobic jar (85% N₂, 15% CO₂). Mating experiment used Columbia Base Agar (Difco Laboratories, Detroit, MI)(CBA) supplemented with 5% sheep blood. Isolates were biochemically defined and verified by SDS-PAGE (Borinski & Holt, 1990). All isolates, except one, showed typical SDS-PAGE profiles for *C. rectus* but had differences indicating that the isolates did not represent a clonal population (data not shown).

Minimum Inhibitory Concentration determination. The agar dilution method was used for the determination of MIC values. The media used for MIC determination was Mueller Hinton Agar supplemented with 5% sheep's blood and formate and fumerate, as described above. Bacteria were suspended in 0.84% NaCl to a 0.5 McFarland standard. Following the National Committee on Clinical Laboratory Standards (1993) guidelines, this suspension (10⁵ cfu/spot) was used to load the a Steers replicator which was used to inoculate all plates. Erythromycin concentrations ranged from 0.5 - 64 µg/ml. Following the NCCLS guidelines, plates were inoculated and incubated at 36.5°C in anaerobic conditions (5% CO₂, 10% H₂ and 85% N₂) and read at 48 hrs. (NCCLS, 1993).

The MIC assays, for testing *Enterococcus faecalis* transconjugants, used Blood Mueller Hinton medium (Difco Laboratories, Detroit, MI) following standard procedures (NCCLS, 1993). All agar dilution MIC plates were incubated at 36.5°C in ambient conditions and read at 24 hrs. (NCCLS, 1993).

DNA Isolation. DNA was extracted from *C. rectus* isolates following a protocol developed for *Mobiluncus* spp. (Spiegel & Roberts, 1984). The protocol was modified by doing only one phenol-chloroform extractions. After one ethanol precipitation instead of two, the DNA pellets were resuspended in 400 μ l TE buffer (10 mM Tris-HCl, 1mM EDTA, pH 8.1) to which 400 mg RNase was added followed by incubation at room temperature for 15 min. DNA from *E. faecalis* transconjugants was extracted as previously described (LeBlanc & Lee, 1979).

Hybridization. Southern blots of uncut DNA from *C. rectus* isolates were hybridized with each of the following intragenic probes: *ermA*, pEM9592, 0.7 kb *SspI*; *ermB*, pJIR229, 0.8 kb *PstI-EcoRI*; *ermC (ermAM)* pBR328:33RV, 0.9 kb *HpaI*; *ermF*, pFD292, 0.45 kb *EcoRI-HindIII*; *ermQ*, pJIR745, 0.43 kb *EcoRI-HindIII*. Hybridization used stringent conditions with either 32 P labeled probes (Roberts & Lansciardi, 1990; Roe *et al.*, 1995a) or random-primed DIG-11-dUTP labeled probes which were detected using an anti-DIG-alkaline phosphatase chemiluminescent substrate (GeniusTM 3 kit, Boehringer Mannheim Biochemica, Indianapolis IN) as described by Roe *et al.* (1995a).

Mating Experiments. Seven *C. rectus* isolates were used as donors to evaluate the mobility of the rRNA methylase determinants. The isolates carried between one and four rRNA methylase genes (Table 11). The *C. rectus* donors were grown on four plates for four days, the growth on all plates were resuspended in Brain Heart Infusion broth (Difco Laboratories, Detroit, MI), while the *E. faecalis* recipient, JH2-2, was grown in GC broth (Difco Laboratories, Detroit, MI) overnight. Equal volumes of the donor and recipient were mixed, plated on CBA plates and incubated for 48 hrs. anaerobically at 36.5 $^{\circ}$ C (Roberts and Lansciardi, 1990). DNase at 50 μ g/ml was added to some of the matings to

verify the transfer of DNA was by conjugation. *E. faecalis* transconjugants were selected on CBA plates supplemented with 10 µg/ml of erythromycin and grown in 5% CO₂. These environmental conditions do not support the growth of *C. rectus*. Transconjugants were confirmed as *E. faecalis* using whole chromosomal DNA probes as previously described (Roberts & Lansciardi, 1990). Southern blot of uncut whole cell DNA was hybridized with the various rRNA methylase probes to identify which genes were present in the transconjugants.

RESULTS

Erythromycin Susceptibility Testing. Seventeen (76%) of the 18 isolates had MIC values of 0.5 - 1 µg/ml for erythromycin (Table 11). One *C. rectus* isolate, EC5-28 had an MIC value of 16 µg/ml for erythromycin (Table 11). Poor growth of all isolates was observed under standard MIC conditions. Results from MIC assays had poor reproducibility between experiments.

Identification of *erm* genes. Seventeen (76%) of the 18 isolates and the one ATCC isolate hybridized with one or more of the five rRNA methylase probes examined (Table 11). Two isolates hybridized with *ermB*, four with *ermC*, including the ATCC isolate, three with *ermQ*, three with *ermB* and *ermC*, one with *ermB*, *ermC* and *ermF*, one with *ermB*, *ermC*, and *ermQ*, two with *ermB*, *ermF*, and *ermQ*, one with *ermB*, *ermC*, *ermF*, and *ermQ* and none with *ermA* (Table 11). All determinants hybridized with the chromosome on Southern blots.

Transfer of rRNA methylase determinants. Six *C. rectus* isolates and the one ATCC strain were used to evaluate the mobility of the erythromycin resistance phenotype.

Erythromycin resistance was transferred at frequencies of 1×10^{-7} to 6×10^{-8} per recipient to *E. faecalis* from all seven *C. rectus* donors. The frequency of transfer did not differ when the matings were performed with or without DNase (data not shown). The minimum inhibitory concentration (MIC) for the *E. faecalis* transconjugants were ≥ 128 $\mu\text{g/ml}$ of erythromycin as compared to the original recipient MIC value of 1 $\mu\text{g/ml}$ of erythromycin using standard conditions (data not shown)(Roe *et al.*, 1995).

The Erm C determinant transferred from all six isolates and the one ATCC strain that were used as donors, while the Erm B determinant transferred from four of five isolates that were used as donors (Table 12). The Erm Q determinant transferred from the one donor examined, but the Erm F determinant did not transfer from either of the two donors examined (Table 12). In donor 30428 matings, only the Erm C determinant transferred in the 12 transconjugants examined. In contrast, donors 30442 and 6711 could transfer both Erm B and Erm C determinants. Mating experiments using donor 30442 yielded, 50% of the transconjugants carrying the Erm B and 50% carrying both Erm B and Erm C determinants. With donor 6711, 12.5% of the transconjugants carried the Erm B, 50% carried the Erm C and 37.5% carried both Erm B and Erm C determinants. With donor MP4-EO5, all six transconjugants carried both the Erm B and Erm C determinants, while with the EC5-4 donor the Erm B determinant was found in 14%, the Erm C in 43% and Erm C and Erm Q in 43% of the transconjugants.

CHAPTER DISCUSSION

When trying to determine the susceptibility of *C. rectus* to erythromycin, it was observed that *C. rectus* grew very poorly under standard MIC conditions. Even though MIC values are reported, they had very poor reproducibility between assays. During a given MIC assay, it was common to observe no growth with several isolates. This

suggests that the MIC conditions used in this study were not optimal for *C. rectus*. Others have also reported difficulty in reproducibility of susceptibility assays with fastidious anaerobes (Thornsberry, 1990).

This study found the rRNA methylase determinants to be present in *C. rectus*. The erythromycin resistance phenotype was transferable from all nine donors evaluated. The rRNA methylase determinants Erm B, C, and Q were mobile with various frequencies depending on the donor *C. rectus* isolate used. The Erm C determinant transferred at the highest frequency of all the rRNA methylase determinants and was transferable from all the donors examined (Table 12). The *ermB* and *ermQ* genes were carried in fewer transconjugants than the *ermC* gene suggesting the frequencies of transfer for the *ermB* and *ermQ* genes might be lower than the transfer frequency for the *ermC* gene. The various rRNA methylase determinants were found to transfer both alone and in pairs from the donor to the transconjugants. Two of the donors (MP4-E05, EC5-4) carried more than two rRNA methylase determinants however, none of the transconjugants carried more than two of the determinants. Whether the Erm F determinant does not transfer from *C. rectus* in general, if this is unique to these two donors, or whether the transfer frequency is significantly lower than transfer of the other Erm determinants and therefore harder to detect is not clear. More studies will be needed to distinguish between these possibilities. It appears that the *ermB* and *ermC* are able to transfer independently in most of the donors. Further studies are need to determine if the rRNA methylase determinants in isolates 30442, MP4-E05 and EC5-4 are able to transfer the *ermB*, *ermC* and *ermQ* independently or if the frequency of transfer is similar for the determinants in these donors.

The ability to move the rRNA methylase determinants suggests they are associated with conjugal elements. Many rRNA methylase genes are associated with

conjugative transposons and conjugative plasmids have been described in *Campylobacter*. The transfer of rRNA methylase determinants occurred at frequencies similar to what we have previously reported for transfer of a tetracycline resistance determinant, Tet M, between a Gram-negative donor and JH2-2 recipient (Roberts & Lanscairdi, 1990). Whether the *C. rectus* can transfer these determinants to other periodontally associated bacteria is of interest.

It has yet to be determined whether the presence of rRNA methylase determinants in *C. rectus* is unique to the Seattle population. However, the ATCC strain which was isolated in 1981 and was not from Seattle carried the Erm C determinant suggesting that this may not be a local phenomenon. In the isolates studied it appears that the rRNA methylase genes are associated with the chromosome. The Erm A determinant was not found in any of the 19 isolates examined. This study broadens the host range for four of the Erm rRNA methylase determinants (*ermB*, *ermC*, *ermF*, and *ermQ*). Based on the high MIC values of the rRNA methylase determinants the *E. faecalis* transconjugants it is apparent that these genes are functioning in the transconjugants and can be selected for through conjugation.

