UiT The Arctic University of Norway

Faculty of Health Sciences

Department of Clinical Dentistry

Antibiotic Resistance in Oral Streptococci

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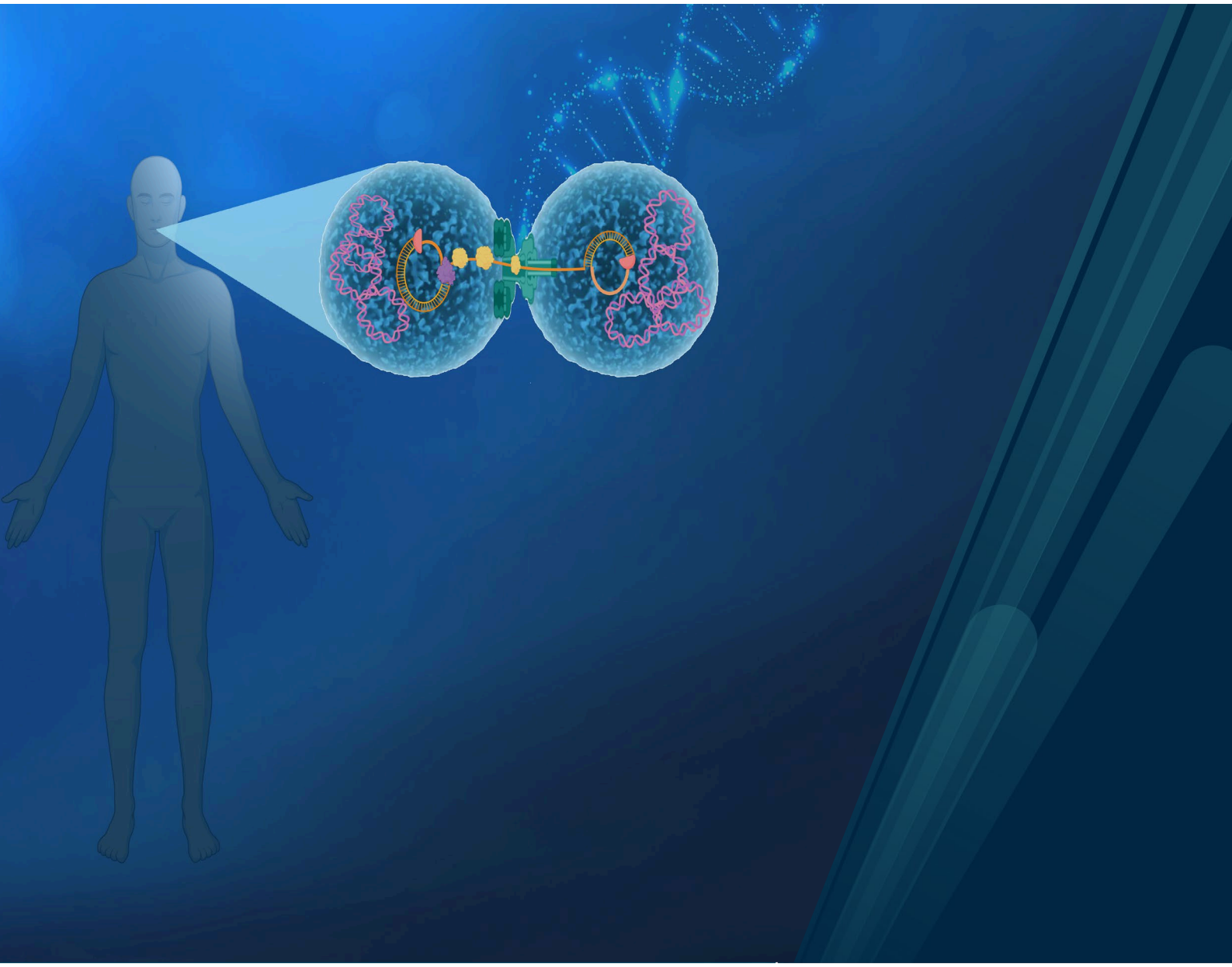
Tracy Munthali Lunde

A dissertation for the degree of Philosophiae Doctor- December 2021



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ACKNOWLEDGMENTS

The present work was conducted at the Faculty of Health Sciences in the Department of Clinical Dentistry. I would like to extend my gratitude to The Arctic University of Norway for providing the resources and opportunity to conduct this work and The Norwegian National Advisory Unit on Detection of Antimicrobial Resistance (K-RES) for providing the clinical bacterial isolates used in this study.

I would first like to extend my uttermost gratitude to my main supervisor and mentor, Mohammed Al-Haroni. Your patience, guidance, and dedication have helped me to not only complete this work but also find great joy and satisfaction in it. Thank you for not only knowing the way but for walking it with me and for always having time to listen to my endless rants before pointing me in the right direction. Albert Einstein defines a leader as "one who out of the clutter brings simplicity, out of the discord, harmony and out of the difficult, opportunity." Thank you for being the true definition of a good leader and for bringing simplicity, harmony, and opportunity my way.

I would also like to thank my co-supervisor, Pål Johnsen, who has successfully seen me through two degrees. Thank you for bringing your infectious curiosity and energy to this work and for taking the time to share your extensive knowledge in evolution studies.

To my co-authors, Adam P. Roberts, Erik Hjerde, and Espen Åberg thank you for contributing to the success of this project. It has been a pleasure working with you.

My Ph.D. journey has had many hills and valleys. Fortunately, I have walked them with many good people. Thank you to everyone who has contributed to this work directly or indirectly and to the people who are or have been a part of the IKO and Oral ecology group during my Ph.D. studies. Special thanks go to Laila for straightening out the endless logistics over the years, to Hege for the chats and encouragement; and to Irene for always having a smile and encouraging words. To Berit, Olga, Ida Sofie, Marwan, Supathep, and Rania; it was always nice to have someone to share the joys of successful conjugation or the frustrations of poor ddPCR droplet separations. Stanislav, I am grateful for the many scientific chats and your continued interest in ICEs even after moving from Tromsø.

During my time in Tromsø, I have had the fortune of meeting some very wonderful people. Some have moved away, and some have stayed, but many have remained present in my life. Special thanks go to Julia, Montse, Jaione, Elizabeth, Irina, Sara, Bjørg, Rodmire, Aunty Sarah and Joe; your interest and encouragement have helped me overcome many challenges. Miss Conny, I am grateful for the many lunches, hugs, sushi dinners, travels, chats, and phone calls. Ane Utnes, thank you for being a great friend. Our many walks and talks have been a good venting arena for all things work and family related. Thank you all for your role in turning Tromsø into my new home.

The support of my family has been instrumental in my success. Special thanks go to my parents, who have always encouraged me to focus on my education. Dad, I wish you were here to see your highest aspirations for me come true. Mum, thank you for always being there and wanting to hear about every little hick-up and success in my life. A big thank you to Lynnette, Lawson, O'Bryan, Line, and Dag Erik.

Finally, I want to thank my beautiful children Alma, Malin, and Leon, for putting up with my long absences and always having a warm hug waiting for me. To Tom, the best husband ever and the love of my life, thank you for your support, encouragement, love, and patience. Thank you for willingly adjusting your work schedule and for holding down the fort so that I could finish this project. You are the best! 😊

Tracy Munthali Lunde

ABBREVIATIONS

AMR - Antimicrobial Resistance
BHI - Brain Heart Infusion
Bp - Base Pair
CI - Circular Intermediate
CN - Copy Number
CR - Circularization Rate
ESBL Extended Spectrum β-Lactamase
ICEs - Integrative Conjugative Elements
CTns - Conjugative Transposons
ddPCR - droplet digital Polymerase Chain Reaction
DNA - Deoxyribonucleic Acid
HGT - Horizontal Gene Transfer
ICE - Integrative Conjugative Element
IME - Integrative Mobilizable Element
int - Integrase Gene
K-RES - National Advisory Unit for Detection of Antimicrobial Resistance
Kb - Kilo Base Pair
µg - Microgram
µl - Microlitre
µm - Micrometer
MGE- Mobile Genetic Element
MIC - Minimal Inhibitory Concentration
mRNA - Messenger Ribonucleic Acid
*npt*II - Neomycin Phosphotransferase II Enzyme
ORF - Open Reading Frames
PCR - Polymerase Chain Reaction
RFLP - Restriction Fragment Length Polymorphism
RNA - Ribonucleic Acid
SNP – Single Nucleotide polymorphism
Ssp - Subspecies
tet (M) -Tetracycline Resistance Gene
tRNA - transfer Ribonucleic Acid
T4SS -Type IV Secretion System Family
WGS -Whole Genome Sequencing
xis - Excision Gene

LIST OF PAPERS

Paper I

Lunde, Tracy Munthali; Roberts, Adam P.; Al-Haroni, Mohammed. Determination of copy number and circularization ratio of Tn*916*-Tn*1545* family of conjugative transposons in oral streptococci by droplet digital PCR. Journal of Oral Microbiology 2019; Volume 11 (1). ISSN 2000-2297.s doi: 10.1080/20002297.2018.1552060.

Paper II

Lunde, Tracy Munthali; Hjerde, Erik; Al-Haroni, Mohammed. Prevalence, diversity and transferability of the Tn*916*-Tn*1545* family ICE in oral streptococci. Journal of Oral Microbiology 2021; Volume 13 (1). ISSN 2000-2297.s 1 - 13.s doi: 10.1080/20002297.2018.1552060

Paper III

Lunde, Tracy Munthali; Åberg, Espen; Tømmerås, Berit; Johnsen, Pål; Al-Haroni, Mohammed. Stability and fitness cost of newly acquired Tn*916* in *S. oralis*. Manuscript

SUMMARY

The oral cavity has rich and diverse microbial communities in which oral streptococci are the most abundant species. As is the case with all microbial-rich environments, the oral cavity has been found to harbor bacterial species that are resistant to antibiotics. The presence of antibiotic resistance genes that are associated with Mobile Genetic Elements (MGE) such as the Tn*916*-Tn*1545* family has been implicated in the increased spread of antibiotic resistance.

The Tn*916*-Tn*1545* family is a group of broad-host-range Integrative Conjugative Elements (ICEs) that encode all the necessary proteins for intracellular transposition and intercellular conjugation. Tn*916* is a prototype of these highly promiscuous conjugative elements, and it has been found in or transferred into a wide range of bacterial phyla. The transfer of Tn*916* is initiated by the formation of a replicative intermediate known as a circular intermediate (CI). The CI, which has been shown to autonomously replicate once it is excised from the chromosome, can either integrate into the same position in the donor, integrate into a different chromosomal location or transfer to a new bacterial cell (recipient). The number of copies of CIs in a bacterial population has an impact on the transfer rate and/or spread of the element, as CIs are a prerequisite for Tn*916* transfer. The work in this study addresses the presence of Tn*916* and CIs in oral streptococci. In addition, we determined the transfer frequencies, stability and fitness cost associated with the acquisition of these elements in the absence of selective pressure.

The work in this thesis indicates that the carriage of the Tn*916*-Tn*1545* family among antibiotic-resistant oral streptococcal isolates collected in Norway in 2005 is 21%. The most prevalent member of this family is wild-type Tn*916,* and CI molecules were detected in all but one of the clinical isolates that harbored the Tn*916*-Tn*1545* family. In terms of diversity, we detected three different members of this family of ICEs, namely, Tn*916*, Tn*6815* and Tn*6816*. Tn*6815* and Tn*6816* are novel elements; therefore, we described the structural composition and transfer potential of these elements. Tn*6815* is a composite element (consisting of Tn*916* and Tn*917*) and thus carries both the tetracycline resistance gene (*tet* M) and the erythromycin resistance gene (*erm* B), whereas Tn*6816* carries a group II intron reverse transcriptase/maturase and part of peptidase P60 inserted between *orf21* and *orf22*. We also investigated the inter- and

intraspecies transfer rate of the three elements and found that this family of elements had transfer rates ranging from 6.0 (± 4.03) × 10^{-9} to 3.5 (± 6.0) × 10^{0} transconjugants per recipient.

Having observed high rates of transfer of the Tn*916*-Tn*1545* family, we were interested in determining the effect that these elements have on the new host (*Streptococcus oralis*) and what happens to both the element and the host in the absence of selective pressure. We therefore endeavored to investigate the fitness cost associated with the acquisition of these elements and the stability of these elements in the naïve host in the absence of selective pressure. The findings of this work showed that Tn*916* was successfully transferred into *S. oralis*. We selected seven transconjugants and investigated the relative fitness cost, the copy number, and the stability of the newly acquired Tn*916.* Upon the introduction of Tn*916*, a reduction in relative fitness ranging from 6% to 25% at time zero (T0) was observed. Within 360 hours of evolution (approx. 500 generations), we observed amelioration of relative fitness cost in all seven transconjugants. To study the evolution of the newly acquired element over a longer period, two transconjugants (TC8.1 and TC10.1) were selected for an additional 240 hours (totaling approx.1000 generations). The stability of the newly acquired ICE in the *S. oralis* host isolates was stable in the absence of selection. Analysis of the number of copies of Tn*916* in the transconjugants using digital droplet PCR (ddPCR) copy number analysis and whole genome sequencing (WGS), indicated no changes during the evolution study. Interestingly, the insertion sites remained the same during evolution, and no SNPs were detected within the elements. Comparisons of the genomes of the Tn*916* carrying populations (non-evolved vs evolved) indicate the presence of multiple SNPs in different locations in the genomes. SNPs in the bacterial chromosome suggest that other secondary mechanisms are responsible for the amelioration of fitness.

We have showcased the relatively high rate at which these elements transfer both inter- and intraspecies and how, in as little as 1000 generations, the fitness cost associated with the acquisition of these elements can be ameliorated. Taken together, the findings of this work highlight the role that Tn*916*-Tn*1545* family elements play in the spread of antibiotic resistance among oral streptococci.

1 INTRODUCTION

For a long time, the discovery of antibiotics has been considered one of the best achievements of modern medicine. The ability of antibiotics to eradicate or reduce bacterial infections has resulted in longer life expectancy and better quality of life (1). However, the increased use and misuse of antibiotics have resulted in the inability to cure uncomplicated bacterial infections due to bacterial adaptation to antimicrobial agents. The issue of antimicrobial resistance (AMR) is now a well-established and fast-growing problem that affects all sectors of human health, including oral health. In recent years, oral health care professionals have seen an increase in the number of antibiotic-resistant strains isolated from the oral cavity (2). The oral cavity harbors more than 1000 different taxa (3). The microbial communities found in the oral cavity are diverse and abundantly complex; thus, antibiotic resistance genes are an expected occurrence. The increase in the number of antibiotic resistance genes in the oral microbiome has been attributed, at least in part, to the continued overuse and misuse of antibiotics (4).

The use of antibiotics by dentists is in most cases related to the treatment of acute oral bacterial infections, some cases of chronic periodontitis, and, although controversial, prophylactic therapy in patients with a risk of developing infectious endocarditis (5-7). Increasing awareness and knowledge on the proper use and the dangers of unnecessary use of antibiotics has led to a reduction in the number of antibiotic prescriptions among general dental professionals (8). A study by Smith *et al.* reported a 4% reduction in the number of antibiotic prescriptions among dentists in Norway in 2010-2016 (9). In Norway, dentists are currently responsible for approximately 5% of the total annual consumption of antibiotics (8).

The treatment of acute dental infections where antibiotic therapy is required can be achieved using beta-lactams, lincosamides, macrolides, metronidazole, and tetracyclines. Tetracyclines are one of the most extensively used antimicrobial agents in clinical settings, and their use has been coupled with increasing numbers of tetracycline-resistant bacteria. Resistance to tetracycline is associated with the presence of tetracycline resistance genes (*tet* genes). Of these *tet* genes, *tet* (M) has the broadest distribution among bacteria (10), which may in part be due to the gene's association with MGEs. One such MGE is Tn*916*, which was first isolated from a multidrug-resistant *Enterococcus faecalis* strain, DS16 (11), and has over the years been isolated in numerous bacterial species, including oral streptococci.

1.1 The oral cavity

The oral cavity is the gateway to the body. In addition to being the main entry point into the gastrointestinal system and the respiratory system, the oral cavity has the second most complex microbial community (the oral microbiome) after the gut (12). The oral microbiome refers to the total genetic material of all the microscopic organisms that are present in the oral cavity, including bacteria, fungi, viruses, archaea and protozoa.

The oral cavity has different surfaces that include soft epithelial cells, hard tooth enamel and the tongue. These surfaces provide different and unique niches that vary in nutrients, pH and physical forces such as saliva or brushing and are thus preferentially colonized by members of the oral microbiome. The complexity and ever-changing conditions in the oral cavity have been shown to influence the preferential species colonization of the different surfaces and the different ecological niches that are present (13). A study by Aas *et al.* illustrated that 34-70 species were detected per individual, whereas Mager *et al.* reported significant variations in the proportions of the 40 species isolated from eight oral soft tissue surfaces (13, 14). These findings show that not only do the number of species present vary between individuals but also the microbial composition varies across time. Despite these variations, stable communities known as climax communities are present in the oral cavity and are responsible for maintaining microbial homeostasis (15).

The microbial abundance and complexity of the oral cavity provides an excellent environment for microbial interactions and the formation of structured communities called biofilms or dental plaque. Biofilms are diverse communities of microorganisms that are highly organized and enclosed in matrix structures (16). They may consist of bacteria, fungi and viruses. The exact composition and diversity of oral biofilms is difficult to determine, as not all species can be cultured, and the interactions between some of these species are far from being fully understood (17). Attempts to understand the role of individual species in oral biofilms have resulted in the identification of a corncob structure comprising of the 10- species as shown in **Figure 1** (18). As shown in this figure, in the formation of oral biofilms, Actinomyces and Streptococcus species are the first bacterial species that attach to the acquired pellicle on the surface of the tooth.

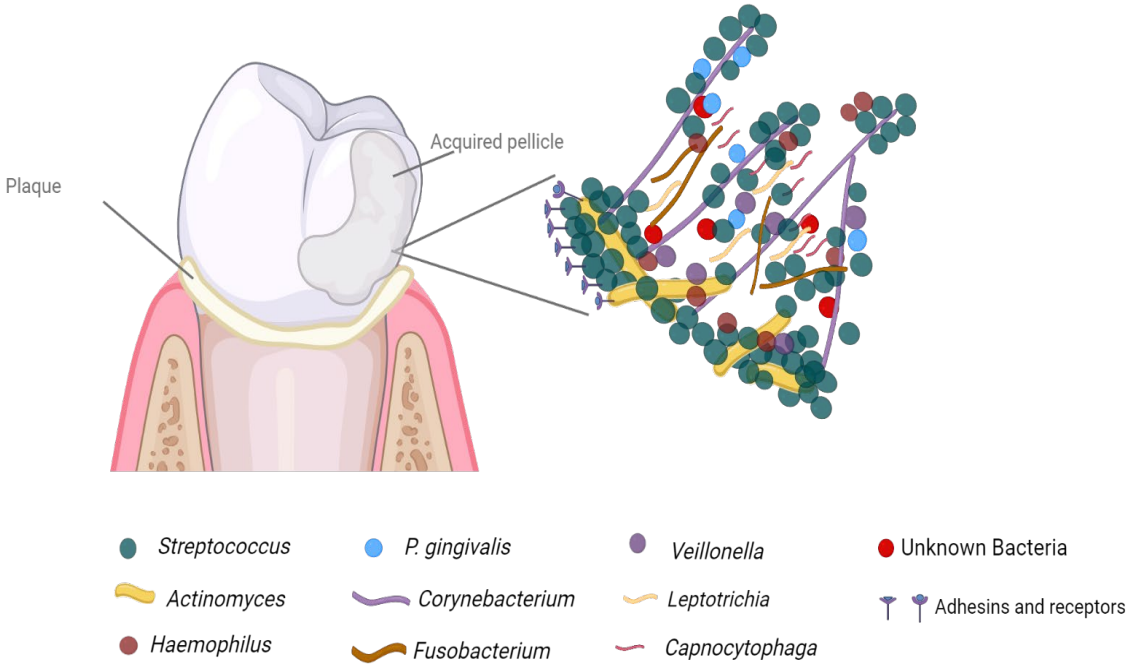

Figure 1: A representation of a biofilm on a tooth's surface. This biofilm is made up of 10- species and illustrates a summary hypothesis of the way that these species interact in the biofilm. As the early colonizers, *Streptococcus* and *Actinomyces* attach to the acquired pellicle on the surface of the tooth. The *Corynebacterium* filaments bind to the *Streptococcus* and *Actinomyces* cells and the distal ends of the filaments provide attachment sites for corncob structure of the growing biofilm. The biofilm includes *Haemophilus*/*Aggregatibacter*, *Neisseriaceae*, *Fusobacterium*, *Leptotrichia*, *Capnocytophaga* and some unknown bacterial species. The scale is not representative and is based on Amman *et al.* (17) and Welch *et al*. (18).

Although the composition of biofilms may vary, the formation of all biofilms occurs due to a series of ordered events that begin with the attachment of free-floating early colonizers to a surface (16). The attachment surfaces include the mucous membrane, epithelia, food particles and teeth. The rate at which planktonic bacteria attach to the surface is influenced by, for example, body fluids such as saliva, which form a conditioning film or acquired pellicle on tooth surfaces.

The formation of dental biofilms involves several distinct stages, including acquired pellicle formation, reversible adhesion between the bacterial surfaces and the pellicle, attachment of secondary colonizers, multiplication and biofilm formation and, in some cases, detachment (19). The predominant initial colonizers in dental biofilms include the genera *Actinomyces* and *Streptococcus* (20). Once *Actinomyces* and *Streptococcus* species have preferentially attached

to the epitope (which may be provided by the epithelial cell membrane, saliva, proteins or glycolipids), they alter the environmental conditions, thus providing more suitable growth conditions for fastidious species such as *Porphyromonas gingivalis, Fusobacterium nucleatum, Treponema* species and *Prevotella intermedia* (20).

These species bind to the developing biofilm by an adhesion-receptor mechanism and facilitate the expansion of the microbial population within the biofilm. The number of species and the composition of oral biofilms vary, but biofilms have been shown to provide bacteria with a microenvironment that favors nutrient sharing, exchange of genetic material, host defense protection, improved metabolism, and increased protection from antibiotics (16). Protection from antibiotics could be attributed to the inability of the antibiotic to penetrate the biofilm, alteration in the bacterial growth rate and slow bacterial metabolism (21).

1.1.1 Streptococci

The term streptococcus has its roots from the Greek word *streptos* (chain) and *kokkos* (berry) and was first used by the Austrian surgeon Theodor Billroth in 1874. He used the term to describe "small organisms as found in either isolated or arranged in pairs, sometimes in chains" (22). However, only a decade later, Rosenbach used the term *Streptococcus* to describe chain-forming cocci isolated from infectious sores in a man (23). The genus *Streptococcus* is a diverse group of gram-positive, catalase-negative, nonmotile cocci that constitute a large part of the normal flora of humans and animals. In humans, streptococci have been isolated from the oral cavity and upper respiratory tract.

1.1.2 Classification of oral streptococci

With more than 110 known *Streptococcus* species belonging to the genus to date (24), the diversity of the genus *Streptococcus* has presented some challenges in the classification of all the species. As early as 1937, attempts were made to classify the genus into "pyogenic," "enterococcus," "lactic," and "viridans" groups based on their hemolytic activity and temperature, sodium chloride and pH tolerances (25).

Over the years, numerous attempts have been made to improve the classification scheme by basing it on phenotypic traits, such as sugar fermentation and growth characteristics in milk (26) and tolerance to heat and sodium chloride (27). Although extensive, these methods have not been sufficient in classifying all oral streptococci due to the heterogeneous nature of the genus and have resulted in strains within the same species testing negative for some common

traits (28, 29). Genotypic methods such as species-specific PCR have proven more successful in classifying oral streptococci (30-32).

DNA sequencing has proven to be a more accurate and reliable way to classify oral streptococci. A genome analysis of 68 representative genomes from the genus *Streptococcus* resulted in a phylogenetic tree, showing relationship positions within the genus (**Figure 2**) (33). Based on Bayesian phylogenetic analysis of the genetic sequences from eight housekeeping genes, oral streptococci have been identified in six of the nine newly determined groups: the mitis, sanguinis, anginosus, salivarius, downei, and mutans groups (34, 35). The oral cavity and upper respiratory tract have the highest abundance of streptococci on the human body, and the mitis group, with 20 currently identified species, is the largest group (35, 36).

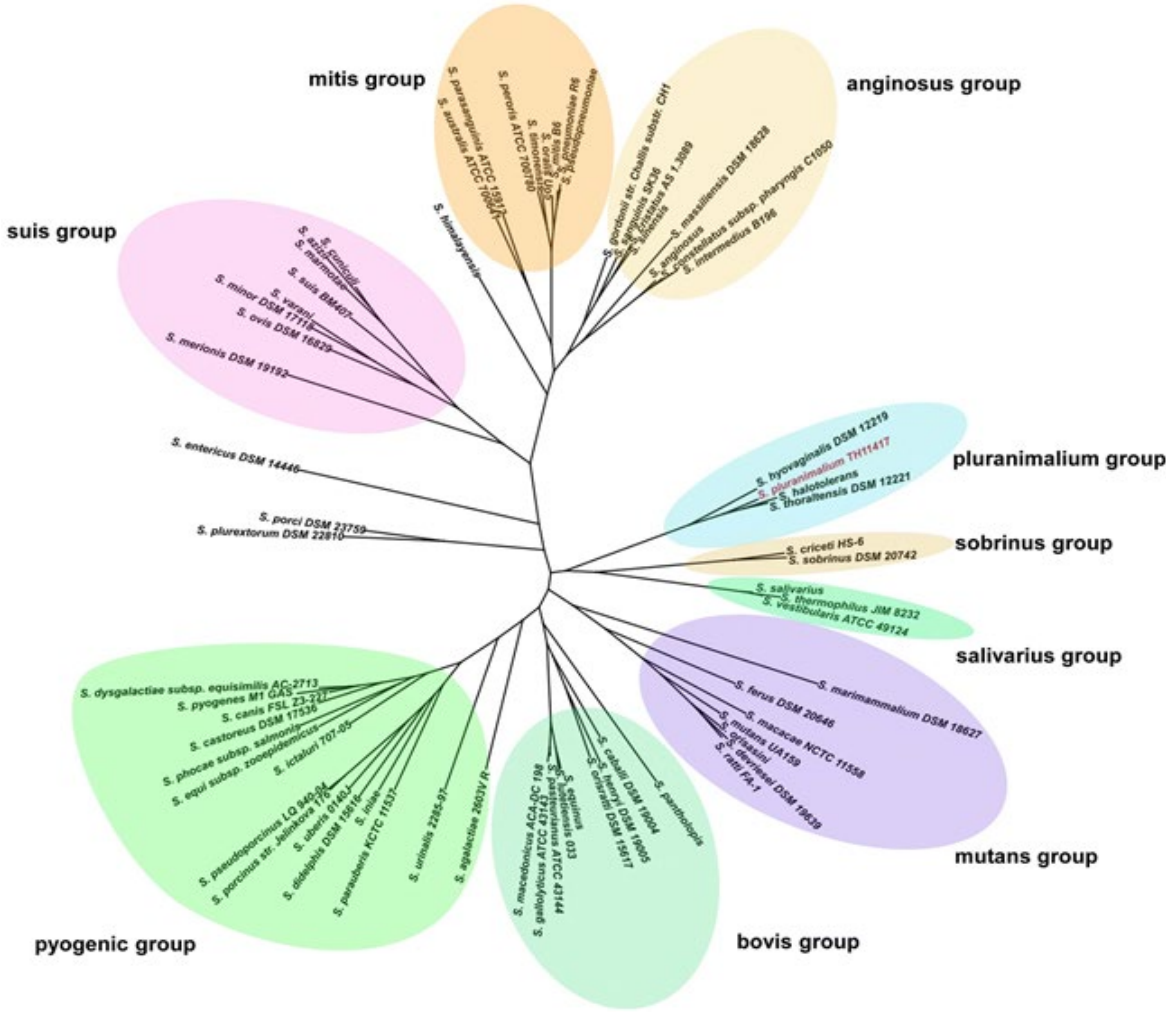

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Figure 2: A phylogenetic tree showing the relationship positions of 68 representative genomes from the genus *Streptococcus*. Courtesy of Pan *et al.* (33).

The term "viridans streptococci," derived from the Latin word *viridis* (meaning green), has been interchangeably used to describe oral streptococci. "Viridans" refers to the green sheen that appears due to the partial hemolysis observed when the bacteria are cultivated on blood agar. The use of this term has, however, proven to be erroneous, as not all oral streptococcal strains have the ability to hemolyze blood, and the degree of hemolysis has been found to have both inter- and intraspecies variation (36, 37).

Streptococcus species are present in virtually all sites in the oral cavity. To survive in the complex and ever-changing oral environment, streptococcal species have established niches that they are well adapted to in the oral cavity. For instance, the species *Streptococcus salivarius* has been found mainly in the saliva and on the tongue; *Streptococcus sanguinis* and *Streptococcus gordonii* have been found on the teeth, whereas *Streptococcus oralis* and *Streptococcus mitis* are associated with multiple sites in the oral cavity (13).

1.1.3 Clinical significance of oral streptococci- Friend or foe

Oral streptococci constitute a substantial part of the oral microbiome of virtually all humans. They are natural inhabitants of the oral tissues and have been shown to inhabit nearly all surfaces in the mouth. Some oral *Streptococcus* species, such as *Streptococcus mutans*, *S. gordonii*, *S. oralis*, and *S. sanguinis*, have been shown to express adhesions that allow them to bind to salivary glycoproteins and bacterial-derived salivary components (32).

The expression of adhesins on the surface of oral streptococci enables them to bind to several substrates within the human body, thus promoting their success as both commensals and opportunistic pathogens (32). Most of these species are found as part of a consortium (biofilm) that, through intricate interactions with the host immune system, helps to maintain homeostasis and a state of health (38). Oral streptococci have generally been regarded as commensal bacteria and are essential in both the innate an adaptive host immune system due to their ability to inhibit the colonization of many bacterial pathogens. For instance, *S. oralis*, *S. mitis* and *S. sanguinis* are some of the peroxigenic oral streptococcal species that inhibit colonization by *S. mutans* and *P. gingivalis*, which can cause dental caries and chronic periodontitis, respectively, by producing hydrogen peroxide (38).

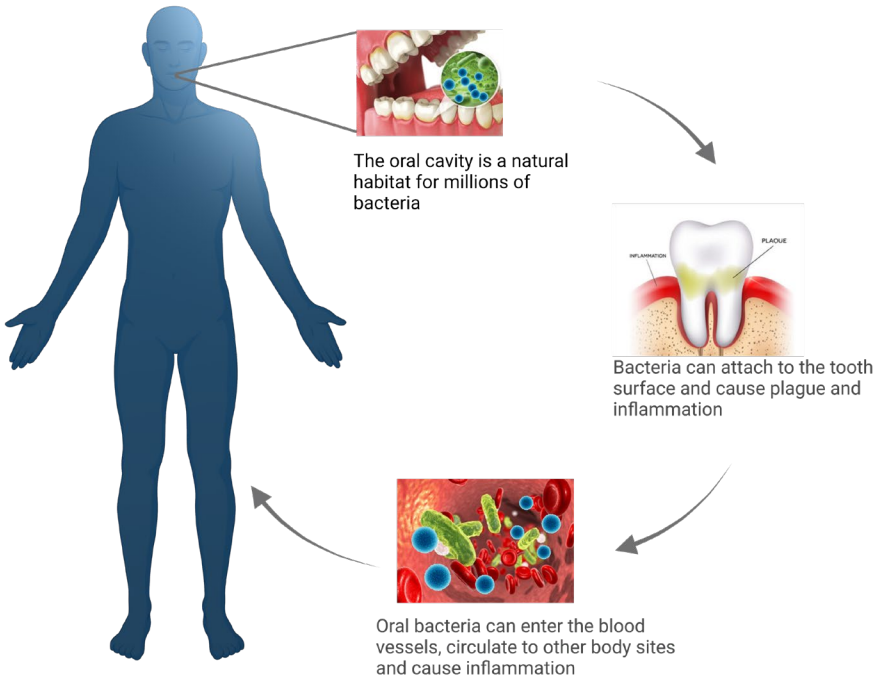

Figure 3: The health status of the oral cavity has the potential to affect the overall health of the body. Bacterial species that are present in the oral cavity can attach to the root surface and form part of biofilms or dental plaque. Alterations in microbial homeostasis or injuries in the gum may lead to inflammation in the gum, which may serve as an entry point for the bacteria into the bloodstream. Once in the bloodstream, the bacterial species can circulate to other body sites and cause inflammation.

However, in recent years, some species have been labeled opportunistic pathogens that can cause invasive infections such as infective endocarditis, septicemia, and pneumonia in neutropenic individuals (37, 39). Changes in oral microbial homeostasis may lead to dysbiosis within the oral microbiota and thus niche changes and a relatively higher microbial load of specific species. This altered state of homeostasis may result in periodontal disease, which may in turn lead to oral bacteria invading the blood vessels in the periodontal tissue before migrating to other body sites, as illustrated in **Figure 3**. Once in the bloodstream, these species migrate to other body sites and cause infections. The invasive potential of oral bacterial species coupled with the oral cavity's exposure to millions of transient bacterial species as they enter the body is indicative of how oral health can affect the overall health of the whole body.

In addition to their possible invasive pathogenicity, oral streptococci are increasing in significance, as the reported number of antibiotic-resistant streptococci is increasing. To date, oral *Streptococcus* isolates that are resistant to the most commonly used antibiotics, including penicillin, beta-lactams, fluoroquinolones and tetracyclines, have been reported (37, 39-41).

1.2 Antibiotic resistance

Antibiotics may be defined as natural, synthetic, or semisynthetic molecules that can inhibit the growth of or kill bacterial cells. The mode of action of antibiotics is termed either bacteriostatic when they slow bacterial growth or bactericidal when the antibiotic kills the bacterial cells. Antibiotics prevent or reduce bacterial cell growth by inhibiting cell wall synthesis, protein synthesis, nucleic acid synthesis, or folic acid synthesis and/or by interfering with membrane permeability.

The discovery of penicillin by Alexander Fleming in 1928 held the promise of good public health and reduced mortality rates. This success was, however, short-lived due to bacteria's natural ability to adapt to and survive in hostile and ever-changing environments. The use of antibiotics resulted in bacteria evolving and developing the ability to survive in the presence of these antibiotics. This ability to survive in the presence of a chemical agent that would typically kill the bacteria is referred to as antimicrobial/antibiotic resistance. As Fleming predicted in 1945, the inconsiderate use of antibiotics has led to the selection and propagation of selected bacterial mutants resistant to antibiotics only a few years after the introduction antimicrobial agents. As illustrated in **Figure 4**, the introduction of an antimicrobial agent is usually followed by the reported emergence of resistance to that antimicrobial agent (42). However, cases of resistance determinants that pre-date the antibiotic launch have been reported, as with the identification of penicillinases before the use of the antibiotic (43).

The emergence of antibiotic resistance among bacterial populations presents one of the most severe challenges of modern medicine. This phenomenon is occurs in both gram-positive and gram-negative bacteria and is associated with more than 30 000 deaths per year in the European union and the European Economic Area (44, 45). There has been an increase in mortality rates due to superficial infections that become complicated. The use of antibiotics as prophylactics to accompany life-saving procedures such as chemotherapy and surgery (including hip replacement and cesarean sections) once promised good survival rates among patients. However, the ever-increasing rates of AMR among bacterial populations are turning these procedures into high-risk and life-threatening procedures. In India alone, more than 58 000 babies have died due to methicillin-resistant *Staphylococcus aureus* (MRSA) and extended-spectrum β-lactamase-related infections (ESBL) (46).

The emergence of bacteria resistant to more than one antibiotic or multidrug-resistant bacteria is increasing globally, with approximately 23 000 people dying annually from multidrug-resistant infections in the US alone (47). In addition to the high mortality rates, AMR infections impose a social and economic burden on society due to large medical care bills and prolonged hospitalization.

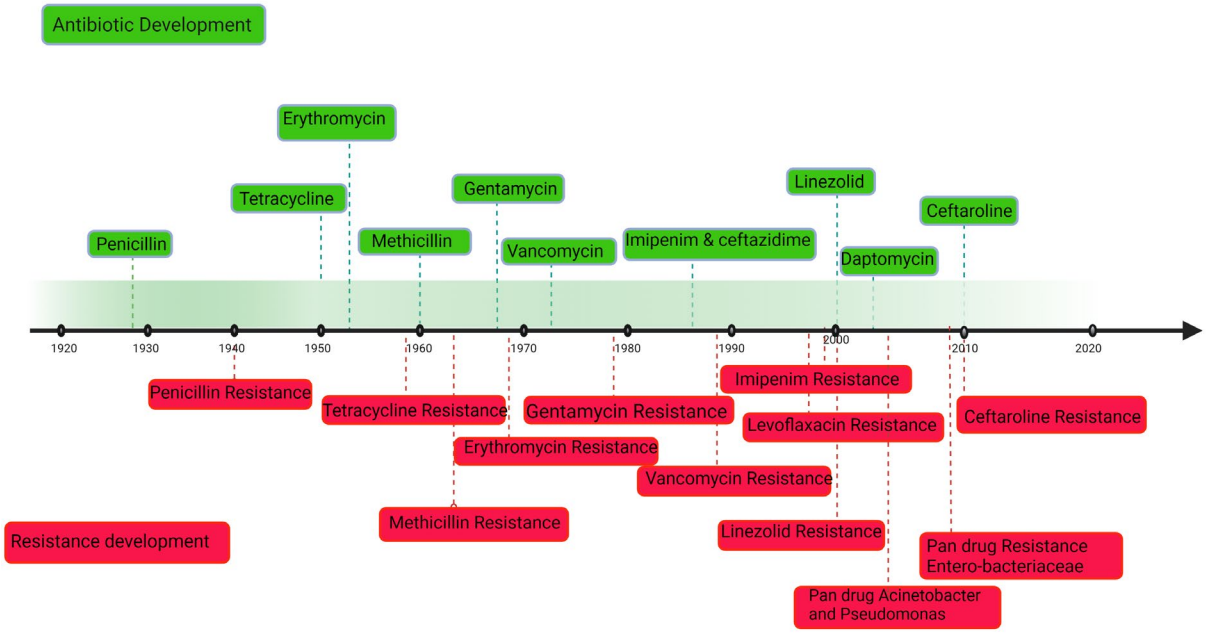

Figure 4: A timeline showing the development of the main classes of antibiotics followed by the emergence of resistance to these antibiotics. The year of antibiotic development is shown above the timeline in green, whereas the year when resistance was first observed for each antibiotic is shown below the timeline in red. Reprinted and adapted with permission from (42).

The ability of bacteria to withstand antibiotics can broadly be classified into two main mechanisms: intrinsic and acquired resistance. Intrinsic or innate resistance is attributed to inherent structural and functional characteristics that render bacterial cells inaccessible to antibiotics. These traits are species specific and are usually related to cell wall features that act as a barrier and prevent the entry of antibiotics. Intrinsic resistance is present in the entire bacterial population and is passed from one generation to the next (inheritable). Acquired resistance, on the other hand, occurs due to mutations or the acquisition of genetic material mediated by horizontal gene transfer (HGT), as discussed in detail in Section 1.4.

1.2.1 Mechanisms of antibiotic resistance

Bacterial cells can survive in the presence of antibiotics by employing one or more of the mechanisms shown in **Figure 5**. These mechanisms include (i) enzymatic inactivation of the antibiotic (ii) structural features that limit permeability of the antibiotic (iii) active reduction of antibiotic concentrations in the cell by either efflux pumps or reduced penetration, and (iv) mutations, post-translational modifications, and protection of the antibiotic target. The latter is responsible for the tetracycline resistance we focused on in **papers I**, **II** and **III**.

In addition to the abovementioned mechanisms, bacteria may aggregate and form biofilms, which have been reported to increase tolerance to antibiotics by up to 1000 times (48).

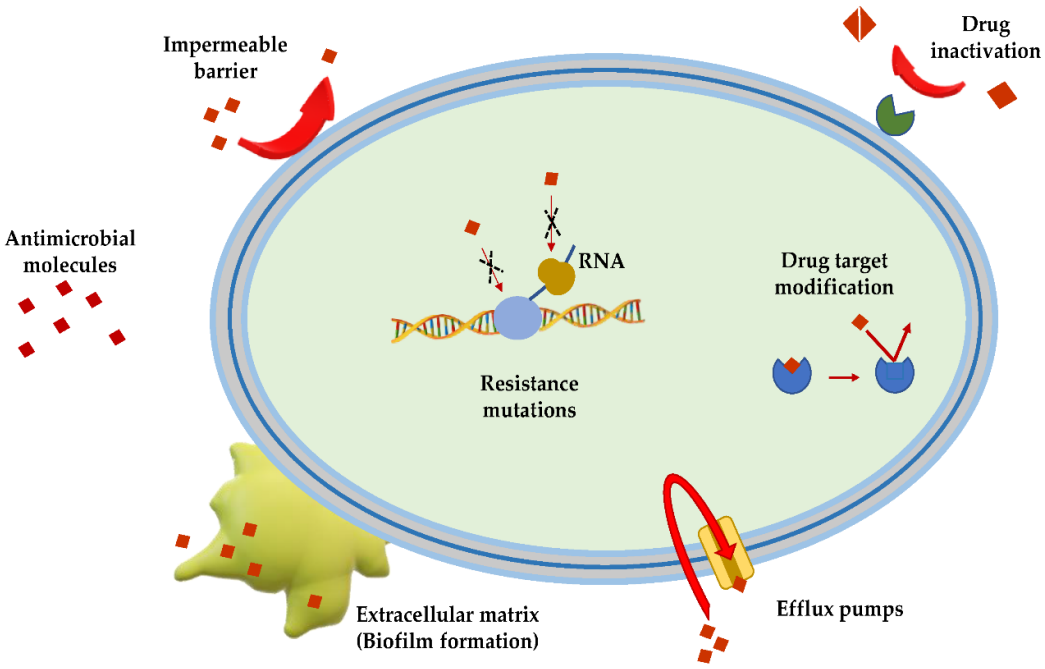

Figure 5: Schematic representation of the mechanisms involved in antibiotic resistance in bacteria. Courtesy of Cappiello *et al.* (49).

1.2.1.1 Tetracyclines

Tetracyclines were introduced to medicine in 1948 as an alternative treatment agent after the emergence of penicillin resistance. As broad-spectrum antibiotics, tetracyclines have been used to treat a wide range of gram-positive and gram-negative bacterial infections. The broad-spectrum activity, low cost of production, and absence of significant side effects have resulted in extensive production and use of tetracyclines. According to Gu *et al.* tetracyclines are the second most produced and consumed antibiotic globally (50). Tetracyclines can be classified

into two main groups based on their mode of action: atypical tetracyclines, which disrupt the membrane of the bacteria, and typical tetracyclines, which prevent bacterial growth by binding to the ribosome and thus inhibit protein synthesis (51). Typical tetracyclines include tetracycline, chlortetracycline, doxycycline, and minocycline. In humans, tetracyclines are used to treat atypical pneumonia, cholera, periodontal infections, acne, and other local and systemic infections (52). In the United States, tetracyclines are not only used to treat bacterial infections but also administered in subtherapeutic amounts in animal feeds to promote growth. (53).

1.2.1.2 Mode of action of tetracycline

Tetracycline is a bacteriostatic agent that crosses the cell membrane by passive diffusion. Once inside the bacterial cell, it inhibits bacterial growth by attaching to the ribosome and blocking protein synthesis, as illustrated in **Figure 6** (54). Tetracycline binds reversibly to the A-site of the 30S ribosomal subunit (which appears to be the primary binding site for tetracycline), thereby interfering with the binding of aminoacyl-transfer RNA (tRNA) and preventing the transport of tetracycline into bacterial cells varies between gram-positive and gram-negative bacteria. In gram-positive bacteria, tetracycline enters the bacterial cell through an energy-dependent active transporter (55), whereas in gram-negative bacteria, tetracycline has been shown to passively diffuse across the outer membrane through the *Omp* F and *Omp* C porins (56, 57).

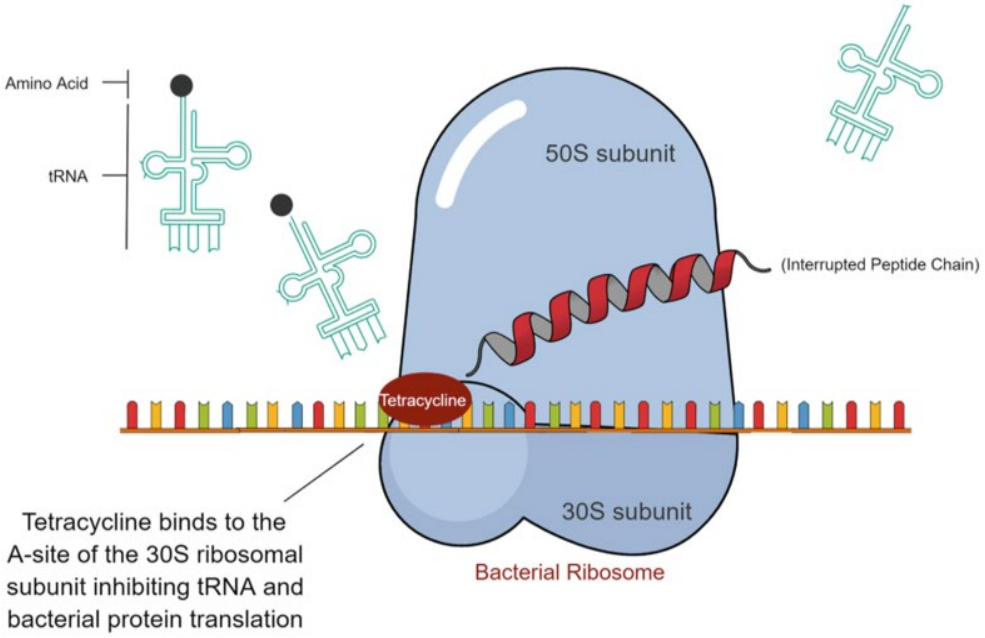

Figure 6: An illustration of the mechanism of action of tetracycline. Reproduced from Reis *et al.* 2020 (54).

1.2.1.3 Resistance to tetracycline

Resistance to tetracycline can be attributed to mutations within the chromosome, leading to increased expression of intrinsic resistance mechanisms or altered ribosomal binding sites. However, the most frequently encountered mechanism of tetracycline resistance is associated with the presence or acquisition of tetracycline resistance genes. The forty-six different genes that have been identified from 126 bacterial genera have been shown to confer resistance to tetracycline by three mechanisms, i.e., efflux pump activity, ribosomal protein protection, and/or enzymatic inactivation (58). In addition to the abovementioned mechanisms, one more undescribed mechanism of resistance is conferred by *tet* (U) (59). Of the 46 tetracycline resistance genes reported, *tet* (O) and *tet* (M) are the most common and well-described genes. The *tet* (M) gene has the broadest distribution among bacteria and has frequently been associated with MGEs (58). The association of *tet* (M) with MGEs coupled with findings showing that this gene is more than 95% identical at the nucleotide level across bacterial species suggests that it has been disseminated among bacterial species by HGT (60).

Despite the reduction in tetracycline usage in humans, it has become evident that reversal of tetracycline resistance within the human microbiome is not easy to achieve. This was evident, for example, in a study carried out by Lancaster *et al.* which reported the presence of tetracycline resistance genes in 46 out of 47 children aged between 4-6 years old who had not received antibiotic treatment in the previous three months (61). In another study by Seville and others, tetracycline resistance genes were detected in all twenty saliva samples collected from a group of volunteers from six European countries that had not received antibiotic therapy in the three months prior to the study (52).

The continued use of tetracycline in the agricultural sector poses a significant challenge, as tetracyclines have been detected in farming effluents and wastewater treatment facilities (62). The presence of these antibiotics in the environment and the continued misuse of this antibiotic can, at least in part, explain the increase in observed levels of tetracycline resistance.

1.2.2 Biological cost of antibiotic resistance

The use of antimicrobial agents may lead to eradication of the targeted bacterial population if the treatment is successful. The introduction of this selective pressure has been shown to correlate with the emergence of antibiotic resistance as bacteria evolve and adapt to the new environmental challenges. Although beneficial in the presence of antibiotics, the development of antibiotic resistance may affect the biological fitness of bacterial cells. The biological fitness of a bacterial species refers to the bacteria's ability to thrive in competitive environments and is defined as the bacteria's ability to survive, reproduce, and be transmitted (63). It may be beneficial (increased relative fitness: >1), neutral (no change in relative fitness: =1) or detrimental (reduced relative fitness: <1). In evolutionary biology, biological fitness is one of the fundamental concepts that reflects the reproductive success of the fitter organisms. In bacterial populations genotypes with lower fitness grow slower and reproduce at lower rates than the competing fitter genotypes which grows and reproduces quicker and thus outcompetes the less fit genotype (64).

The fitness of a bacterial population may be measured as absolute fitness or relative fitness. Absolute fitness refers to the total performance of a genotype, whereas relative fitness measures how well one genotype performs relative to another. Relative fitness is mainly used to measure bacterial fitness and can be expressed as generation time or growth rate calculated from optical density readings of a monoculture. Relative fitness (w) is mainly used to measure bacterial fitness and is expressed as the relative rate of growth of one genotype to another and can be calculated from optical density readings of the log phase of a monoculture. Relative fitness may also be determined by head-to-head competition between the two genotypes (65).

Biological fitness can be seen as a function of natural selection as changes that impose a high fitness cost tend to lead to loss of the change or replacement of the change by a less costly one. The persistence and retention of the acquired genetic material are dependent on several factors, including the biological cost, the genetic advantage of the acquired material, and the host's ability to ameliorate the cost via genetic compensation mechanisms associated with retaining the acquired traits (66, 67).

The reduction in relative fitness of a bacterial population can be associated with the acquisition of resistance determinants (as described in detail in this thesis). It is, however, worth noting that

acquired resistance determinants may not be the only source of observed fitness costs in a bacterial population. For instance, a reduction in biological fitness was observed in *S. aureus* after introducing sub-MIC volumes of vancomycin which induced the expression of the *vanA* operon and resulted in a resistant phenotype (68). In addition, spontaneous mutations, such as those found in the *rpo* B of rifampicin-resistant *E. coli* clinical isolates, have also been shown to impose fitness costs on bacterial cells (69, 70).

In the case of AMR, the acquisition of additional genetic material may reduce the survival and reproductive success of the bacterial cell compared to those of its susceptible counterpart. It has long been shown that the acquisition of resistance genes by HGT may lead to reduced fitness in a population due to, among other factors, increased energy requirements associated with gene expression and replication of the acquired genes (71). In the presence of selective pressure, the acquired resistance determinant although costly for the host is beneficial as it allows the host to survive. Once the selective pressure is removed, one would expect that the "more fit" susceptible bacteria to outcompete their resistant counterparts or for the resistant bacteria to loss the acquired gene and revert to the susceptible fitter phenotype. This is, however, not always the case, as the reversal of antibiotic resistance seems complex and hard to accomplish. The persistence of resistance may be linked to reduced, negligible or low fitness cost associated with the acquired genes (72-76). In cases where the acquired DNA imposes high fitness costs on the host, reciprocal or compensatory adaptations have been shown to ameliorate the cost and restore fitness (72, 77, 78) (**paper III**). Experimental evolution studies indicate that mutations on the chromosome or within the acquired DNA may be used as a strategy to restore fitness within a bacterial population (77, 79). The fitness cost, rate of compensatory mutations, and quantity of antibiotics present in a bacterial population are some of the factors that influence the frequency and persistence of AMR in a bacterial population (80). The abovementioned factors are indicative of retention of resistance within bacterial populations, even in the absence of selective pressure, and are a cause for concern.

1.3 Horizontal gene transfer (HGT)

HGT is a natural phenomenon that was first demonstrated in pneumococci by Griffith in the early 1900s (81). In HGT, the lateral transfer of genetic material occurs between bacteria regardless of relatedness. It has, therefore, long been known to be an important force in the evolution of bacteria and has been identified as a way for bacterial populations to acquire and spread new traits or resistance determinants (82-84). The rich diversity and abundance of bacterial species found in the human body, especially in dense microbial environments such as the intestines, have been shown to frequently exchange DNA by HGT (85). The oral cavity and bacterial populations therein are exposed to multitudes of transient species, and the bacteria can thus acquire genetic material by HGT, as has been shown to occur in the gut (86, 87).

The genus *Streptococcus* displays extreme evolutionary plasticity due to its high levels of gene gain and loss (88). The observed genetic diversity has been attributed mainly to HGT events that are thought to occur in biofilms (89). An example of the central role that HGT plays in generating diversity in this genus was shown by Choi *et al.* who showed abundant gene flow between *Streptococcus pyogenes* and *Streptococcus dysgalactiae* (90). In addition, findings that showed that more than half of the species-specific genes in *Streptococcus* have possibly been acquired by HGT serve to only highlight the importance of HGT in these species (83, 91). Genes acquired by HGT contribute to the evolutionary plasticity of the bacterial population and can generally be divided into two main groups: genes that play a role in pathogenesis and virulence and/or genes that play a role in bacterial niche maintenance (83). In the oral cavity, the role of HGT in niche maintenance can be showcased by the ability of *S. mutans* species to survive under low-pH conditions due to the lateral acquisition of multiple genes related to transport and carbohydrate metabolism (83).

HGT within streptococcal species is of especially great concern, as it has been shown to facilitate the transfer of resistance determinants from pathogenic isolates to commensal isolates. Chi *et al.* for instance, demonstrated that the increase in penicillin-resistant streptococci may be attributed to the transfer of regions of the *pbp2x* gene between *S. pneumoniae*, *S. oralis*, and *S. mitis* by recombination (92).

To date, four main mechanisms of HGT have been well described, i.e., transduction, transformation, membrane vesicles (MVs) and conjugation. **Figure 7** shows a summarized illustration of the mechanisms of HGT.

1.3.1 Transduction

The transfer of genetic material from one bacterial cell to another mediated by bacteriophage particles is referred to as transduction. Transduction can occur in a wide range of bacteria, and as the host range is determined by the receptor recognition sites on the viruses, bacteriophages may transduce single or multiple phyla (93). Transduction may allow the transfer of any bacterial gene, in which case it is referred to as generalized, or it may be specialized, in which case only specific bacterial genes have the potential for transfer (94). In addition to the above-mentioned modes of phage transfer, a recent study describes an additional natural and potentially powerful mode of phage mediated gene transfer in *S. aureus* (95). Lateral transduction can facilitate the transfer of several hundred kbs of genetic material and thus forms a portal for genetic exchange among hosts (95). Bacteriophages can transfer whole plasmids, entire genes, and/or pieces of chromosomes between hosts. Several studies have reported the presence of bacteriophages in samples isolated from the oral cavity (96, 97), indicating that transduction may occur in the oral cavity. With more than fifty known phages that can infect oral *Streptococcus* species (including *S. mitis*, *S. mutans*, *S. oralis*, *S. salivarius*, and *S. sobrinus*) (98), transduction can be deduced to play a role in the transfer of genetic material in these species. The limited knowledge regarding the role bacteriophages play in the spread of AMR in oral streptococci has not hampered the growing interest in using phages to target oral bacteria. Szafrański *et al.* reported that phages can infect oral bacteria and have been successfully used to target and penetrate oral biofilms consisting of *A. naeslundii* and *Streptococcus* spp. (98).

1.3.2 Transformation

The process of transformation involves the active uptake of foreign DNA from the surroundings of a bacterial cell. Donor cells do not have to be viable for transformation to occur, as free DNA from cell lysis is sufficient for the process (99). For transformation to occur, recipient cells have to be competent, a physiological state that allows for an active uptake and incorporation of foreign DNA into recipient bacteria (100). The state of competence may be constitutive, as is the case with *Neisseria gonorrhea*, or may be regulated by the gene products of the competence

regulation operon, as is the case in *S. pneumoniae*, *S. gordonii*, *S. mitis*, *S. oralis*, and *S. sanguinis* (100-103). Once taken up, DNA may be integrated into the chromosome of the recipient cell by homologous recombination, a process that requires similar or identical DNA sequences in the donor and recipient cells. The success of homologous recombination events increases with increased homology between the donor and the recipient, a characteristic that is common in streptococci (104). Several oral streptococcal species can undergo transformation, with *S. gordonii* being considered the archetype (90). In vitro studies have shown that competent *S. gordonii* DL1 could actively take up plasmid-derived DNA in the mouth by transformation (105, 106). These studies showcase homologous recombination as a potential mechanism for the acquisition and integration of DNA sequences when relatively short regions (458 bp) of homology are present in both the free DNA and the chromosome of *S. gordonii* DL1 (106).

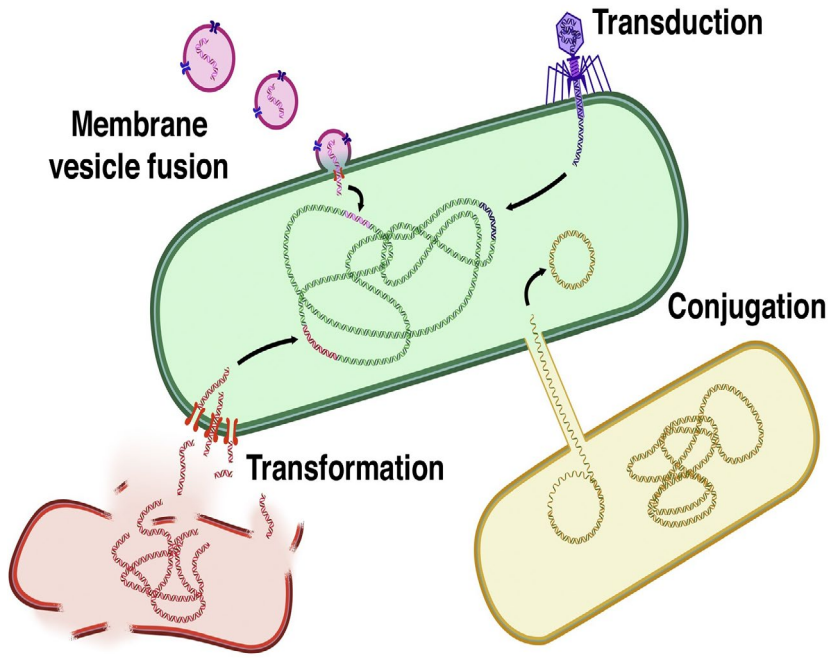

Figure 7: The four mechanisms of Horizontal gene transfer that mediate the transfer of both chromosomal and extra-chromosomal DNA in bacteria. In **Transformation**, extracellular free DNA is taken up by a viable bacterial cell. In **Conjugation**, mobile genetic elements including plasmids and ICEs can be transferred from the donor to the recipient after the bacterial cells establish physical contact. The transfer of genetic material that is mediated by phages is called **Transduction** whereas **Membrane vesicles** are lipid bilayer enclosed vesicles that bud off from the cell and can transfer DNA to another cell. Curtesy of Mclnnes *et al*. (107).

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Bacteria in the oral cavity tend to form biofilms (as discussed in Section 1.1). The conditions in these biofilms (including the abundance and diversity of bacterial cells) provide an excellent environment for the exchange or uptake of genetic material, as has been shown by Li *et al.* (108). In the study mentioned above, Li *et al.* demonstrated that when grown in biofilms, *S. mutans* cells were hyper transformable and could be transformed 10- to 600-fold more readily than planktonic cells. They also illustrated how biofilms may act as reservoirs of antibiotic resistance genes when dead bacterial cells act as donors of resistance determinants (108).

The role of transformation in the transfer of antibiotic resistance determinants in oral streptococci was evident as early as the 1980s, when three *S. mutans* strains acquired streptomycin resistance via natural transformation (109). Transformation has been shown to occur between oral bacteria from different genera; it has, for example, been demonstrated to occur between oral streptococci and *Treponema* species, which are gram-negative bacteria associated with pathogenesis of periodontal disease (110). The presence of genes related to restriction endonucleases that play a role in monitoring the foreign DNA that is acquired by transformation in *S. pneumoniae* is an indication of the important role that HGT plays in these species (83).

Transformation is thought to be a key player in the transfer and spread of antibiotic resistance in the oral cavity due to the abundance of bacterial species, the availability of free DNA from cell lysis, and the presence of competent bacteria in the oral cavity (60).

1.3.3 Membrane Vesicles

Membrane vesicles (MVs) are tiny spherical, bi-layered membrane structures that range from 10 to 500 nm in diameter (111). The presence of MVs in numerous bacterial species suggests that they play an important role in the survival and plasticity of bacterial species. MVs have been shown to contain a variety of cargo, including nucleic acids, enzymes, virulence factors, and antibiotic resistance proteins (112-118). Although commonly produced by gram-negative bacteria, MVs have been shown to occur in a few gram-positive bacteria, including *Bacillus subtilis*, *S. aureus* (119), and *S. mutans* (117). In *S. mutans*, membrane vesicles have been shown to play a role in the transfer of genetic material by transferring extracellular DNA into developing biofilms (117). The genetic material found in MVs can be of chromosomal (such as those reported in *P. gingivalis* (120)), viral and plasmid origin (118, 121, 122). The size of this genetic material may vary from small fragments to large fragments of up to 370kb (111).

1.3.4 Conjugation

In conjugation, the transfer of genetic elements from the donor bacterium to the recipient requires that both the donor and the recipient are living and that they are in physical contact with each other. Conjugation plays a vital role in the acquisition and spread of antimicrobial resistance genes among bacterial populations, as it has been shown to occur in both closely related and distant bacterial species. In conjugation, the spread of resistance determinants is mediated by two types of broad-host-range elements: conjugative plasmids and/or integrative and ICEs, such as Tn*916.* Conjugative plasmids and ICEs have been isolated from many different oral bacterial species, and both the inter- and intraspecies transfer of these MGEs has been reported by numerous studies (11, 123-130). Experimental studies have shown that oral streptococcal species can act as donors and/or recipients of both conjugative plasmids and ICEs. For instance, the transfer of erythromycin resistance between *S. gordonii* and *E. faecalis* in an *ex vivo* tooth model was mediated by the conjugative plasmid pAM81 (131). Another study reported the *in vivo* transfer of the ICE Tn*6002*, which conveys resistance to tetracycline and macrolides, from *S. oralis* to *S. cristatus* (132). A study by Roberts *et al.* reported the transfer of the conjugative transposon Tn*5397* from nonoral *B. subtilis* to a mixed oral biofilm consisting of *Streptococci, Actinomyces* spp., *Lactobacillus* spp. and *Veillonella* spp. (129). The study mentioned above illustrates the role that conjugation can potentially play in transferring genetic material in the oral cavity. Ciric *et al.* reported the presence of Tn*916*-like elements isolated

from 48 minocycline-resistant oral *Streptococcus* isolates cultured from pooled saliva. The authors of this study said that 66.7% of these elements were able to form detectable CI molecules (the first step in conjugative transfer), thus indicating their potential to excise the host chromosome prior to transfer to other cells by conjugation (123).

The presence of conjugative plasmids and ICEs in the diverse communities of biofilms in the oral cavity provides an optimal conjugation environment.

1.4 Mobile genetic elements

Mobile genetic elements (MGEs) are fragments of DNA that encode the proteins required to facilitate their own mobility. MGEs generate genetic diversity within bacterial populations, as they can mediate the transfer of genetic material between bacterial cells and/or bacterial genomes. This renders them key contributors to the emergence and spread of AMR among bacterial species (133). As MGEs move around bacterial genomes, they may disrupt the genome structure and alter the functionality and expression of chromosomal genes, thus it comes as no surprise that MGEs may impart a biological cost to the host cell. However, as MGEs usually harbor genes that are beneficial or advantageous to the host, such as antimicrobial resistance genes, the biological cost of a MGE may be "tolerated" by the host. Of the known mechanisms of HGT, two are mediated by MGEs, namely, conjugation and transduction (see Sections 1.4.1 and 1.4.3 for details). Several MGEs have been identified to date, and the most common MGEs include plasmids, integrons, transposable elements, and integrative conjugative elements (ICEs).

1.4.1 Plasmids

Plasmids are extrachromosomal DNA molecules that can replicate autonomously and usually can be transferred between cells by conjugation machinery. As key vectors of HGT, plasmids are abundant and play an active role in the evolution of bacteria. They have been found to vary in size, ranging from less than one kilobase (kb) to approximately 500 kb (134). The core backbone of plasmids harbors genes that encode for their transfer between cells, whereas the accessory regions may include genes coding for virulence, detoxification, and antibiotic resistance (135). The presence of plasmids carrying erythromycin resistance genes in streptococci has been reported in several studies (136, 137).

1.4.2 Transposable elements

Transposons are a diverse group of MGEs that have been defined as a "specific DNA segment that can repeatedly insert into one or more sites in one or more genomes" (138). Thus, these elements can move from one genomic location by excision and insert into another genomic location or cell. Based on their mechanisms of transposition, transposable elements can be divided into two main classes: integrative mobilizable elements (IMEs) and ICEs. In contrast to ICEs (described in detail in Section 1.5.3 below), IMEs do not possess genetic material to facilitate their movement and thus depend on other MGEs for transposition.

1.4.3 Integrative Conjugative Elements

ICEs or conjugative transposons (CTns) are fragments of DNA that are integrated into the host chromosome but have the ability to excise, forming a CI that can then be transferred into another chromosomal site or genome prior to reintegration (11, 139). Since their discovery, the family of ICEs has been found to be a diverse group of MGEs that vary remarkably in size (from approximately 18 kb to 500 kb), and they have been identified in numerous gram-positive and gram-negative bacteria (84, 139). Furthermore, despite their tendency to often be integrated into the chromosome and thus passively propagate during host replication and cell division, some ICEs, including ICEBs1 and Tn*916*, have been reported to replicate autonomously (130, 139, 140).

ICEs have a common conjugation machinery or a backbone structure that includes distinct modules responsible for maintaining, disseminating, and regulating the ICE (118). This machinery primarily facilitates the mobilization of ICEs. However, it can facilitate the movement of other elements that are not mobile when ICEs form composite elements with IMEs that are inserted at the *att* site of the ICE (139, 141). These composite elements use the conjugation machinery of the ICE to move both elements, and if regions of homology are present within these composite elements, recombination may occur, resulting in the generation of ICE diversity (139). ICE diversity may also be generated when ICEs join to form composite tandem elements, as has been shown to occur in *E. faecalis* (tandem accretion of Tn*5801* and Tn*6647* to form Tn*6648*) (142).

The conjugative transfer of ICEs and plasmids in most prokaryotes involves a translocation device comprised of a type IV secretion system (T4SS), coupling protein, and relaxase (135, 143, 144). The integration of ICEs into the host genome is facilitated by three unrelated families of recombinases; serine integrases, DDE transposases, and tyrosine integrases (145, 146). Whereas DDE recombinase does not frequently require specific integration sites, serine and tyrosine integrases usually facilitate site-specific recombination (139). With the exception of Tn*916*, which tends to show low integration specificity, tyrosine integrases generally tend to catalyze the integration of ICEs into several site-specific regions of the genomes, such as the 3' end of tRNA and the 3' and 5' ends of housekeeping genes (145). The recombination modules of most members of the Tn*916*-Tn*1545* family are made up of two genes (i.e., the *int* and *xis*); however, some elements harbor other forms of recombinases, such as Tn*1116* and Tn*5397,* which carry a single serine recombinase gene (*tndx*) instead of *int* and *xis* genes (147, 148).

For these site-specific enzymes to recognize and act on the attachment site, the ICE has to be in a circular form, which leads to the fusion of the *attL* and *attR* (which flank the left and right regions of the linear ICE, respectively) and results in the formation of the *attI* site on the circular ICE, as shown by the illustration of the circularization and insertion of Tn*916* in **Figure 8.** The recombinase cleaves the circular element at the *attI* site and the bacterial chromosome at the *attB* site and facilitates the integration of the element into the host genome, resulting in the restoration of the *attL* and *attR* sites on the flanking regions of the element (139, 144, 145, 149, 150).

In addition to the three modules, ICEs can carry cargo genes, such as genes that code for heavy metal resistance, pathogenicity, biofilm formation, symbiosis, and antibiotic resistance (139, 151). The presence of ICEs involved in the transfer of multiple antibiotic resistance determinants in beta-hemolytic streptococcal species was found as early as 1981 (152). The following section focuses on one of the most studied families of ICEs: the Tn*916*-Tn*1545* family.

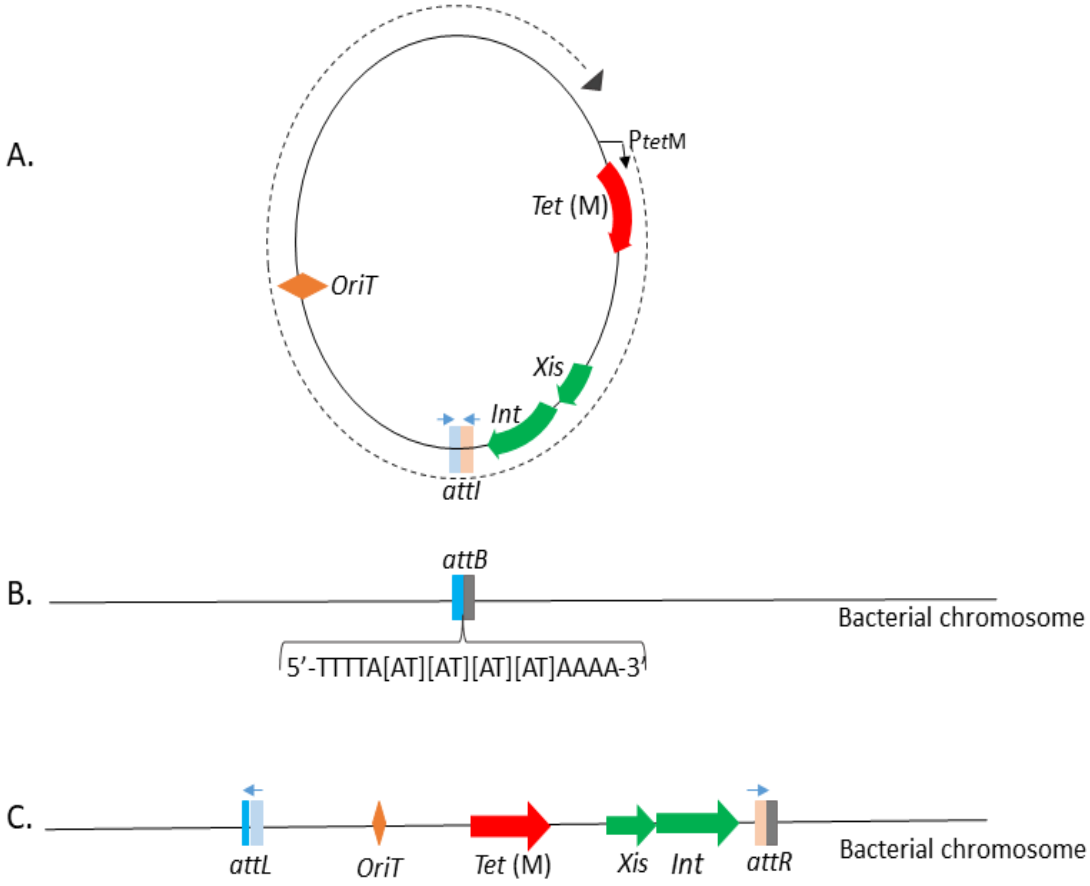

Figure 8: A schematic illustration of the Tn*916* circular intermediate and the mechanism of insertion in the host target site. A. The transcription of genes downstream from P*tetM* in the CI of Tn*916* and the formation of the *attI* site, which can be amplified by primers shown by blue arrows. B shows the A: T rich attachment site (*attB* site) in the bacteria prior to insertion of the element. C illustrates the positions of the attachment sites after integration of the element at the *attB* site in the host chromosome and the *attL* and *attR*. As illustrated by the blue arrows facing opposite directions, the primers cannot produce an amplicon when the element is integrated in the genome. This figure is based on the works of Celli and Trieu-Cout (1998) (153), Roberts and Mullany (2009) (154) and Johnson and Grossman (2015) (139).