Erythromycin resistance has been reported for *Campylobacter jejuni* and *Campylobacter coli* (Yan & Taylor, 1991). The resistance mechanism for three *C. jejuni* isolates and one *C. coli* isolate was determined to be constitutive and chromosomally mediated (Yan & Taylor, 1991). A second study failed to show the presence of either the Erm B or Erm C determinant in 29 erythromycin-resistant isolates of *C. jejuni* or *C. coli* (Ryan & Tenover, 1988). Even though erythromycin resistance has been known to be present in the genus *Campylobacter*, none of this resistance has been reported to be due to the presence of rRNA methylase determinants.

This is the first time within the genus *Campylobacter* that a known rRNA methylase determinant has been found and this finding increases the distribution of these genes. However, it is still unknown what effect the expression of these genes will have on the treatment of periodontal disease.

Table 11. Susceptibility to erythromycin and distribution of rRNA methylase determinants in *C. rectus*

Isolate	MIC µg/ml	Erm A	Erm B	Erm C	Erm F	Erm Q
MP4-D06	1	-	-	-	-	+
MP4-E05 ^b	1	-	+	+	+	-
MP4-K05	1	-	-	-	-	+
MP4-J04	0.5	-	-	-	-	-
MP4-M06	1	-	+	-	+	+
MP4-S05	0.5	-	+	-	+	+
EC5-4 ^b	ND ^c	-	+	+	+	+
EC5-28	16	-	+	+	-	+
EC2-14	ND	-	-	-	-	-
5216	1	-	-	-	-	+
6711 ^b	1	-	+	+	-	-
30442 ^{a,b}	1	-	+	+	-	-
30428 ^{a,b}	1	-	+	+	-	-
30443 ^a	1	-	+	-	-	-

Table 11. Continued

30444 ^a	1	-	+	-	-	-
31514 ^b	ND	-	-	+	-	-
31517	1	-	-	+	-	-
31226	1	-	-	+	-	-
ATCC33238 ^b	ND	-	-	+	-	-

^a Isolates from the same patient

^b Isolates used as donors

^c ND = Not Done

All MP4 isolates are from different patients

Table 12. Erythromycin resistance transfer to *E. faecalis* transconjugants

Donor	Donor Determinants	Number of Transconjugants Tested	Determinant Transferred
30442	Erm B, C	2	Erm B
		2	Erm B, Erm C
30428	Erm B, C	12	Erm C
31514	Erm C	6	Erm C
ATTC 33238	Erm C	6	Erm C
6711	Erm B, C	1	Erm B
		4	Erm C
		3	Erm B, C
MP4-E05	Erm B, C, F	6	Erm B, C
EC5-4	Erm B, C, F, Q	1	Erm B
		3	Erm C
		3	Erm C, Q

CHAPTER 5

Tetracycline resistance in *Treponema denticola*.

BACKGROUND

Members of the subgingival anaerobic microbial flora contribute to the progression of periodontal disease (Page, 1986). Treponemes are normally found in very low numbers in the oral cavity of periodontally healthy individuals, but increase dramatically in diseased periodontal pockets (Armitage *et al.*, 1982; Simonson *et al.*, 1988).

Tetracycline and its derivatives are commonly used as an adjunct in the treatment of periodontitis (Genco, 1991; Slots & Rams, 1990). Tetracycline inhibits bacterial growth by interacting with the 30S ribosomal subunit which inhibits bacterial protein synthesis (Roberts, 1994). At least seventeen different tetracycline (Tet) resistance determinants have been characterized to date (Roberts, 1994). These determinants encode one of three mechanisms of resistance; efflux, ribosomal protection and enzymatic inactivation (Olsvik & Tenover, 1993; Roberts, 1994).

Recently we have shown that *Actinobacillus actinomycetemcomitans*, another periodontal pathogen, carries the determinant on a conjugative plasmid (Roe *et al.*, 1995a). This prompted further investigations of other periodontal pathogens. We found that a mixture of oral treponemes isolated from a periodontal patient could grow in the presence of 2 µg/ml tetracycline (peak levels in the sulcus for tetracycline are 4 µg/ml). Therefore, it was of interest to determine if these *Treponema denticola* isolates as well as isolates from other periodontal patients carried any of the known Tet determinants. In this study, we examined sixteen *T. denticola* isolates for the presence of known tet determinants. These isolates come from five different geographical locations and were isolated between 1980 and 1995. We present, for the first time, information

demonstrating the presence of a previously described tetracycline resistance determinant in the genus *Treponema*.

MATERIALS AND METHODS

Bacterial Isolates and growth conditions. Four ATCC isolates (35404, 35405, 33520, 33521) were obtained from the American Type Culture Collection (Rockville, MD) were also included. ATCC strains 35404 and 35405 were isolated in Canada in 1985 and ATCC strains 33520 and 33521 were isolated in Maryland in 1980. Clinical isolate GM-1 came from Micheal Sela, Hebrew University, Jerusalem, Israel, the E and E' came from Richard Ellen, University of Toronto, Toronto, Canada and ST10 came from Lloyd Simonson, Naval Dental Research Center, Illinois. All isolates were from periodontally diseased sites. We evaluated four isolates from one periodontal patients, and four mixtures of treponemes from four additional periodontal patients. Patient isolates were collected between 1991-1995, from five patients seen in the Graduate Periodontal Clinic at the University of Washington in Seattle, Washington. *T. denticola* was isolated from representative subgingival plaque samples as previously described (Leschine & Canale-Parola, 1980; Sela *et al.*, 1988). All routine growth of treponemes was done with supplemented trypticase Soy media, GM-1 media, in an anaerobic chamber with a gas mixture of 5% CO₂, 10% H₂, and 85% N₂, as previously described (Blakemore & Canale-Parola, 1976). *Actinobacillus actinomycetemcomitans*, *Haemophilus influenzae* and *Escherichia coli* were grown on 3.5% Brain Heart Infusion Agar supplemented with 10 µg/ml of hemin, 10 µg/ml of L-histidine, and 2 µg/ml of nicotinamide adenine dinucleotide (Difco Laboratories, Detroit, MI). The *Campylobacter rectus* was grown on formate/fumerate supplemented Brucella Base Blood Agar Media (Difco Laboratories, Detroit, MI), as previously described by Tanner (1987). For matings, agar media was

supplemented with one or more of the following antibiotics; rifampin (10 or 25 $\mu\text{g/ml}$) streptomycin (50 or 250 $\mu\text{g/ml}$), and/or tetracycline (10 $\mu\text{g/ml}$) as needed.

DNA Extraction. Each isolate passed into larger volumes until growth was achieved in 500 ml of GM-1 media (Blakemore & Canale-Parola, 1976) for 4 to 6 days in the anaerobic chamber. The bacteria were harvested by centrifugation at 14,000 X g at 4°C for 15 min. The bacterial pellets were resuspended in phosphate buffered saline (0.120 M NaCl, 8.1 mM $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 1.5 mM KH_2PO_4 , 2.7 mM KCl, pH 7.4), centrifuged at 14,000 X g at 4°C for 15 min and then stored frozen at -20 °C. The pellets were resuspended in lysing buffer (25% RNase free sucrose, 50 mM Tris, 1 mM EDTA, pH 8.0) to which 1.6 mg/ml lysozyme (0.25 Tris pH 8.0) was added. The suspension was incubated on ice for 15 min after which a 1/6 volume of Triton-X 100 solution (1.0% Triton X, 60 mM Na_2EDTA , 50 mM Tris, pH 8.0) was added. The suspension was mixed, and incubated at 55 °C for one hr. Equal volume of distilled water and 0.21 mg Proteinase K (Sigma Chemical Co., St. Louis, MO) were added and incubated at 37°C for 2 hrs. The samples were extracted with an equal volume of phenol followed by extraction with an equal volume of chloroform, and then the DNA was ethanol precipitated. The DNA pellets were resuspended in 100 μl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.1) to which 100 mg RNase was added followed by incubation at room temperature for 15 min. The DNA was then stored at -20°C until needed.

Hybridization. Oligonucleotide probes were used for hybridization of Southern blots of *T. denticola* DNA. These probes used are listed in Table 13. All oligonucleotide probes were labeled using the nonradioactive Genius™ kit labeling system (Boehringer Mannheim Biochemica, Indianapolis IN) following manufacturer's instructions.

Hybridizations were done under stringent conditions and all wash temperatures were 50°C below the melting temperature of the oligonucleotide probe. Detection followed manufacturer's instructions as previously described by Roe *et al.* (1995a).

A Tet B plasmid probe was used to confirm the presence of the Tet B determinant in PCR gels (Roe *et al.*, 1995a). This probe was also labeled using the nonradioactive Genius™ kit labeling system (Boehringer Mannheim Biochemica, Indianapolis IN) following manufacturer's instructions. These were used to confirm the presence of the Tet B PCR product from the PCR assay as previously described (Pang *et al.*, 1994; Roberts *et al.*, 1993). Hybridizations were done under stringent conditions and detection followed manufacturer's instructions.

Growth in Tetracycline supplemented Media. The treponemes were passaged twice after 4 days of growth in GM-1 media supplemented with 10 µg/ml of Rifampicin after initial isolation. They were then diluted 1:5 into fresh GM-1 media supplemented with tetracycline and without tetracycline. Tetracycline concentrations were 0.5, 1, 2 and 4 µg/ml. Growth was determined by visual turbidity, the optical density recorded and by darkfield microscopy. No standard protocol exists for determining susceptibility for *T. denticola* or spirochetes in general (National Committee for Clinical Laboratory Standards, 1993).