1.4.3.1 Tn*916*-Tn*1545* family

The Tn*916*-Tn*1545* family is one of the most studied families of ICEs in bacteria and has been shown to have a broad host range in both gram-negative and gram-positive bacteria (155). This family of elements, although broad, has structural similarities, as illustrated in **Figure 9**. Tn*916* is considered the paradigm of this family, and members of this family have a structural organization similar to that of Tn*916*. In the years following the discovery of Tn*916*, many elements with the same conjugation and transcription regulation modules have been reported. Thus, these modules are regarded as the core sequence of the family. The presences of the *tet* (M), *int,* and *xis* genes has been used as the main screening criteria for members of this family. These screening criteria capture members of the Tn*916*-Tn*1545* family, as shown in a retrospective study of 27 streptococcal species that revealed that all but one of the Tn*916* family elements present in this sample collection carried integrase genes that were identical or almost identical to the Tn*916* integrase (156). This finding suggests that using the *int* and *xis* genes for screening, although adequate, may lead to the omission of some members from this family of elements. This is already evident in elements found to have variations in the recombination module, such as Tn*6000* and Tn*5386*, which have *int6000* and *int5386,* respectively (157, 158), and in Tn*1116* and Tn*5397,* which have *tndx* instead of the Tn*916 int* gene (147, 148).

Although members of this family of elements primarily harbor tetracycline resistance genes in the accessory module, other resistance determinants instead of or in addition to *tet* genes have been identified (**paper II**). These include *erm* (B) in Tn*3872*, Tn*6002*, Tn*6003*, and Tn*2017* and *Aph*-3 in Tn*1545*, as shown in **Figure 9** (159, 160). Other variations in the accessory module include *qrg* and *merA*, which encode proteins mediating resistance to biocides and heavy metals, respectively, that have been identified in Tn*916*-Tn*1545* family elements (161). The presence of multiple resistance determinants in these elements results in increased resistance genes, thus leading to the persistence and spread of both the elements and the resistance determinants.

The presence of Tn*916*-Tn*1545* family elements in oral streptococcal species has been reported in several studies and is summarized in Table 1 below.

Table 1: Tn*916*-Tn*1545* family elements in oral streptococcal species

Element	**Resistance determinants (reference)**	**Size (K bp)**	**Presence in oral streptococci and ref.**	**Transferable**
Tn*916*	*tet* (M) (162)	18.032	*S. oralis, S. mitis, S. gordonii, S. anginosus, s. salivarius, S. sanguinis* (123, 163)	yes
Tn*919*	*tet* (M) (164)	15	*S. sanguis* (164)	yes
Tn*917*	*erm* (B) (165)	5.3	*S. oralis, S. mitis, S. anginosus, S. sanguinis* (166)	yes
Tn*1545*	*erm B, aph-3, tet M* (160)	25.3	*S. oralis, S. mitis, S. sanguis* (167)	yes
Tn*6000*	*tet S* (157, 168)	33.2		
Tn*6002*	*erm B, tet M* (132)	20.9	*S. oralis, S. mitis, S. salivarius, S. sanguinis, S. infantis* (166)	yes
Tn*6003*	*tet* (M), *erm* (B)(169)	25.1		
Tn*3872*	*tet* (M), *erm* (B)(170)	23.3	*S. oralis, S. mitis, S. constellatus, S. salivarius, S. sanguinis* (171)	yes
Tn*2009*	*tet* (M), *mef E* (124)	7.8* (partial sequence)	*S. mitis, S. salivarius* (166, 171)	
Tn*2010*	*tet* (M), *erm* (B), *mef E* (172, 173)	26.4	*S. mitis* (166)	
Tn*2017*	*tet* (M), *mef E, erm* (B) (174)	28.5	**Beta-hemolytic streptococci (175)	
Tn*6087*	*tet* (M), *antiseptic resistance, CTAB* (123)	21.1	*S. australis, S. oralis* (123)	yes
Tn*6815*	*tet* (M), *erm* (B) (127)	23.3	*S. mitis* (127)	yes
Tn*6816*	*tet* (M) (127)	20.8	*S. constellatus* (127)	yes
Tn*3704*	*tet* (M), *erm* (B) (176)	20.3	*S. anginosus* (176)	yes
Tn*3705*	*tet* (M), *erm* (B) (176)	52.0	*S. anginosus* (176)	yes

*Only partial sequence available: accession number AF376746
**Species not specified

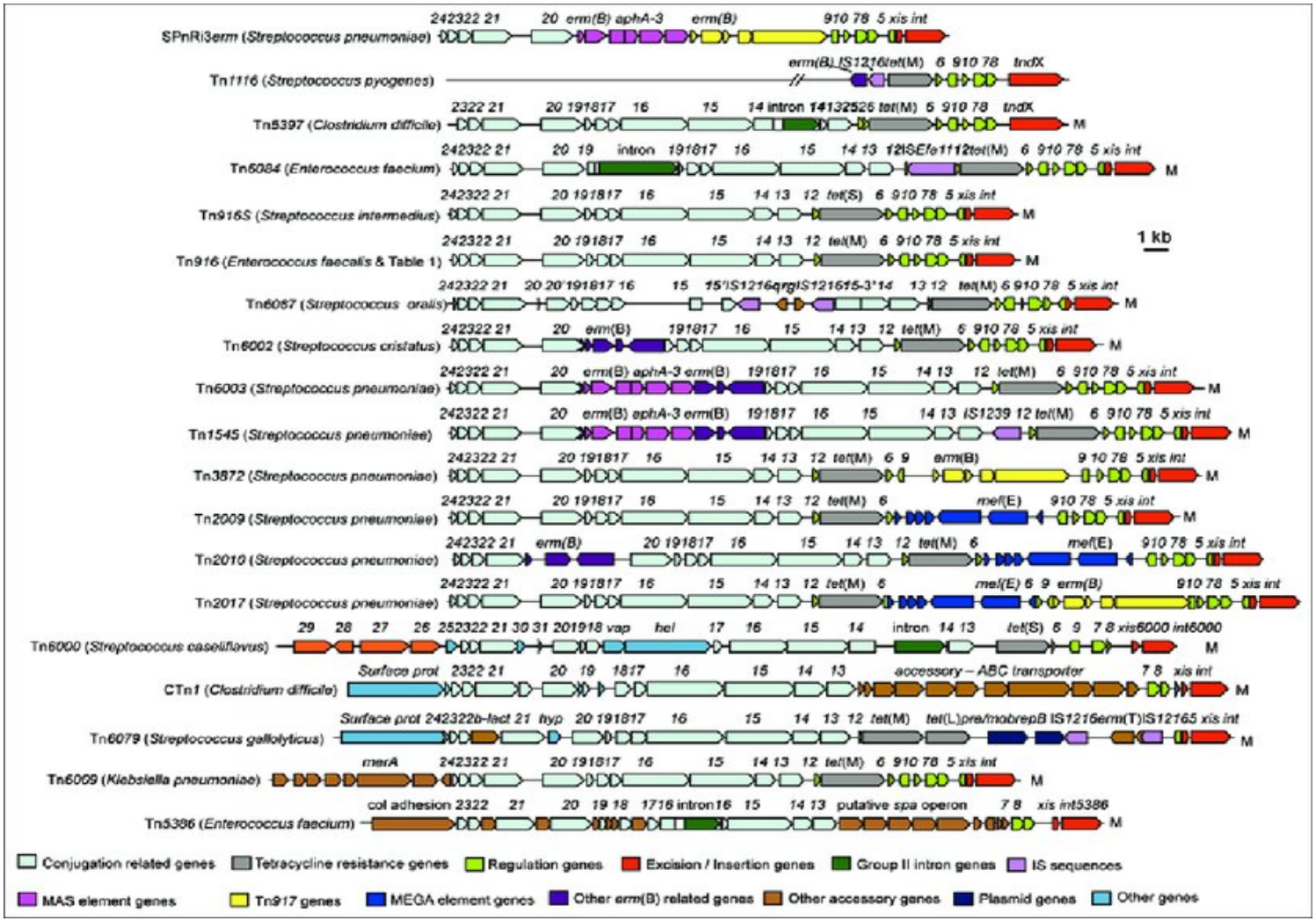

Figure 9: A figure showing the similarities in the structures of 14 members of the Tn*916*-like family in various host species. The names of the elements and the host species are given to the left of the figure, whereas the arrows represent ORFs and the direction of transcription. The key legend specifies the modules and accessory regions that are present in the elements. Reproduced with permission from Ciric *et al.* (155).

1.4.4 Tn*916*

Tn*916* (accession no. U09422.1) is an 18-kb broad-host-range ICE that is widespread in a large number of commensal and pathogenic bacteria (11, 177, 178). It was first isolated from *E. faecalis* strain DS16 in the late 1970s and was shown to transfer tetracycline resistance to *E. faecalis* strain JH2-2 (11). The element has been shown to contain all the proteins necessary for its intracellular transposition and intercellular conjugation (151), and these are structured as 24 open reading frames (ORFs) (listed in Table 2) and are arranged in functional modules. As shown in the simplified schematic illustration in **Figure 10,** the functional modules that are responsible for conjugative transfer (shown in blue) span the left half of the element, whereas the modules responsible for transcriptional regulation, excision and insertion reactions (recombination) and accessory functions span the right half of the element (154).

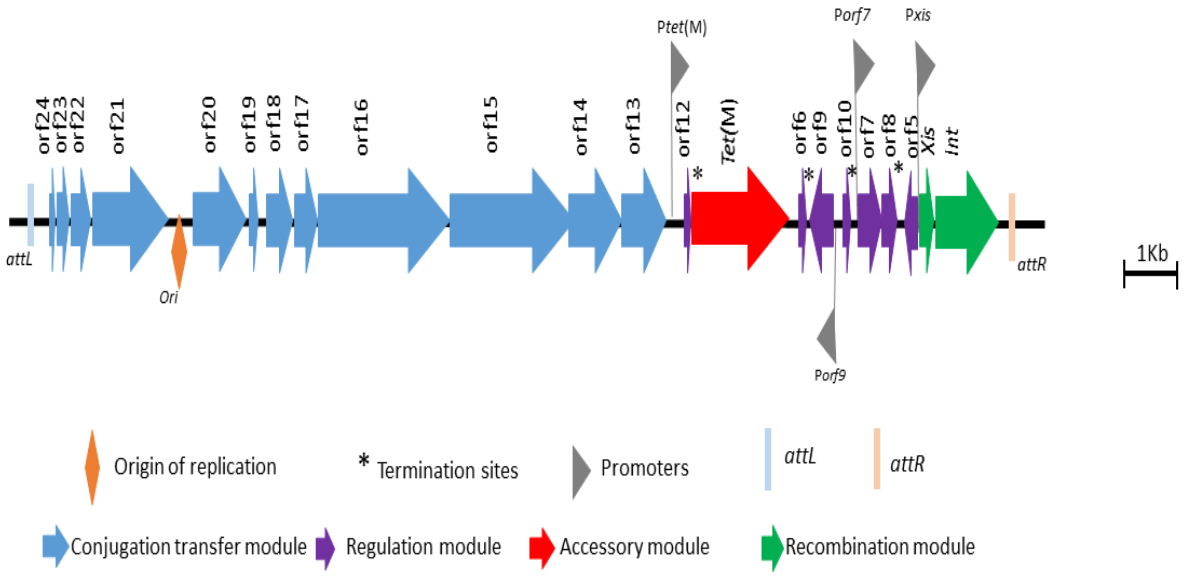

Figure 10: A simplified schematic illustration of the structure of Tn*916*. The conjugation module is shown in blue, the transcription regulation module (in purple), the recombination module (in green), and the accessory module (in red). The figure also shows the origin of transfer (*OriT*) as an orange diamond, terminators as asterisks, promoters (P*tetM*, P*orf9*, P*orf7*, and P*xis*) as gray triangles, and the attachment sites *attL* and *attR* as blue and peach rectangles, respectively. The direction of the arrows illustrates the orientation of the genes. This figure is an adaptation from the works of Roberts and Mullany, 2009 (154).

Initially, Tn*916* was thought to solely depend on the host for replication; however, recent studies have shown that Tn*916* can self-replicate (**paper I**) (130, 163). This ability is believed to, in part, contribute to the element's vast host range when compared to other MGEs, such as plasmids. Tn*916* is particularly important from a clinical perspective, as it mediates the transfer

of antibiotic resistance among a wide range of environmental, commensal, and pathogenic bacteria, including oral streptococci (166). From an evolutionary standpoint, Tn*916* is important because it provides variation in bacterial populations by:

i. Disruption of chromosomal host genes due to its insertion in A:T rich regions
ii. Introduction of foreign DNA that is present in the coupling sequence
iii. Deletion of host DNA upon excision
iv. Incorporation of antibiotic/antiseptic resistance that is present on the element into the host genome
v. Mobilization or transactivation of other Tn*916* elements or MGEs in the same cell

Tn*916* has been reported in both gram-positive and gram-negative bacteria, and in the former case, the donor and recipient strains do not need to be from the same species or even the same genus (179). Although the element can insert into a wide variety of targets in different hosts, it is known to display a preference for A:T-rich regions (154, 180) (**paper III**). The preference for A:T-rich regions in the host genome is thought to impose a low fitness cost for the host and has been proposed to increase the mobilization of the element (181). In contrast to that of other MGEs, the integration of Tn*916* into the host genome does not produce duplications or replications of the target sequence (179).

The ability of Tn*916* to move from bacterial strains that were not previously thought to conjugate, such as *B. subtilis* (182) (**paper II** and **III**), renders it a crucial factor in the dissemination of antibiotic resistance genes (154).

1.4.4.1 Mechanism of transfer (excision)

The transposition of Tn*916* within its bacterial host genome or from one donor strain to a recipient strain has been shown to occur via a Rec-independent excision-integration system that results in the formation of a replicative intermediate known as a CI (141). The integration and excision of Tn*916*-like elements are facilitated by the integrase gene (*int*) and the excisionase gene (*xis*), which are located on the right end of the element (183). The *int* gene belongs to the family of tyrosine recombinases, whereas the *xis* gene is a small protein of 67 amino acids (158).

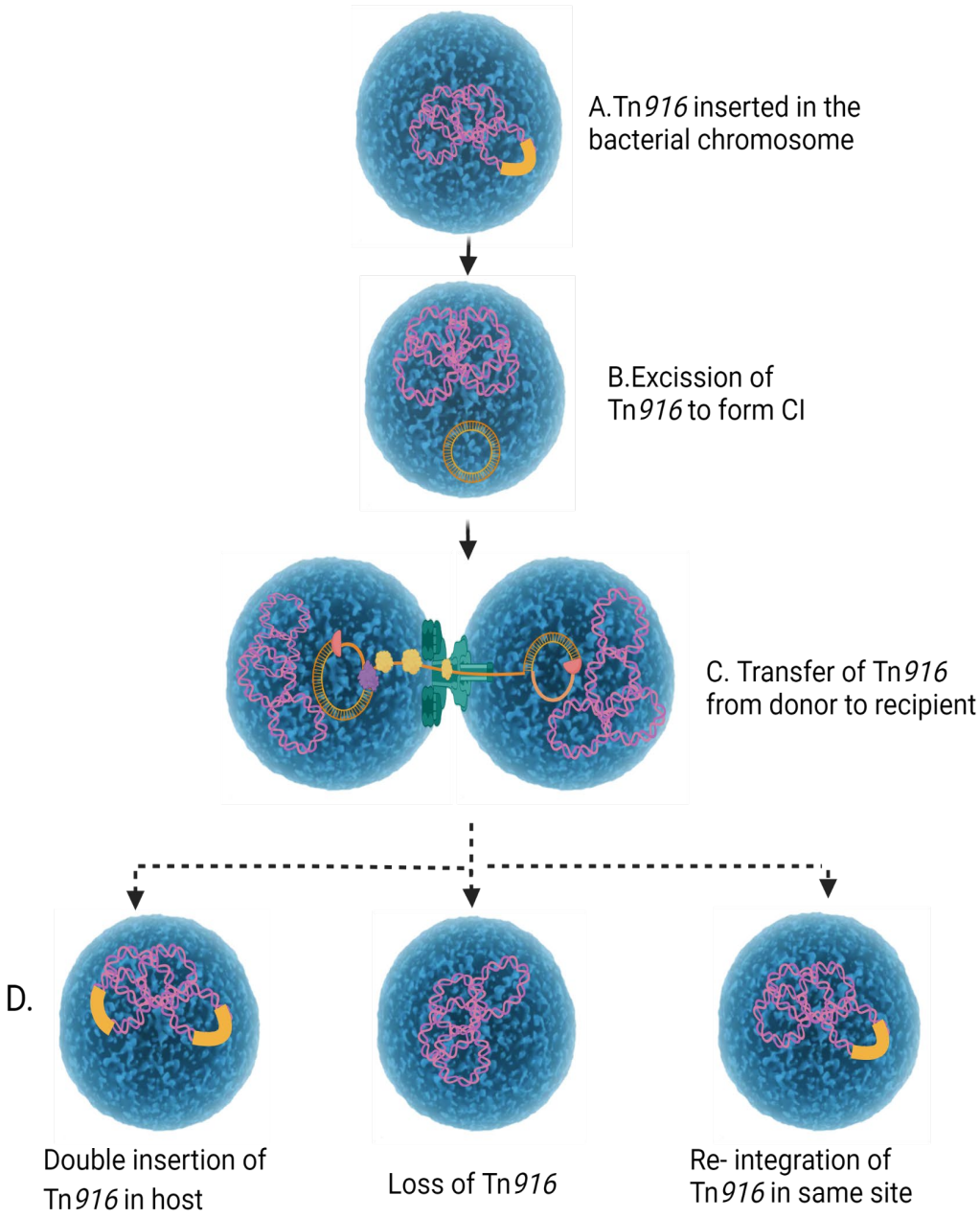

Figure 11: A schematic representation of the life cycle of Tn*916*. A. Tn*916* is incorporated in the chromosome of the donor cell. B. Tn*916* excises from the donor cell to form a CI. C. The rolling circle replication of Tn*916* in which the relaxase (orange half-moon) binds to the free 5' end after recognizing and nicking the *ori*. The CI is unwound by the helicase (purple protein), and the single-stranded DNA is protected by single-stranded binding protein (yellow proteins) during transfer through from the donor to the recipient via the T4SS (shown in green (116). D. The possible fates of the replicated Tn*916* include (i) reintegration into two different sites in the donor cell, (ii) loss of the element from the cell and (iii) reintegration of the element into same insertion site in the donor chromosome.

For excision and integration to occur, both *int* and the *xis* are required, but the integrase alone can facilitate integration (184). The *int* has two DNA-binding domains, which are the N-terminus and the C-terminus (185). The N-terminal domain is smaller and binds to the short DR-2 (186), and the C-terminal domain is larger and is responsible for creating staggered cuts at the end of the CTn (179).

The first step in the transposition of Tn*916* involves the creation of staggered cleavages that produce six base pair heteroduplex ends at each end of the CTn (141, 179). The transfer process begins when the relaxase (*orf20*) nicks the origin of conjugal transfer, *oriT* (187, 188). *oriT* is a 466-bp long sequence found in the noncoding region between *orf21* and *orf22* (187). It has been shown to contain six sets of inverted repeats and a *nic* site where *orf20* cleaves (187, 188). Upon excision from the genome, the ends of the CTn have a six-base-pair heteroduplex sequence from the donor genome, which is referred to as the coupling sequence. The heteroduplex ends of the CTn join to form a double-stranded molecule called the CI (182). The transfer of the CI from the donor to the recipient is thought to occur via a single strand that is replicated and subsequently integrated into the recipient's genome (180, 189).

The excision of the ICE initiates the transfer of an ICE to form a CI. The transfer of the CI from the donor cell to the recipient, as illustrated in **Figure 11**, occurs in a manner similar to that of plasmid transfer across the T4SS, which is attached to the surface of the donor cell (135, 139). The rolling circle replication of the CI is initiated at the *oriT* by the relaxase, which remains bound to the single strand of the CI as it is transferred to the recipient cell and undergoes recircularization, as illustrated in **Figure 11C**. Upon recircularization, the ICE can be integrated into the host genome, can replicate autonomously, is lost from the donor cell, or can reintegrate into different sites in the donor cell. If integrated, the integration site is determined by the integrase gene present on the ICE. As seen in other ICEs (190), in addition to autonomous replication, transfer to a new cell, and integration into a new site, the other possible fate of the CI may include loss from the bacterial population, as depicted in **Figure 11D**.

1.4.4.2 The Regulation of Tn*916*

The transcriptional regulation of Tn*916* has not yet been thoroughly tested experimentally. The available information regarding this process is based on a proposed model of regulation presented by Su *et al.* (191). The model suggests that the regulation of Tn*916* starts with the transcription of *orf12* from the promoter P*tet(M)* and includes *orf9*, *orf7,* and *orf8*. O*rf12* is an 86-bp open reading frame that encodes a rare amino acid that slows translation by forming a stem-loop structure when the ribosome lags behind the RNA polymerase. The slow translation coupled with the lack of charged tRNA that occurs in the absence of tetracycline causes the ribosome to pause on the leader peptide of *orf12*, resulting in the termination of most transcription (191). As shown in **Figure 12**, in the absence of tetracycline, it has been proposed that P*orf9* initiates the transcription of *orf9* and produces an antisense RNA that leads to the downregulation of the promoter P*orf7*. The downregulation of P*orf7* results in lower transcription of the *orf7*, *orf8*, *orf5, int*, and *xis* genes, which in turn leads to lower excision of the element. In the presence of tetracycline, an accumulation of tRNA occurs in the cell, as tetracyclines inhibit most ribosomes. Ptet*(M)*, located upstream of *tet* (M), as shown in **Figure 12**, initiates the rapid transcription of *orf12*.

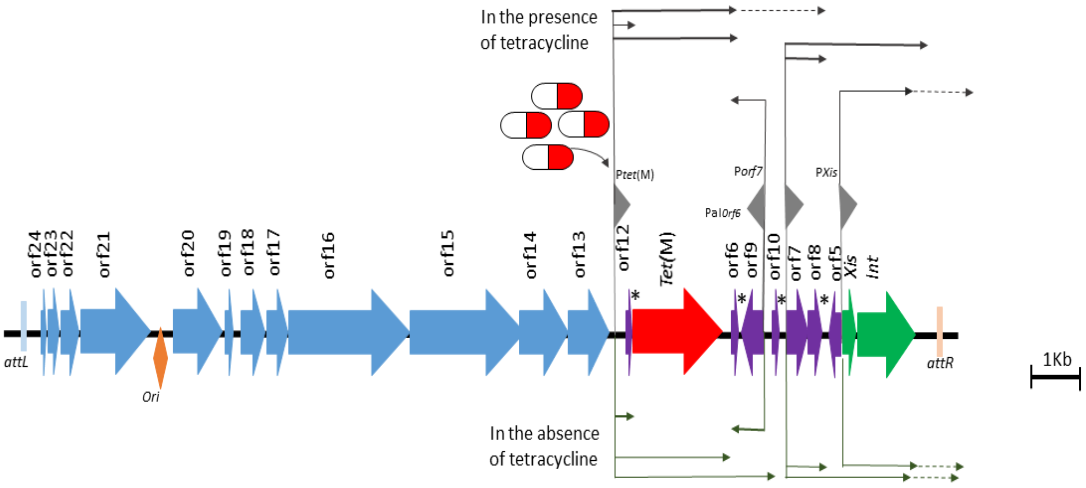

Figure 12: An illustration of the regulatory mechanism and insertion of Tn*916*. The structural organization of Tn*916* is shown by the blocked arrows, and *OriT* is shown by the orange diamond. Transcriptional activity in the presence (above the blocked arrows in black) and absence of tetracycline (below the blocked arrows in green). Positions of putative promoters and terminators are indicated by gray triangular flags and asterisks, respectively. Horizontal arrows of different thicknesses (wide or narrow) represent transcripts with varying transcription levels (higher or lower, respectively). This figure is based on the works of Celli and Trieu-Cout (1998) (127) and Roberts and Mullany (2009) (154).

The available charged tRNA prevents the formation of stem-loop structures in *orf12*. The absence of stem-loop structures allows RNA polymerase to continue transcription into *tet* (M) and downstream open reading frames. The increased levels of tetracycline in the cell have been hypothesized to downregulate *orf9* due to the production of an antisense *orf9* mRNA transcript by RNA. Lower levels of antisense RNA result in lower levels of *orf9*, which in turn leads to the upregulation of P*orf7.* This increases the transcription of the *orf7*, *orf8*, *orf5, int*, and *xis* genes and consequently increases the rate of recircularization and excision of the element. In addition to P *tet(M)*, P*orf9*, and P*orf7*, Tn*916* has another promoter, P*xis*, which regulates the transcription of the recombination module (192).

The increased excision of Tn*916* from the bacterial chromosome may be trigger by changes in the bacterial cell or changes in the environment. For instance the presence of sub-minimal inhibitory concentrations of tetracycline (192) (**paper II**) promotes the transfer of the CTn. Other factors that may influence the transfer of Tn*916* include lack of ICE repressors, as is the case when Tn*916* is newly acquired (139) and activation of the SOS response system due to DNA damage or host cell stress. Roberts and Mullany, in their work from 2009, hypothesized that any damage in a bacterial cell that relates to translation leads to higher transcription activity in the elements due to the accumulation of charged tRNA molecules (154).

Numerous Tn*916*-like elements have been identified and characterized over the years. Although they have been found to vary in size and composition, the regulatory region tends to be conserved, suggesting that this region is a vital component in the transcriptional regulation of these elements (154, 193).

Table 2: The open reading frames (ORFs) in Tn*916*

ORF	Gene product/closest homolog	Position	Function	Reference
Orf24	Hypothetical protein	194..313		
Orf23	Hypothetical protein similar to MbeA of ColE1	336..650		(188)
Orf22	Hypothetical protein	666..1052		
Orf21	DNA translocase FtsK	1081..2466	DNA segregation during cell division	(194)
Orf20	Relaxase/endonuclease	2861..3850	Endonuclease that cleaves DNA at *oriT*	(188)
Orf19	Replication initiation factor	3893..4114		(195)
Orf18	*Ard* anti-restriction protein	4231..4728	Transposon immunity to DNA restriction modification	(196)
Orf17	*Tcp*E family protein	4703..5209		(195)
Orf16	AAA-like protein domain	5193..7640		
Orf15	Hypothetical protein similar to VirB6	7643..9907		
Orf14	*Iap*/ putative endopeptidase p60 precursor	9816..10817	NLP/P60 extracellular lipoprotein	
Orf13	Conjugative transposon protein *Tcp*C	10832..11746	ATP-binding domain in an ABC	(195)
Orf12	*tet* (M) leader peptide	12021..12107	*tet* (M) leader peptide	
Orf11	*tet* (M)	12123..14042	Tetracycline resistance	
Orf10		14946..15017		
Orf9	Anerobic benzoate catabolism transcriptional regulator	14388..14741	Proposed repressor of Porf7 (191)	(195)
Orf8	Helix-turn-helix domain protein	15665..15895		
Orf7	RNA polymerase sigma factor	15195..15668	Proposed to upregulate Porf7 (191)	(195)
Orf6		14140..14328		
Orf5	Hypothetical protein	16121..16372		
Orf4	Int-Tn	16884..17858	Transposase from the transposon Tn*916*	(183)
Orf3	Int-Tn	16773..17858	Transposase from the transposon Tn*916*	(183)
Orf2	Int-Tn	16641..17858	Transposase from the transposon Tn*916*	(183)
Orf1	Xis-Tn	16356..16559	Excisionase	(183)

1.5 Oral streptococci as reservoirs of antibiotic resistance genes

The diversity, high microbial load, and presence of biofilms in the oral cavity provide an ideal scenario for the exchange of genetic materials. In addition to being the most dominant species in the oral cavity, oral streptococci can actively acquire and exchange genetic material from related bacteria that are present in the same environment (197). The natural ability of streptococcal species to take up DNA has long been thought to contribute to the transfer of resistance determinants. As early as 1997, Reichmann *et al.* illustrated the interspecies transfer of cefotaxime resistance genes from *S. oralis* and *S. mitis* to *S. pneumoniae* by transformation (198). In subsequent years, other studies have shown the transfer of resistance genes to and from *Streptococcus* spp. The global increase in the number of penicillin-resistant *S. pneumoniae* isolates has been attributed to interspecies gene transfer and recombination of the penicillin-binding protein gene *pbp* from *S. mitis* and *S. oralis* (199). In addition to the transfer of penicillin resistance genes, in vitro transfer of fluoroquinolone resistance determinants by transformation has been shown to occur among *S. pneumoniae*, *S. oralis S. sanguis*, *S. constellatus* and *S. mitis* (200). Distinguishing oral streptococci from *S. pneumoniae* is important in the clinical setting and has been done using optochin-impregnated discs, which produce a zone of inhibition in *S. pneumoniae* but not in oral streptococci. The work by Fenoll *et al.* demonstrated that *S. pneumoniae* can take up *S. oralis* DNA by transformation and become resistant to optochin, making it difficult to distinguish *S. pneumoniae* from *S. oralis*.

Over the years, numerous studies have reported the presence of AMR genes in oral streptococci, including those that confer resistance to penicillin, macrolides, other beta-lactams, and tetracyclines (37, 39-41). A study by Lancaster *et al.* reported that tetracycline resistance genes were detected in the oral cavities' of 46/47 children, and 65% of these were associated with streptococcal species. Further analysis of the subjects in this study showed that 56% had additional resistance determinants to either erythromycin, penicillin, and/or ampicillin (61). Another study showed that streptococci were predominantly associated with the persistent carriage of *tet* (M) genes in the oral cavity of 15/18 children aged between 4 and 6 years for 12 months (201).

The presence of these resistance determinants in oral streptococci can serve as a source of resistance for both pathogenic and commensal bacteria present in the oral cavity and/or biofilms. The association of the resistance determinants and the presence of MGEs in

streptococci has been implicated in the spread of resistance determinants. The abovementioned study by Lancaster *et al.* revealed that the highly predominant *tet* (M) genes found in that study were associated with a MGE from the Tn*916*-Tn*1545* family (201). Although other MGEs, such as plasmids (pSI01) (202) and insertion sequences (IS861) (203), have also been found in association with antibiotic resistance genes in streptococcal species, the most commonly reported MGEs associated with antibiotic resistance in oral streptococci are nevertheless ICEs. For instance, Tn*5801*, which confers resistance to tetracycline, has been reported in *S. mitis* B6 (204) and *S. oralis* (166), whereas Tn*917*, which harbors the erythromycin resistance gene, has been reported in *S. oralis*, *S. mitis*, *S. sanguinis* and *S. anginous* (166). There have been reports of multiple resistance determinants on the same MGE, such as *tet* (M) and *erm* (B) on Tn*3872*, which has been identified in *S. salivarius S. oralis, S. mitis, S. constellatus, S. salivarius,* and *S. sanguinis* (170, 171), and Tn*6002*, which has been identified in *S. mitis*, *S. oralis* and *S. sanguinis* and harbors *erm* (B) and *tet* (M) (166). The presence of multiple resistance determinants on these ICEs and their ability to excise and transfer inter- and intracellularly further boost the reservoir potential of these species.

In addition to carrying multiple resistance determinants, some isolates have been shown to carry conjugative mega-like sequences, which have been associated with increasing numbers of erythromycin-resistant strains that carry *mef* (A/E) genes (166). These elements have not only been shown to move from oral streptococcal isolates to *S. pyogenes* and *S. pneumoniae* (205, 206) but also been found to harbor elements that are related to Tn*916* and other conjugative elements, such as ICE*St3*, in *S. salivarius* (171).

The abundance of oral streptococcal species in the oral cavity and the natural ability to take up and incorporate DNA from their surroundings, coupled with the increased presence of resistance determinants and MGEs in these species, underscore the role that oral streptococcal species play in genome plasticity and dissemination of resistance determinants (35, 36).

2 AIMS AND OBJECTIVES OF THIS THESIS

The overall aim of the study was to determine the presence, molecular diversity, stability, and fitness cost of the Tn*916*-Tn*1545* family that were present in oral streptococci. We aimed to determine the number of elements present in each clinical isolate prior to investigating the transferability, stability and fitness cost associated with the acquisition of the Tn*916*-Tn*1545* family of conjugative elements.

The objectives of this study included:

- Determining the diversity of Tn*916*-Tn*1545* family elements in the Norwegian oral streptococcus sample collection from 2005
- Determining the transferability, transfer rates and fitness cost associated with Tn*916*-Tn*1545* family elements in oral streptococci
- Investigating the fitness cost associated with the acquisition of Tn*916* and the time required, if any, to ameriolated fitness costs

The specific aims of thesis were:

Paper I: Establish an assay to determine and detect the presence, copy number and circularization ratio of the Tn*916*-Tn*1545* family in oral streptococci by ddPCR

Paper II: Determine the prevalence, diversity, and transferability of the Tn*916*-Tn*1545* family in oral streptococci

Paper III: Investigate the stability and fitness cost of the newly acquired Tn*916* in *S. oralis*.

3 MATERIALS AND METHODS

Oral streptococcal species are mostly isolated from the oral cavity; however, if and when the mucosal barrier is damaged, they can be isolated from the bloodstream. We chose to investigate the prevalence, diversity, stability, and transferability of the Tn*916*-Tn*1545* family in oral streptococci, as this family plays a role in the spread of antibiotic resistance determinants in oral bacteria. The articles (**papers I** and **II**) and the manuscript (**paper III**) give a detailed description of the methodology we employed in this study. A brief overview of some of the materials and methods used in this study is, however, given below.

3.1 Biological material

The bacterial strains used in this study are part of the collection of clinical oral streptococcal isolates collected by Norwegian hospitals and submitted to the National Competence Service for the Identification of Antibiotic Resistance (K-RES). These samples were selected based on the knowledge that they are resistant to one or more antibiotics. The sample collection of 100 clinical strains consisted of *S. mitis* (n= 42), *S. oralis* (n= 22), *S. sanguinis* (n=7), *S. salivarius* (n=7), *S. anguinus* (n=7), *S. gordonii* (n=2), *S. constellatus* (n=2), *S. intermedius* (n=1), *S. mutans* (n=1) and nine unclassified streptococcus species. In addition to these clinical isolates, the isolates *B. subtilis* BS34A (accession number NZ_LN680001.1) and *B. subtilis* BS49 (accession number NZ_LN649259.1) were included in this work as experimental controls.

The work in **papers II** and **III** includes the kanamycin-resistant isolates derived from the type strains *S. oralis* ATCC 35037, *S. mitis* ATCC 49456, *S. sanguinis* ATCC 10556, *S. mutans* ATCC 25175 and *S. gordonii* ATCC 10558, respectively. The derivatives were obtained by introducing the EZ-Tn*5* <*kan-2*> transposon kit (Lucigen, Middleton, WI) by mutagenesis (see **paper II** for details). The isolate *S. pneumoniae* ATCC 49616 was included as a positive control for MIC testing.

3.2 Minimal inhibitory concentration (MIC) testing

MIC testing was performed to determine the resistance profiles of these isolates and to shed more light on the resistance patterns of oral streptococcal isolates. The antibiotics that were used in these tests included penicillin, linezolid, trim-sulfa, cefuroxime, cefotaxime, gentamycin, ciprofloxacin, meropenem, tetracycline, erythromycin and clindamycin. E-tests were performed according to the instructions from BioMérieux SA, Marcy l'Etoile, France, and the results were interpreted according to the standards set by the European Committee for

Antimicrobial Susceptibility Testing (EUCAST) (www.eucast.org) and the Clinical and Laboratory Standards Institute (CLSI) (https://clsi.org/). The results from the MIC tests are included in Table S1 in the appendix.

3.3 Genome sequencing

The genomic DNA that was subjected to sequencing in this study was extracted using the modified Marmur extraction procedure as described by Salva-Serra *et al.* (207). In **paper II**, the Illumina NextSeq 550 platform and the Pacific Biosciences Sequel instrument (PacBio) with Sequel Polymerase v3.0, SMRT Cells v3 LR and Sequencing Chemistry v3.0 were used to generate the sequences. Canu was used to produce hybrid assemblies (208) prior to reordering the assemblies/contigs using Abacas 1.3.1 (209), with Tn*916* (U09422.1) as a guiding reference. PROKKA version 1.12 (210) was used to predict the protein coding regions in the assembled genomes. In **paper III**, we used paired- end illumina sequencing to obtain WGS of both the evolved and the non-evolved bacterial populations. In addition, we created preformed hybrid assembly of the long PacBio reads and the short illumina reads for one triplicate of each population using Canu (208). The short read assemblies were analyzed with Breseq (211) to check for SNPs in the assembled genomes.

4 METHODOLOGICAL CONSIDERATIONS

The compilation of this thesis is based on the use of numerous molecular biology methods. Detailed descriptions of the experimental materials and procedures are provided in the attached **papers I**, **II** and **III**. This section presents a brief description of the rationale, advantages, caveats, and limitations of some of the methods included in this study.

4.1 Detection of Tn*916*-Tn1545 family

The Tn*916*-Tn*1545* family is a broad family of conjugative transposons (CTns) that have a modular structure, as described in **Figure 10**. The conjugation and recombination modules are highly conserved within this family of elements and have thus been used to identify members of this family. The detection of the Tn*916*-Tn*1545* family routinely involves screening for *tet* (M), *int*, and *xis* genes. These three genes are good markers for the detection of Tn*916*-Tn*1545* family members, as they have been identified in nearly all members of the family. Polymerase chain reaction, an *in vitro* method that amplifies short fragments of DNA with a known sequence, is the most common way of detecting the *tet* (M), *int*, and *xis* genes. Therefore, we used the genotypic presence of all three genes as the criteria for inclusion in the Tn*916*-Tn*1545* family in this study. Although the use of the *tet* (M), *int*, and *xis* genes to detect the Tn*916*-Tn*1545* family is standard practice, this strategy may lead to overlooking some members of the family that have variations in the accessory module and recombination module. For instance, Tn*6000* and Tn*5386* have different *Int* genes that have been designated *int6000* and *int5386*, respectively (157, 158). In addition, although *tet* (M) is the most frequently identified gene in the accessory module, other resistance markers, such as *tet* (S), have been observed in CTns such as Tn*6000* and Tn*916S* (157, 168, 212). Some members of this family have acquired additional resistance determinants, such as Tn*1545* and Tn*6003*, which both have kanamycin and erythromycin resistance genes in addition to *tet* (M) (159, 160). To address this limitation, we screened our samples for both the phenotypic presence of tetracycline resistance and *tet* (S). All samples that had the phenotype and/or genotype for tetracycline resistance were subjected to additional screening using a long PCR that amplified the entire element in two fragments. The primers designated Long A, which amplifies the region between 38 bp and 9884 bp of Tn*916*, and Long B, which amplifies positions 9824 bp to 17947 bp of Tn*916*, were used to amplify these two fragments. The abovementioned primers are designed to detect and amplify two large fragments in many members of the Tn*916*-Tn*1545* family, including Tn*916*, Tn*6002*, Tn*6003*, Tn*1545*, Tn*3878*, Tn*2009*, Tn*2010*, Tn*2017*, Tn*6084* and Tn*6079* (123).

4.2 Restriction Fragment Length Polymorphism

Variation in homologous fragments of DNA, known as polymorphisms, can be used to distinguish DNA sequences if the fragments have different restriction enzyme sites. This principle is used in restriction fragment length polymorphism, where restriction endonuclease digestion is used to selectively cut a DNA molecule at the specific restriction site, thereby generating restriction fragments of varying lengths that can be visualized on an agarose gel. RFLP is an inexpensive tool that has been applied in DNA profiling, genotyping, gene mapping, paternity testing and genetic disorder identification (213). One of the uses of RFLP in microbiology is the identification of single-nucleotide polymorphisms (SNPs), which are single-nucleotide changes at a DNA locus (214).

In this study, we employed the use of RFLP to determine whether the amplified region of the Tn*916*-Tn*1545* family elements was identical to the wild-type Tn*916* or varied in sequence. To achieve this, we amplified the 9846 bp 5' fragment (Long A) and the 8123 bp 3' fragment (Long B) from the isolates that carried both the *int* and *tet* (M) genes (as described above). These fragments were digested with *HincII,* a 6-bp cutter that recognizes the sequence 5'-GTYRAC-3' and cleaves after Y-3 (215). In wild-type Tn916, *HincII* generates five fragments in Long A and three fragments in Long B.

RFLP is a rapid, inexpensive, and sensitive tool that has many applications in microbiology. However, the sensitivity of the method and its ability to detect the presence of SNPs resulted in inaccurate classification of 3/6 isolates harboring the Tn*916*-Tn*1545* family. The three elements that were incorrectly classified had SNPs that introduced new restriction sites and thus generated restriction patterns that were different from those of the wild type. DNA sequence analysis of these elements revealed that these elements were 99% identical to the wild-type Tn*916*. These findings showcase a limitation of this method when applied to the classification of the Tn*916* family. It is imperative that other tools, such as genome sequencing, be used to support this technique.

4.3 Determination of copy number and circularization rate by droplet digital PCR

The number of transferable CTns in a bacterial population is of great interest, as it is reasonable to infer that the abundance of transferable CTns influences not only the spread of resistance determinants but also the biological fitness and stability of a bacterial population (216). Our efforts to determine the copy number (CN) of Tn*916*-Tn*1545*-like elements led to the development of an assay that can easily and accurately determine the copy number of Tn*916*-Tn*1545*-like elements in *E. faecium*, *B. subtilis* and oral streptococci using ddPCR, as described in **paper I**.

ddPCR is considered the third generation of PCR and can be used to achieve high-precision quantification of target DNA. The system uses water-oil emulsion technology combined with microfluidics to distribute the DNA sample into 20 000 droplets. Each of the 20 000 droplets acts as an individual PCR "tube" in which separate individual PCRs occur. During annealing, the probe binds, which consists of a reporter and quencher, to the target sequence, as illustrated in **Figure** 12. The reporter part of the probe is cleaved from the quencher only during extension and thus emits fluorescence. Droplets that emit fluorescence are designated positive, and those that do not emit fluorescence are considered negative.

The number of copies of the Tn*916*-Tn*1545* family in each bacterial population was determined by ddPCR. This approach has, to the best of our knowledge, not been tried prior to this study; therefore, method optimization and verification had to be performed as described in detail in **paper I**. The general principle applied in the determination of copy number was the use of a single copy housekeeping gene as a reference for the number of genes present in the bacterial population. We then determined the number of elements present in the same population and reported the CN as a ratio of the elements per bacterial genome.

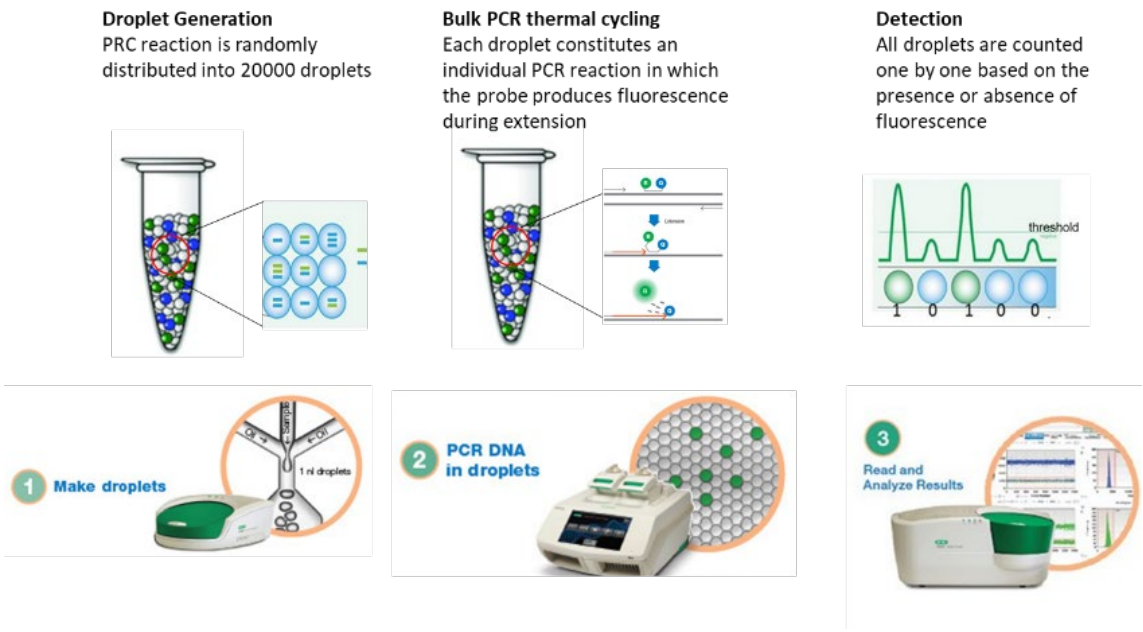

Figure 12: The general workflow of ddPCR. The image has been modified and is based on an image from the Droplet Digital ™ PCR Application guide (Bio–Rad).

The α-amylase gene, *amyE* was used as the reference housekeeping gene, as it is a single-copy, highly conserved gene that has sufficient species-specific variations in the primer/probe regions (163). For strain specificity, species-specific primers and probes were designed that amplified the variable region of *amyE* in each species (**paper I**; **Figure 2S** in the Appendix). The presence and number of copies of the Tn*916*-Tn*1545* family were determined by primers and probes that anneal to the *tet* (M) gene and the *int/xis* region.

The transfer of Tn*916* is initiated by the formation of a CI (as described in detail in Section 1.5.4.1). To form the CI, the element excises from the chromosome, and the left and right fragments of the element combine to form a circular molecule. The use of primers that anneal to the left and right side of the element ensures that PCR amplicons and fluorescence from the probe are produced only when the molecule is in circular form (see **Figure 7**). The rate at which CIs are formed is termed circularization ratio (CR) and was determined by measuring the number of CIs per bacterial population or element.

4.4 Transfer and stability of Tn*916*-Tn*1545* family

The Tn*916*-Tn*1545* family has all the necessary genetic material required to move from one genetic location to another by excision and integration. The ability to detect and determine these movements is important, as it provides some insight into the rate of transfer and the stability of these elements in a bacterial population. In this study, we investigated the transferability and stability of these elements by conjugation and evolution analysis, respectively.

4.4.1 Conjugation

Conjugation is an efficient means of transferring CTns from a donor to a recipient cell, and it has been shown to readily occur in streptococci. The biological material used in this study included isolates that harbor the Tn*916*-Tn*1545* family. The presence of these elements, however, does not give any indication as to how long the element has been present in the bacteria, the stability or the fitness cost associated with the element. To investigate and understand the mechanisms involved in the rate of transfer and the stability and the fitness cost of the elements, the Tn*916*-Tn*1545* family had to be introduced into naïve oral streptococcal isolates. The naïve oral streptococcal isolates we chose to work with were ATCC strains that contained the kanamycin resistance gene (*nptII*) as a selective marker. We chose to introduce the kanamycin resistance gene (*nptII*) via mutagenesis with Tn*5* as described in **paper II**, as this would provide a neutral and controlled naïve background. The fitness cost of the introduced Tn*5* was assessed, and only isolates whose growth rates were not affected by the introduction of Tn*5* were used as recipients in the conjugation experiments. As direct contact is essential for conjugation, the Tn*916*-Tn*1545* family was introduced by filter mating experiments as described in detail in **paper II**. The observed number of transconjugants per recipient was reported as the transfer frequency. Both inter- and intraspecies conjugation experiments were conducted to shed some light on the role that species selection plays in the transfer and spread of the Tn*916*-Tn*1545* family.

4.5 Evolution and fitness studies

The presence of MGEs in a bacterial population, in addition to accelerating the evolution of the population, may come with substantial consequences for the host cell. It is well known that the acquisition of additional genetic material by a bacterial cell may affect the growth rate, reproduction and ultimately the survival of the bacterial cell. The tendency of MGEs to selfishly use the host's resources for their maintenance and transfer may be related to reduced rates of growth and reproduction of the host cells. For this reason, growth rates can be used to infer the fitness of a bacterial population (63). In cases where a MGE carries genes that offer a phenotypic advantage for the host (such as antimicrobial resistance genes the host cell may disregard or compensate for the cost related to the maintenance of these genes. Several studies have investigated the transfer of Tn*916* in both gram-positive and gram-negative bacteria. Once the transfer is complete, very little is known regarding the events that lead to the maintenance, stability and/or consequent transfer of the element in this new background.

In this study, we introduced Tn*916* elements into naïve clinical and laboratory isolates. The rationale behind this approach was to investigate and observe the biological cost that would result from the introduction of Tn*916*. Studies have shown that the removal of selection pressure does not necessarily lead to the loss of resistance determinants. Considering this fact, we used an evolution study to elucidate the fitness cost associated with acquiring a Tn*916* element, the changes that occurred within the first 500 generations after element acquisition and the compensatory mutations that occurred to ameliorate the fitness cost in the absence of selective pressure. The biological fitness observed in this study was based on growth rate measurements that are obtained from the log phase. Despite this being the standard method for calculating growth rates, it is important to note that these findings do not reveal the entire picture of the population dynamics, as the data from the lag phase and the stationary phases are not included.

5 SUMMARY OF PAPERS

Paper I

Determination of the copy number and circularization ratio of the Tn*916*-Tn*1545* family of conjugative transposons in oral streptococci by droplet digital PCR

The presence and increase of AMR determinants in oral streptococci continue to present challenges in the treatment of bacterial infections in the health sector. The association of these resistance determinants with MGEs, such as the Tn*916*-Tn*1545* family of conjugative transposons (CTns), has, in part, been shown to be responsible for this increase in AMR. Variation in the copy number (CN) of Tn*916*-Tn*1545* elements and the circularization ratio (CR) may play an important role in the propagation of ARGs carried by these elements. In this study, we illustrated that ddPCR is a rapid, easy and reliable tool that can be used in the determination of the CN and CR of Tn*916*-Tn*1545* elements in oral streptococci. The findings from this study showed that the Tn*916*-Tn*1545* elements are present as a single copy in the genomes of the 10 selected oral streptococcal isolates and that these CTns are generally stable in the absence of selective pressure. In addition to determining the CN, we also illustrated that ddPCR can be used to determine the linkage between two genetic targets, an attribute that is useful in distinguishing colocalization of resistance determinants on the same MGE. In addition to illustrating that ddPCR can be used to detect the number of CIs per bacterial genome, we have also demonstrated that ddPCR can be used to demonstrate the autonomous replication of Tn*916* by detecting increments in the CI molecules in comparison to the reference gene. Based on this principle, the work in this paper illustrated that the number of CI molecules of Tn*916*-Tn*1545* elements increased in the presence of sub-MIC levels of tetracycline. We also showed that the CR varies between and within bacterial species in both the absence and presence of tetracycline.

Paper II

Prevalence, diversity, and transferability of the Tn*916*-Tn*1545* family ICE in oral streptococci

The increasing levels of tetracycline resistance in oral streptococci are of great concern primarily due to the abundance of these species in the oral cavity and the carriage of MGEs, such as the Tn*916*-Tn*1545* family. The spread of the tetracycline resistance gene *tet* (M) in a bacterial population can be facilitated by the transposition of the Tn*916*-Tn*1545* family. In this study, we screened 100 Norwegian clinical oral streptococcal isolates for the presence and diversity of the Tn*916*-Tn*1545* family. In addition, we investigated the transferability of these elements and their associated transfer frequencies. We observed that *S. mitis* was the most prevalent species in this collection, representing 42% of the isolates. The tetracycline resistance gene *tet* (M) was detected in 24% of the 100 isolates, but only 21% harbored members of the Tn*916*-Tn*1545* family elements. The most prevalent Tn*916*-Tn*1545* family member element present in this collection of samples was Tn*916*, which was present in 15/21 isolates. In addition to wild-type Tn*916*, we identified two novel elements, Tn*6815*, which harbors the tetracycline resistance gene *tet* (M) and erythromycin resistance gene *erm* (B), and Tn*6816*, which harbors a 1,663 bp insertion that includes the *ltr* A gene.

We analyzed the resistance profile of this collection of samples and found that although most strains were resistant to more than two antibiotics, only 16% (n = 4) of the tested isolates were resistant to four of the five tested antibiotics. The MIC of tetracycline ranged from 0.190 to 96 μg/ml, whereas the MIC of erythromycin ranged from 0.023 to >256 μg/ml. The MICs of penicillin, gentamycin and clindamycin were found to be 0.016 to 1.5 g/ml, 0.16 to 32 μg/ml, and 0.094 to > 256 μg/ml, respectively.

With the aid of filter mating experiments, we were able to show both inter- and intraspecies transfer of Tn*916*-Tn*1545* elements with transfer frequencies ranging from 6.0 (± 4.03) × 10^{-9} to 3.5 (± 6.0) × 10^{0} transconjugants per recipient. In general, the observed transfer frequencies in this study can be considered relatively high, thereby suggesting that these clinical isolates may be more efficient in spreading Tn*916* despite having a low CR.

Paper III

Stability and relative fitness cost of newly acquired Tn*916* elements in *Streptococcus oralis* (Manuscript)

MGEs such as ICE are vital players in transferring genetic material by conjugation and can thus spread antibiotic resistance genes between and across bacterial species.

The cost of a newly acquired Tn*916* in a naïve *S. oralis* isolate among seven transconjugants ranged from 6% to 25% at T0. During an evolution experiment, the relative fitness cost in all the transconjugants was reduced within 500 generations. In addition to amelioration of the cost of carrying the element, in the *S. oralis* host retained the ICE in the absence of selection for more than 1000 generations. Analysis of the number of copies of Tn*916* in TC4.1, TC4.2, TC4.6, TC6.1, and TC8.1 gave indication that the average number of Tn*916* detected by ddPCR was 3 copies per genome. In transconjugants TC6.2 and TC10.1, the average copy numbers were 2 and 1, elements per genome, respectively. Based on both ddPCR copy number analysis and WGS, the number of copies of Tn*916* did not change during the evolution study. Interestingly, the insertion sites remained the same during evolution, and no SNPs were detected within the elements. Comparisons of the genomes of the Tn*916* carrying populations (non-evolved vs evolved) indicate the presence of multiple SNPs in different locations in the genomes. SNPs in the bacterial chromosome suggest that other secondary mechanisms are responsible for the amelioration of fitness.

6 GENERAL DISCUSSION

The increased presence of antimicrobial resistance genes in bacterial populations is a significant threat to human health. There is a growing need to understand the underlying factors that promote the evolution, selection, and spread of antibiotic resistance. Defining how these antibiotic resistance determinants are transferred and how we can prevent or slow down this process is vital in combating the spread of antibiotic resistance. Recent efforts to combat the rise of antibiotic resistance include increased awareness of the proper use of antibiotics among all health practitioners, identifying novel antibiotic treatment options, surveillance of antibiotic usage, and monitoring the development and spread of antibiotic resistance. As a contribution to monitoring the development and spread of antibiotic resistance, this study investigates the prevalence of the Tn*916*-Tn*1545* family and the factors that influence the transfer and stability of these ICEs in oral streptococci.

The presence of ICEs in antibiotic resistant oral streptococcal isolates has become a significant concern. Furthermore, the increased availability of microbial genomes illustrates that ICEs outnumber conjugative plasmids in bacterial populations and currently render them the most abundant self-replicating conjugative elements in prokaryotes(217, 218). The presence of one of the most studied ICEs, Tn*916*, in oral bacteria has been reported by numerous studies (52, 60, 123, 127, 128, 164, 167, 201, 219). However, despite the known promiscuity of the Tn*916*-Tn*1545* family, little is known about the prevalence, diversity, and stability of these elements in oral streptococci. Moreover, less is known regarding the factors that influence and promote the transferability of the Tn*916*-Tn*1545* family. The present study addresses the role these ICEs play in the spread of resistance among isolates by investigating the prevalence, diversity, stability, transferability, and fitness cost of the Tn*916*-Tn*1545* family in a collection of clinical oral streptococci.

Prevalence of the Tn*916*-Tn*1545* family

The Tn*916*-Tn*1545* family has contributed to the dissemination of *tet* (M) and *erm* (B) genes in many bacterial species. Although the Tn*916*-Tn*1545* family elements have been reported in the oral cavity, little is known regarding their prevalence in clinical oral streptococcal isolates. In **paper II**, we report a prevalence of 21% for Tn*916*-Tn*1545* family members in a collection of 100 antibiotic-resistant oral streptococcal isolates. This study used the *tet* (M), *int,* and *xis* genes as the inclusion criteria for the Tn*916*-Tn*1545* family. According to the RFLP and WGS data, the

most common element in our sample material was wild-type Tn*916*. In contrast to the findings by Ciric *et al.* (110) that reported Tn*3872* as the most prevalent Tn*916*-like element (39.6%) followed by Tn*916* (31%), the results from our study demonstrate that the wild type Tn*916* was the most prevalent as it was present in 71% of the samples. The study mentioned above reports the presence of the Tn*916*-Tn*1545* family in 48 samples, but no information is given regarding the number of isolates that did not harbor the Tn*916*-Tn*1545* family. Consequently, it is not easy to compare the prevalence of the Tn*916*-Tn*1545* family between these two studies.

Interestingly, the highest occurrence of Tn*916* was observed in *S. oralis* with a prevalence of 41% (**paper II**), while the most prevalent species (41%) in the isolate collection in this study was *S. mitis*, of which only 26.2% harbored the Tn*916*-Tn*1545* family. The observed variations in species carriage raise the question of whether the elements have a species preference, a notion that lays outside the scope of this study but requires further investigation.

Diversity of the Tn*916*-Tn*1545* family

The Tn*916*-Tn*1545* family is a diverse group of ICEs identified in more than 30 bacterial genera, including oral streptococcal species (220). In this study, we identified three different members of the Tn*916*-Tn*1545* family, namely Tn*916*, Tn*6815*, and Tn*6816*. Tn*6815* and Tn*6816* are novel elements first reported in this work (**paper II**). When compared to the findings from Ciric *et al.* (123) that reported four different members of the Tn*916*-Tn*1545* family in a collection of 48 oral streptococcal isolates, the presence of three different elements in the 21 isolates carrying the Tn*916*-Tn*1545* family elements suggests a higher diversity in our collection of samples. In addition, the strict inclusion criteria used in this study may have resulted in the exclusion of some members of the Tn*916*-Tn*1545* family that do not carry *tet* (M) and that have other recombination genes instead of the *int* gene (as mentioned in section 1.5.3.1). For instance, we observed that 17% of the isolates carried *int* but not *tet* (M), and an additional 3% carried *tet* (M) but not *int*. In retrospect, these isolates should have been subjected to further screening, as they may have increased the observed diversity of this family of elements in our study.

It should be noted that the limited number of studies focusing on the diversity of these elements in oral streptococci renders it challenging to discuss with certainty how diverse the Tn*916*-Tn*1545* family is in these species. In addition, variations between the sample materials and

collection methods render it challenging to compare the findings from different studies. For instance, the study mentioned above by Ciric *et al.*, which reports the presence of four different Tn*916*-Tn*1545* family elements in a collection of 48 minocycline-resistant oral streptococci, uses saliva samples from 19 healthy human volunteers as the source of the streptococcal isolates (123), whereas we analyzed clinical antibiotic resistant isolates.

Recent advancements in bioinformatics provide an essential tool to investigate the role oral streptococci play in disseminating of resistance determinants. The findings in our study illustrate the importance of using bioinformatics to support other molecular biology tools. For instance, the RFLP results indicated that 6 out of the 21 isolates harboring Tn*916* did not harbor wild-type Tn*916*. However, Sequence analysis of these elements demonstrate that three of these elements are, in fact, wild-type Tn*916* with SNPs that result in altered restriction digestion patterns (**paper II**).

These findings indicate that relying on merely one approach to determine the diversity of ICEs may result in inaccurate results and the exclusion of some elements, thus underrepresenting the diversity and abundance of these elements. The findings from our study also highlight the importance of combining multiple approaches (molecular and bioinformatic strategies) that complement each other to obtain a clear and more accurate picture regarding the diversity of ICEs.

The CN and CR of Tn*916*-Tn*1545* family elements

The use of molecular biology tools such as PCR has been instrumental in determining the abundance of genes in bacterial populations. Although reliable and versatile, these methods do not shed light on the number of elements present in a bacterial population. The development of a ddPCR assay that detects the number of Tn*916*-Tn*1545* elements present and indicates the CR of the bacterial population is vital in understanding some critical factors (such as the CN and CR) that influence the transfer potential of the ICEs. In **paper I**, we designed and implemented a ddPCR assay that quickly and accurately determines the CN and CR of the Tn*916*-Tn*1545* family elements in a bacterial population. Comparing the detected number of elements to those specified in fully sequenced isolates verified the accuracy of this assay. The ICE copy number and the CR are essential factors as ICE transfer is likely to increase with the number of elements in a bacterial population. In addition, the presence of more than one element

in a bacterial cell provides additional prerequisite elements that can excise and form more Cis. In **papers I** and **III**, we demonstrate that the isolates with more than one element have higher CI numbers.

Interestingly, the work in **paper II** illustrates that the number of elements is not the only factor influencing the rate of Tn*916* transfer. Paper II's intra- and interspecies conjugation experiments show variations in transfer frequencies among the different recipients even when the same donor is used. These findings concur with earlier findings of variations in transfer frequencies among different donors (221). Furthermore, variations were observed within the same recipient when different donors were used. Interestingly, we observed that the laboratory strain *B. subtilis* BS49, which has two ICE copies and had a higher CR than the other donors, had the lowest transfer frequencies in this study. These findings suggest that the transfer of the Tn*916*-Tn*1545* family elements is influenced by other factors beyond the donor, recipient, CR, and copy number of the element.

In **paper I**, we demonstrate that sub-MIC levels of tetracycline influence the CR of this family of elements. The observed increase in the CR is in accordance with findings from previous studies that reported increases in the excision of Tn*916* at sub-MIC antibiotic levels (222). The increased excision rate of Tn*916* in the presence of sub-MIC levels of tetracycline is of particular importance, as it indicates one factor that increases the transferability of the element. This implies that the continued use of tetracycline in agriculture and aquaculture, which leads to low levels of this antibiotic in the environment, may facilitate higher rates of Tn*916* transfer among bacterial populations.

Our work demonstrates that the clinical oral streptococcal isolates have a lower CR than the laboratory isolates in both the presence and absence of selection (**paper I**). It was, therefore, intriguing that we observed higher transfer frequencies in clinical oral streptococci than in the laboratory strains (**paper II**). As we examined clinical isolates, we cannot determine when they acquired the ICEs or how long the ICEs have coexisted with the host cell. Tn*916* has been shown to replicate autonomously. However, like most ICEs, its transfer requires that the host cell physically connects with the recipient and establishes the conjugation transfer machinery for the element. Establishing the conjugation transfer machinery and expression of conjugation genes are energy requiring processes that may be detrimental to the host (139, 223, 224). Therefore, we hypothesize that some host-ICE interactions are in play that result in lower

excision levels in clinical isolates. Lower excision levels would create an equilibrium of sorts where the host uses less energy on the transfer of the element and maintains the resistance phenotype, while allowing the ICE to play its natural role of transferring horizontally but at lower rates, and thus at reduced biological costs for the host (225). We further speculate that clinical isolates have established a mechanism to reduce the potential of losing elements via HGT, hence the lower excision rates, thereby maintaining the elements and transferring them vertically. This hypothesis, although plausible, requires further investigation, a notion that we have attempted to address in **paper III** by investigating the evolution of ICE-containing isolates.

One of the significant concerns related to the transfer of the Tn*916*-Tn*1545* family is the presence of two or more resistance determinants on the same ICE. Reports of streptococcal isolates harboring elements with more than one resistant determinant are common and listed in Table 1. In our collection of isolates, we only identified one isolate that carried a Tn*916*-Tn*1545* family element with more than one resistance gene: Tn*6815* (**paper II**). Based on the WGS results, the newly characterized Tn*6815* is a composite element (composed of Tn*916* and Tn*917*) and harbors genes that confer resistance to tetracycline and erythromycin. In addition to carrying Tn*916* and Tn*917*, Tn*6815* was transferable by conjugation through filter mating experiments. Interestingly, in contrast to other elements that have insertions downstream of *tet* (M), such as Tn*2009* and Tn*3872* (124), Tn*6815* was transferable by conjugation to several recipient strains. We further describe that the novel Tn*6815* carries two truncated copies of *orf9. Orf9* has been proposed to produce an antisense RNA that leads to the downregulation of the promoter P*orf7* (191). As described in section 1.4.4, the product of *orf9* is thought to suppress the expression of the *orf7*, *orf8*, *orf5, int*, and *xis* genes. In the absence of a functional *orf9* gene, higher levels of excision may be seen as the transcription of the *orf7*, *orf8*, *orf5, int*, and *xis* genes increases due to the lack of suppression of P*orf7*. We speculate that the absence of a complete *orf9* gene in Tn*6815* may play a role in the observed ability of the element to transfer via conjugation. We further speculate that the presence of two copies of truncated *orf9* may be due to other HGT events such as recombination. Although difficult to prove, it is plausible that multiple HGT events can coincide in bacterial populations (226). The transferability of this element is an indication of the potential role that members of the Tn*916*-Tn*1545* family play in the spread of multi-resistance determinants among bacterial populations.

Transfer frequency and the implications of using clinical isolates

The presence of MGEs, such as the Tn*916*-Tn*1545* family, have been implicated in the increasing number of antibiotic-resistant oral streptococcal isolates. This work illustrates the transfer of the Tn*916*-Tn*1545* family within the same species, between different species, and between clinical oral streptococcal isolates (**paper II**). In the isolates where conjugation was detected, the transfer frequencies ranged from 6.0 (± 4.03) × 10^{-9} to 3.5 (± 6.0) × 10^{0} transconjugants per recipient. A search of the literature showed that Tn*916* has been successfully transferred to *S. gordonii, S. salivarius*, and *S. sanguinis* isolates with conjugation frequencies ranging from 10^{-8} to 10^{-5} (128, 164). Of the 28 successful transfers observed in our study, 20 of the filter mating experiments showed higher transfer frequencies than previously reported.

Interestingly, the higher transfer frequencies were all associated with clinical isolates with a low CR and only one Tn*916*-Tn*1545* family element. The low CR suggests that the observed higher transfer frequencies may be due to other factors in the clinical isolates that allow for higher transfer efficiency despite having a low CR. It is also possible that the observed higher frequencies in the clinical isolates may be due to adaptations within the host or ICE that drive ICE stability within bacterial populations. This line of thought, although plausible, requires further studies for substantiation.

In **paper II**, *B. subtilis* BS49 was used as a study control for the transfer of Tn*916*. As this strain harbors two elements and has been reported to have a high CR (9.4% per bacterial population; **paper I**), we expected to see high transfer frequencies in the conjugation experiments involving this isolate. Surprisingly, *B. subtilis* BS49 yielded the lowest number of transconjugants per recipient, suggesting that the transfer of the Tn*916*-Tn*1545* family is not only influenced by the number of CIs present in the population but also by the species involved. We further speculated that despite providing cell-to-cell contact between the donor and the recipient cells during conjugation, other unknown barriers may exist that led to the observed low number of transconjugants. The observed variations in transfer frequencies between the donors are in agreement with the findings reported by Clewell *et al.* in their work from 1995, which reported: "while transfer frequencies range from $<10^{-9}$ per donor to $>10^{-4}$, each strain has a consistent characteristic donor potential" (221).

The observed intra- and interspecies transfer of the Tn*916*-Tn*1545* family is further evidence of the promiscuity of this family of ICEs. However, it is worth noting that the conditions in the oral cavity and the clinical setting are very different from a filter mating conjugation experiment. The use of one donor and one recipient in the conjugation experiments cannot mirror what happens in the body where billions of bacteria are in contact. One can, however, speculate that the presence of numerous recipients would lead to even higher numbers of transferred elements unless the oral cavity has environmental and host-specific factors that influence the rate of transfer of these elements.

Oral streptococci as reservoirs of resistance

The 100 oral streptococcal isolates used in this study are known to be resistant to one or more antibiotics. MIC testing with the selected 11 antibiotics revealed that 10% of these isolates were resistant to more than one antibiotic and that 5% were resistant to four or more antibiotics (see Table S1 in appendix). Of the tested antibiotics, meropenem was the only antibiotic we did not observe phenotypic resistance to within the studies isolates. The most prevalent resistance phenotype was tetracycline resistance, which was present in 23% of the isolates, followed by erythromycin resistance, which was observed in 15% of the tested isolates. Although only one isolate was found to carry a Tn*916*-Tn*1545* family element that harbors both *tet* (M) and *erm* (B), it is interesting that a total of seven isolates were found to be resistant to both tetracycline and erythromycin. This finding implies that the presence of other members of this family may not have been detected in this study due to

(i) the strict inclusion criteria that were used,
(ii) the fact that these resistance determinants are chromosomally located, or
(iii) the fact that the resistance determinants are located on unidentified elements.

The prevalence of penicillin resistance was low, with only two isolates (*S. mitis* SM28 and *S. mitis* SM70) having MICs of 6 and 4 μg/ml, respectively. Despite the low prevalence of penicillin resistance in these isolates being a positive finding, the high prevalence of erythromycin resistance overshadows these observations. The presence of erythromycin resistance genes in oral streptococci has substantial consequences for patients, primarily those allergic to penicillin, as erythromycin is often used as the treatment alternative.

The observed penicillin resistance coupled with the high levels of resistance to multiple antibiotics among the *S. mitis* isolates in this collection raises some concerns, especially as *S. mitis* species are closely related to *S. pneumoniae*, which has been shown to have high levels of antibiotic resistance, including penicillin resistance. However, as we did not detect any penicillin resistance determinants on the ICEs we investigated, we speculate that other modes of HGT independent of the Tn*916*-Tn*1545* family may be responsible for the spread of penicillin resistance in the oral microbiota.

The presence of multiple genetically linked antibiotic determinants on an ICE offers co-selection for multiple antibiotics and thus exacerbates the problem of antibiotic resistance(65). For instance, erythromycin is an antibiotic that is commonly used in agriculture (to treat gram-positive infections) and in humans (to treat respiratory tract infections). Over the last few decades, there has been an increase in not only the number of erythromycin resistant clinical isolates but also tetracycline resistant clinical isolates, including oral streptococci (227). The combined presence of tetracycline and erythromycin resistance in streptococci is usually associated with the presence of both genes on the same MGE and has been reported to occur most frequently in *S. oralis* and *S. sanguinis* (219, 228). Therefore, the presence of these determinants on the same elements implies that reduced use of tetracycline will not reduce the presence of tetracycline resistance genes as long as erythromycin is used as a treatment option, as erythromycin will co-select for both determinants. The presence of MGEs and resistance determinants and the ability of these species to exchange genetic material via HGT render them critical players in the spread of antimicrobial resistance genes and thus as reservoirs of antibiotic resistance.

Fitness cost and stability of the Tn*916*

ICEs, like plasmids are transferred by conjugation and through similar mechanisms (rolling circle replication). It has, however, been suggested that the fitness cost and evolutionary trajectories of these MGEs may be different (72). Little is currently known regarding the evolution and cost of ICEs in bacterial populations in general. In **paper III**, we examined the relative fitness cost of a newly acquired ICE and reaffirm that high biological costs accompany the acquisition of Tn*916*. In the light of these facts, one cannot help but wonder why these elements are abundant in bacterial populations, especially in the absence of selective pressure. Based on the results from this evolution study, it is plausible that the high prevalence of Tn*916* elements may be attributed to the rapid reduction of the fitness cost of carrying these elements as seen in this study. Reductions in fitness cost may be attributed to several factors, including:

(i) loss of the element,
(ii) change in the insertion site,
(iii) adaptative mutations, and
(iv) changes in the regulation of conjugation module (once integrated into the host chromosome, the conjugation transfer genes are switched off, which is thought to reduce the burden on the host) (72).

We anticipated the reduction in relative fitness to corelate with element loss, especially in cases where more than one copy of the same element was present. To our surprise, not only were the ICEs retained in the absence of selection pressure, but more than one copy of Tn*916* was still present after 600 hours of passage (>1000 generations). In addition, we find it intriguing that the bacteria retained multiple copies of the same element. Unlike **paper II,** in which we reported two copies of identical Tn*916*-like elements in a clinical isolate, the isolates in question here were constructed in the laboratory. Cases of laboratory-constructed isolates carrying multiple copies of ICEs have been reported in the past; for instance, the sequenced *B. subtilis* isolate BS49, which we used as a reference strain in **papers I** and **II**, has two copies of Tn*916* in different orientations (195). Previous studies have reported that it is not common to find copies of two identical Tn*916*-like elements on the same chromosome (229). Therefore, it may be speculated that carrying multiple identical Tn*916* elements, as seen in this experimental study, may be influenced by other bacterial- and host-related factors *in vivo*.