Polymerase Chain Reaction. Ten to 50 ng of DNA or 1 µl of a proteinase K treated whole bacterial sample was used as a template for the Tet B, Tet M/O/S and Tet K/L assay as previously described (Pang *et al.*, 1994, Roberts *et al.*, 1993, Roe *et al.*, 1995a). Proteinase K treated samples were prepared as previously described (Roberts *et al.*, 1993). Both positive and negative controls were included with each reaction. The PCR

products were run on a 1.5% agarose gel stained with ethidium bromide and visualized. Southern blots were made and hybridized to confirm positive PCR products.

Sequencing. The Tet B PCR product from a representative Tet B positive isolate, 304-2, was sequenced. The PCR product was extracted from the agarose gel using the Qiagen DNA extraction kit (Qiagen, Chatsworth, CA). This DNA was used in the Taq Dye DeoxyTM terminator cycle sequencing kit (Applied Biosystems, Foster City, California) and the product was read on a model 373A sequencer (Applied Biosystems) as previously described (Pang *et al.*, 1994). The PCR generated Tet B sequence was compared with Tn10 (GenBank accession number V00611) using FASTA from Genetics Computer Group (GCG Inc., Madison, WI).

Mating Experiments. *A. actinomycetemcomitans* 9501 resistant to rifampin and streptomycin (Roe *et al.*, 1995a), *H. influenzae* Rd 8 resistant to rifampin and streptomycin (Roe *et al.*, 1995a), *E. coli* HB101 resistant to streptomycin, and *C. rectus*, ATCC strain 33238 (Roe *et al.*, 1995b) were chosen as recipients for mating experiments with tetracycline resistant *T. denticola*. Both the clinical isolate 304-12 and the ATCC strain 33520 isolate were used as donors. All recipients were grown on Brain Heart Infusion Agar (Difco, Laboratories, Detroit, MI) except *C. rectus* which was grown on Brucella Base Blood Agar Media (Difco, Laboratories, Detroit, MI) and the *T. denticola* isolates which were grown in GM-1 media (Blakemore & Canale-Parola, 1976). Donor and recipient isolates were grown separately to a density of approximately 10^8 organisms/ml. A 1:1, 1:3, 1:5 ratio of recipient to donor volumes were mixed, spun at 9,000 X g for 10 min, resuspended in new GM-1 medium to produce clumps rather than a disperse suspension. This was then incubated for 48 hrs at 36.5°C in anaerobic chamber.

Selection of transconjugants was done on 4 and 8 $\mu\text{g/ml}$ of tetracycline in 5% CO_2 with the exception of the *C. rectus* matings, which were plated under anaerobic conditions. The number of recipients was determined as was the viability of the *T. denticola* donor in each mating experiment. *T. denticola* donors were examined under darkfield microscopy to examine mobility which occurs only with viable cells. Since *T. denticola* is extremely fastidious, growing only in anaerobic conditions and do not grow well on solid media, these two characteristics were used to select for the recipients. All mating experiments were successfully completed at least four times.

RESULTS

Identification of Tetracycline resistance. We examined a fresh mixed culture of oral treponemes isolated from periodontal patient 304 and found that they could grow in the presence of a maximum concentration of 2 $\mu\text{g/ml}$ tetracycline. Peak levels in the sulcus are typically 5 times blood levels after a single dose of tetracycline (Gordon *et al.*, 1981) and ≥ 4 $\mu\text{g/ml}$ tetracycline is typically found. We also found that a number of periodontal pathogens and commensal species from patient 304 were resistant to tetracycline (data not shown). Therefore, we wondered if the oral treponemes from this patient might carry know Tet determinants. We separated individual *T. denticola* isolates from the mixture and DNA was prepared from four selected isolates (304-2, 304-4, 304-10, 304-12). Southern blots of *T. denticola* DNA were prepared and hybridized with oligonucleotide probes which detect Tet B, Tet M, O and S. All four of the isolates hybridized with the Tet B probe in the area of the chromosome (Figure 4). To verify the presence of this determinant we performed PCR on the four isolates and found that all four isolates gave PCR products of 528 bp which specifically hybridized with the Tet B probe (Figure 5). No PCR products were seen with the Tet M/O/S PCR assay.

We selected one of the clinical isolates, 304-2, for sequencing the PCR product. Over the 270 bp which were sequenced from the PCR product there was 90% DNA sequence identity between the *T. denticola* PCR product and the *tetB* gene (Figure 6). Only the *tetB* gene from *Tn10* has been sequenced. Therefore, it is not clear what amount of DNA sequence variability occurs in different *tetB* genes. However, different *tet* genes have been distinguished from each other by their inability to cross-hybridize with other genes under stringent conditions (Levy *et al.*, 1989; Roberts, 1994; Roberts, 1996). Genes which do not cross-hybridize but are related such as *tetM*, *tetO*, and *tetS* can share 75% DNA sequence homology and similar amino acid sequence homology (Roberts, 1994). In contrast, seven different *tetM* genes have been sequenced from a diverse group of species. These sequences can be found in GenBank and have been shown to share $\geq 90\%$ DNA sequence homology (Roberts, 1996, unpublished observations).

Since the four positive isolates came from a single periodontal patient we wanted to examine other isolates, as well as isolates from different locations to look at the distribution of the Tet B determinant in the *T. denticola* population. We examined four ATCC isolates, four isolates from other geographical locations (GM-1, E, E', and ST-10) and four mixtures of oral treponemes from other Seattle patients. Plasmids were found in ATCC 33520, as previously described (Listgarten *et al.*, 1993; MacDougall *et al.*, 1992), but not seen in any of the other isolates (Figure 4). Southern blots of whole cell DNA was hybridized with the labeled oligonucleotide probes which detect the various Tet determinants listed in Table 13. Isolates GM-1, ATCC 35405 and ATCC 33520 were positive for the Tet B determinant but not with the other Tet probes used (Table 14). All Tet B positive isolates, including ATCC 33520, hybridization in the chromosomal region of the Southern blot (Figure 4). Isolates were reconfirmed as carrying the Tet B

determinant using the PCR assay. Those strains negative for *tetB* by Southern blot hybridization were also negative by PCR assay (data not shown). None of the isolates were positive by Southern or PCR for *tetM*, *tetO* or *tetS*.

We examined whether we could show a correlation between the presence of the *tetB* gene and the ability to grow in the presence of tetracycline broth. We compared the isolates from patient 304 with that of ATCC 35404 which did not carry any of the *tet* genes tested. All the isolates had been stored in liquid nitrogen for extended time periods. Isolates were placed in broth with varying concentrations of tetracycline (0.5-4 µg/ml) and the growth was visualized and the optical density recorded for seven days. No reproducible differences were found. To account for inducible regulation of most *tetB* genes (Roberts, 1996) we pregrew the isolates in 0.1 µg/ml of tetracycline for 24 hrs prior to growth in tetracycline supplemented media. There was a trend for the clinical isolate to grow better in the tetracycline supplemented broth than ATCC 35404 but the differences were not reproducible.

Mating experiments. We used two different *T. denticola* isolates as donors and *A. actinomycetemcomitans*, *H. influenzae*, *E. coli*, and *C. rectus* as recipients. The *H. influenzae* recipients died in the presence of the *T. denticola* donor though by itself it was able to survive 48 hrs under anaerobic conditions. The other recipients did survive in the presence of the *T. denticola*. However, we were unable to show transfer of the *tetB* gene from the two *tetB* gene carrying *T. denticola* isolates (304-2, and ATCC 33520) into any recipient after multiple experiments.

CHAPTER DISCUSSION

In this report, we examined 12 isolates and four ATCC strains of *T. denticola* for the presence of known Tet determinants. Five isolates (42%) and 2 ATCC strain (50%) carried the Tet B determinant and seven isolates (58%) and two ATTC strains (50%) carried none of the Tet determinants examined in this study (Table 14). These isolates came from a variety of different geographic locations and were isolated over 15 years. This suggests that the *tetB* genes are widely distributed in the *T. denticola* population and have been in *T. denticola* for at least 16 years. The Tet B determinant appeared to be located in the chromosome. In other species, the Tet B determinant has been found in the chromosome (Roberts, 1994). However, we were not able to move the *tetB* gene from any of the isolates examined.

We could not establish a reproducible correlation between *T. denticola*'s ability to grow in tetracycline supplemented media and the presence of the *tetB* gene. However, we have found similar lack of correlation between minimum inhibitory concentration assays for tetracycline and the presence of the *tetB* gene in *A. actinomycetemcomitans* (Roe, 1995a). Others have also noted the problems with the lack of reproducibility of susceptibility testing with anaerobes, especially those which are fastidious like *T. denticola* (Thomsberry, 1990). Without a susceptibility assay, it is not possible to determine or predict the affect, if any, the presence of the Tet B determinant might have in the treatment of disease with *T. denticola* isolates carrying this determinant.

Prior to this study Stapleton, Stamm and Bassford (1985) published a review on treponemal diseases and suggested that antibiotic resistance may be the cause for treatment failures in *T. pallidum* and *T. pertenue* associated disease. In another study with *Treponema hyodysenteriae*, an intestinal treponeme, four (12.5%) of 32 isolates were found to be resistant to penicillin and produced β -lactamases (Tompkins *et al.*,

1987). None of the penicillin resistant isolates carried plasmids, and the β -lactamases genes were not mobile (Tompkins *et al.*, 1987).

The ATTC 33520 isolate has a cryptic 2.6 kb plasmid pTD1, but the Tet B probe did not hybridize with the plasmid. Since the plasmid has been sequenced and not found to carry the Tet B determinant our results correlate with sequence data (MacDougall *et al.*, 1992; MacDougall & Daint Giron, 1995).

The data presented here extends the host range of the *tetB* gene into spirochetes. Previously, the Tet B determinant has been found in the periopathogen *A. actinomycetemcomitans*, *Haemophilus ssp.* and *Moraxella catarrhalis*, as well as many enteric species (Roberts, 1994; Roberts, 1996; Roberts *et al.*, 1991; Roe, 1995a). However, this is the first time that the Tet B determinant has been described in a strict anaerobic bacteria. The *tetB* gene could provide a usable antibiotic resistant marker for *T. denticola* genetics and perhaps other spirochetes.