As stated above, the transfer of more than one copy of Tn*916* by conjugation is not uncommon and has previously been reported in various studies, including Clewell *et al.* (221). In **paper III**, we demonstrate that varying numbers of Tn*916* can be transferred into a naïve isolate and that this transfer comes with a fitness cost. The observed fitness cost may be attributed to various factors, including the presence of conjugation and accessory modules as these modules have been observed to impose a fitness cost on the host in integrons and other elements that carry a T4SS (216). Additionally, once acquired and integrated into the host genome, ICEs use the host's molecular machinery (e.g., ribosomes) and energy resources for replication, which may stress the host cells and lead to a reduction in the growth rate (230, 231). Another factor that may lead to the reduction in fitness is the presence of ssDNA in the cells as Tn*916* migrates into the recipient cell. As demonstrated with plasmids, the presence of ssDNA in a cell leads to the activation of the SOS response system, which slows cell division, and is manifested as a temporary reduction in fitness (232). The observed fitness cost may also be attributed to the insertion site of the ICE especially in cases where functional genes are disrupted (as seen by the insertion of Tn*916* in an A:T rich region of the *Com D* gene in transconjugant TC10.1).

Our data from the evolution experiments demonstrates a reduction in Tn*916* fitness cost over time. A possible explanation for the observed reduction in Tn*916* fitness cost may be altered gene expression levels of the integrases and/or resistance genes as has been reported in *Acinetobacter baylyi* (233), *Enterococcus* ssp. (234) and in resistant *E. coli* isolates (235). There is, however, a need for more experimental research to determine whether gene expression plays a role in reducing the fitness cost of Tn*916* and, if so, what that role is.

The observed retention of the elements in the absence of selective pressure despite the fitness cost is intriguing. We expected to see the element loss in the population, especially where CR were observed, and fitness costs were substantial. We, for instance, anticipated that if cell division occurs after the excision of Tn*916* from the genome, some of the bacterial cells would lose the ICE. Loss of the ICE would lead to a fitness advantage in the absence of selective pressure and the ICE-free cells would dominate the population. As this did not occur, we speculate that Tn*916* possesses a stabilization mechanism such as the plasmid-like type II partition system found in the SXT/R391 ICEs (236). The possibility of a stabilization mechanism that promotes ICE stability calls for further research.

7 CONCLUDING REMARKS

One of the goals in AMR research is to find ways to slow down the spread of antibiotic resistance among bacterial populations. To accomplish this goal, there is need to understand the resistance landscape and the factors that promote the spread of resistance determinants. The work in this thesis sheds light on the prevalence of the Tn*916*-Tn*1545* family among oral streptococcal isolates. In addition to establishing that Tn*916* is the most prevalent member of this family present in this collection, we identified two novel members of the Tn*916* family Tn*6815* and Tn*6816* in *S. mitis* and *S. constellatus*, respectively. We demonstrate that ddPCR can be used to determine the copy number of the Tn*916*-Tn*1545* family elements and to quantify the CR of the elements present in the bacterial population. Furthermore, we provide evidence of inter- and intraspecies transfer of the Tn*916*-Tn*1545* family and show that once acquired, Tn*916* is stable for more than 1000 generations.

Taken together, the findings in this thesis demonstrate that ICEs are commonly associated with AMR in oral streptococci and that they are readily transferred with relatively high transfer frequencies. It is evident that once introduced into a new background, Tn*916* comes with a biological cost that is ameliorated with time in the absence of selection pressure. The relatively rapid reduction in fitness cost, the stability, and the persistence of Tn*916* may thus be the driving forces behind the abundance of this family of ICEs in streptococcal species and bacterial populations.

8 FUTURE PROSPECTIVES

Based on the findings from this work, it is evident that the Tn*916*-Tn*1545* family plays a role in the spread of resistance among oral streptococcal species. To determine how these ICE impact the oral microbiome, there is a need to investigate the prevalence and diversity of these elements in the oral cavity of health individuals.

The *tet* (M), *int*, and *xis* genes are used as inclusion criteria in the Tn*916*-Tn*1545* family. However, as we illustrated in **paper II**, this may lead to the under-representation of ICEs in bacterial populations. It would therefore be interesting to determine whether including the conserved conjugation module of the Tn*916*-Tn*1545* family in the screening criteria coupled with the use of multiple molecular and bioinformatics tools would lead to the detection of more members of this family of ICEs. If successful, this would provide a more accurate picture of the abundance of these elements.

This study reports the transferability and stability of the Tn*916*-Tn*1545* family in oral streptococcal species. The work in **papers I** and **II** illustrate that multiple factors influence the transfer of these elements. It should, however, be noted that transfer rates reported here are based on conjugation between two mono-species. To fully understand the transfer rates and stability of these ICEs, there is a need to conduct these studies in conditions that include host specific factors and resemble the oral cavity, such as biofilms.

Considering the substantial fitness cost associated with the acquisition of the Tn*916* and the rapid amelioration observed in **paper III**, it is evident that there are mechanisms in play that alleviate the observed fitness costs. Therefore, a testable hypothesis would be that the reduction in fitness is related to altered gene expression levels at the Tn*916* and chromosomal levels. It would be interesting to conduct transcriptomics analysis on these isolates to determine gene expression levels.

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Paper I

JOURNAL OF ORAL MICROBIOLOGY
2018, VOL. 11, 1552060
https://doi.org/10.1080/20002297.2018.1552060

Taylor & Francis
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ORIGINAL ARTICLE

Determination of copy number and circularization ratio of Tn*916*-Tn*1545* family of conjugative transposons in oral streptococci by droplet digital PCR

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ABSTRACT

Background: Tn916 and Tn1545 are paradigms of a large family of related, broad host range, conjugative transposons that are widely distributed in bacteria and contribute to the spread of antibiotic resistance genes (ARGs). Variation in the copy number (CN) of Tn916-Tn1545 elements and the circularization ratio (CR) may play an important role in propagation of ARGs carried by these elements.

Objectives and Design: In this study, the CN and CR of Tn916-Tn1545 elements in oral streptococci were determined using droplet digital PCR (ddPCR). In addition, we investigated the influence of tetracycline on the CR of Tn916-Tn1545 elements.

Results: The ddPCR assay designed in this study is a reliable way to rapidly determine CN and CR of Tn916-Tn1545 elements.

Conclusions: Our data also suggest that Tn916-Tn1545 elements are generally stable without selective pressure in the clinical oral Streptococcus strains investigated in this study.

ARTICLE HISTORY
Received 22 March 2018
Revised 12 November 2018
Accepted 19 November 2018

KEYWORDS
Oral streptococci; Tn*916*-Tn *1545* family; antibiotic resistance; mobile genetic elements (MGEs); droplet digital PCR

Introduction

The oral cavity is among the most microbiologically diverse environments in the human body and has been shown to contain over 1100 different bacterial species [1] of which *Streptococcus* species are the most abundant [2]. Although the majority of the *Streptococcus* species are not considered pathogenic, some species such as *Streptococcus mutans* are responsible for oral diseases and others, such as viridans group streptococci, can cause infections (such as pneumonia, endocarditis, and intra-abdominal infection) at other body sites [3].

There has been an increase in the number of antibiotic-resistant streptococcal strains over the last few decades [4], and recent studies suggest that the oral cavity functions as a reservoir for transferable antibiotic resistance genes [5–8] including genes encoding resistance to macrolides [9], beta-lactams, and tetracyclines [10]. One of the most common tetracycline resistance genes within oral isolates and metagenomes is *tet*(M) [8,11]. The broad distribution of *tet*(M) has frequently been linked to its association with mobile genetic elements (MGE) from the Tn*916*-Tn*1545* family of conjugative transposons/ Integrative Conjugative Elements [ICEs] [12–16].

The Tn*916 conjugative transposons/ICE*

Tn*916* (accession number; U09422.1) is an 18-kb broad host range ICE [17] first isolated from *Enterococcus faecalis* DS16 [18]. Tn*916* contains 24 ORFs (open reading frames) which are arranged in functional modules. These modules are responsible for conjugal transfer, transcriptional regulation, excision and insertion reactions (transposition), and accessory functions such as antibiotic and antiseptic resistance [17]. The transfer of Tn*916* from a donor cell to a recipient cell involves the excision of the element from its original replicon to form a circular intermediate (CI) molecule [19], which has also recently been shown to autonomously replicate [20]. Tn*916* and many related elements of the Tn*916*-Tn *1545* family are frequently able to insert into multiple sites within a host genome [21].

Variations in ICE CN (copy number) may have an impact on their stability (of the ICE and the host genome) and conjugation potential thereby influencing the level of antibiotic resistance within bacterial populations [22–24].

Evaluation of the CN of the Tn*916*-Tn*1545*-like elements in oral streptococci can be achieved by Restriction Fragment Length Polymorphism (RFLP) followed by southern blot hybridization [25], full genome sequencing and assembly [26], and by real-time quantitative PCR (qPCR) [20]. The first two methods are not only time consuming and labor intensive but they also require high quantities of pure, high molecular weight DNA. The qPCR has become a common method in determining CN of target genes [27], however it does have some

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Supplemental data for this article can be accessed here.

limitations such as performance variation in and between assays [28] and artificial qPCR data resulting from samples with low target concentration but high levels of impurity [29]. These limitations can be overcome by droplet digital PCR (ddPCR) as it has been shown to produce more precise and reproducible results when compared to qPCR [30]. In the QX200 ddPCR (Bio-Rad, USA), a single PCR reaction is divided into approximately 20,000 droplets which are treated as individual reactions. Each reaction contains the relevant forward and reverse primer, the probes to detect the target gene and template DNA molecule.

In this study, we present an assay that rapidly reports CN of integrated and CI of Tn*916*-Tn*1545*-like elements in various clinical oral *Streptococcus* species. In addition, the CR of CI in the study strains is reported (percentage of CI molecules detected within the bacterial population as a function of the total number of host genomes).

Materials and method

Control strains

The fully sequenced *Bacillus subtilis* BS34A (NZ_LN680001.1), *B. subtilis* BS49 (NZ_LN649259.1), *Enterococcus faecium* OrEc1, and *E. faecium* OrEc2 derivatives containing different CN of Tn*916* were used as control strains in ddPCR (Table 1).

Clinical oral streptococcus strains

A selection of 10 antibiotic resistant oral *Streptococcus* strains collected by the National Advisory Unit for Detection of Antimicrobial Resistance (K-RES), University Hospital of North Norway were used in this study. These 10 strains tested PCR positive for *tet*(M) and the Tn*916* integrase (i*ntTn*) and excisionase (*xisTn*) genes. The 10 strains were further identified at the species level using MALDI-TOF. MALDI-TOF identification was carried out at the National Advisory Unit for Detection of Antimicrobial Resistance (K-RES) laboratories, University Hospital of North Norway. These strains were used in the determination of CN and CR of Tn*916*-Tn*1545* like elements in oral streptococci.

Bacterial cultivation

B. subtilis and *E. faecium* strains were cultivated on Luria-Bertani (LB) agar at 37°C under aerobic conditions whereas the oral *Streptococcus* strains (Table 1) were cultivated in anaerobic conditions on Todd Hewitt (TH) agar at 37°C overnight using the Anaerocult® System (Merck, Germany).

Determination of tetracycline MIC

The MIC of tetracycline for the oral streptococci was determined by E-test (BioMerieux, France) on Mueller-Hinton agar supplemented with 5% sheep blood and interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (www.eucast.org). The *S. pneumoniae* ATCC 49619 was included in all the runs as a positive control.

DNA extraction and DNA concentration measurement

The QIAcube (Qiagen, Hilden, Germany) automated system was used to extract DNA with a preprogramed protocol using the QIAamp DNA Mini Kit (Qiagen, Germany) to obtain DNA from all bacterial strains in this study according to the manufacturer's instructions. The quality and yield of extracted genomic DNA were

Table 1. Bacterial strains used in this study.

Bacteria	Relevant properties (MIC Tet)	Reference or Source
***B. subtilis* BS34** (Control strain containing one copy of Tn*916*)	*Tetracycline resistant bacterium (32*µg/ml)	[26]
B. subtilis BS49 (Control strain containing two copies of Tn*916*)	*Tetracycline resistant bacterium(48*µg/ml)	[26]
***E. faecium* OrEc1** (Control strain containing five copies of Tn*916*)	*Tetracycline resistant (96*µg/ml) (Transconjugant)	This study
***E. faecium* OrEc2** (Control strain containing one copy of Tn*916*)	*Tetracycline resistant (48*µg/ml) (Transconjugant)	This study
S. pneumonia (control strain for Tetracycline MIC)	*Tetracycline susceptible* (≤1µg/ml)	ATCC 49619
S. mitis SM28	*Tetracycline resistant* clinical isolate *(64*µg/ml)	This study
S. mitis SM29	*Tetracycline resistant* clinical isolate *(32*µg/ml)	This study
***S. sanguinus* SS33**	*Tetracycline resistant* clinical isolate *(24*µg/ml)	This study
***S. sanguinus* SS41**	*Tetracycline resistant* clinical isolate *(32*µg/ml)	This study
***S. oralis* SO44**	*Tetracycline resistant* clinical isolate *(24*µg/ml)	This study
***S. oralis* SO47**	*Tetracycline resistant* clinical isolate (32µg/ml)	This study
***S. oralis* SO62**	*Tetracycline resistant* clinical isolate (4µg/ml)	This study
***S. oralis* SO67**	*Tetracycline resistant* clinical isolate (48µg/ml)	This study
***S. gordonii* SG71**	*Tetracycline resistant* clinical isolate (32µg/ml)	This study
***S. oralis* SO74**	*Tetracycline susceptible* clinical isolate (2µg/ml)	This study

analyzed by agarose gel electrophoresis before determining the DNA concentration with the Qubit 3.0 fluorometer (Life Technologies, USA) according to the manufacturer's instructions.

Conventional PCR

Conventional PCR was conducted with primers listed in Table 2 and depicted in Figure 1S (see supplementary data). All reactions were performed in a final volume of 25 µl containing 12.5 µl of 2 x Dream Taq Green PCR master mix (Thermo Scientific, USA), 1 µl each of the forward and reverse primer (20 µM), 2.5 µl DNA sample, and 8 µl water. The PCR conditions were: initial denaturation at 94°C for 10 min, 30 cycles of 94°C for 1 min, 56°C for 1 min and 72°C for 2 min, and a final extension step at 72°C for 10 min. A volume of 5 µl was analyzed with a 1-KB DNA ladder on a 1% agarose gel containing GelRed™ (Biotium, USA) for visualization of the amplicon.

Genetic linkage between *tet*(M) and intTn and xisTn

In order to analyze the genomic proximity between *tet* (M) and *intTn* and *xisTn*, indicating that the genes are located on the same genetic element, linkage analysis by a duplex ddPCR was carried out. Intact bacterial cells were boiled for 5 min in molecular grade water to obtain a DNA template. The concentration of the DNA was determined using the Qubit 3.0 fluorometer (Life Technologies) and accordingly the optimal DNA amount was used for further analysis. The digestion reactions were performed in a 20 µl reaction mixture, which contained 2.5 µl of the bacterial DNA, 10 U of restriction enzyme, 2 µl of 10 x buffer, and water. The digestion was conducted at 37°C for 4 hrs prior to inactivation at 80°C for 20 min. The restriction enzymes used were *Bsu*RI (which does not cleave Tn*916* between *tet*(M) and *intTn* and *xisTn* genes) and *Hinc*II which cuts Tn*916* between the *tet*(M) and *xisTn* at position 14,934 bp in U09422.1. The *Hinc*II enzyme was used as a control for the genetic linkage analysis. The initial linkage between *tet*(M), *intTn*, and *xisTn* was automatically calculated by the Quantalife™ software as a 'linkage' score. This is the estimate of the total number of molecules (copies/µl) in the assay that contain fragments on which the two targets are physically linked. The linkage percentage is calculated by normalizing the linkage score for differences in DNA input between the two assays as described by Roberts et al. [31]. The linkage percentage (L%) between *tet*(M), *intTn* and *xisTn* was calculated as follows: $L\% = (2\lambda_{tet(M)\ intTn/xisTn\ \text{regions}}/(\lambda_{tet(M)} + \lambda_{intTn/xisTn\ \text{regions}})) \times 100$, where L% is the normalized linkage score, $\lambda_{tet(M),\ intTn/xisTn\ \text{regions}}$ is the concentration of *tet* (M) and *intTn* and *xisTn* contributed to the *tet*(M)-*intTn* and *xisTn* genes droplets linkage, $\lambda_{tet(M)}$ is the average number of *tet*(M) copies per one droplet and $\lambda_{intTn/xisTn\ \text{regions}}$ is the average number of *intTn* and *xisTn* genes copies per one droplet.

Evaluation of amyE, intTn, and xisTn genes as a representative gene and representative region for genome CN and Tn916 CN, respectively

The known genome size of *B. subtilis* BS49 and different amounts of DNA inputs based on Qubit 3.0 flourometer (Life Technologies) readings were used to evaluate the suitability of *amyE* as a representative gene for detection of the genome CN in ddPCR.

The formula used to calculate the expected genome CN of *B. subtilis* BS49 with a DNA input ranging from 0.6 pg to 40 pg in the ddPCR reaction mixture is as follows: Genome equivalents/copies = A/HGW where A is the input DNA concentration and HGW is the genome weight of the bacterial genome that was

Table 2. Sequence of primers and probes used in the study.

Target	Forward primer	Reverse primer	Probe sequence	Amplicon size	Label (chlorophore)	Annealing temperature
Tet(M)	GGT TTC TCT TGG ATA CTT AAA TCA A	CCA ACC ATA CAA TCC TTG TTC AC	ATG CAG TTA TGG AAG GGA TAC GCT A	88bp	HEX/FAM	56°C
amyE B. subtilis	TGC AGA CGG AAT TTA CAC	CCG AGT CAT TAT ATA AAC CA	ACG GAT ACA ACC AAC GCA AA	146bp	HEX	56°C
Circular Intemidate (CI)ddpcr	CGT GAA GTA TCT TCC TAC A	GAC CTT GAT AAA GTG TGA TAA	AAT ACT CGA AAG CAC ATA GAA TAA GGC	167bp	FAM/HEX	56°C
intTn and *xisTn* regions	ATA CTC CCA TAC AGT CAA TAG TCC	AGT TCC ACC CCT GCA TGG	CCG TCG CAGGCA ATG AGT ATG GCT	88bp	FAM	56°C
amyE S. sanguinus	GGC GGA TGT CTA GGA GTT TAT C	TGG ATT GCC TTG CGT CTT	TTG GGC AAA TTC TCC GCT AAT GCC	67bp	FAM	56°C
amyE S. *oralis*	GGC ATC ATA GTC TGT ACC TGT G	AAC GGC TGG ACT CAC TTT AC	ACC AGT GCC AGT GGA AGT CAT TGT	96bp	FAM	56°C
amyE S. *mitis*	GCA TCC AAG CGG AAA CC	GAC CTA GAC TTT AAA CAT CCT GAA	TTT CCA TGA ACC AGT CAG CCC AGT	98bp	HEX	56°C
amyE S. *gordonii*	ATA AAT ACC AGA GCG TCG ACT T	CTA CTG CTA TTT CTG AAC CCT TTA TG	CAG TTC CAG TGA AAT GAT ACC AAT GCC A	149bp	FAM	56°C
amyE E. faecium	GAT TCG GAA CGA TGG AAG AT	GCG ATA CGG GCT TTC TTT AG	TTC AAA CCA TTG ATG CTG ATC CGA A	148bp	HEX	56°C
Circular Intermediate (CI) PCR	CGT GAA GTA TCT TCC TAC A	AC CTT GAT AAA GTG TGA TAA	N/A	166bp	N/A	56°C

calculated according to the genome size in Mb multiplied by 0.001096.

In addition, the *intTn* and *xisTn* in *E. faecium* OrEc1 and *B. subtilis* BS49, which harbor five and two copies of Tn*916* respectively, were evaluated for being a representative region in Tn*916* for determination of the CN of Tn*916*-Tn*1545* like elements by ddPCR. The primers and probes used for the *amyE* and the *intTn* and *xisTn* genes were designed and labeled with either FAM or HEX as listed in Table 2.

Calculation of the CN of Tn916-*Tn1545*-like elements and their CR by ddPCR

The QX200™ Droplet Digital ™ PCR system (Bio-Rad, Pleasanton, CA) was used in the current study to determine the CN of Tn*916*-Tn*1545*-like elements in the genome. In addition, the CR of Tn*916*-Tn*1545*-like elements that formed the CI in the *B. subtilis, E. faecium* and *Streptococcus* species populations were evaluated. The primers and probes used in the ddPCR assays in this study are listed in Table 2. The reaction mixture for CN experiments consisted of 10 µl ddPCR ™ Supermix for Probes (No dUTP), 1 µl of 20 x *intTn*/*xisTn* regions (target gene) primers and probes, 1 µl 20 x of *amyE* primers and probe, 0.5 µl restriction enzyme (5 Units per reaction), 8 µl water, and 60 pg DNA template.

A total volume of 21 µl of the reaction mixture was transferred into the sample well of the cartridge, and 70 µl of droplet generation oil was applied to the correspondent oil well prior to placing the gasket over the cartridge and transferring it into the droplet generator. After droplet generation, 40 µl of the sample emulsion was transferred into a 96-well PCR plate (Eppendorf, Germany) and then sealed with pierceable foil (Bio-Rad). PCR amplification was done in a C1000™ Thermal cycler (Bio-Rad). In all experiments, a non-template control (NTC) and positive controls were used to rule out any primer dimer or contamination issues. The amplification parameters consisted of an initial activation step at 95°C for 10 min followed by 40 cycles of 95°C for 30 s, and varying the annealing temperature (depending on the primers annealing temperatures as listed in Table 2) for 30 s. An additional inactivation step at 98°C for 10 min was used at the end of the cycles. The temperature ramp was set to 2°C per second and the lid was heated to 105°C. Upon completion of the PCR, the 96-well plate was transferred to the QX200 Droplet Reader (Bio-Rad) and the generated data were analyzed using the QuantaSoft software version 1.7.4.0971 (Bio-Rad). The threshold to distinguish positive droplets from the negative ones was set for each reaction automatically by the software if not stated otherwise. If needed, further analysis of the data was done using the QuantaSoft™ PRO software (version 1.0).

The CN of Tn*916*-Tn*1545*-like elements per bacterial genome was calculated by using the ratio between the Tn*916* target region, that is *intTn* and *xisTn* and the single copy reference gene (*amyE*). For strain specificity, the variable region of the reference gene was used to design species-specific primers and probes for the studied species (Figure 2S in the supplementary data). The accession numbers of the *amyE* used in the current study are given in Table 3. In the CR experiments, the reaction mixture was prepared as described above with the exception of the primers and probes used, which in this case, only produced a signal if and when the element was in the circular form. The sequence of the primers, probes, product size and annealing temperatures are shown in Table 2.

The CR was measured by calculating the percentage of the CI molecules detected within the bacterial population, that is the number of detected copies of CI molecules in the bacterial population to the number of bacterial genomes represented by the *amyE* CN in the same population. Based on screening for the presence of CI by conventional PCR, seven oral *Streptococcus* strains; *S. oralis* (n = 4), *S. mitis* (n = 2), and S. *gordonii* (n = 1) were selected for the CR analysis. In addition to investigate the CR, this study also assessed the effect of varying the tetracycline concentration below the MIC levels on the excision of Tn*916*-Tn*1545*-like elements in the control strains, that is *E. faecium* and *B. subtilis*, and the oral *Streptococcus* strains.

It has been recently reported in *B. subtilis* that Tn*916* can replicate autonomously [20]. In order to determine whether the element in our strains was replicating autonomously we compared the ratio of detected CI to the detected copies of bacterial genome, represented by *amyE* CN.

DNA sequencing of the promoter region upstream of *tet*(M)

DNA Sanger sequencing was used to investigate the DNA sequence of the promoter region upstream of *tet*(M) in oral streptococci. In brief, two primers (Table 2) were designed to yield a PCR fragment of 595 bp that covers the promoter region upstream of *tet*(M). The PCR fragment was subjected to BigDye terminator v 3.1 (Thermo Scientific) cycle sequencing prior to DNA sequencing by capillary electrophoresis using the SeqStudio sequencing platform (Thermo Scientific,). Sequencing data were aligned against the wild type Tn*916* using the Lasergene Molecular Biology Suite software (DNASTAR, USA).

Table 3. Accession numbers and genetic regions of the reference gene *amyE*.

Bacterial species	Accession number	*amyE* genetic region
B. subtilis	NZ_LN680001	327604..329583
E. faecium	CP012522.1	1785531..1787153
Streptococcus sanguinis	CP000387	1041272..1042738
Streptococcus oralis	FR720602	723983..725431
Streptococcus mitis	FN568063	702427..703881
Streptococcus gordonii	CP000725	1119068..1120519

Results

Bacterial strains

The 10 oral *Streptococcus* species. included in this study were identified by MALDI-TOF as follows: *S. mitis* (n = 2), *S. sanguinis* (n = 2), *S. oralis* (n = 5), and *S. gordonii* (n = 1).

Evaluation of amyE as a representative gene for genome CN by ddPCR

The accuracy and sensitivity of using the *amyE* as a reference gene for detecting genome CN by ddPCR was achieved by analyzing varying concentrations of *B. subtilis* BS49 DNA (obtained from cultures that were cultivated in the absence of selection pressure). *B. subtilis* BS49 is known to harbor two copies of Tn*916* [26] and, as shown in Figure 1, the detected CN of Tn*916* using *amyE* as a reference gene over a two-fold increase in DNA concentration was on average 2.00, SD 0.24. The detected CN of Tn*916* measured up to the expected theoretical CN even at a low DNA input of 0.6 pg/µl.

Determination of the CN of Tn916-Tn1545 -like elements

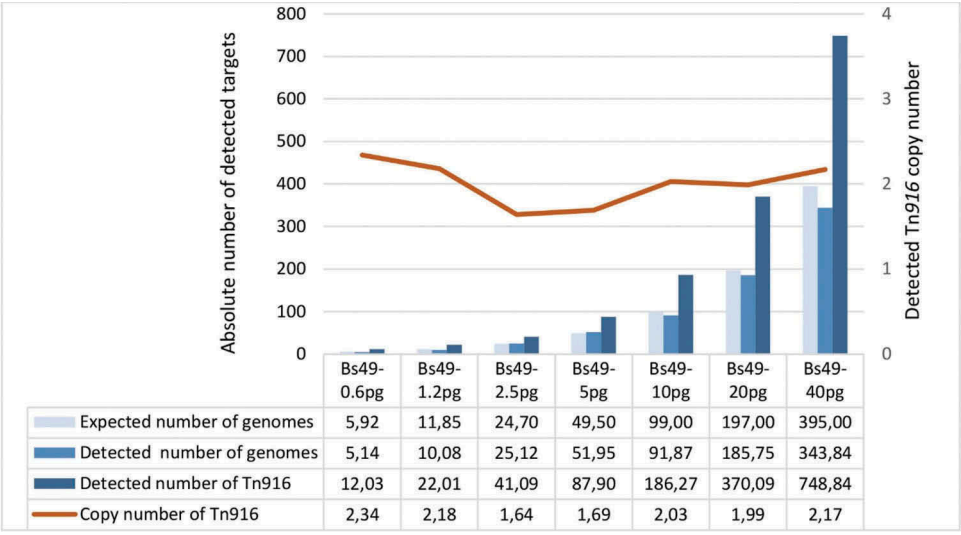
Prior to determining the CN of Tn*916*-Tn*1545*-like elements in *Streptococcus* species, we used previously published genome data of *B. subtilis* BS34A and *B. subtilis* BS49 (which contain one and two copies of Tn*916*, respectively) [26] and *E. faecium* OrEc1 and *E. faecium* OrEc2 (sequenced transconjugants produced in our laboratory which contains five copies and one copy of Tn*916*, respectively) to validate our ddPCR CN determination assay. In *B. subtilis* BS49 and *E. faecium* OrEc1 the observed number of Tn*916* corresponded to the expected CN (2 and 5 respectively). In *B. subtilis* BS34A, the observed CN was 0.72 as more *amyE* were detected than *intTn/xisTn*. The validated CN determination assay was used to screen a panel of 10 oral streptococci for determination of the CN of Tn*916*-Tn*1545*-like elements. Figure 2 shows that all the tested oral streptococci strains harbored only one copy of a Tn*916*-Tn*1545*-like element.

Linkage between tet(M) and intTn and xisTn

The linkage percentage between *tet*(M) and *intTn* and *xisTn* regions, which represents the percentage of droplets containing both targets suggesting that they are physically linked on the same fragment of DNA. This linkage percentage ranged from 88% to 6% in the bacterial cells that were digested with *Bsu*RI as shown in Figure 3(c). In the control samples that were digested with *Hinc*II which not only cuts between the *tet(M)* and the *intTn* and *xisTn* genes but produces six fragments in Tn*916*, we observed a 3–10-fold drop in linkage (ranging from 2–26%). Figure 3(a, b) illustrates that the reduction in droplets that contain the double targets; *tet*(M) and the *intTn* and *xisTn* genes when digested with the two targets are physically delinked.

The CR of Tn916-Tn1545-like elements

The CN of the circular form of Tn*916*-Tn*1545*-like elements and the CN of *amyE* in the bacterial population were used to calculate the CR, that is the percentage of CI molecules detected within the bacterial population. Based on our findings, the values of CI vary among



	Bs49-0.6pg	Bs49-1.2pg	Bs49-2.5pg	Bs49-5pg	Bs49-10pg	Bs49-20pg	Bs49-40pg
Expected number of genomes	5,92	11,85	24,70	49,50	99,00	197,00	395,00
Detected number of genomes	5,14	10,08	25,12	51,95	91,87	185,75	343,84
Detected number of Tn916	12,03	22,01	41,09	87,90	186,27	370,09	748,84
Copy number of Tn916	2,34	2,18	1,64	1,69	2,03	1,99	2,17

Figure 1. An illustration of the number of copies of *amyE* and *IntTn* and *XisTn* regions detected in *B. subtilis* BS49. *B. subtilis* BS49 has one copy of *amyE* and two copies of Tn*916* that are represented by *IntTn* and *XisTn* regions. The light blue bars show the theoretical single copy gene number of a 4.2 MB genome as calculated by QuantaSoft. Linearity was maintained across an increase of DNA concentration by two folds with the lowest input of 0.6 pg/µl and the highest input of 40 pg/µl.

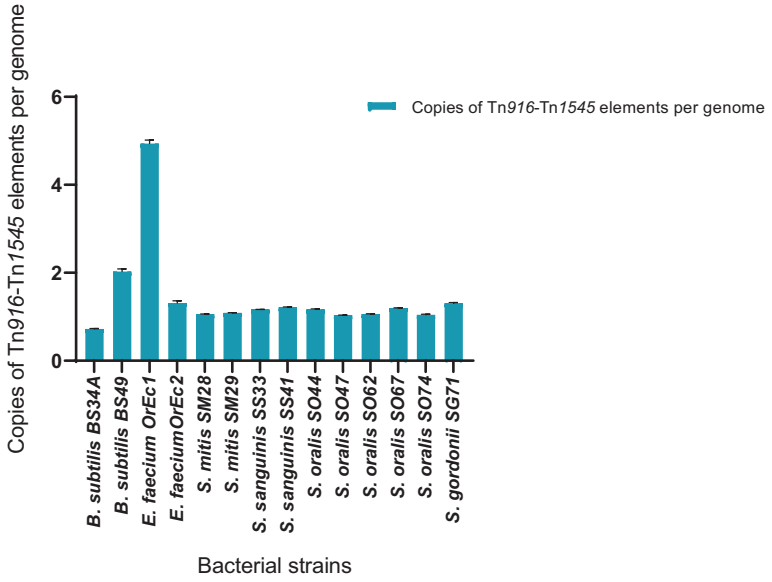


Figure 2. A graphical illustration of the copy number of Tn*916*-Tn1545 family detected in bacteria involved in this study. The bars represent the copies of the Tn*916*-Tn*1545* family per bacterial genome. The error bars represent 95% confidence intervals.

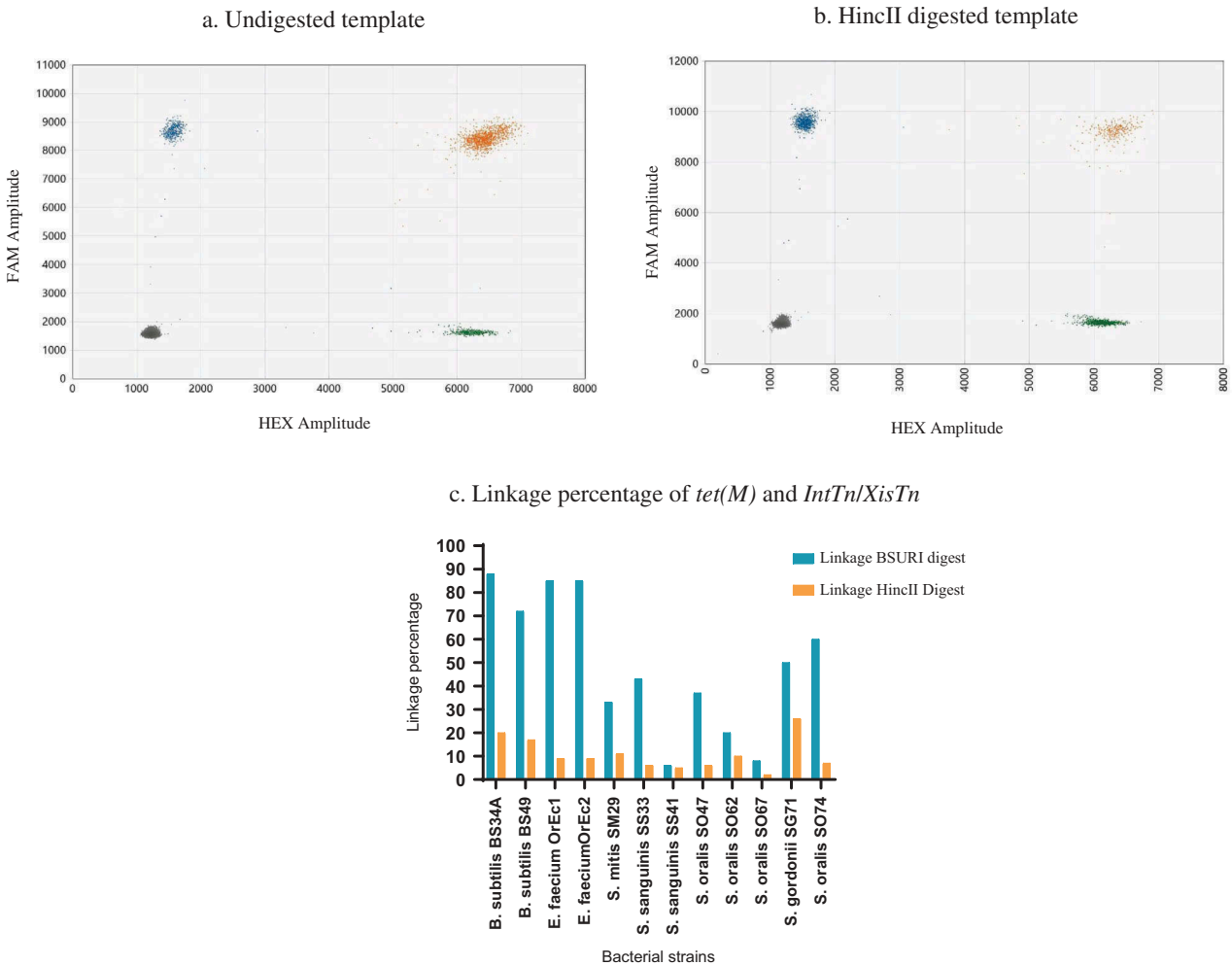


Figure 3. An illustration of Linkage between *tet*(M) and *IntTn/XisTn* regions. 3(a, b) show 2-D amplitude plot in which each axis represents the amplitude axis of either FAM or HEX. The blue droplets represent FAM targets (*tet(M)*), the green droplets represent HEX targets (IntTn/XisTn regions), the orange droplets are these that contain both FAM and HEX, and the gray represents the droplets with no target molecules. Image 3(a) shows the droplets distribution of undigested *B. subtilis* BS49 whereas as image 3(b) illustrates the target distribution when the template is digestion with *Hinc*II which cut between *tet(M)* and IntTn/XisTn regions in Tn*916*. 3C shows linkage percentage between *tet*(M) and IntTn/XisTn regions. The blue bars represent the linkage percentage between *tet*(M) and IntTn/XisTn regions. The orange bars show the drop of the linkage percentage when the two targets have been physical separated by restriction enzyme digestion.

the bacterial species. In the selected clinical oral *Streptococcus* species, that is *S. oralis, S. sanguinis*, and *S. mitis*, the CR of Tn*916*-Tn*1545*-like elements was influenced by the presence and concentration of tetracycline. In the absence of tetracycline, the CR ranged from 0% to 0.036% while in the presence of 5 and 10 µg/ml tetracycline, the observed CR ranged from 0.004% to 0.17% and from 0.008% to 3.19%, respectively (Figure 4(a)). Interestingly, in *E. faecium* OrEc1, *B. subtilis* BS34A and *B. subtilis* BS49, the levels of CI were higher than in oral streptococci and influenced by the presence and concentration of tetracycline. In the absence of tetracycline, the observed CRs were, 9.9%, 0.4%, and 9.7% for *E. faecium* OrEc1, *B. subtilis* BS34A and *B. subtilis* BS49, respectively. In the presence of 5 µg/ml tetracycline the detected levels of CI increased to 11.8%, 9.8%, and 244% for *E. faecium* OrEc1, *B. subtilis* BS34A, and *B. subtilis* BS49, respectively (Figure 4(b)). When *E. faecium* OrEc1 was cultivated in the presence of 10 µg/ml tetracycline, it was observed that the percentage of CI molecules detected within the bacterial population exceed 50%. In both *B. subtilis* BS34A and *B. subtilis* BS49, the percentage of CI molecules detected within the bacterial population were 113% and 239%, respectively, exceeding the number of bacterial genomes that were detected in the assay.