Table 13. Tet resistance Oligonucleotide Probes

Tet B	TB1 - 5' CAG TGC TGT TGT TGT CAT TAA 3'	(Kariuki <i>et al.</i> , 1992)
	TB2 - 5' GCT TGG AAT ACT GAG TGT AA 3'	(Roe <i>et al.</i> , 1995)
Tet M/O/S	M6 - 5' GTT TAT CAC GGA AGT GCA A 3'	(Roberts <i>et al.</i> , 1993)
	C T	
	M4 - 5' GGA GCC CAG AAA GGA TTC GG 3'	(Roberts <i>et al.</i> , 1993)
	T	
Tet K/L	TL32 - 5' CCT GTT CCC TCT GAT AAA 3'	(Pang <i>et al.</i> , 1994)
	TL2 - 5' CAA ACT GGG TGA ACA CAG 3'	(Pang <i>et al.</i> , 1994)
	A T	

**Table 14. The distribution of tetracycline
determinants in *Treponema denticola***

Isolate	Tet B determinant
304-2 ^a	+
304-4	+
304-10	+
304-12	+
313	-
314	-
330	-
331	-
GM-1	+
E	-
E'	-
ST10	-
ATCC 35404	-
ATCC 35405	+
ATCC 33520	+

Table 14. Continued

ATCC 33521

-

^a Tet B gene has been sequenced

304-2, 304-4, 304-10, 304-12 were isolated from the same patient which we demonstrated as a mixture were able to grow in 2 µg/ml of tetracycline. Determined by Southern of purified DNA and verified by PCR.

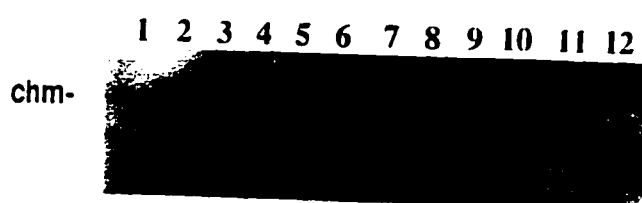


Figure 4. Southern blots of a 0.7% agarose gel of whole-cell DNA which had been hybridized with labeled Tet B oligonucleotide probes. Lane 1, 304-2; lane 2, 304-4; lane 3, 304-10; lane 4, 304-12; lane 5 and 6, ATCC 35405; lane 7 ATCC 33520; lane 8, ATCC 33521; lane 9, blank; lane 10, pUC13; lane 11, blank; lane 12, pRT11 with cloned *tetB* gene. Chm, chromosomal region.

1 2 3

-528

Figure 5. Southern blot of an agarose gel of amplification products from Tet B PCR assay hybridized with the labeled Tet B probe. Lane 1, ATCC 33520; lane 2, 304-2; lane 3, pRT11 with cloned *tetB* gene. Base pairs are indicated on the right.

CHAPTER 6

Tetracycline and erythromycin resistance in oral commensal *Neisseria*

BACKGROUND

The members of the genus *Neisseria* are Gram-negative diplococcus which include, commensal species such as *Neisseria perflava/sicca* and *Neisseria mucosa*, as well as pathogens such as *Neisseria gonorrhoeae* and *Neisseria meningitidis*. The commensal *Neisseria* spp. are common members of the human normal flora, some of which colonize the oral cavity. There have been very few studies that have evaluated the impact of antibiotic therapy on the normal flora, and even less with regards to the impact on the commensal *Neisseria* spp. In contrast, much research has been done with acquisition of antibiotic resistance by *N. gonorrhoeae* (Morse *et al.*, 1986; Morse *et al.*, 1988; Roberts & Knapp, 1988; Roberts *et al.*, 1977).

The first β -lactamase plasmids, conferring resistance to penicillin, were identified in *N. gonorrhoeae* isolates in the 1970's (Elwell *et al.*, 1977). This finding led to a change in the recommended first line therapy antibiotic for the treatment of gonorrhoea from penicillin to tetracycline (Elwell *et al.*, 1977). When this change was made, resistance soon emerged to high level of tetracycline (16 to 64 $\mu\text{g/ml}$) (Morse *et al.*, 1986; Wilson *et al.*, 1976). This tetracycline resistance was due to the insertion of the Tet M determinant into the 24.5 MDa conjugative plasmid commonly found in *N. gonorrhoeae* to create a 25.2 MDa conjugative plasmid. The Tet M transposon was deleted in the process (Morse *et al.*, 1986; Wilson *et al.*, 1976). The Tet M determinant

codes for a protein that confers tetracycline resistance by protecting the ribosome from the action of tetracycline. Knapp *et al.* (1988) reported the presence of similar 25.2 MDa Tet M determinant carrying plasmids in *N. meningitidis*, *Kingella denitrificans* and *Eikenella corrodens*. These isolates came from oropharyngeal samples of patients attending the DeKalb County Sexually Transmitted Disease Clinic. This study also reported the presence of the Tet M determinant in oral *N. perflava/sicca* and *Neisseria mucosa*. However, the *N. perflava/sicca* and *N. mucosa* were shown to carry incomplete Tet M determinants in their chromosome (Knapp *et al.*, 1988) and thus were not movable by conjugation. Based on this information, the authors suggest that the commensal *Neisseria* spp. most likely did not acquire the Tet M determinant from *N. gonorrhoeae* but rather from other bacteria which originally carried a complete Tet M transposon (Knapp *et al.*, 1988). A later study examined the mobility of the 25.2 MDa plasmid in *N. meningitidis*, *K. denitrificans* and *E. corrodens* (Roberts & Knapp, 1988). The plasmid was mobile from all three donor species into *N. gonorrhoeae*, *Neisseria cinerea*, *N. meningitidis*, *Neisseria lactamica*, *N. mucosa*, *N. subflava*, *N. flava*, and *Haemophilus influenzae* but not mobile into *Moraxella catarrhalis* (Roberts & Knapp, 1988). This indicated that the commensal *Neisseria* spp. are able to acquire and maintain the 25.2 MDa Tet M plasmid.

In this study, we evaluated commensal *Neisseria* spp. for the presence of penicillin, erythromycin and tetracycline resistance from both the original STD clinic 1988 study and from Seattle periodontal patients. Resistance was evaluated using minimum inhibitory concentration (MIC) assays and DNA probes which recognize

previously described tetracycline and erythromycin resistance determinants. The presence of β -lactamase, which inactivates penicillin, was detected using a chromogenic cephalosporin assay (Kammer *et al.*, 1975). The mobility of erythromycin resistance determinants in the commensal *Neisseria* spp. was also evaluated.

MATERIAL AND METHODS

Bacterial isolates. We examined 26 isolates: *N. perflava/sicca*, *N. flava* and *N. mucosa* (Table 15). The WA clinical isolates were collected from the periodontal pockets of 9 Seattle patients with periodontitis seen at the Graduate Periodontics Clinic at the University of Washington between 1991 and 1995. The GA isolates were from oropharyngeal specimens from 14 patients attending the DeKalb County Sexually Transmitted Disease Clinic in 1986 (Roberts & Knapp, 1988). The NRL isolates obtained from Dr. Knapp and were part of the *Neisseria* Reference Laboratory isolates and represent type strains (Roberts & Knapp, 1988).

Media. GC base or GCP broth (GCK; Difco Laboratories, Detroit, MI) supplemented as previously described (Roberts & Falkow, 1977) was used for routine culture of *Neisseria* spp. and *Enterococcus faecalis*. Mueller Hinton medium (Difco Laboratories, Detroit, MI) was used for minimum inhibitory concentration (MIC) assay of all *Neisseria* spp. and *N. meningitidis* transconjugants as recommended by National Committee on Clinical Laboratory Standards for aerobic bacteria (NCCLS, 1993). The *E. faecalis*

transconjugants were tested on Mueller Hinton medium supplemented with 5% sheep blood as recommended by NCCLS (1993).

Minimum Inhibitory Concentration determination. The MIC assays were determined by agar dilution, following NCCLS guidelines (1993). Bacterial isolates were suspended in 0.84% NaCl to a 0.5 McFarland standard and diluted 1:10. This dilution was inoculated onto agar plates by a Steers replicator giving a final concentration of 10^4 cfu/spot (NCCLS, 1993). The antibiotic concentrations tested were the following: erythromycin, 0.25 - 64 $\mu\text{g/ml}$; tetracycline, 0.25 - 64 $\mu\text{g/ml}$; and penicillin, 0.25 - 64 $\mu\text{g/ml}$. MIC plates were incubated at 36.5°C for 24 h in 5% CO₂ (NCCLS, 1993).

Neisseria spp. transconjugants were tested on Mueller Hinton medium and *E. faecalis* transconjugants were tested on Mueller Hinton medium supplemented with 5% sheep's blood, using the same parameters as stated above (NCCLS, 1993). Plates were incubated at 36.5°C for 24 h in ambient air for the *E. faecalis* transconjugants (NCCLS, 1993).

Assay of β -lactamase activity. The presence of β -lactamase activity was detected using a rapid chromogenic cephalosporin substrate method described by Kammer *et al.* (1975). A 500 mg/ml solution of Nitrocephan powder was mixed with a turbid bacterial suspension was mixed in a ration of 1:3 (bacteria: solution). Reactions were read after 15

min and were considered positive if there was a color change from yellow to red and negative if there was no color change.