DNA sequencing of the promoter region upstream of *tet*(M)

The DNA sequencing results show distinct 58 bp deletions in two *S. oralis* strains, a 27 bp and 12 bp

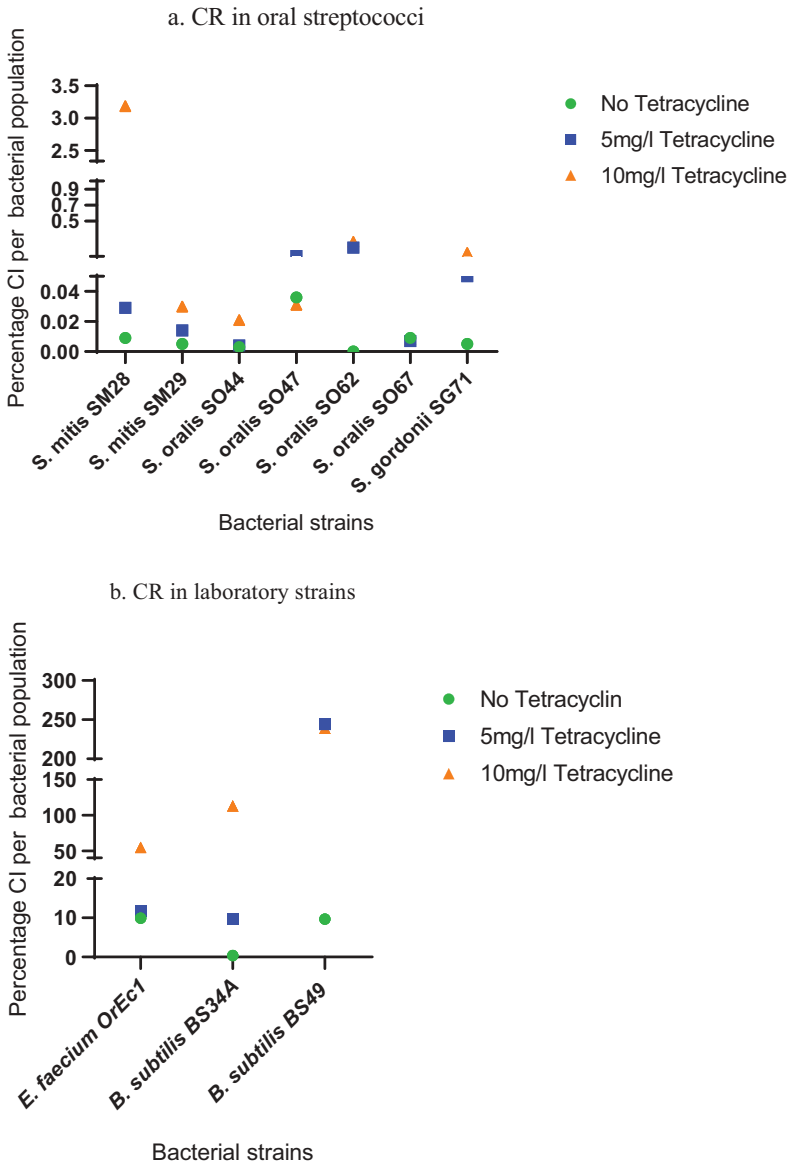


Figure 4. An image indicating percentage of CI per bacterial population. (a) CR in *E. faecium* OrEc1, *B. subtilis* BS34A, *B. subtilis* BS49. (b) CR in oral streptococci. The green circles represent the CR in the absence of tetracycline whereas the blue squares and the orange triangles represent the CR in the presence of 5 mg/ml and 10 mg/ml tetracycline, respectively.

deletion in other two *S. mitis* strains, and multiple SNPs in the upstream of *tet(M)* in the other oral *Streptococcus* strains included in the CR experiment compared to the wild type sequence of Tn*916*. The deletions effectively removed the predicted large terminator structure responsible for transcriptional attenuation (Supplementary data, Figure 3S).

Discussion

Antibiotic resistance in oral streptococci is an ever-growing problem [32]. Advances in molecular biological techniques and detection methods of resistance genes, have increased our knowledge of factors contributing to the propagation of MGEs carrying resistance genes in bacterial populations. The CN determination of MGEs carrying resistance genes in oral streptococci is of particular interest, as high CN might influence the propagation and spread of resistance due to availability of more than one element in any given genome. Furthermore, the presence of more than one copy of MGEs might influence bacterial biological fitness [33] and hence the reversibility of resistance. Our attempt to determine CN of MGEs, such as Tn*916*-Tn*1545*-like elements, is part of ongoing work to determine the biological cost of these elements in oral streptococci. This work has led to the development of an assay that can easily and accurately determine the CN of Tn*916*-Tn*1545*-like elements in *E. faecium, B. subtilis* and oral streptococci using ddPCR. We tested the sensitivity and reproducibility of our assay by analyzing varying amounts of input DNA that contained a predetermined number of target regions per genome. Our results illustrate that ddPCR is a sensitive and highly specific tool that can be used to determine CN of MGEs. The reproducibility and precision, even at very low input DNA concentration (0.6 pg/µl) is promising as it allows for analysis of samples with low DNA target concentrations and may be applicable therefore to analysis of bacteria directly from saliva samples and other body fluids.

Four sequenced bacterial strains; *B. subtilis* BS34A (NZ_LN680001.1), *B. subtilis* BS49 (NZ_LN649259.1) *E. faecium* OrEc1, and *E. faecium* OrEc2 (unpublished data) were used to determine the accuracy of ddPCR in detecting multiple copies of Tn*916*-Tn*1545*-like elements. In *B. subtilis* BS49, *E. faecium* OrEc1, and *E. faecium* OrEc2, we were able to accurately detect the expected number of elements using *amyE* as a chromosomally located, single copy, reference gene. In *B. subtilis* BS34A however, the ratio between Tn*916*-Tn*1545*-like elements (represented by the *intTn*/*xisTn* genes) and the reference gene *amyE* was below one copy (approximately 0.75). The lower ratio may be explained by the chromosomal positioning of the two targets in relation to the origin of replication. In *B. subtilis* BS34A, the *amyE* gene (327,604–329,583 bp) is situated closer to the origin of replication in comparison to Tn*916* which is in position 1,886,552–1,904,583 bp. The closer proximity of *amyE* to the origin of replication may result in more targets of the reference gene due to the occurrence of multiple replication forks within a cell prior to cell division, as has been previously reported in *B. subtilis* [34].

A few studies have reported the presence of more than one copy of Tn*916*-Tn*1545*-like elements in clinical strains [35–37]. Rice et al. (2005) reported that the presence of multiple copies Tn*916*-Tn*1545* elements in clinical strains is rare [38]. The current study supports such findings as all our clinical oral streptococci contained one copy of Tn*916*-Tn*1545*-like elements.

The ability to detect linkage between two genetic targets; the likelihood that two genetic targets are in physical proximity of each other, is fundamental to our understanding of the likelihood of horizontal gene transfer of resistance genes, especially if the linked genetic target to the resistance determinants is responsible for mobility. Several Tn*916*-Tn*1545*-like elements that confer resistance to more than one antibiotic have been reported [2,36,39–40]. The ability to determine whether or not these resistance genes are on the same mobile element will shed light on the prevalence of these resistance determinants and the likelihood for co-transfer. In our study, we illustrate that ddPCR can be used to determine the linkage between *tet*(M) and *intTn* and *xisTn* genes of Tn*916* in *B. subtilis* BS49, *E. faecium*, and in oral streptococci. Whilst most Tn*916*-Tn*1545* elements share an extremely high degree of sequence homology we acknowledge that subtle variations in the DNA sequence of the genetic target for ddPCR will affect the efficiency of the reaction. This can be overcome by using multiple sets of primers to detect the element itself and previously published primers for various resistance genes based on conserved regions within these genes.

The ratio between CI and the number of bacterial genomes present in the bacterial population was used to investigate the CR of the elements. The basal rate of CI formation varied between and within species. In *B. subtilis* BS34A, *B. subtilis* BS49, and the *E. faecium* OrEc1, the CR ranged from 0.4% to 9.9% in the absence of tetracycline. In oral *Streptococcus* strains, the CR in the presence of tetracycline (5 µg/ml and 10 µg/ml) was observed to range from 0.004% to 3.19% (Figure 4(a)). In contrast, the CR in *B. subtilis* and *E. faecium* strains in the presence of the same tetracycline concentrations showed higher numbers of CI in the bacterial population. It can be hypothesized that the observed lower CR in oral streptococci may be due to the fact that these are clinical isolates containing Tn*916*-Tn*1545*-like elements that have evolved a mechanism to reduce the level of excision to reduce any fitness cost associated with acquisition of these elements, a theory that requires further investigation for these strains. Sequencing of DNA upstream of *tet*

(M) shows several SNPs and deletion when compared to the wild type sequence of Tn916. The presence of deletions results in the removal of the predicted large terminator structure responsible for transcriptional attenuation upstream of tet(M). The removal of the large terminator structure would suggest that these isolates would not respond to tetracycline in the same way as it is hypothesized for the wild type Tn*916*.

Another possible explanation for the low CR of Tn*916*-Tn*1545* in oral streptococci is that *tet*(M) is not present on the Tn*916*-Tn*1545* like elements in some strains (e.g. *S. sanguinis* SS41 in Figure 3(c)).

In both *B. subtilis* strains, cultivation in the presence of 10 µg/ml tetracycline resulted in levels of CI that were higher than the number of bacterial genomes detected. This suggests that at this concentration of tetracycline, Tn*916* is undergoing autonomous replication; a phenomenon that has been recently demonstrated [20]. The observed increase in CI presumably occurred because tetracycline could result in increased mobility of Tn*916* as shown by Scornec et al. [41]. Introduction of even higher concentrations of tetracycline did not result in more CR in our bacterial populations, but rather we observed a decrease of CI number in the bacterial population where the concentration of tetracycline reached higher values, although still under the MIC values for these resistant strains (Table 1). The latter observation could be attributed to an overall effect on protein synthesis thus leading to slower or nearly diminished replication [12].

It is reasonable to assume that the more copies of the Tn*916*-Tn*1545* family present in the genome, the more CI molecules would be present in the bacterial cell. This assumption was true when CI levels in *B. subtilis* BS49 strain; containing two copies of Tn*916*, were compared to CI levels in *B. subtilis* BS34A, which harbors only one copy of Tn*916*. This is consistent with findings from previous studies [42].

In this study, we demonstrate that ddPCR can be used to study CN and CR of the Tn*916*-Tn*1545* family in oral streptococci with and without the presence of antimicrobial challenge. In addition to detection of the CI, we have also demonstrated that ddPCR can be used to detect an increase in the CN of the target molecule compared to another, as would happen if the CI of Tn*916* was autonomously replicating. The minimal skills requirements, and flexibility, requirements for small amounts of DNA sample, and good reproducibility illustrate the potential that ddPCR carries for the advancement of studying MGEs like Tn*916*-Tn*1545* family and antibiotic resistance.

Acknowledgments

The current study was supported by the Department of Clinical Dentistry (IKO), UiT The Arctic University of Norway. We thank the Norwegian Surveillance System for Antimicrobial Drug Resistance (NORM) for their partial financial support. We are also grateful to the National Advisory Unit for Detection of Antimicrobial Resistance (K-RES), University Hospital of North Norway for providing the majority of the strains used in this study and to Bjørg Christina Haldorsen for the excellent technical support. The publication charges for this article have been funded by a grant from the publication fund of UiT The Arctic University of Norway.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

The publication charges for this article have been funded by a grant from the publication fund of UiT The Arctic University of Norway

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Paper II

JOURNAL OF ORAL MICROBIOLOGY
2021, VOL. 13, 1896874
https://doi.org/10.1080/20002297.2021.1896874

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Prevalence, diversity and transferability of the Tn*916*-Tn*1545* family ICE in oral streptococci

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ABSTRACT

Background: The Tn*916*-Tn*1545* family of Integrative Conjugative Elements (ICE) are mobile genetic elements (MGEs) that play a role in the spread of antibiotic resistance genes. The Tn*916* harbors the tetracycline resistance gene *tet*(M) and it has been reported in various bacterial species. The increase in the levels of tetracycline resistance among oral streptococci is of great concern primarily due to the abundance of these species in the oral cavity and their ability to act as reservoirs for antibiotic resistance genes.

Methods: In the current study, we screened 100 Norwegian clinical oral streptococcal isolates for the presence and diversity of the Tn*916*-Tn*1545* family. In addition, we investigated the transferability the elements, and the associated transfer frequencies.

Results: We observed that 21 isolates harboured the Tn*916*-Tn*1545* family and that two of these elements were the novel Tn*6815* and Tn*6816*. The most prevalent member of the Tn*916*-Tn*1545* family observed in the Norwegian clinical oral streptococcal isolates was the wild type Tn*916*.

Conclusion: The detection of other members of this family of ICE and varying transfer frequencies suggests high versatility of the Tn*916* element in oral streptococci in Norway.

ARTICLE HISTORY
Received 24 September 2020
Revised 1 February 2021
Accepted 25 February 2021

KEYWORDS
Integrative Conjugative Elements (ICEs); oral streptococci; mobile genetic elements; antibiotic resistance; conjugation; tetracycline resistance; ddPCR; Tn*6815*; Tn*6816*

Introduction

The oral cavity is the second highest bacterial populated area of the human body [1]. With more than 110 approved species, streptococci are the most abundant species in the oral microbial communities [2]. In general, oral streptococci are classified as commensal bacteria and constitute part of the oral microbiota of virtually all humans. Some strains, however, have been shown to have pathogenic abilities, causing invasive infections such as infective endocarditis, septicemia, and pneumonia in neutropenic individuals [3,4]. In addition to the possible invasive pathogenicity, oral streptococci are gathering significance as they have been implicated in being reservoirs of antibiotic resistance genes [5–9], and carry substantial resistance genes as part of the 'oral resistome' [10]. Sequence analysis of evolutionary important genes in oral streptococci suggests that the presence of antibiotic resistance genes in these species are due, at least in part, to Horizontal Gene Transfer (HGT) [11]. One of the mechanisms of HGT involves the transfer of mobile genetic elements (MGEs) such as Integrative Conjugative Elements (ICE).

ICE constitute a very large and diverse group of MGEs of which the Tn*916*-Tn*1545* family is one of the largest members. The members of this family are by definition, self-transferable genetic elements that can exist as circular intermediates or as part of a chromosome [12–14]. Although very diverse, the elements in the Tn*916*-Tn*1545* family have Tn*916* in the backbone structure with the similar conjugative transfer, transcriptional regulation and recombination modules. Whereas Tn*916* has the *tet*(M) gene in the accessory function module, other antibiotic genes have been observed in other members of this family such as the presence of *tet*(S) in Tn*6000* [15,16] and Tn*916S* [16,17]; *erm*(B) in Tn*3872* [18]; *tet*(M) and *mef*(E) in Tn*2009* [19]; and *tet*(M), *erm*(B) and *mef*(E) in Tn*2017* [20]. Tn*916*, which was first isolated from *Enterococcus faecalis* DS16 [21], is a well-documented prototype of the Tn*916*-Tn*1545* family. It is the smallest member of the Tn*916*-Tn*1545* family and has been found in 35 different bacterial genera [22,23]. The wide spread of Tn*916*, is in part, attributed to the presence of integrase and excision genes, which enable the ICE to excise (cut) itself from one location and integrate itself in another location. The transcription regulation module of Tn*916* tightly regulates the excisionase and integrase, which are located at the 3′end of the element. This module consists of orf5, orf6, orf7, orf8, orf9, orf10

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and orf12, and the importance of this module is reflected by being highly conserved [24]. It has been proposed that transcription starts from a promoter that is upstream of orf12 and runs through orf12, *tet* (M) and the downstream Open Reading Frames (ORFs) [25]. The transcription of these ORFs not only leads to the self-mobilization of the element but also increases the levels of *tet*(M), which enables the bacteria to survive in the presence of tetracyclines.

Tetracyclines, like all other antibiotics in Norway, are regulated and controlled by prescription. In 2018, tetracyclines were the second most prescribed group of antibiotics in Norway, accounting for 26% of all defined daily doses for systemic antibacterial agents [26]. Although the use of antibiotics continues to decrease in Norway, cases of antibiotic resistant bacterial isolates continue to appear [26]. This increase, coupled with the higher number of antibiotic resistance related infections highlight the need to understand the underlying factors that promote the spread and stability of tetracycline in bacterial populations.

Recent approaches to study the oral microbiome have re-enforced the abundance of *Streptococcus* ssp. in the oral cavity and the high prevalence of antibiotic resistance genes. A study by Almeida et al. 2020 [27] found that 72% of the samples isolated from the oral microbiota had at least one antibiotic resistance gene, whilst a study by Christensen and Sørensen found that more than 90% of the saliva samples analyzed carried two antibiotic resistance genes [28]. In another study, analysis of 342 clinical oral streptococci found that 44% were resistant to tetracycline, whereas 23.1% were resistant to a combination of erythromycin, tetracycline and ofloxacin [29].

The growing number of studies investigating the prevalence of resistance genes in oral streptococci illustrate the role these species play in the spread of antibiotic resistance genes. There are, however, few studies that have investigated the diversity and transferability of the Tn*916*-Tn*1545* family. In this study, we investigated the presence of the *tet*(M) carrying ICE belonging to the Tn*916*-Tn*1545* family in antibiotic resistant oral streptococci collected in Norway. We analyzed the diversity and transferability of these ICEs to determine the role that the Tn*916*-Tn*1545* family plays in the spread of these resistance determinants.

Material and methods

Bacterial strains

The bacterial strains used in this study were part of the oral streptococci collection strains obtained from Norwegian hospitals and submitted to the National Competence Service for the Identification of Antibiotic Resistance (K-RES). The 100 clinical strains used in this study consisted of *Streptococcus mitis* (n = 42), *Streptococcus oralis* (n = 22), *Streptococcus sanguinis* (n = 7), *Streptococcus salivarius* (n = 7), *Streptococcus anginosus* (n = 7), *Streptococcus gordonii* (n = 2), *Streptococcus constellatus* (n = 2), *Streptococcus intermedius* (n = 1), *Streptococcus mutans* (n = 1) and nine unclassified streptococcal species. In addition, the type strains *S. oralis* ATCC 35037, *S. mitis* ATCC 49456, *S. sanguinis* ATCC 10556, *S. gordonii* ATCC 10558, *S. pneumoniae* ATCC 49616, *Bacillus subtilis* BS34A (NZ_LN680001.1) and *B. subtilis* BS49 (NZ_LN649259.1) were included in this study as experimental controls.

Construction of kanamycin resistant oral streptococcal strains

The type strains *S. oralis* ATCC 35037, *S. mitis* ATCC 49456, *S. sanguinis* ATCC 10556 and *S. gordonii* ATCC 10558 were subjected to mutagenesis using the EZ-Tn*5* < *kan-2*> transposons kit (Lucigen, Middleton, WI) according to the manufacturer's instruction. In brief, the competent oral streptococcal cells were prepared as described by Smith et al. [30]. The EZ-Tn*5* < *kan-2*> transposon was introduced into the competent cells by electroporation using the MicroPulser Electroporator (Bio-Rad, Pleasanton, CA, USA) according to manufacturer´s instructions. The kanamycin resistant isolates *S. oralis* SOK10, *S. mitis* SMK7, *S. sanguinis* Sg10, *S. mutans* M8 and *S. gordonii* G3 were used as recipients in the Tn*916*- Tn*1545* family transferability assay.

Assessment of Minimal Inhibitory Concentrations (MIC) of Tn916 carrying isolates

The MIC to tetracycline, erythromycin, penicillin, gentamicin, and clindamycin of the clinical oral streptococcal isolates were determined using the E-test according to the manufacturer's instructions (BioMérieux SA, Marcy l'Etoile, France). In short, a single colony was used to prepare a fresh overnight culture in BHI broth. Bacterial cultures with a turbidity of 0.5 McFarland were inoculated onto the Mueller-Hinton-F agar supplemented with 5% horse blood, prior to the addition of the respective antibiotic strips. The agar plates were incubated in an aerobic atmosphere at 37°C for 24 hours. The isolate *S. pneumoniae* ATCC 49616 were used as a control strain. The results were interpreted according to the standards set by the European Committee for Antimicrobial Susceptibility Testing (EUCAST) (www.eucast.org) and the Clinical and Laboratory Standards Institute (CLSI) (https://clsi.org/).

Characterization of the Tn916-Tn1545 family found in oral streptococci

The genomic DNA used in the conventional Polymerase Chain Reaction (PCR) and ddPCR was extracted using the QIAcube automated system (Qiagen, Hilden, Germany) according to the manufacturer's instructions. To investigate the presence of the Tn*916*-Tn*1545* family in oral streptococcal strains, PCR was performed as previously described [31–33]. The presence of the *tet* (M) gene and the integrase and excisionase regions (*Int* and *Xis* genes) were determined using the primers listed in Table 1. The PCR reaction was performed in a volume of 25 µl which contained 12.5 µl DreamTaq 2X (Thermofisher Scientific), 0.5 µM of each specific primer, 8 µl of water and 2.5 µl DNA template. Subsequently, the samples that tested positive for both these targets were subjected to Long PCR using Long A primers (amplify the region between 38 bp and 9,884 bp of Tn*916*) and Long B primers (amplify position 9,824 bp to 17,947 bp of Tn*916*). These primers (listed in Table 1) were designed to detect and amplify two large fragments in Tn*916*, Tn*6002*, Tn*6003*, Tn*1545*, Tn*3878*, Tn*2009*, Tn*2010*, Tn*2017*, Tn*6084* and Tn*6079* [34]. In brief, the long PCRs were performed using Platinum SuperFI (Thermofisher Scientific) in a total volume of 25 µl. The PCR reaction mix contained 12.5 µl Platinum SuperFI mix, 5 µl Enhancer, 1 µl of each primer (10 pmol/µl), 3.5 µl water and 2 µl DNA template. The PCR reactions were run according to the manufacturer's instructions. The two long PCR amplicons were subjected to restriction fragment length polymorphism (RFLP) by enzymatic digestion with *HincII* as previously described by Ciric et al. 2012 [34] .

Next generation sequencing of the Tn916-Tn1545 family found in oral streptococci

The extracted streptococcal DNA for the NGS was obtained using the modified Marmur extraction procedure as described by Salva-Serra et al. [35]. Based in their RFLP pattern, we selected six oral streptococcal isolates for NGS using the Illumina Nextseq 550 platform and the Pacific Biosciences Sequel instrument (PacBio) applying Sequel Polymerase v3.0, SMRT cells v3 LR and Sequencing chemistry v3.0. The Illumina Nextseq 550 was conducted at the Genomics Support Center, Tromso whereas as the PacBio sequencing was performed at the NorSeq (Oslo). Hybrid assemblies were generated using Canu [36]. The assemblies/contigs were re-ordered using Abacas 1.3.1 [37] with Tn*916* (U09422.1) as a guiding reference. The protein coding regions in the assemblies were predicted using PROKKA version 1.12 [38] and the similarities and differences between the assembled elements and the Tn*916*-Tn*1545* family were assessed using Easy fig [39]. BLAST was used to align the assembled elements to the wild type Tn*916*.

Circularization ratio of the Tn916-Tn1545 family in oral streptococci

The circularization ratio (CR) of the Tn*916*-Tn*1545* family was determined by investigating the presence of the circular intermediate (CI) in the selected isolates. The presence of CI was determined by conventional PCR as previously described [40]. The primers used in this PCR are designed to produce an amplicon only when the element has been excised from the chromosome and the left and right junctions of the element join to form the CI. The CR was determined by ddPCR as previously described by Lunde et al. [41] and expressed as a ratio of CI to the number of the Tn*916*-Tn*1545* family elements that were present in a bacterial population.

Transferability and conjugation frequency of the Tn916-Tn1545 family in oral streptococci

The *in vivo* transferability and conjugation frequency of the Tn*916*-Tn*1545* family was

Table 1. Details of primers and probes used.

Target	Forward primer	Reverse primer	Probe sequence and label (chlorophore)	Amplicon size	Annealing temperature	Reference
tet(M)	GTR AYG AAC TTT ACC GAA TC	ATC GYA GAA GCG GRT CAC	N/A	633bp	55°C	[31]
Int and *Xis* genes	CGC CAAAGG GTC TTG TAT ATG	GCT GTA GGT TTT ATC AGC TTT TGC	N/A	650bp	58°C	[50]
Long A (position 38–9,884 bp)	GGA CTT ATC CAC TTT ATC AAG G	AAA CAG AAG CAG TGA GAA GA	N/A	9806bp	58°C	[51,52]
Long B (Position 9,824–17,947 bp)	GAA AAC TTT AGT GAT TGG TGG	CTG TAG GAA GAT ACT TCA CG	N/A	8123bp	58°C	[53]
Circular intemidate (conventional PCR)	CGT GAA GTA TCT TCC TAC A	AC CTT GAT AAA GTG TGA TAA	N/A	166bp	56°C	[54]
Circular intemidate (ddPCR)	CGT GAA GTA TCT TCC TAC A	GAC CTT GAT AAA GTG TGA TAA	AAT ACT CGA AAG CAC ATA GAA TAA GGC FAM/HEX	167bp	56°C	[41]
Int and Xis (ddPCR)	ATA CTC CCA TAC AGT CAA TAG TCC	AGT TCC ACC CCT GCA TGG	CCG TCG CAGGCA ATG AGT ATG GCT FAM	88bp	56°C	[41]

Table 2. Bacterial strains used in the inter- and intra-species transfer of the Tn*916*-Tn*1545* family to clinical and reference oral streptococci.

Strain	Background and relevant phenotype	Reference
Bacillus subtilis BS49	Laboratory strain::Tn*916*, Tet^R	[55]
S. sanguinis Ssg41	Clinical isolate:: Tn*916*, Tet^R	[41]
S. oralis SO52	Clinical isolate:: Tn*916*, Tet^R	[41]
S. mitis SM28	Clinical isolate:: Tn*6815*, Tet^R	[41]
S. oralis SO32	Clinical isolate, $Genta^R$	This study
S. gordonii G3	ATCC 10558::Tn*5*, Kan^R	This study
S. mutans M8	ATCC 25175::Tn*5*, Kan^R	This study
S. mitis SMK7	ATCC 49456::Tn*5*, Kan^R	This study
S. oralis SOK10	ATCC 35,037::Tn*5*, Kan^R	This study
S. sanguinis Sg10	ATCC 10556::Tn*5*, Kan^R	This study

determined by filter mating experiments as previously described by Roberts et al. [42]. In brief, the donor and recipient isolates were cultivated on BHI agar plates with selection (according to the species selective markers, as listed in Table 2). The overnight cultures were prepared from single colonies and incubated at 37°C for 20 hours prior to mixing the donor and the recipient species in a 1:1 ratio. The mixed cultures were centrifuged at 1,000 g for 5 minutes and the supernatant was discarded. The cells were suspended in 1 ml of BHI broth and then 100 µl of the cell suspension was plated on a cellulose nitrate filter (0.45 µM, Sartorius Stedim Biotec; Germany). The conjugation experiments were conducted in three biological replicates and the generated transconjugants were screened phenotypically (on selective agar) and genotypically (by PCR) to verify the presence of the Tn*916*-Tn*1545* family elements. The conjugation frequency of the Tn*916*-Tn*1545* like elements was accessed both inter- and intra-species.

Results

Distribution of the tet(M) gene and Int and Xis genes in oral streptococcal isolates

The 100 oral streptococcal isolates used in this study were screened for the presence of the Tn*916*-Tn*1545* family using primers that detect the *tet*(M) gene and the integrase/excisionase regions (*Int* and *Xis* genes) of these elements. A total of 24 isolates harbored *tet*(M) whereas 38 isolates carried the integrase/excisionase. The most dominant species in this study was *S. mitis* (n = 42) and the prevalence of *tet*(M) in these isolates was 26.2% (n = 11). *S. oralis* and *S. sanguinis* had a *tet* (M) prevalence of 41% (9/22) and 28.6% (2/7), respectively. Of the seven *S. salivarius*, seven S. *anginosus* and three *S. gordonii* isolates that were tested, only one isolate of each species had *tet*(M). In *S. constellatus*, only one of the two studied isolates were found to harbor the tetracycline resistance gene. The *tet*(M) gene was not detected in the tested *S. intermedius* and *S. mutans* isolates. In total, 21% (21/100) tested positive for both the *tet* (M) gene and the integrase/excisionase region and these were considered to harbor Tn*916*-Tn*1545* like elements. The species distribution of both *tet*(M) and the integrase/excisionase regions is illustrated in Figure 1.

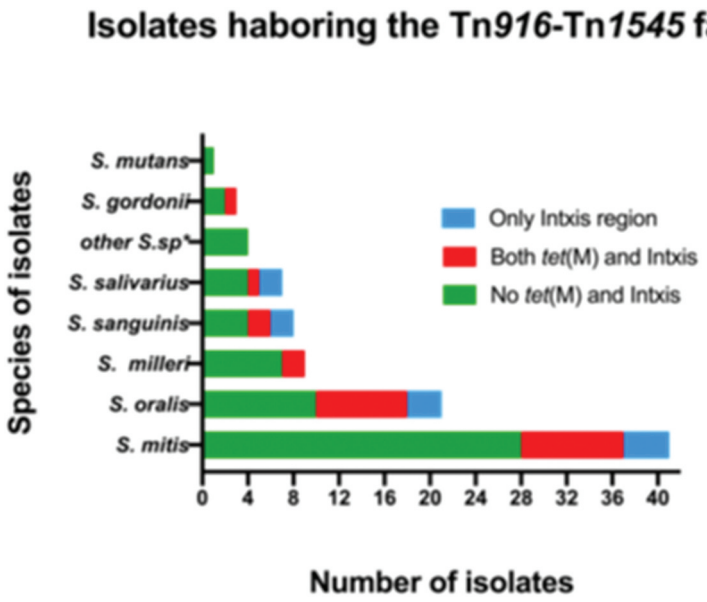

Figure 1. A graph illustrating the species distribution of the oral streptococci isolates included in this study. The isolates that tested PCR negative for the presence of *tet*(M), *Int* and *Xis* genes are shown in green whereas isolates that were PCR positive for *tet*(M), *Int* and *Xis* genes are illustrated in red. The blue bars indicate isolates that only tested positive for *Int* and *Xis*. S.sp*; other streptococcal species including *S. anginosus, S. constellatus* and *S. intermediate* and member of the *S. milleri* group.

Assessment of minimal inhibitory concentrations of Tn916 carrying isolates

The antibiotic resistance profile of the 21 isolates harboring the Tn*916*-Tn*1545* family were assessed by determining the MIC to tetracycline, erythromycin, penicillin, gentamicin and clindamycin, and the result are shown in Table 3. The MIC to tetracycline ranged from 0.190 to 96 µg/ml. All isolates that carried the *tet*(M) gene were phenotypically resistant to tetracycline with the exception of isolate *S. oralis* SO04 where the *tet*(M) gene was silent and the detected MICs were below the resistance threshold (≤ 2 µg/ml) as determined by EUCAST. The MIC values to erythromycin ranged from 0.023 to >256 µg/ml and of the 21 isolates, five were classified as resistant to erythromycin with MIC values ranging from 3 to > 256 µg/ml. The MIC to penicillin, gentamycin and clindamycin where found to be 0.016 to 1.5 g/ml, 0.16 to 32 µg/ml and clindamycin 0.094 to > 256 µg/ml, respectively. Although most strains were resistant to more than two antibiotics, only 16% (n = 4) of the tested isolates were resistant to four of the five tested antibiotics. The isolate *S. mitis* SM28 was the only isolate found to be phenotypically resistant to penicillin with a MIC of 6 µg/ml.

Characterization of the Tn916-Tn1545 family found in oral streptococci

The Tn*916*-Tn*1545* family elements were characterized by amplifying two fragments that covered a large portion of the 18 kb wild type Tn*916*; Long A (position 38 bp to 9,844 bp) and Long B (position 9,824 to 17,947 bp). When subjected to RFLP analysis, the large amplicons indicated that 15 out of the 21 isolates harbored the wild type Tn*916*. The RFLP pattern of isolates *S. oralis* SO62, *S. oralis* SO67, *S. mitis* SM74, *S. oralis* SO90, *S. mitis* SM28 and *S. constellatus* SC99 varied from the wild type Tn*916* (as shown in Supplementary Figure S1). This suggested that these isolates potentially harbor other members of the Tn*916*-Tn*1545*family and were thus selected for further analysis with NGS.

Next generation sequencing of the Tn916-Tn1545 family found in oral streptococci

Analysis of the NGS resulted in the assembly of complete ICE in all of the isolates with the exception of isolate *S. oralis* SO62. The assembled elements are available under the Bioproject 660235 on NCBI. Sequence comparisons of the wild type Tn*916* with the entire length of Tn*916*-Tn*1545* like elements found high similarity in the isolates *S. mitis* SM74 and *S. oralis* SO90 of 98.8% and 98.8%, respectively. Analysis of the *S. oralis* SO67 assembled genome, showed the presence of two Tn*916* elements. The elements were completely identical and showed 99.8% sequence identity to Tn*916* with 99% query coverage (17,994/18,032). The resulting variations in the RFLP patterns compared to the wild type Tn*916* were found to be due to SNPs and *in silico* digestion of the sequenced elements confirmed the obtained RFLP pattern.

In *S. mitis* SM28, an insertion of 5,265 bp was observed respectively as shown in Figure 2. Sequence comparison of the assembled element in S. *mitis* SM28 showed 99.3% similarity with ICESpn22664 (accession number HG799489.1) [43]

Table 3. Antibiotic resistance profile of oral streptococcal isolates harboring the Tn*916*-Tn*1545* family collected in Norway.

Isolate	Tetracycline µg/ml	Erythromycin µg/ml	Penicillin µg/ml	Gentamycin µg/ml	Clindamycin µg/ml
S. sanguinis SS33	24 (R)	0.047 (S)	0.190 (S)	6 (IE)	0.125 (S)
S. sanguinis SS41	32 (R)	0.016 (S)	0.047 (S)	1.5 (IE)	0.160 (S)
S. oralis SO1	32 (R)	>256 (R)	0.016 (S)	16 (IE)	>256 (R)
S. oralis SO4	0.190 (S)	0.125 (S)	0.023 (S)	12 (IE)	0.190 (S)
S. oralis SO30	24 (R)	0.047 (S)	0.094 (S)	8.00 (IE)	0.094 (S)
S. oralis SO44	24 (R)	3 (R)	1 (I)	24 (IE)	0.094 (S)
S. oralis SO47	32 (R)	0.047 (S)	0.125 (S)	32 (IE)	0.094 (S)
S. oralis SO52	32 (R)	0.094 (S)	0.064 (S)	24 (IE)	0.125 (S)
S. oralis SO62	2 (S)	6 (R)	0.047 (S)	24 (IE)	0.75 (R)
S. oralis SO67	64 (R)	0.047 (S)	0.125 (S)	12 (IE)	0.094 (S)
S. oralis SO69	96 (R)	6 (R)	1.500 (I)	32 (IE)	0.125 (S)
S. oralis SO90	2 (S)	0.125(S)	0.064 (S)	12 (IE)	0.32 (S)
S. mitis SM2	48(R)	>256 (R)	0.094 (S)	0.16 (IE)	>256 (R)
S. mitis SM28	64 (R)	>256 (R)	6 (R)	6 (IE)	>256 (R)
S. mitis SM29	32 (R)	0.047 (S)	0.047 (S)	1.5 (IE)	0.064 (S)
S. mitis SM74	2 (S)	0.064 (S)	0.380 (I)	24 (IE)	0.094 (S)
S. mitis SM81	48 (R)	0.032 (S)	0.190 (S)	6 (IE)	0.125 (S)
S. gordonii SG71	24 (R)	0.047 (S)	0.016 (S)	4 (IE)	0.064 (S)
S. anginosus SA46	12 (R)	0.094 (S)	0.047 (S)	12 (IE)	0.094 (S)
S. salivarius SSv51	96 (R)	0.064 (S)	0.023 (S)	12 (IE)	0.125 (S)
S. constellatus SC99	16 (R)	0.023 (S)	0.094 (S)	3 (IE)	0.047 (S)

S = Susceptible; I = Intermediate; R = Resistant; IE = Intrinsic Resistance.

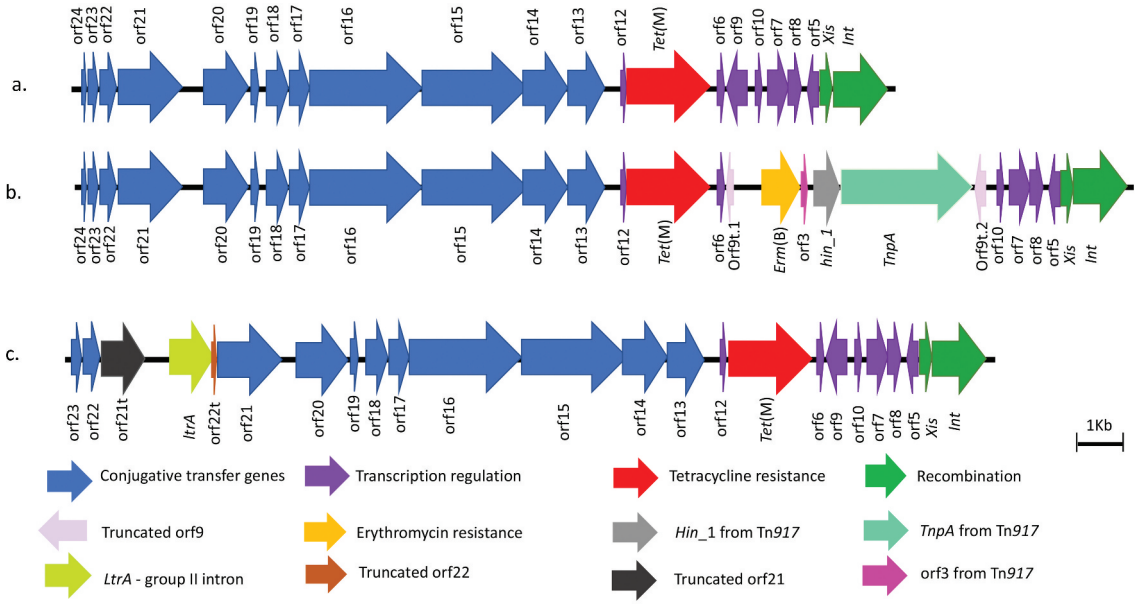

Figure 2. A schematic representation of the structure of Tn*916* and the Tn*916*-Tn*1545* family elements. The image in A. is Tn*916* from *E. faecalis* DS16 (accession number U09422.1); B. illustrates Tn*6815* from *S. mitis* SM28 (accession number LR828204.); and C. Tn*6816* from *S. constellatus* SC99 (accession number LR828206.1). The element Tn*6815* carries the *erm*(B) *hin_1*, orf3 and *TnpA* Transposase from Tn*917* inserted between two truncated regions of orf9 (orft.1 and orft.2). The element in *S. constellatus* SC99 has been designated Tn*6816* and it carries group II intron reverse transcriptase/maturase, truncated orf21 and orf22 and a complete copy of orf21 inserted upstream of orf20.

with a coverage of 98%. The element was also found to harbor the *erm*(B), orf3, hin_1 and *tnpA* (a transposase) genes which are inserted within orf9 and are located downstream of *tet*(M). The Blast results of this region showed high similarity (99.89%) to Tn*917* (accession number M11180.2). This suggested that Tn*6815* is a composite element in which Tn*917* has been inserted into position 14,525 bp of Tn*916* and extends to position 19,790 bp. Downstream of position 19,790 bp, the element was identical to the 3′end of Tn*916*. This element has been given a new name, Tn*6815*, and registered in the Transposon registry [44]. The sequence of the novel Tn*6815* is available on NCBI under the accession number LR828204.1

BlastN alignment between LR828204 (Tn*6815*) and U09422 showed that Tn*916* covered 77% of Tn*6815* with two matches going from 1 to 14,524 to the left of the 5,266 bp insertion and 19,790 to 23,299 right of the insertion. The 5′end of the alignment ranged from 1 to 14,524 in both elements and was 99% identical whereas the 3′end after the insertion was 98% identical to Tn*916* and corresponded to positions 14,519 bp to 18,032 bp in Tn*916*. The Tn*6815* carried two truncated orf9 segments which have been designated orf9t.1 and orft.2. The orf9t.1 was 136 bp and lacked 218 bp of orf9 and the orf9t.2 lacked the first 131 bp of the 5′end but had the remaining 222 bp of the 3′end. The right side of the alignment started at 14,519 (which is 131 bp within orf9) and covered the rest of Tn*916*. The Tn*6815* also carried a 5 bp repeat of orf9 on either side of the 5,266 bp insertion. BlastN analysis showed that the insertion was 99% identical to Tn*917* (accession number M11180.2) and that three ranges were present in Tn*6815*. The first range started at 90 bp and went to 5,355 bp, which is the entire sequence of Tn*917*. The second range was from 92 bp to 162 bp (corresponded to 15,988 bp to 16,058 bp in Tn*6815*) and the third range was from 1,550 bp to 1,623 bp which corresponded to 14,524 bp to 14,597 bp in Tn*6815*. These last two ranges were 96% identical (68/71bp similar) indicating that there was a repeated segment with Tn*917*.

In *S. constellatus* SC99, an element harboring a 1,663 bp insertion was observed. The insertion was located 401bp before the end orf21 and corresponded to the *ltrA* gene, a group II intron reverse transcriptase/maturase and part of peptidase P60. The *ltrA* gene was followed by 91bp of the 3′end of orf22 (lacking 295 bp of orf22) and a complete copy of orf21. BlastN analysis indicated that the element had two copies of orf21and orf22. The first copy of orf21 was truncated and only 1,061 bp long. It has been designated orf21t and was located upstream of the inserted *ltrA* and downstream of the first and complete copy of orf22. The second copy of orf21 was complete and located downstream of *ltrA*. Sequence comparison of this insertion showed 99.9% similarity and 100% coverage with the *ltrA* gene found in the *Enterococcus avium* strain 352 chromosome (accession number CP034169.1). As no matches of 99% or more were obtained from sequence comparison analysis, this element has been designated a new name, Tn*6816*, and registered in the

Transposon registry [44]. The sequence of the novel Tn*6816* is available on NCBI under the accession number LR828206.1

Circularization ratio of the Tn916-Tn1545 family in oral streptococci

The presence of CI was first determined by conventional PCR and CR was determined by ddPCR. In the absence of selective pressure, the conventional PCR results indicated the presence of the CI molecules in all the tested isolates with the exception of *S. mitis* SM74. The quantification of the CI molecules in the bacterial population by ddPCR was found to range from 0% in two isolates to 0.2% per element as indicated in Figure 3. Interestingly, the three highest levels of CI observed in this study were from *S. mitis* isolates. This coupled with the fact that *S. mitis* is one of the most abundant streptococcus species in the oral cavity warranty the need to further investigate the evolution of Tn*916* in *S. mitis*.

Transferability and conjugation frequency of the Tn916-Tn1545 family in oral streptococci

The *in vivo* transferability of the Tn*916*-Tn*1545* family was investigated by filter mating experiments in which both laboratory and clinical isolates were used as donor strains. The strain *B. subtilis* BS49 was included in this study as a control strain for the transfer of Tn*916* as this element is known to be transferable with a CR of 9.7% [41], and exists as two copies [45]. To evaluate the transferability of Tn*916*-Tn*1545* elements in the clinical setting, we used clinical isolates harboring the wild type Tn*916* as donor strains in the conjugation experiments. Of the 30 filter mating experiments, 28 yielded transconjugants with frequencies that are summarized in Table 4. The bacterial strain *B. subtilis* BS49 was able to transfer Tn*916* to five out of the six recipients. Interestingly, the isolate in which no transfer was detected (*S. oralis* SO32) is a clinical isolate whereas the other recipients are laboratory strains. In the successful conjugation experiments, the transfer frequency of Tn*916* from *B. subtilis* BS49 ranged from 6.0 (± 4.03) × 10^{-9} to 1.5 × 10^{-1} (±0.25). The frequencies of transfer ranged between to 4.0 (± 3.0) × 10^{-7} to 3.5 (± 6.0) × 10° per recipient when the clinical isolate *S. sanguinis* Ssg41 harboring Tn*916* was used as a donor. The transfer of Tn*916* from the clinical isolate *S. oralis* SO52 was observed to ranged from 8.4 × 10^{-3} (±0.01) to 4.7 (± 4.1) × 10^{-1}. The transfer frequency of Tn*6815* ranged between 7.4(± 9.7 × 10^{-6} and 5.8 (± 3.6) × 10^{-2}

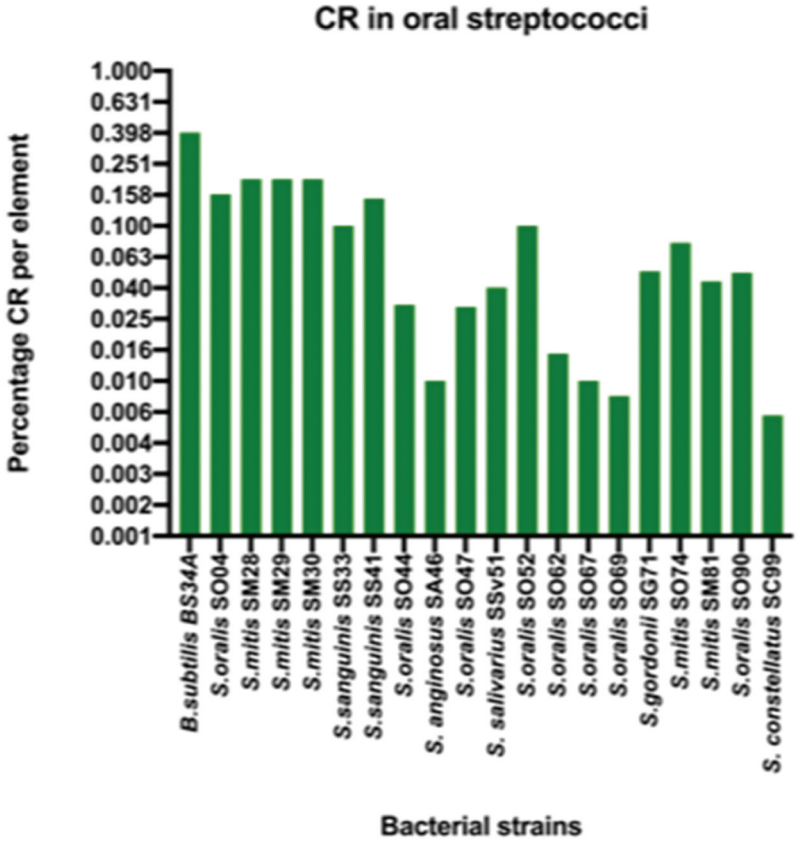

Figure 3. A graph illustrating the percentage of circularization rate (CR) in oral streptococci. *B. subtilis* BS34A was used as a positive control for the excision of Tn*916* and shows the highest levels of CR. The clinical oral streptococci isolates have CR that ranges from zero to 0.25 percentage CR per element.

Table 4. Transfer frequencies of the Tn*916*-Tn*1545* family in oral streptococci per recipients.

Donor (element)	*S. oralis* SOK10	*S. mitis* SMK7	*S. sanguinis* Sg10	*S. gordonii* G3	*S. mutans* M8	*S. oralis* SO32
Bacillus subtilis BS49; Tet^R ::Tn*916*	4.7 (± 8.1) × 10^{-6}	6.0 (± 4.03) × 10^{-9}	2.9 (± 4.9) × 10^{-5}	1.5 (±0.25) × 10^{-1}	3.2 (± 4.1) × 10^{-6}	Not detected
S. sanguinis Ssg41; Clinical isolate Tet^R :: Tn*916*	3.5 (± 6.0) × 10^{o}	1.7 (± 1.8) × 10^{-1}	1.0 (±0.09) × 10^{-6}	1.0 (± 1.7) × 10^{-3}	3.0 (± 4.6) × 10^{-6}	4.0 (± 3.0) × 10^{-7}
S. oralis SO52; Clinical isolate Tet^R :: Tn*916*	3.1 (± 2.3) × 10^{-2}	2.1 (± 1.2) × 10^{-2}	6.2 (±0.09) × 10^{-2}	4.7 (± 4.1) × 10^{-1}	6.2 (±0.09) × 10^{-2}	8.4 (±0.01) × 10^{-3}
S. mitis SM28; Clinical isolate Tet^R ::Tn*6815*	2.9 (± 3.1) × 10^{-3}	2.5(± 3.9) × 10^{-3}	7.4 (± 9.7) × 10^{-6}	1.4 (± 3.5) × 10^{-3}	3.3 (± 5.4) × 10^{-2}	5.8 (± 3.6) × 10^{-2}
S. constellatus SC99; Clinical isolate :: Tet^R Tn*6816*	2.2 (± 1.9) × 10^{-3}	1.2 (± 2.1) × 10^{-2}	1.5 (± 2.7) × 10^{-1}	1.0 (± 1.6) × 10^{-1}	1.8 (± 2.0) × 10^{-3}	Not detected

Tet^R – Tetracycline resistant

whereas for Tn*6516*, the transfer frequencies ranged between below detection in *S. oralis* SO32 to 1.5 (± 2.7) × 10^{-1} in *S. sanguinis* Sg10.

The random selection of twenty colonies from the resulting transconjugants per filter mating confirmed the presence of both the *tet*(M) and the *Int* and *Xis* genes of Tn*916* by conventional PCR. In addition, growth of the transconjugants on BHI agar plates containing both selection makers, i.e. tetracycline and kanamycin, verified the transfer of the Tn*916*-Tn*1545* family to the recipient cells. Sequence analysis of the *tet* (M) promoter region in all the selected clinical isolates, which is known to influence the rate of transfer, showed nucleotide changes as shown in Figure 4.

Discussion

Oral streptococci are the most abundant bacterial species in the oral cavity and are regarded as commensal bacteria. *Streptococcus* species can, however, cause serious infections if they enter the blood stream. There has been an increase in the number of antibiotic resistant oral streptococci thus the aim of this study was to determine the prevalence of the Tn*916*-Tn*1545* family in a collection of oral streptococci from Norway. The presence of *tet*(M) was detected in 24% the 100 oral streptococcal isolates. This rate of carriage is within the previously reported prevalence of 21% and 29.6% of *tet*(M) in oral streptococci [46,47]. The above-mentioned studies were from 1984 and 2017 respectively, therefore it is interesting that despite the time span, different geographic locations and changes in usage of antibiotics, the carriage of *tet*(M) appears to be similar. Of the 100 investigated isolates, the Tn*916*-Tn*1545* family was detected in 21% of the isolates with the highest prevalence per species occurring in *S. oralis* (41%). *S. mitis* was the most prevalent species in this study (41%), however only 26.2% of the isolates harbored the Tn*916*-Tn*1545* family. This raises the question of whether the elements have species preference or not, a notation that requires further study. Analysis of the diversity of Tn*916*-Tn*1545* in our study, revealed

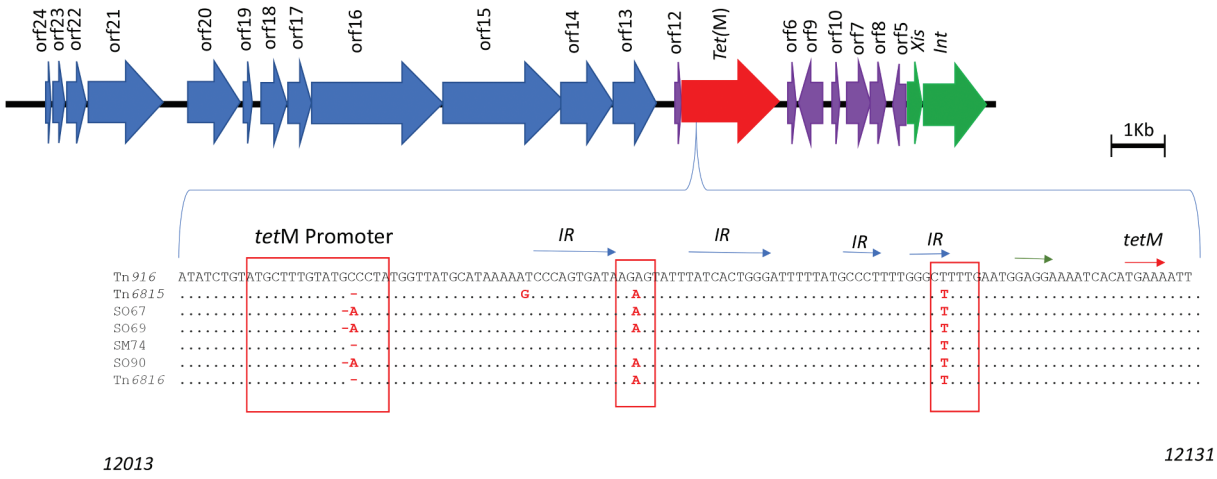

Figure 4. A nucleotide alignment of the promoter region in Tn*916* and six oral streptococci clinical isolates highlighting single nucleotide changes in the clinical isolates. The dots indicate nucleotides that are similar to the reference Tn*916* (KM615585), whereas the red nucleotides indicate SNPs. The red boxes indicate seemingly conserved SNPs, the blue arrows; inverted repeats (IR), the green arrow; the ribosomal protein binding site, and the red arrow; the start of the tetracycline resistance gene *tet*M. The numbers in the bottom right and left corners of the image indicate the nucleotide position in the reference *E. faecalis* DS16 (U09422.1). Tn*6815* and Tn*6816* are novel transposons that have been isolates from the clinical isolates *S. mitis* SM28 and *S. constellatus* SC99.

a total of three different elements with the most occurring being Tn*916*. The other two elements are new members in the Tn*916*-Tn*1545* family, i.e. Tn*6815* and Tn*6816*. Other studies have documented a diversity of four elements in a collection of 48 oral streptococci where the most frequent occurring was Tn*3872* [34].

The presence of both tetracycline and erythromycin resistance genes in Tn*6815* and *ltrA* in Tn*6816* is an indication of the role that these elements play in the elasticity of the bacterial genome and HGT. Although the diversity of these elements in our study seems low, it should be noted that based on the inclusion criteria set for further analysis for the Tn*916*-Tn*1545* family excluded the analysis of 14 oral streptococci that tested positive only for the integrase/excisionase regions and not *tet* (M). It can be speculated that including these samples in this study might have increased the diversity of the detected elements.

Based on the RFLP analysis, the most prevalent form of the Tn*916*-Tn*1545* family observed was the prototype Tn*916*, which was present in 15 isolates. Interestingly, despite carrying the same element, the 15 isolates had different MIC and lower levels of the CI when compared to the wildtype of *B. subtilis* BS34. As in many other clinical isolates, the variations in the level of resistance to tetracycline may be attributed to several factors such as the presence of other tetracycline resistance genes in the chromosome and/or the strength of the *tet* promoter [25]. Analysis of the *tet*(M) promoter regions in the sequenced isolates (Figure 4) illustrates some of the SNPs that we identified. The fact that these SNPs were observed in nearly all the analyzed clinical isolates, and since it has been hypothetized that their presence is due to beneficial evolutionary advantages requires further analysis and investigations .

Although all but one of the Tn*916* like elements found in this study harbored only one resistance gene, the antibiotic resistance profile indicated that five isolates were resistant to erythromycin and that four of the tested isolates were resistant to four of the five tested antibiotics. This may suggest the presence of other MGE or the presence of other resistance genes within the bacterial chromosomes and further highlights the role that oral streptococci play in the spread of antibiotic resistance among bacteria.

The six isolates that were identified as having *Tn916*-like elements based on the observed difference in the RFLP were further investigated by NGS. The results from the NGS showed that in three of the six isolates (isolates *S. oralis* SO67, *S. mitis* SM74 and *S. oralis* SO90), the observed differences in the RFLP are due to SNPs and/or mutations that result in the introduction of a new *HincII* digestion target site. According to the sequences of these isolates, they were more than 99% similar to the prototype Tn*916*. These observations highlighted the need to consider the suitability of the using the RFLP method as a way of distinguishing Tn*916* from Tn*916*-like elements. In the isolate *S. oralis* SO67, assembly of the genome, suggested the presence of two identical copies of Tn*916* in two different chromosomal locations. According to our knowledge, this is the first time that two identical copies of Tn*916* have been identified in two different positions in the same chromosome of an oral streptococcal clinical isolate.

S. mitis is one of the most predominant oral *Streptococcus* species in the oral cavity. The isolate *S. mitis* SM28 was found to harbor the novel Tn*6815* which contained the *erm*(B) gene inserted downstream to *tet*(M). The presence of *erm*(B) and *tet* (M) genes rendered *S. mitis* SM28 the only isolate found to have two resistance genes in the same element in this study. Tn*6815* harbored two truncated copies of orf9. In spite of the absence of a complete orf9, Tn*6815* was able to form CI and excise from the genome suggesting that an alternative mechanism is regulating the excision process, as has been suggested to occur in other Tn*916*-like elements [23]. The 5,265 bp insertion downstream from the *tet*(M) gene resulted in an element that was 99% identical to an ICE that has been previously isolated from *S. pneumonia* [43]. In contrast with the other previously identified elements that carry insertions downstream from the *tet*(M) gene such as Tn*2009* and Tn*3872* [19], the Tn*6815* in *S. mitis* SM28 was transferable to other oral streptococcal strains (Table 4). In addition to carrying both the *tet*(M) and the *erm*(B) genes, *S. mitis* SM28 was also shown to be resistant to penicillin, which is usually the first drug of choice for oral streptococcal infections. The ability of Tn*6815* to transfer to other species, further supported previous findings that oral streptococci play an active role in the dissemination of resistance genes by being reservoirs of antibiotic resistant genes.

The formation of the CI is the first step in the transfer of the Tn*916*-Tn*1545* elements, hence the presence of the CI and CR may indicate the functionality of the ICE to disseminate associated genes, including resistance genes. To show that the element is transferable, we conducted *in vivo* inter- and intra-species conjugation assays. Interestingly, we were able to show both inter- and intra-species transfer of Tn*916*-Tn*1545* elements with transfer frequencies ranging from 6.0 (± 4.03) × 10^{-9} to 3.5 (± 6.0) × 10° (Table 4). Conjugation frequencies of Tn*916* to *S. gordonii, S. salivarius* and *S. sanguinis* have previously been reported to range from 10^{-8} to 10^{-5} [48,49]. Although some of the observed conjugation frequencies reported in this study were comparable to those previously reported, 20/28 of the observed conjugation frequencies were considered higher than what has been previously

reported [48,49]. Interestingly all the higher observed conjugation frequencies where observed when clinical isolates where used as donor suggesting that clinical isolates may be more efficient in spreading Tn*916* despite having a lower CR. In spite of the bacterial isolate *B. subtilis* BS49 having two copies of Tn*916* and having higher CR than the clinical oral streptococci in this study, it was observed that the *B. subtilis* BS49 yielded the lowest number of transconjugants compared to the studied oral streptococci. In this study, we observed varying transfer frequencies of the Tn*916*-Tn *1545* elements within the same recipients when different donors were used in the filter mating and vice versa. These findings suggested that other factors than the donor, recipient, number of elements and/or the number of CI influence the rate at which the Tn*916*-Tn*1545* family elements are transferred within and between species.

The clinical oral *Streptococcus* isolates were all found to have a low CR. Sequence analysis of the *tet* (M) promoter region in all the selected clinical isolates revealed the presence of some SNPs (Figure 4). As this region has been shown to influence the transcription and transfer of the element, the observed SNPs might be responsible for the observed low levels of CR.

The presence of the Tn*916*-Tn*1545* family in commensal oral streptococci raises concern as it highlights the role that these abundant species may play in the increasing rates of antibiotic resistance. In this study, 21% of the investigated oral streptococci have been found to harbor a diverse elements of the Tn*916*-Tn*1545* family, with the highest prevalence occurring among *S. mitis* isolates. One of these *S. mitis* isolates was found to harbor a new element, i.e. Tn*6815* that had two resistance genes, i.e *tet*(M) and *erm*(B). Tn*6815* is transferable to other species with a relatively high transfer frequency. These findings underscore the role of *S. mitis* plays in the spread of antibiotic resistance within and across bacterial species. There is a need to further investigate the factors that contribute to the stability of the Tn*916*-Tn*1545* elements in oral streptococci.

Acknowledgments

The current study was supported by the Department of Clinical Dentistry (IKO), UiT The Arctic University of Norway. Our gratitude goes to the National Advisory Unit for Detection of Antimicrobial Resistance (K-RES), University Hospital of North Norway for providing the majority of the strains used in this study, and to Bjørg Christina Haldorsen, Berit Tømmerås and Ida Sofie Furuholmen for the excellent technical support. The publication charges for this article have been funded by a grant from the publication fund of UiT The Arctic University of Norway.

Disclosure statement

The authors have no conflict of interest.

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Paper III

Stability and relative fitness cost of newly acquired Tn*916* in *S. oralis*

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Abstract

The increase in antibiotic resistant oral streptococcal isolates is a growing problem boosted by promiscuous mobile genetic elements such as the integrative conjugative element Tn*916.* These ICEs have been implicated in generating genome plasticity and spreading antibiotic-resistant determinants among these bacterial species. The acquisition and maintenance of resistance determinants via MGE, although beneficial to the host cell when challenged with selective pressure, may impose an additional burden on the host cell. At present, little is known about the fitness cost, stability, and evolution of Tn*916* upon introduction into a naïve species. In this study, we introduce Tn*916* by filter mating into naïve bacterial isolates before determining the relative fitness cost of the acquired ICE. We observed relatively high fitness costs (ranging from 6% to 25%) upon acquisition of 1 to 3 copies of the ICE. Evolution experiments demonstrate that an initial fitness cost of acquiring Tn*916* is rapidly alleviated (within 500 generations) without altering the element structure, copy number, or insertion site of the elements. SNPs in the bacterial chromosome suggest that other secondary mechanisms are responsible for the amelioration of fitness.

Keywords

Oral streptococci, Integrative Conjugative Elements (ICEs), experimental evolution, fitness cost, mobile genetic elements, antibiotic resistance stability, tetracycline resistance

Introduction

Horizontal Gene Transfer (HGT) plays a vital role in the evolution and genetic adaptability of bacterial populations to environmental changes (1, 2). Conjugation, one of the mechanisms of HGT, has been shown to facilitate the transfer of large fragments of DNA, such as genes that code beneficial traits, including virulence, ecological interactions, and antibiotic resistance genes(3). As key players in conjugation, plasmids, and Integrative Conjugative Element (ICEs) contribute significantly to HGT among the bacterial population (2, 4). The importance of ICEs in HTG is becoming more transparent with the increased availability of microbial genomes, highlighting that ICEs outnumber conjugative plasmids in bacterial populations, and currently rendering them the most abundant self-replicating conjugative elements in prokaryotes (5, 6). The conjugative transfer of ICEs is facilitated by the presence of a type IV secretion system (T4SS), a relaxase, and type IV coupling protein, all of which are encoded by the genes on the ICE (7). ICEs exist as part of the host genome or as a circular intermediate (after excision), which can then be transferred into a different chromosomal site or genome before re-integration (8, 9) . The processes of excision, integration, and or duplication of ICEs not only result in alterations in the bacterial genome (10) they tend to increase genome plasticity of the bacterial population (11-14).

The presence of ICEs in Streptococcus, as reported by several studies (11, 15-20), makes it increasingly clear that ICEs play a significant role in increased genome plasticity and the spread of antibiotic-resistant determinants. ICEs belonging to various families have been identified in numerous streptococcus species, including the members of the Tn*916*-Tn*1545* family (15, 21, 22). Tn*916*, is an 18kb broad host range ICE found in many commensal and pathogenic bacteria (8, 23, 24). The genes in Tn*916* are arranged in modules responsible for conjugation, regulation, antibiotic resistance, and recombination (25). The integrase and excisionase genes (which make up the recombination module) enable the element to undergo intracellular transposition and intercellular conjugation (26) into A-T rich regions of the recipient genome (11), and the tetracycline resistance gene *tet* (M) offers a selective advantage in environments with tetracycline.

The acquisition and maintenance of resistance determinants via MGE, although beneficial to the host cell when challenged with selective pressure, may impose an additional burden on the host cell (27, 28). This fitness cost, may be expressed as a reduction in growth rates,

virulence, or transmission, which in turn affect the bacteria's ability to survive, reproduce, and be transmitted (29, 30).

Whereas our understanding of evolution, selection and maintenance of mobile genetic elements such as plasmids and integrons has been greatly improved over the last decade (31-36) the evolutionary dynamics through which ICEs adapt to their bacterial hosts are largely unknown (4, 37). Here we introduce Tn*916* from *Bacillus subtilis* BS49 into a naïve *S. oralis* strain ATCC 35037 and measure the fitness cost associated with harboring the element in seven transconjugants. We observed relatively high fitness costs upon acquisition of 1 to 3 copies of the ICE followed by ameliorated during the evolutionary experiment. Through this study we demonstrate that the acquisition of Tn*916* imposes an initial substantial cost on the host and that this cost is rapidly alleviated without altering element structure, copy number or insertion site.

Materials and Methods

Construction of background strain

The bacterial isolates used in this study are derivatives of *Streptococcus oralis* ATCC 35037. As S. *oralis* ATCC 35037 has no selective markers, the kanamycin-resistant gene (*npt II* was introduced into the isolates by mutagenesis with Tn*5* transposon (Lucigen, Middleton, WI) and used as recipients in the conjugation studies. Tn*5* transposon randomly integrates the *ntp II* gene into the chromosome of the host and thus provides a selective marker. In brief, competent cells of the oral streptococci strain, S. *oralis* ATCC 35037, were prepared as described by Smith et al. (38) before introducing Tn*5* by electroporation using (Bio-Rad, Pleasanton, CA, USA). The kanamycin resistant isolate was selected as recipient strain for the transfer of Tn*916* by conjugation as it had the most similar growth dynamics to the original S. *oralis* ATCC 35037 isolate.

Construction of Tn916 transconjugants.

The isolate *Bacillus subtilis* BS49 (NZ_LN649259.1) harbors two copies of Tn*916* and was used as a donor strain in the conjugation study. The filter mating experiments were used to construct transconjugants as previously described (39). In brief, the donor and recipient isolates were cultivated on BHI agar plates with selection (tetracycline 8µg/ml and kanamycin 25µg/ml, respectively). The overnight cultures were prepared from single colonies and

incubated at 37 °C for 20 hrs before mixing the donor and the recipient species in a 1:1 ratio. The mixed cultures were centrifuged at 1000 g for 5 minutes before discarding the supernatant. The pelleted cells were suspended in 1 ml of BHI broth, and then 100 µl of the cell suspension was plated on a cellulose nitrate filter (0.45 µM, Sartorius Stedim Biotec; Germany). To investigate the stability, relative fitness cost, and copy number of the transferred elements, we selected the transconjugants TC4.1, TC4.2, TC4.6, TC6.1, TC6.2, TC8.1 and TC10.1. The successful transfer of Tn*916* into the recipient isolates was verified by phenotypic (cultivation on selective agar) and genotypic (PCR) methods.

Relative Fitness and Stability of Tn916

The relative fitness and stability of Tn*916* were performed in parallel and initiated from the same fresh overnight cultures. In brief, the donor and recipient isolates were cultured in pre-warmed 2ml BHI in three biological replicates. After the overnight incubation at 37°C with shaking at 150 rpm, 20µl of the culture was inoculated into 1980 µl of fresh BHI. Of the new inoculated culture, 250 µl of the culture was transferred to a 96-well plate for growth rate experiments in triplicate, whereas the remaining volume was used to make freeze stocks.

Relative growth rates between transconjugants and ancestral strains (calculated from OD_{600} measurements) were used to infer the relative fitness of the newly acquired transconjugants. To access whether the initial costs of Tn*916* carriage was reduced during experimental evolution, relative growth rates of the evolved Tn*916*- containing and Tn*916*-free strains were used to determine the relative fitness of the evolved isolates. All experiments were performed in 96- well microtiter plates in triplicate. The plates were incubated at 37 °C in the *Multiskan™ GO* (ThermoFisher) with a 10-minute kinetic interval and continuous low shaking. The observed OD600 measurements were used to determine the growth rates using the GrowthRates v.3.0 software (40). The experiments were performed in three biological replicates and three technical replicates, and all results had a correlation coefficient, R greater than 0.98.

The stability of the elements was determined by plating the evolved isolates on selective plates (BHI agar plates with eight µg/ml tetracycline). The stability of Tn916 in transconjugants TC4.1, TC4.2, TC4.6, TC6.1, and TC6.2, was accessed after 360hrs (approximately 500 generations) whereas in transconjugants TC8.1 and TC10.1, the stability assay was preformed

after 360 hours and 600hrs (corresponding to more than 1000 generations). The experiments were performed in biological triplicates, and the stability of the elements was verified by screening the colonies on the selective plates for the integrase (*int*), excisionase (*xis*) and *tet* (M) genes.

Determination of Tn916 copy number

The number of copies of the ICE Tn*916* conjugated from *B. subtilis* BS49 into the S. *oralis* ATCC 35037 transconjugants were determined by droplet digital PCR (ddPCR) as previously described by Lunde et al. (41). Briefly, primers and probes (Table 1) that amplify the *int* and *xis* genes in Tn*916* were used to determine the number of elements present in the bacterial population, whereas primers and probes that amplify the *Amy E* gene were used as the reference for the number of bacterial cells present in the sample. The ddPCR were run in a total volume of 21µl consisting of 10µl ddPCR TM Supermix for Probes (No dUTP), 1µl of 20X IntTn/xisTn regions (target gene) primers and probes, 1µl 20X of *Amy E* primer and probes, 0.5 µl restriction enzyme (5 Units per reaction), 8 µl water and 1µl DNA template. The ddPCR amplifications were performed in a C1000TM Thermal cycler (Bio-Rad, USA). In addition to determining the number of ICEs in the bacterial population, the copy number of the ICE in eight single colonies were also determined.