Mating experiments. Recipients used for the mating experiments were: *E. faecalis* JH2-2 resistant to rifampin 25 µg/ml and fusidic acid 25 µg/ml (Roberts *et al.*, 1992), *N. meningitidis* NRL9205 (serogroup A) resistant to streptomycin 250 µg/ml and rifampin 20 µg/ml and *N. mucosa* CTM 1.1 resistant to streptomycin 250 µg/ml and rifampin 20 µg/ml (Roberts & Knapp, 1988). The donor *Neisseria* spp. isolates were susceptible to rifampin and streptomycin and carried the Tet M determinant and various rRNA methylase determinants. After 24 hrs growth on plates, the donor and recipient isolates were resuspended in 0.5 ml of GCP broth to form a turbid suspensions $>10^8$ /ml, mixed together and plated on a plain GC plate (Roe *et al.*, 1995a). The mixture was incubated at 36.5°C in 5% CO₂ for 24 hrs. JH2-2 transconjugants were selected on media containing rifampin 10 µg/ml and erythromycin 10 µg/ml. *N. meningitidis* transconjugants were selected on media containing streptomycin 150 µg/ml and erythromycin 10 µg/ml.

The *E. faecalis* transconjugants were confirmed on media supplemented with streptomycin 250 µg/ml, and using a chromosomal DNA probe which is specific for *E. faecalis* (Roberts & Lanciardi, 1990; Roberts & Brown 1994). All *N. meningitidis* transconjugants were confirmed on media supplemented with rifampin 20 µg/ml.

Pulsed field gel electrophoresis (PFGE) was done to confirm all *N. meningitidis* transconjugants using a previously described protocol for *Neisseria gonorrhoeae* (Xia *et al.*, 1995). The isolates were digested with *Xba*I and run on a 1% LMP agarose gel with an initial time of 1 sec and a final time of 15 sec at 175 Volts for 17 hrs. This digest gave banding patterns containing several large bands (97.0-242.5 kb) and numerous small bands. PFGE patterns of band migration of the transconjugants were compared to the donor and recipient. The transconjugants were considered real if the transconjugant PFGE banding pattern was the same as the recipient isolate PFGE banding pattern.

DNA hybridization. DNA was extracted from commensal *Neisseria* spp., *N. meningitidis* transconjugants and *E. faecalis* as previously described (Roberts & Moncla, 1988; Spiegel & Roberts, 1984; Roe *et al.*, 1995a). Uncut whole-cell DNA was visualized on a 0.7% agarose gel stained with ethidium bromide, viewed under long-wave UV light and processed into Southern blots. The rRNA methylase gene probes used were representatives of the five most commonly found classes (Table 7). The tetracycline resistance probes used detected the Tet M, Tet O, Tet B and Tet Q determinants listed in Table 4. Fragment DNA probes, as previously described (Roe *et al.*, 1995a), were labeled using the random-primed DIG-11-dUTP chemiluminescent GeniusTM 3 kit and detected using lumiphos (Boehringer Mannheim Biochemina, Indianapolis USA). Hybridization under stringent conditions and detection followed the manufacturer's instructions as previously described by Roe *et al.* (1995a). Positive and

negative controls were included in each Southern blot. Each isolate and each transconjugant examined were tested at least twice on two different Southern blots.

RESULTS

Identification of Macrolide Resistance. Using the agar dilution method, the susceptibility of the *Neisseria* spp. to erythromycin was evaluated. The MIC values of these isolates ranged from 4 to 32 $\mu\text{g/ml}$ for erythromycin (Table 16). The NCCLS (1993) criteria for susceptibility of aerobic bacteria to erythromycin are as follows; susceptible $\leq 0.5 \mu\text{g/ml}$, intermediate 1-4 $\mu\text{g/ml}$, and resistant $\geq 8 \mu\text{g/ml}$. Using these standards, none of the isolates were susceptible to erythromycin, eight (31%) had intermediate susceptibility and the remaining 18 (69%) were resistant MIC $\geq 8 \mu\text{g/ml}$ to erythromycin.

Southern blot were hybridize with rRNA methylase genes and the results are summarized in Table 16. The Erm B determinants was found in 14 (48%) isolates and was the most common. The other four determinants were present as follows; Erm C in 11 (38%), Erm F in 5 (17%), Erm A in 2 (7%) and Erm Q in one (3%). Eight (28%) of the isolates hybridized with one *erm* gene, seven (24%) of the isolates hybridized with two *erm* gene, and four (13%) of the isolates hybridized with three *erm* genes (Table 16). The majority of the *erm* genes were found to hybridize with the chromosome. The exceptions; 10004, CTM 5.4, and CTM 8.1, where the Erm C determinant hybridized with a plasmid carried by these isolates. Ten (34%) of the *Neisseria* spp. did not carry any of the methylase genes tested in this study.

When the presence of the rRNA methylase determinant was compared to the MIC values, 14 isolates (48%) had MIC values ≥ 8 $\mu\text{g/ml}$ for erythromycin also had an rRNA methylases gene present. Seven of the isolates (24%) had MIC values of ≥ 8 $\mu\text{g/ml}$ for erythromycin, which indicated resistance but did not carry any of the five rRNA methylase determinant used as probes. With intermediate MIC values; five isolates (17%) carried an rRNA methylase determinant and three isolates (10%) did not carry one of the five rRNA methylase determinant examined. None of the isolates examined had MIC values that were indicative of susceptibility to erythromycin.

Identification of Tetracycline Resistance. The MIC values to tetracycline ranged from 1 to 32 $\mu\text{g/ml}$ (Table 16). The NCCLS criteria for susceptibility of aerobic bacteria to tetracycline are as follows; susceptible ≤ 4 $\mu\text{g/ml}$, intermediate 8 $\mu\text{g/ml}$, and resistant ≥ 16 $\mu\text{g/ml}$ (NCCLS, 1993). According to these criteria, nine (35%) of the isolates were susceptible to tetracycline, two (8%) were intermediate and the remaining 15 (57%) were resistant MIC ≥ 16 $\mu\text{g/ml}$ of tetracycline.

The Tet M determinant was the only tetracycline resistance determinant found in the *Neisseria* spp. examined. The Tet M determinant was present in 17 (57%) out of the 29 isolates. None of the isolates examined carried the Tet O, Tet B or Tet Q determinants.

The tetracycline MIC data was compared to the presence of the Tet M determinant to see if any patterns emerged. There was a 87.5% correlation between an MIC of ≥ 16 $\mu\text{g/ml}$ to tetracycline and the presence of the Tet M determinant. There

were two isolates that had MIC values of ≥ 16 $\mu\text{g/ml}$ to tetracycline, which indicated tetracycline resistance but did not carry the Tet M determinant. There were also two isolates that had MIC < 16 $\mu\text{g/ml}$ but carried the Tet M determinant, one would be classified as intermediate (30423) and one would be classified as susceptible (31212). The MIC assays were repeated and the same values were obtained.

Identification of Penicillin Resistance. For the determination of susceptibility to penicillin, the MIC values ranged from < 0.25 to > 16 $\mu\text{g/ml}$ (Table 16). The NCCLS (1993) criteria for susceptibility of aerobic bacteria to penicillin are as follows; susceptible ≤ 0.12 $\mu\text{g/ml}$, intermediate 0.25-2 $\mu\text{g/ml}$, and resistant ≥ 4 $\mu\text{g/ml}$ (NCCLS, 1993). Based on this criteria, one (4%) of the isolates was susceptible to penicillin, 24 (92%) were intermediate and the remaining one (4%) was resistant to ≥ 4 $\mu\text{g/ml}$ of penicillin.

Using the chromogenic cephalosporin assay, the presence of a β -lactamase enzyme was detected in one (4%) of the isolates. The β -lactamase positive isolate was *N. perflava sicca* (30423) was cultured more recently (1991). The presence of a β -lactamase, was associated with an MIC value of ≥ 4 $\mu\text{g/ml}$ for penicillin. While isolates without the β -lactamase had MIC values ≤ 2 $\mu\text{g/ml}$ for penicillin which was indicative susceptibility resulting in a 100% correlation between the MIC data and the presence of the β -lactamase.

Transfer of erythromycin resistance. The mobility of the Tet M in these isolates was not evaluated (Roberts & Knapp, 1988). Three *Neisseria* spp. were examined for their ability to transfer of the Erm^r phenotype. Frequencies ranged from 10⁻⁷ to 10⁻⁸/recipient with *E. faecalis* as recipient and from 10⁻⁶ to 10⁻⁷ /recipient with the *N. meningitidis* as recipient. The Erm F determinant was transferred in one of the three donors used. The Erm C determinant was transferred in two of the three donor isolates used (Table 17). The Erm B determinant was transferable from one of the three donors examined. Matings were done at least twice and only a portion of the transconjugants were characterized; these are described in Table 17. None of the three donors appeared to be able to move the rRNA methylase determinant to the *N. mucosa* recipient CTM 1.1 at a detectable frequency <10⁻⁹/recipient.

CHAPTER DISCUSSION

The data from this study indicates that commensal *Neisseria* spp. can and do carry β -lactamase, *tetM*, and/or rRNA methylases genes. The β -lactamase activity was only found in one (30423) recent isolate (1991). This β -lactamase carrying isolate had an MIC value of 8 μ g/ml for penicillin which would be classified as resistant following the NCCLS guidelines (NCCLS, 1993).

The Tet M determinant is the only tetracycline resistance found in these isolates and is the only one previously found in tetracycline resistant *Neisseria* spp. (Roberts &

Moncla, 1988). However, there were two isolate (10502 & 3201) that had MIC values for tetracycline of $\geq 16 \mu\text{g/ml}$ but did not carry the Tet M determinant. These isolates could carry another tetracycline resistance determinant that was not evaluated in this study or carry a novel tetracycline determinant. Of the isolates that carried the Tet M determinant, thirteen would be classified as resistance, one (30423) would be classified as intermediate and one (31212) would be classified as susceptible. Further studies will be necessary to determine if the Tet M determinant in isolates classified as susceptible or intermediate are functional. However, there is a general agreement between the NCCLS guidelines and the presence of Tet M determinant.

Using the current guidelines for the susceptibility of aerobes to erythromycin, none of the isolates in this study would be classified as susceptible. A variety of rRNA methylase determinants were found in 66% of the isolates (Table 16). When the MIC values was evaluated of isolates carrying rRNA methylase determinants 48% of the isolates had MIC values indicating resistance and 17% had intermediate MIC values. Further investigation is needed to determine if other erythromycin resistance mechanisms or other rRNA methylase genes are present in these isolates. Based on the current data there is a poor correlation between the MIC values and the presence of rRNA methylase genes.

This study demonstrates that erythromycin resistance is present in commensal *Neisseria* spp. and can be transferred to both closely related *N. gonorrhoeae* and *N. meningitidis* (Gram-negative) and unrelated *E. faecalis* (Gram-positive) recipients (Table 17). The Erm B determinant was the most common in *Neisseria* spp., followed by Erm C

and then the Erm F determinants. With the exception of three isolates, the rRNA methylase determinants were found to be associated with the bacterial chromosome. All three *Neisseria* spp. donors tested, could transfer the Erm^r phenotype. The Erm B, Erm C and Erm F determinants were transferable from commensal *Neisseria* spp. (Table 17). In the three donor *Neisseria* spp. tested (10915, 33006, 30423) could transfer the Erm^r phenotype suggesting the rRNA methylase genes were associated with a conjugative transposons.

Further studies are needed to determine the effects the presence of these antibiotic resistance determinants will have on the human host. It also needs to be determined if the commensal bacteria can act as a reservoir for resistance mechanism, which would have implications as to how antibiotic resistance will be dealt with in the future.

Table 15. Number of *Neisseria* spp. isolates examined

Species	Number	GA	WA	NRL
<i>N. perflava/sicca</i>	18	10	7	1
<i>N. flava</i>	2	1		1
<i>N. mucosa</i>	6	3	2	1

GA - From the DeKalb County Sexually Transmitted Diseases Clinic in Georgia

(collected in 1988)

WA - From the University of Washington (collected from 1991 to 1995)

NRL - From the *Neisseria* Reference Laboratory

Table 16. MIC values and antibiotic resistance determinants in *Neisseria* spp.

Isolate	MIC ($\mu\text{g/ml}$)			Hybridization ^b		
	Pen	Tet	Erm	Pen	Tet	Erm
<i>N. perflava/sicca</i>						
CTM 1.2	<0.25	1	4	none	none	C
CTM 2.4	2	16	8	none	M	none
CTM 4.1	0.5	16	8	none	M	none
CTM 4.3	0.5	16	8	none	M	B
CTM 5.8	1	16	8	none	M	none
CTM 7.2	1	16	4	none	M	B
CTM 8.2	0.5	16	4	none	M	none
CTM 11.4	2	16	4	none	M	none
CTM 18.4	0.5	16	4	none	M	C,F
CTM 21.1	0.5	16	4	none	M	none
10004	2	16	16	none	M	B,C
10915	1	16	16	none	M	B,C,F
30423	8	8	16	Yes	M	B,C
31212	0.5	4	16	none	M	B,C
3201	1	4	16	none	none	none

Table 15. Continued

33006	0.5	8	4	none	none	B,C,F
33107	2	1	8	none	none	B
NRL 45	1	2	4	none	none	B
<i>N. flava</i>						
CTM 5.4	0.5	1	16	none	none	B,C,F
NRL 69	1	1	8	none	none	F
<i>N. mucosa</i>						
CTM 2.2	1	16	8	none	M	B,C
CTM 3.4	0.5	16	8	none	M	none
CTM 8.1	0.5	16	8	none	M	B,C
10502	2	>16	8	none	none	C
2701	1	2	16	none	none	none
NRL 76	2	1	8	none	none	B

^bTet: tetracycline determinants; Erm: erythromycin determinants; Pen: penicillin resistance due to the presence of a β -lactamase enzyme

Table 17. Distribution of Erm^r and Tet^r determinants in representative transconjugants from different experience

Recipient	Donor	Donor Erm ^r Determinants	Transconjugants		
			Number of Tested	Determinants Erm	Frequency
<i>E. faecalis</i> - JH2-2					
	10915	B,C,F	1	F	1.3 X 10 ⁻⁸
			5	C, F	1.3 X 10 ⁻⁸
	33006	B,C,F	2	C	3.4 X 10 ⁻⁷
<i>N. meningitidis</i> - 9205					
	30423	B,C	8	B	3.4 X 10 ⁻⁶
	33006	B,C,F	6	C	1.7 X 10 ⁻⁶
<i>N. mucosa</i> - CTM1.1					
	30423	B,C			Not Mobile
	33006	B,C,F			Not Mobile
	10915	B,C,F			Not Mobile

CHAPTER 7

Conclusion

SUMMARY OF FINDINGS

In summary, four oral bacterial genera were evaluated for the presence of antibiotic resistance genes, specifically: *Actinobacillus actinomycetemcomitans*, *Campylobacter rectus*, *Treponema denticola*, and commensal *Neisseria* spp. These bacteria were shown to carry a variety of previously described tetracycline, β -lactamase and/or erythromycin resistance genes. The mobility of some of these determinants was also determined by conjugal mating experiments. The findings presented here extend the distribution and mobility of several previously described tetracycline and erythromycin resistance determinants into new bacterial species.

SUSCEPTIBILITY

No standards have been established for the determination of antibiotic susceptibility of periodontal pathogens (Baker *et al.*, 1985; NCCLS, 1993). Therefore, the dissertation work started by comparing all the antibiotic susceptibility conditions reported in the literature in an attempt to determine the most appropriate conditions for antibiotic susceptibility testing with *A. actinomycetemcomitans*. It was determined that growth on Brain Heart Infusion media incubated in 5% CO₂ at 48 hrs was the most reproducible of all the conditions evaluated. Similar experiments were done with *C. rectus* using Mueller Hinton Blood Agar media supplemented with formate and fumerate; however, the results had poor reproducibility and some isolates did not grow on control

plates without antibiotic under minimum inhibitory concentration assay conditions. This suggests that the optimal conditions for MIC assays with *C. rectus* have not been determined.

When the susceptibility results were compared to the presence of erythromycin resistance genes, the susceptibility to erythromycin did not correlate with the presence of rRNA methylase genes in *C. rectus* or *A. actinomycetemcomitans*. However, there was an 82% correlation between *A. actinomycetemcomitans* ability to grow in the presence of ≥ 2 of tetracycline with regular streaking and the presence of the Tet B determinant in that isolate. However, when the MIC data was compared to the NCCLS breakpoints for *Haemophilus influenzae*, the closes related species which has breakpoints established, there was no correlation between the MIC value and the presence of the Tet B determinant.

Since *T. denticola* grows poorly on solid media, agar dilution assays were not feasible. Susceptibility assays for *T. denticola* were analyzed using the broth dilution method. However, the results were not reproducible. This is not the first report of difficulties in establishing reproducible assays for the determination of susceptibility of anaerobes (Thornsberry, 1990). This problem is more evident with the more fastidious anaerobic species; however, this does not explain the difficulties with *A. actinomycetemcomitans* which is a fastidious facultate bacteria.

It is vary apparent that there is very poor correlation between the MIC values in these organisms and the presence of antibiotic resistance determinants. This suggests that appropriate breakpoints need to be established and it needs to be determined if these

antibiotic resistance determinants are functional in these bacterial species. Therefore, until further studies are done using more isolates that do not carry antibiotic resistance determinants, it can not be determined what background levels of susceptibility are for these species and what appropriate breakpoints should be. The data presented here is a starting point; however, for all the periodontal pathogens, a larger number of isolates will need to be evaluated before enough data are collected to be able to determine the appropriate breakpoints for these species.

It has been suggested the appropriate breakpoint for the periodontal pathogens should be 2 to 4 fold lower than the highest concentration achievable in the gingival sulcus (Baker *et al.*, 1983). This is an appropriate starting point for trying to establish breakpoints. However, it also needs to be determined if the MIC values for the oral bacteria *in vitro* correlate with that is seen with the antibiotic *in vivo*. It has yet to be determined what effect plaque formation has on the expression and effectiveness of antibiotic resistance determinants. It is possible that bacteria in a biofilm rather than in isolation on agar media have different MIC values due to their surrounding environment. It also needs to be determined if these antibiotic resistance genes are fully expressed in these species. The establishment of standards for antibiotic susceptibility and breakpoints is necessary in order to be able to detect the presence of antibiotic resistance in oral bacteria spp. and monitor these resistant isolates.

Minimum inhibitory concentrations assay conditions have been established by NCCLS for aerobic bacteria. These standards recommend the use of Mueller Hinton media which provided adequate growth conditions and reproducible results for

susceptibility testing of the commensal *Neisseria* spp.. Based on the data presented here, the breakpoints for penicillin and tetracycline seem to predict the presence or absence of genes coding for resistance. However, as seen with *C. rectus* and *A.*

actinomycetemcomitans there is poor correlation between MIC values for erythromycin and the presence of rRNA methylase determinants. Whether the established erythromycin breakpoints for aerobic bacteria are appropriate for the commensal *Neisseria* spp. has yet to be determined. More isolates and more of the commensal *Neisseria* spp. will need to be evaluated in order to be able to attempt to determine appropriate breakpoints.

THE PRESENCE OF TETRACYCLINE RESISTANCE DETERMINANTS

After the susceptibility assays were completed, the presence of a previously described tetracycline resistance determinants were examined. The Tet B determinant was found in 18 (82%) of the 22 *A. actinomycetemcomitans* isolates examined. Concordance between the MIC data and the ability to grow on tetracycline supplemented media was seen in 18 (82%) out of the 22 *A. actinomycetemcomitans* isolates. The four non-concordance isolates carried the *tetB* gene and were able to grow on antibiotic supplemented media at higher concentrations than indicated by MIC. *Haemophilus* and *Actinobacillus* are members of the family *Pasteurellaceae* (Moore *et al.*, 1986; Roberts, 1990). Since the Tet B determinant has been previously described in *Haemophilus* spp. and *Pasteurella* spp., finding the Tet B determinant in *A. actinomycetemcomitans* was not surprising (Morse *et al.*, 1986; Roberts, 1990; Roberts, 1996). However, the MIC

values of *A. actinomycetemcomitans* isolates carrying the Tet B determinant were lower than *H. influenzae* isolates carrying the Tet B determinant. The Tet B determinant has also been previously reported to be present in *Moraxella catarrhalis* and many enteric species (Roberts, 1990; Roberts 1994; Roberts, 1996). This makes the Tet B determinant the most common tetracycline resistance gene in Gram-negative species (Roberts, 1996).

The Tet B determinant in *A. actinomycetemcomitans* was found to be mobile and could be transferred to a tetracycline susceptible *A. actinomycetemcomitans* isolate. The *tetB* gene was also transferable from *A. actinomycetemcomitans* to a *H. influenzae* recipient. Most studies which have found the *tetB* gene in Gram-negative species, report the *tetB* gene to be associated with a conjugative plasmid (Roberts, 1989). Based on the frequency of transfer and the hybridization patterns, the *tetB* gene in *A. actinomycetemcomitans* also appears to be located on a conjugative plasmid. Plasmids were observed in some preparations of the original isolates. This finding is also supported by the fact that plasmids have been previously reported in *A. actinomycetemcomitans* (LeBlanc *et al.*, 1993; Olsvik & Preus, 1989). All *A. actinomycetemcomitans* donor isolates were also resistant to erythromycin and carried rRNA methylase genes. Three out of the five *A. actinomycetemcomitans* donors transferred both the *tetB* gene and various rRNA methylase determinants 50% of the time to the *H. influenzae* recipient even though erythromycin was used for the selection. This observation could be due to a physical link between the two antibiotic resistance determinants in some isolates however, this seems unlikely since these two resistance mechanisms appear to be located on two different types of mobile elements. It would

seem more likely that some isolates have transfer frequencies that are similar for both mobile elements and the two resistance mechanisms are transferred independently.

The Tet B determinant found in 7 (44%) of 16 *T. denticola* isolates evaluated. The Tet B determinant appeared to be located in the chromosome of all the *T. denticola* positive isolates. The hybridization of the Tet B probe with the chromosome was also seen with the ATTC 33520 strain which carries a 2.6 kb plasmid, pTD1 (MacDougall *et al.*, 1992; MacDougall & Girons, 1995; Reedy *et al.*, 1994). The results of mating experiments done with *T. denticola* indicate the Tet B determinant was non-mobile. This finding correlates with previous studies, with other species, which found that when the Tet B determinant was located in the chromosome it was non-mobile (Roberts & Smith, 1980; Roberts *et al.*, 1991; Roberts, 1996). This is not the first report of antibiotic resistance in spirochete, previously, *Treponema hyodysenteriae*, an intestinal spirochete was found to have a non-mobile chromosomally located β -lactamase, causing penicillin resistance (Tompkins *et al.*, 1987). However, the protein and gene conferring this resistance were not further characterized (Tompkins *et al.*, 1987).

Previous studies have reported the presence of the Tet M determinant, a ribosomal protection mechanism, to be responsible for the tetracycline resistance found in *N. mucosa* and *N. perflava/sicca* (Roberts & Moncla, 1988; Knapp *et al.*, 1988). These clinical isolates came from patients who were being treated for adult periodontitis in the University of Washington Graduate Periodontics Clinic or visited Sexual Transmitted Disease Clinic. To date clinical isolates of the commensal *Neisseria* spp.

have been shown to carry the Tet M determinant in the chromosome where it appears to be non-mobile (Knapp *et al.*, 1988; Roberts & Knapp, 1988; Roberts & Moncla, 1988).

All of the *C. rectus* isolates evaluated in this study were susceptible to tetracycline by MIC, none could grow in the presence of tetracycline and none carried the tetracycline resistance determinants used in this study. Previous studies have reported tetracycline resistance in *Campylobacter coli* and *Campylobacter jejuni* was due to the presence of the Tet O determinant (Taylor & Courvalin, 1988). Several possible reasons that no tetracycline resistance was found in this study are a small sample size, all of the isolates except for the ATCC strains came from Seattle and *C. rectus* is very different than *C. coli* and *C. jejuni*.

Recent studies regarding the determinants that are responsible for tetracycline resistance in bacterial isolates from periodontal patients further support the findings of this dissertation. Hartley *et al.* (1984), Olsvik *et al.* (1995), Roberts & Moncla (1988) and Roberts and Lanciardi (1990) report the Tet M determinant to be present in tetracycline resistant Gram-negative bacteria from the oral cavity, specifically commensal *Neisseria* and *Fusobacterium*. Guiney and Bovic (1990) reported the presence of the Tet Q determinant in tetracycline resistant isolates of *Prevotella intermedia*. Olsvik *et al.* (1994) reported tetracycline resistance was due to the presence of the Tet Q determinant located in the chromosome of the following oral anaerobic species; *Prevotella loescheii*, *Prevotella melaninogenica*, *Prevotella nigresceus*, *Prevotella veroralis*, *Mitsuokella dentalis* & *Capnocytophaga ochracea*. A recent paper by Lacroix and Walker (1996) confirms the findings of the two previously discussed papers. This study found the Tet Q

determinant to be present in only Gram-negative species, specifically *Prevotella* ssp. and *Bacteroides* ssp. growing on 4 µg/ml of tetracycline. Of the 210 tetracycline resistant isolates examined, 15% had the Tet Q determinant and 60% had the Tet M determinant.

THE PRESENCE OF ERYTHROMYCIN RESISTANCE

In *A. actinomycetemcomitans*, 24 (96%) of the isolates carried at least one rRNA methylase determinant; however, no correlation was found between the number of determinants present and the MIC value of the isolate. Further studies are need to determine if the rRNA methylase determinants are full expressed and the effectiveness of the methylase in this species. This information will help in the determine the relevance of these determinants in *A. actinomycetemcomitans*. Most isolates (32%) were found to carry at least two different rRNA methylase genes. The frequency of occurrence was as follows going from most prevalent to least prevalent; Erm B, Erm C, Erm F, Erm Q and Erm A. Of the five determinants present in *A. actinomycetemcomitans*, four of the determinants were transferable to *H. influenzae*, with the Erm A determinant being non-motile. The *ermB* and *ermC* genes were more commonly transferred than the *ermF* and *ermQ*. When similar matings were done with *E. faecalis*, only the *ermB* and *ermC* genes were transferred to this recipient. All of the transconjugants had MIC values ranging from 32 to 256 µg/ml for erythromycin increasing from 4 µg/ml for the *H. influenzae* recipient and 1 µg/ml for the *E. faecalis* recipient. A conjugative transposon located in the chromosome is the mechanism most likely responsible for the mobility of the rRNA methylase genes in *A. actinomycetemcomitans*.

The MIC data for the *C. rectus* isolates have poor reproducibility and needs to be further evaluated. In *C. rectus*, 17 (89%) of the isolates carried one or more different rRNA methylase genes. The Erm B, Erm C, Erm F, and Erm Q determinants were present, while the Erm A determinant was absent. The *ermB* and *ermC* genes were the most commonly transferred to donors. However, with *C. rectus*, the Erm F determinant was found to be non-mobile in the two isolates tested. The Erm Q determinant was mobile in the one isolate evaluated, but was only transferred with the Erm C determinant. The rRNA methylase determinants carried by *C. rectus* appeared to be associated with a conjugative transposons.

The rRNA methylase genes were also found in 19 (65%) of the 29 commensal *Neisseria* spp. isolates. All five groups of rRNA methylase determinants were present in *N. perflava sicca*. With the *N. flava* isolates only the Erm B, Erm C, Erm F determinants were found; however, this finding is based the evaluation of two isolates. With the six *N. mucosa* isolates examined, only the Erm B and Erm C determinants were present. The rRNA methylase genes hybridized with the chromosome in the majority of the isolates examined. With three of the isolates, the Erm C was found in the plasmid, which other rRNA methylase genes in the same isolate were located in the chromosome. The commensal *Neisseria* spp. commonly carried one (28%) or two (24%) different rRNA methylase genes. The Erm B, Erm C and Erm F determinants were found to be transferable. These determinants could be transferred to *Neisseria meningitidis*, *Neisseria gonorrhoeae*, and *E. faecalis*. The rRNA methylase determinants in the commensal *Neisseria* spp. appear to be associated with a conjugative transposons.

FINAL COMMENTS

After evaluating the data presented in this dissertation and considering other studies that have been previously published, it has been shown that several periodontal pathogens and commensal *Neisseria* spp. carry known tetracycline and erythromycin resistance determinant. Many of the tetracycline and erythromycin resistance determinants were associated with conjugative elements, either plasmids or conjugative transposons. This suggests that the periodontal pathogens and commensal species can act as a reservoir for resistance determinants. It has also been demonstrated that these genes are mobile in these species, meaning that they can be transferred to any other bacterial species that can accept them. This could have implications into antibiotic therapy in patients carrying these genes, beyond the oral cavity.

It is not known what effect the presence of these antibiotic resistance determinants will have on antibiotic therapy. Slots and Rams (1990) have suggested the best result from the presence of these determinants is they will have no effect on antibiotic therapy. However, they hypothesize the worst outcome of the presence of antibiotic resistance determinants in periodontal pathogens is that they will make the disease progression worse.

Since mobile antibiotic resistance determinants can be present in periodontal pathogens, there is a need for a rapid and easily interpreted assay to detect the presence of these resistance determinants in patients who need antibiotic therapy. It has been suggested that cultures be taken of all patients that are going to receive antibiotic therapy

(Slots & Rams, 1990). By knowing the organisms present and their susceptibility to antibiotics, either based on what has been previously published or by susceptibility assays of the isolates, the clinician will be able to determine which antibiotic therapy is the most appropriate for the individual patient. However, few clinicians have access to a facility that is able to identify and culture all of the organisms that colonize the periodontal pocket. In addition, for the laboratory to culture these sites, it is very expensive and time consuming process.

In conclusion, it is now known that previously described antibiotic resistance determinants are present in periodontal pathogens and the normal oral flora species. It is also evident that many of these determinants are mobile. Therefore, it seems wise to proceed with the use of antibiotics with great caution. It seem logical to use antibiotics when they are really needed and have been shown to be efficacious, so that the patient is only exposed to antibiotics when antibiotic therapy will really help the patient.

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Education:

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Senior Thesis: Genetic variability in *Camassia quamash*
1992-1993 M.S. University of Washington (Pathobiology)
Masters Thesis: Erythromycin resistance in *Actinobacillus actinomycetemcomitans*
1993- present Ph.D. University of Washington (Pathobiology)
Dissertation: Antibiotic Resistance in Oral Flora

Position and Training:

- 1989 Research Assistant, Willamette University, Salem, OR,
1989 Research Assistant, Oregon Department of Agriculture,
Salem, OR
1989-1990 Teaching Assistant, Willamette University, Salem, OR
1991-1992 Clinical Technician I; University of Washington,
Chlamydia Lab, Seattle, WA
1992 Research Technician I; University of Washington,
Department of Pathobiology, Seattle, WA
1994 Teaching Assistant, University of Washington,
Department of Pathobiology, Seattle, WA
1994 Anaerobic Bacteriology for the Clinical Laboratory and
Chamber Training - Anaerobe Systems
1995 National Science Foundation Summer Workshop on
Bacterial Physiology - University of Georgia

Scientific Experience and Skills:

Molecular and Cellular Biology:

DNA isolation, hybridization, development and use of PCR assays, DNA sequencing, GCG data base analysis, pulsed field gel electrophoresis, conjugal matings, DNA recombination techniques, cell culture, multi-locus protein enzyme electrophoresis and analysis, protein preparation, SDS-Page, florescent microscopy

Medical Microbiology:

Knowledge of medical microbiology, isolation and identification of human oral flora organisms(aerobes and anaerobes), experience growing anaerobes (including Treponemes and Methanogens), Minimum Inhibitory Concentration assays

Research Interests:

Bacterial Genetics, Bacterial Pathogenesis, Bacterial Antibiotic Resistance Determinants, Bacterial Gene Exchange, Plasmids

Fellowships:

University of Washington Periodontal Training Fellowship, 1994-1996.

Memberships:

Alliance for the Prudent Use of Antibiotic
 American Society of Microbiology
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Publications:

1. **Roe, D.E., A. Weinberg and M.C. Roberts.** 1995. Characterization of erythromycin resistance in *Campylobacter rectus*. *Clinical Infectious Diseases*. 20(suppl 2):370-371.
2. **Roe D.E., P.H. Braham, A. Weinberg, and Roberts, M.C.** 1995. Characterization of tetracycline resistance in *Actinobacillus actinomycetemcomitans*. *Oral Microbiology and Immunology* . 10:227-232.
3. **Roe, D.E., A. Weinberg and M.C. Roberts.** 1995. Mobile rRNA methylase genes in *Campylobacter rectus*. *Journal of Antimicrobial Chemotherapy*. 36:738-740.
4. **Wasteson, Y., D.E. Roe, K. Falk and M.C. Roberts.** 1996. Characterization of antibiotic resistance in Norwegian *Actinobacillus pleuropneumoniae* isolates. *Veterinary Microbiology*. 48:41-50.
5. **Roe, D.E., M.C. Roberts, and A. Weinberg.** 1996. Mobile rRNA methylase genes coding for erythromycin resistance in *Actinobacillus actinomycetemcomitans*. *Journal of Antimicrobial Chemotherapy*. 37:457-464.

6. Roberts, M.C., W.O. Chung and **D.E. Roe**. Tet B and Erm F antibiotic determinants in *Treponema denticola*. *Antimicrobial Agents and Chemotherapy*. 40:1690-1694.

Abstracts:

1. **Flanagan, D.E.** Genetic variability in *Silene douglassi*. Northwest Undergraduate Research Conference. Pacific University, Forest Grove, OR. April 1990.
2. Roberts, M.C., **D.E. Roe**, P. Braham and A. Weinberg. Characterization of tetracycline resistance in *Actinobacillus actinomycetemcomitans*. International Association for Dental Research. p. 204 Chicago IL. March 10-14, 1993.
3. Weinberg, A., P. Braham, W.F. Ammons, **D.E. Roe**, and M.C. Roberts. Antibiotic resistance in the oral microflora of an RPP patient. International Association for Dental Research. p. 204 Chicago IL. March 10-14, 1993.
4. Weinberg, A., M.C. Roberts, P. Braham and **D.E. Roe**. Antibiotic resistance in periodontal pathogens and other oral bacteria. University of Washington School of Dentistry Research Celebration. May 12, 1993.
5. **Roe, D.E.**, M.C. Roberts, P. Braham and A. Weinberg. Characterization of erythromycin resistance in *Actinobacillus actinomycetemcomitans*. Abstracts of the Annual Meeting of American Society of Microbiology. p. 21. Atlanta, GA. May 16-20, 1993.
6. Roberts, M.C., **D.E. Roe**, P. Braham and A. Weinberg. Mechanisms of resistance in the oral microflora of the periodontitis patient. *Molecular Mechanisms of Drug Resistance*. p. 38. Albany NY Sept. 9-12, 1993.
7. **Roe, D.E.**, M.C. Roberts, Y. Pang and A. Weinberg. Characterization of erythromycin resistance in *Actinobacillus actinomycetemcomitans*. Abstracts of the International Society of Dental Researchers. p. 153. Seattle, WA. March 10-13, 1994
8. **Roe, D.E.**, M.C. Roberts, Y. Pang and A. Weinberg. Characterization of erythromycin resistance in *Actinobacillus actinomycetemcomitans*. University of Washington School of Dentistry Research Celebration. April 26, 1994.
9. **Roe, D.E.**, A. Weinberg, and M.C. Roberts. Characterization of erythromycin resistance in *Campylobacter (Wollinella) rectus*. Anaerobe Society of the Americas. Marina del Rey, CA. July 29-31, 1994
10. **Roe, D.E.**, A. Weinberg, and M.C. Roberts. Antibiotic resistance in *Campylobacter (Wollinella) rectus*. Abstracts of the American Society of Dental Researchers. p. 198. San Antonio, TX. March 8-12, 1995

11. **Roe, D.E.**, A. Weinberg, R.J. Wallace Jr. and M.C. Roberts. Antibiotic resistance in oral gram negative bacteria. Interscience Conference on Antimicrobial Agents and Chemotherapy. p. 64. San Francisco, CA. September 17-20, 1995.
12. **Roe, D.E.**, A. Weinberg, R.J. Wallace Jr. and M.C. Roberts. Antibiotic resistance in oral gram negative bacteria. University of Washington School of Dentistry Research Celebration. September 27, 1995.
13. Hudson, C.R., **D.E. Roe**, M.C. Roberts, and F.C. Gherardini. Identification of an erythromycin resistance gene from *Borrelia burgdorferi*. Gordon Conference, January 21-26, 1996.
14. Chung, W.O., **D.E. Roe**, A. Weinberg, and M.C. Roberts. Tet B and Erm F antibiotic determinants in *Treponema denticola*. Abstracts of the International Society of Dental Researchers. San Francisco, CA. March 13-17, 1996.
15. Hudson, C.R., **D.E. Roe**, M.C. Roberts, and F.C. Gherardini. Identification of an erythromycin resistance gene from *Borrelia burgdorferi*. Abstracts of the Annual Meeting of American Society of Microbiology. New Orleans, LA. May 19-23, 1996.
15. Chung, W.O., **D.E. Roe**, and M.C. Roberts. Tet B and Erm F antibiotic determinants in *Treponema denticola*. Impact of Bacterial Antibiotic Resistance on Oral Health: What is the relevance. Seattle, WA September 5-7, 1996.
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Grants:

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Teaching:

- 1989 Teaching Assistant - Introduction to Biology
- 1989 Teaching Assistant - General Microbiology
- 1990 Teaching Assistant - Introductory to Biology
- 1994 Teaching Assistant - Resistance Mechanisms and Their Impact on Public Health

Invited Participation:

- 1996 Indiana State University, Department of Oral Biology, Indianapolis, IN,
 "Antibiotic Resistance in Periodontal Pathogens."
 1996 University of Rochester, Department of Dental Research, Rochester, NY,
 "Antibiotic Resistance in Periodontal Pathogens."

University Services:

- 1993-1996 Committee on Alcohol and Substance Awareness
 1993-1994 Student Representative for the School of Public Health,
 Curriculum Committee
 1993-1994 Student Representative for the School of Public Health,
 Student Affairs Committee
 1994 Volunteer at IADR
 1994 Ad Hoc Planning Committee for Hac Ed Pavilion
 1994-1995 Senator for the Department of Pathobiology for the Graduate and
 Professional Student Senate
 1994-1996 Hall Health Advisory Committee
 1995 Volunteer for the University of Washington Health Science Center
 Open House
 1996 Speaker for series "Introduction to the Health Professions", representing
 the Department of Pathobiology

Other Services:

- 1991-1992 United Armed Forces Association, Organizational Committee
 1992 USS Nevada Women's Softball Team
 1995-1996 Volunteer at Sarvey Wildlife Rehabilitation Center