Table 1: Primer and probe sequences, amplicon size, chlorophore label, and annealing temperature

Target	**Forward primer**	**Reverse primer**	**Probe sequence**	**Amplicon size**	**Chlorophore**	**Annealing temperature**
Amy E S. *oralis*	GGC ATC ATA GTC TGT ACC TGT G	AAC GGC TGG ACT CAC TTT AC	ACC AGT GCC AGT GGA AGT CAT TGT	96bp	FAM	56℃
IntTn and xisTn regions	ATA CTC CCA TAC AGT CAA TAG TCC	AGT TCC ACC CCT GCA TGG	CCG TCG CAGGCA ATG AGT ATG GCT	88bp	HEX	56℃
tet (M)	GTR AYG AAC TTT ACC GAA TC	ATC GYA GAA GCG GRT CAC	N/A	633bp	N/A	55℃
IntTn and xisTn regions	CGC CAAAGG GTC TTG TAT ATG	GCT GTA GGT TTT ATC AGC TTT TGC	N/A	650bp	N/A	58℃

DNA isolation and sequencing

The modified Marmur extraction procedure, as described by Salva-Serra *et al.* (42), was used to extract total bacterial DNA from bacterial isolates included in this study. The evolved and non-evolved bacterial populations were subjected to WGS using the Illumina Nextseq 500 platform to generate short-read paired-end illumina sequences. One of the three triplicate populations were sequenced using the Pacific Biosciences Sequel instrument (PacBio) using Sequel Polymerase v3.0, SMRT cells v3 LR and Sequencing chemistry v3.0, to generate long PacBio reads. Hybrid assemblies of the long PacBio reads and the short illumina reads were created using Canu (43) and Breseq (44) analysis of the short read assemblies revealed SNPs in the assembled genomes. Sequence comparisons of the assembled Tn*916* elements with the wild type Tn*916* were preformed using blast and visualized as blast rings.

Results

Strain construction

To investigate the fitness cost, stability, and evolution of Tn*916*, we introduced Tn*916* into newly constructed kanamycin-resistant *S. oralis* isolates. The introduction of Tn*916* into the naïve isolates resulted in several transconjugants, of which TC4.1, TC4.2, TC4.6, TC6.1, TC6.2, TC8.1, and TC10.1 were selected for further investigation.

Relative fitness

The fitness costs of newly acquired Tn*916* (T_0) ranged from 6% - 25% in the transconjugants obtained in this study. After 500 generations of experimental evolution the cost of carrying Tn*916* was reduced in all evolved transconjugants. As illustrated in Figure 1, at T_0 the reduction in relative fitness in TC4.1, TC6.1, TC6.2, TC8.1 and TC10.1 was statistically significant in the one-sample t-test; two tailed when compared to the ancestor (see supplementary data Table S1). TC4.2 and TC4.6 displayed a reduction in fitness which was not significantly different (P= 0.07). The cost of acquiring the ICE was ameliorated during 500 generations. TC8.1 and TC10.1 were selected for an additional 240hrs ending in a total evolution period of approximately 1000 generations in these samples. The fitness cost of Tn*916* in isolates TC8.1 and TC10.1 and the varying relative fitness costs during the 600hrs of evolution are depicted in Figure 2.

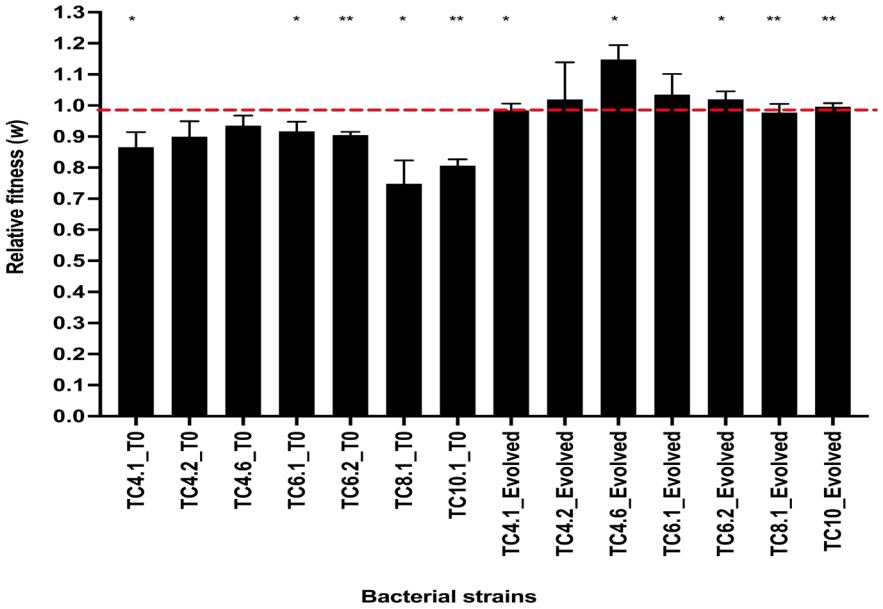

Figure 1: Relative fitness of Tn*916* in transconjugants TC4.1, TC4.2, TC4.6, TC6.1, TC6.2, TC8.1, and TC10.1 obtained from comparing the exponential growth rates of the ancestor and the Tn*916* carrying transconjugants. The bars represent the relative fitness (*w*) at T_0 (before evolution) and at evolved (after approximately 500 generations) and the error bars indicate ± s.e.m. The red dotted line indicates the fitness of the ancestral isolate. The significance of the changes in relative fitness (*w*) are indicated by asterisks (*P* =*<0.05. **<0.01; one-sample *t*-test, two-tailed).

Stability and Copy number

The stability of the newly acquired ICE in the *S. oralis* host isolates was determined by screening for *int* and *xis* genes in the transconjugants after cultivation in the absence of tetracycline for a minimum of 500 generations. The ddPCR results confirmed the presence and number of copies of the ICE. In the transconjugants TC4.1, TC4.2, TC4.6, TC6.1, and TC8.1 the average number of Tn*916* detected by ddPCR was 3 copies per genome. In transconjugants TC6.2 and TC10.1, the average element copy numbers per genome were 2 and 1, respectively (Figure 3).

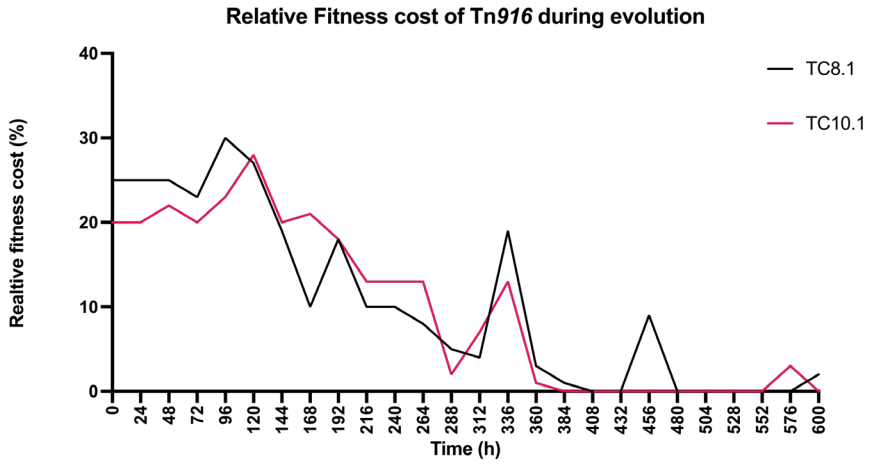

Figure 2: The reduction of relative fitness cost during 600hrs after the acquisition of Tn*916*. The graph shows the fluctuations in relative fitness cost observed in *S. oralis* transconjugants TC8.1 and TC10.1 upon the introduction of Tn*916* and the changes in relative fitness that occur throughout the 600hrs.

Analysis of single colonies of Tn*916* after 360hrs and 600hrs of evolution (in TC8.1) indicates that the number of elements remained constant during this time frame, except for one colony, TC8.1_2_T360, in which 2 copies per genome were observed instead of the expected 3 copies as shown in supplementary Figure S3. Analysis of the copy number of TC8.1 and TC10.1 at the three-time points show that the number of ICEs is constant.

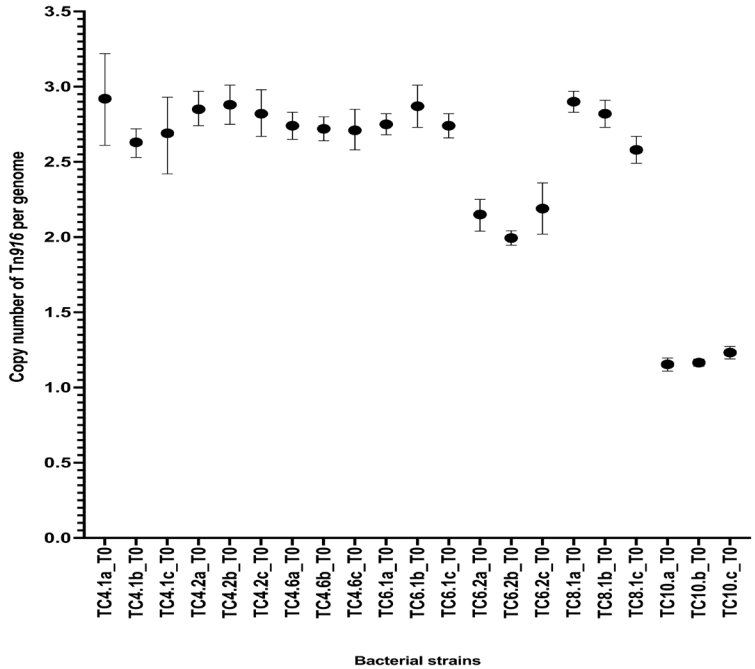

Figure 3: The number of copies of Tn*916* detected by ddPCR in seven transconjugants TC4.1, TC4.2, TC4.6, TC6.1, TC6.2 TC8.1 and TC10.1, at time T0. The copy number was determined from three independent clones of each transconjugant, and the bars represent the mean with standard deviation from three replicates.

Bioinformatics

The sequencing of three biological replicates of the two isolates at the three selected time points resulted in 18 sequenced populations. Analysis of these assemblies indicated the presence of one copy of Tn*916* in TC10.1 and three copies in TC8.1 in the populations. A closer look at the assembled genomes revealed that the elements were inserted in AT-rich regions of the genomes at positions listed in Table 2. Based on the assembled genomes of the evolved populations, the number of Tn*916* observed and the insertion sites did not change during evolution. Attempts to analyze the sequence of the three elements in TC8.1 proved futile as none of the derived Pacbio sequences adequately covered the entire 18kb element regions. Assembly of the TC10.1 isolate verified the presence of one element which did not display any changes within the element when compared to the wildtype Tn*916* as shown in Figure 4. Attempts to identify secondary mutations sites within the host genome proved unsuccessful as we were unable to assemble the full genome of the ICE-free host. Comparisons of the genomes of the Tn*916* carrying populations (non-evolved vs evolved) indicate the presence of multiple SNPs in different locations in the genomes. Interestingly, in all the populations SNPs within the Aminopeptidase N/Response regulator *ArlR*, Ribonuclease Z and Signal transduction histidine-protein kinase *ArlS* genes (see supplementary data Figure S5).

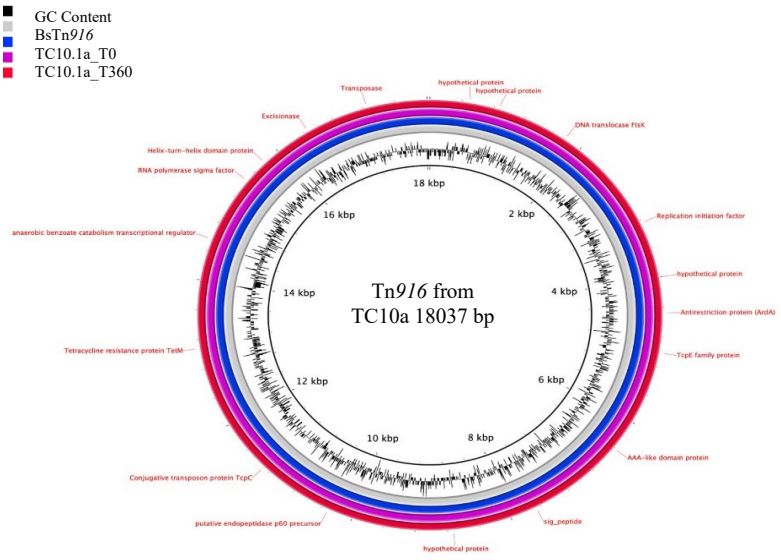

Figure 4: Blast ring of Tn*916* in the reference strain *B. subtilis* BS49 versus the elements in the assembled genomes of TC10.1a at T0, T360 and T600. The sequence of the assembled Tn916 elements was found to be identical to the wild type Tn916 element from *B. subtilis* BS49.The blue ring represents the element assembled from TC10.1a_T0; the pink represents the element from TC10.1a_T360; and the red ring represents the element from TC10.1a_T600.

Table 2: Insertion sites of Tn916 in the assembled genomes

Isolate	Insertion position of Tn*916* bp	Left border gene	Left border gene product	Right border gene	Right border gene product
TC8.1	31596	A*cp* P_1	Acyl carrier protein (219 bp after start of gene)	*Acp* P_1	Acyl carrier protein (645 bp before the end gene)
TC8.1	169822	*Gap* A	Glyceraldehyde-3-phosphate dehydrogenase	*Rlu* A1	Ribosomal large subunit pseudouridine synthase C
TC8.1	1132432	*	hypothetical protein	*	hypothetical protein
TC10.1	1927834	*Com* D	Signal transduction histidine kinase (930bp after start of gene)	*Com* D	Signal transduction histidine kinase (410bp before the end of gene)

*Inserted within a hypothetical protein

Discussion

Molecular mechanisms contributing to genome plasticity include point mutations, genome rearrangements, mobile genetic elements, and horizontal gene transfer (45). The ability to integrate genetic material like mobile genetic elements allows bacteria to modify their genomes, leading to an increase in genetic variability and population survival under changing environmental conditions (46). However, integration of genetic materials, such as MGEs, could also come with additional biological cost to the host that could be disadvantageous, especially in mixed bacterial populations.

In order to investigate the fitness cost, stability and evolution of Tn*916*, we introduced Tn*916* into a new construction kanamycin resistant *streptococcus oralis*. In this report we show that the acquisition of Tn*916* elements imposes a fitness cost on the naïve *S. oralis* isolate ranging from 6% to 25% across the seven transconjugants. We further show that this burden is mitigated with experimental evolution period of 500 generations. It has been predicted that in comparison to plasmids, ICEs should impose a lower fitness cost on the host due to their reduced HGT capacity and lower introduction of foreign DNA into the host (4). Here we show data that contradicts this predication as the observed fitness costs are substantial, and interestingly not different from those observed by Starikova et al. after the introduction of Tn*916* into naïve *E*. *faecium* 64/3 strain (47).

The absence of selection pressure was previously thought to encourage the loss of MGEs, especially in cases where they imposed a burden on the host cell. However, this line of thought has been proved inaccurate, as studies have shown that once acquired, bacterial populations tend to maintain the resistance determinants and instead employ compensatory mutations that restore fitness while maintaining the resistance determinants (48-50). The sequencing data from this study indicates that the ICE structure and insertion site are not altered during the tested evolution period. While some studies shown that fitness cost of acquiring a MGE may be alleviated by mutations within the element (51) our findings suggest that restoration of fitness is not accompanied by any nucleotide changes within Tn*916*. The mitigation of fitness of a MGE by secondary mutations in other parts of the genome is not unheard of and was recently shown to occur *E.coli* (52). Interestingly, analysis of the assembled genomes shows numerous SNPs including SNPs within the two-component system, consisting of

the *ArlS and ArlR* genes. Two-component systems are sophisticated regulatory and signaling systems composed of a histidine kinase and a response regulator (53). They enable bacteria to sense changes in their environment and adapt their gene expression accordingly (54). SNPs in the *ArlS and ArlR* genes suggest that these genes may play a role in the observed restoration of fitness.

As the findings in this study reaffirm, high biological costs accompany the acquisition of ICEs. In the light of these facts, one cannot help but wonder why these elements are abundant in bacterial populations, especially in the absence of selective pressure. Based on the results from this evolution study, it is plausible that the high prevalence of Tn*916* elements may be attributed to the rapid reduction of the fitness cost of carrying these elements.

Regardless of the absence of selective pressure and the biological costs accompanying the acquisition of ICEs, the Tn*916* family elements appear to be stably maintained in the *S. oralis* populations. The absence of changes within the ICE suggests that other mechanisms are responsible for the observed reduction in fitness cost. It is, for instance, plausible that ICEs implore similar mechanisms as those seen in integrons (the downregulation of the integrase genes) (47) to reduce the cost of harboring ICEs. This intriguing notion warrants further investigation.

Conclusion

These findings indicate that once acquired, Tn*916* imposes a fitness cost in *S. oralis* that is alleviated in less than 500 generations. Tn*916* elements are present in the population after 1000 generations without selection pressure and can thus be deemed stable in *S. oralis* isolates. Furthermore, the number of elements and insertion sites are not altered during the evolution experiment. Taken together, the relatively rapid reduction in fitness cost, stability, and persistence of Tn*916* may be the driving forces behind the abundance of this family of ICEs in *streptococcus* species. They, at least in part, explain the continued increase of Tn*916*-Tn*1545* family elements among bacterial populations despite the reductions in the overall use of tetracycline.

Acknowledgment The current study was performed at the Department of Clinical Dentistry (IKO), UiT- The Arctic University of Norway. We are grateful to the Norwegian National Advisory Unit on Detection of Antimicrobial Resistance (K-RES) for providing the clinical bacterial isolates used in this study.

The authors have no conflict of interest.

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Supplementary data

Table S1: Relative Fitness effect of Tn*916* carrying *S. oralis* transconjugants during 360hrs of evolution

Sample	T0 mean Relative Fitness (w)	SD T0	SEM	P Value	Significance
TC4.1	0.865915	0.048327	0.0279	0.0407	*
TC4.2	0.899749	0.049174	0.0284	0.0717	ns
TC4.6	0.93515	0.032621	0.0188	0.075	ns
TC6.1	0.916485	0.031125	0.018	0.0433	*
TC6.2	0.904918	0.010497	0.0061	0.004	**
TC8.1	0.74802	0.074544	0.043	0.0280	*
TC10.1	0.806319	0.020256	0.0117	0.0036	**
TC4.1_Evolved	0.984044	0.03826	0.0221	0.0332	*
TC4.2_Evolved	1.019147	0.119941	0.0692	0.2268	ns
TC4.6_Evolved	1.1478	0.046579	0.0269	0.0156	*
TC6.1_Evolved	1.034466	0.066851	0.0386	0.0924	ns
TC6.2_Evolved	1.019647	0.02623	0.0151	0.0170	*
TC8.1_Evolved	0.977297	0.027464	0.0159	0.0047	**
TC10.1_Evolved	0.99572	0.011731	0.0013	0.0013	**

P =*<0.05. **<0.01; ns= non-significant; one-sample *t*-test, two-tailed.

Table S2: Relative fitness cost of Tn*916* in S. oralis and standard deviation

	TC4.1		TC4.2		TC4.6		TC6.1		TC6.2		TC8.1		TC10.1	
Time (hr)	RF	SD	RF	RF	SD	RF	SD	RF	SD	RF	SD	RF	SD	
24	0.87	±0.05	0.89	0.75	±0.08	0.80	±0.05	0.94	±0.03	0.92	±0.03	0.90	±0.01	
48	0.83	±0.06	0.81	0.75	±0.01	0.78	±0.02	0.88	±0.04	0.83	±0.11	0.79	±0.1	
72	0.83	±0.04	0.83	0.77	±0.04	0.80	±0.03	0.83	±0.03	0.96	±0.07	1.04	±0.15	
96	0.83	±0.02	0.83	0.70	±0.06	0.77	±0.04	0.83	±0.03	0.96	±0.04	1.04	±0.001	
120	0.91	±0.01	0.96	0.73	±0.04	0.72	±0.05	0.97	±0.03	1.02	±0.11	0.93	±0.05	
144	0.98	±0.05	1.08	0.81	±0.04	0.82	±0.06	0.97	±0.06	1.04	±0.002	0.95	±0.001	
168	0.87	±0.06	0.99	0.9	±0.02	0.79	±0.02	0.94	±0.07	1.01	±0.001	1.01	±0.001	
192	0.92	±0.01	1.01	0.83	±0.01	0.83	±0.03	1.03	±0.02	0.98	±0.02	0.84	±0.12	
216	0.91	±0.08	1.04	0.87	±0.08	0.85	±0.02	1.01	±0.03	1.06	±0.01	0.99	±0.04	
240	0.90	±0.06	1.00	0.96	±0.09	0.94	±0.02	1.02	±0.01	0.97	±0.02	0.96	±0.03	
264	0.91	±0.05	1.02	0.92	±0.01	0.88	±0.04	1.06	±0.03	1.15	±0.12	1.04	±0.02	
288	0.95	±0.07	0.97	0.95	±0.02	0.94	±0.06	1.03	±0.04	1.03	±0.14	0.97	±0.06	
312	1.03	±0.05	1.03	1.03	±0.08	1.06	±0.07	1.15	±0.06	0.97	±0.1	0.92	±0.03	
336	0.95	±0.06	1.01	0.93	±0.02	0.94	±0.08	1.06	±0.05	0.94	±0.07	0.95	±0.02	
360	0.98	±0.04	1.01	0.99	±0.03	0.99	±0.12	1.15	±0.05	1.03	±0.07	1.02	±0.03	

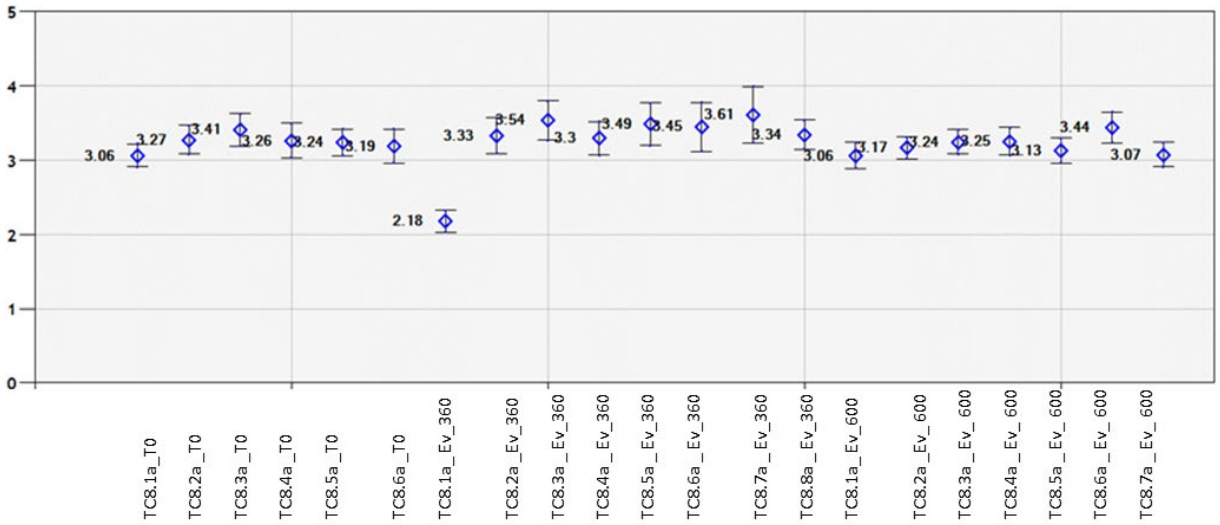


Figure S3: The number of copies of Tn*916* detected in multiple colonies of TC8.1 at time points T0, T360, T600. In TC8.1, three elements are detected at all three-time points, except for sample TC8.1a after 360 hours where only two copies were detected.

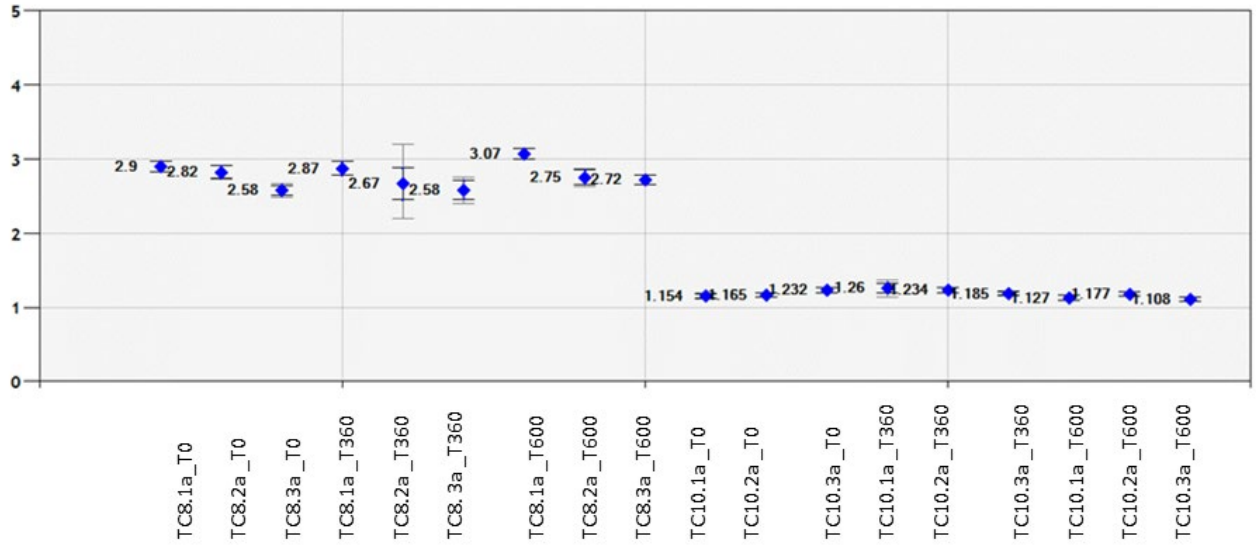


Figure S4: The number of copies of Tn*916* detected In TC8.1 and TC10.1 at time points T0, T360, T600. In TC8.1, three elements are detected at all three-time points, whereas one element was observed in TC10.1. The number of Tn*916* was at T0, T360 and T600.

TC10.1a_Evolved

breseq version 0.36.0
mutation predictions | marginal predictions | summary statistics | genome diff | command line log

Marginal read alignment evidence

seq id	position	ref	new	freq	score (cons/poly)	reads	annotation	genes	product
1	727,274	1	.	T	39.4%	80.8 / inf	193	intergenic (+58/-52)	*pepN/arlR_2*	Aminopeptidase N/Response regulator ArlR
1	728,968	0	G	A	38.3%	155.6 / 184.6	168	D327N (GAT→AAT)	*arlS*	Signal transduction histidine-protein kinase ArlS
1	1,282,143	0	G	C	26.2%	234.6 / 41.0	103	V189V (GTC→GTG) ‡	*rnz*	Ribonuclease Z
1	1,282,146	0	A	G	23.8%	176.5 / 33.6	102	V188V (GTT→GTC)	*rnz*	Ribonuclease Z
1	1,282,144	0	A	T	20.8%	214.8 / 22.8	102	V189D (GTC→GAC) ‡	*rnz*	Ribonuclease Z

TC10.1b_Evolved

breseq version 0.36.0
mutation predictions | marginal predictions | summary statistics | genome diff | command line log

Marginal read alignment evidence

seq id	position	ref	new	freq	score (cons/poly)	reads	annotation	genes	product
1	728,968	0	G	A	23.6%	412.9 / 116.6	204	D327N (GAT→AAT)	*arlS*	Signal transduction histidine-protein kinase ArlS
1	1,635,400	0	T	C	21.3%	232.1 / 17.8	128	V1715A (GTT→GCT)	*bag*	IgA FC receptor
1	1,289,239	0	A	G	21.2%	262.9 / 33.0	163	S382P (TCA→CCA)	*lytB_3*	Putative endo-beta-N-acetylglucosaminidase

TC10.1c_Evolved

breseq version 0.36.0
mutation predictions | marginal predictions | summary statistics | genome diff | command line log

Marginal read alignment evidence

seq id	position	ref	new	freq	score (cons/poly)	reads	annotation	genes	product
1	715,597	0	C	T	70.3%	284.7 / 168.9	199	L888L (CTA→TTA)	*t0_00699*	hypothetical protein
1	814,141	0	C	A	69.2%	189.6 / 196.7	182	A31S (GCT→TCT)	*t0_00792*	hypothetical protein
1	815,162	0	A	.	66.8%	221.9 / inf	202	intergenic (-27/-129)	*t0_00793/glmU*	hypothetical protein/Bifunctional protein GlmU
1	729,185	0	G	C	64.2%	216.7 / 218.0	177	G399A (GGC→GCC)	*arlS*	Signal transduction histidine-protein kinase ArlS
1	1,635,400	0	T	C	25.5%	166.7 / 26.1	95	V1715A (GTT→GCT)	*bag*	IgA FC receptor

Figure S5: Mutation predication in the assembled genomes of TC10.1a, b and c after 360 hours of evolution.

APPENDIX

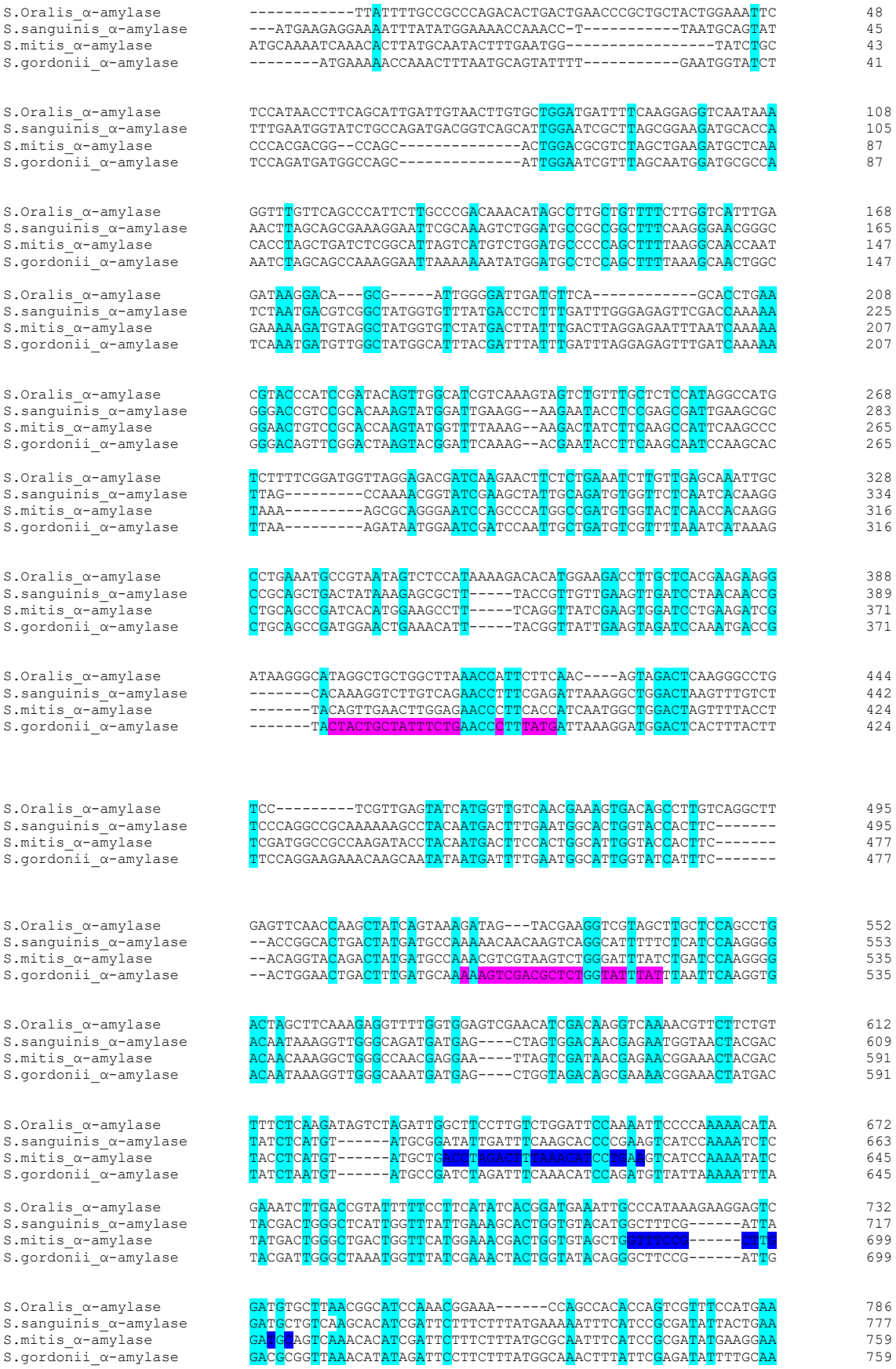
S1

ID	Strain	Source	Laboratory	**Penicillin G**	**Linezolid**	**Trim-sulpha**	**Cefuroxime**	**Cefotaxime**	**Gentamicin**	***Tet*racycline**	**Erythromycin**
SO1	*Streptococcus oralis*	Blood culture	Rikshospitalet	0.016	0.500	0.250	<0,016	0.016	16.00	32.00	>256
SM2	*Streptococcus mitis*	Blood culture	Rikshospitalet	0.094	0.750	1.000	0.125	0.094	0.160	48.00	>256
SO3	*Streptococcus oralis*	Blood culture	Rikshospitalet	0.016	0.500	0.250	<0,016	0.016	16.000	0.094	0.125
SO4	*Streptococcus oralis*	Blood culture	Rikshospitalet	0.023	1.000	0.380	<0,016	0.016	12.000	0.190	0.190
SSg5	*Streptococcus sanguis*	Blood culture	Rikshospitalet	0.023	0.750	1.000	0.023	0.016	8.000	0.125	0.125
SSv6	*Streptococcus salivarius*	Blood culture	Rikshospitalet	0.023	0.750	0.380	<0,016	0.023	24.000	0.125	0.190
SSmgr7	*Streptococcus milleri grupp*	Blood culture	Rikshospitalet	0.023	0.500	3.000	<0,016	0.016	16.000	0.064	0.125
SM8	*Streptococcus mitis*	Blood culture	Rikshospitalet	0.023	0.750	0.250	<0,016	0.012	16.000	0.125	0.190
SM9	*Streptococcus mitis*	Blood culture	Rikshospitalet	0.016	1.000	0.250	0.023	0.016	12.000	0.125	0.190
SO10	*Streptococcus oralis*	Blood culture	Rikshospitalet	0.023	0.750	0.500	<0,016	0.016	12.000	0.125	0.125
SO11	*Streptococcus oralis*	Blood culture	Rikshospitalet	0.012	0.750	0.250	0.016	0.012	12.000	0.094	0.125
SA12	*Streptococcus anginosus*	Blood culture	Rikshospitalet	0.016	1.000	0.380	0.023	0.016	12.000	0.125	0.125
SM13	*Streptococcus mitis*	Blood culture	Rikshospitalet	0.016	0.750	0.750	0.016	0.016	6.000	0.125	0.125
SM14	*Streptococcus mitis*	Blood culture	Rikshospitalet	0.016	1.000	0.38	0.016	0.016	12	0.25	0.19
SSg15	*Streptococcus sanguis*	Blood culture	Rikshospitalet	0.047	1.000	0.38	0.064	0.023	16	0.19	0.19
SSv16	*Streptococcus salivarius*	Blood culture	Rikshospitalet	0.047	1.000	0.25	0.047	0.023	12	0.19	0.125
SM17	*Streptococcus mitis*	Blood culture	Rikshospitalet	0.016	0.750	0.38	0.023	0.023	6	1	0.023
SM18	*Streptococcus mitis*	Blood culture	Rikshospitalet	0.125	0.750	0.125	0.250	0.064	16	0.19	0.047
SM19	*Streptococcus mitis*	Blood culture	Rikshospitalet	0.125	0.750	0.38	0.250	0.094	3	0.25	0.094
SSv20	*Streptococcus salivarius*	Blood culture	Rikshospitalet	0.012	1.000	0.16	0.250	0.19	4	0.19	0.094
SM21	*Streptococcus mitis*	Blood culture	St. Olavs hospital	0.032	0.500	0.750	0.023	0.023	2.000	0.190	0.094
SM22	*Streptococcus mitis*	Blood culture	St. Olavs hospital	0.023	0.500	0.190	0.047	0.016	4.000	0.125	0.047
SM23	*Streptococcus mitis*	Blood culture	St. Olavs hospital	0.750	0.750	0.094	0.750	0.190	3.000	0.250	0.190
SM24	*Streptococcus mitis*	Blood culture	St. Olavs hospital	0.016	0.500	12.000	0.016	0.006	1.500	0.190	0.094
SO25	*Streptococcus oralis*	Blood culture	St. Olavs hospital	0.012	0.750	0.125	0.023	0.012	3.000	0.094	0.047
SO26	*Streptococcus oralis*	Blood culture	St. Olavs hospital								
SO27	*Streptococcus oralis*	Blood culture	St. Olavs hospital	0.016	0.500	32.000	0.023	0.012	4.000	0.064	0.064
SM28	*Streptococcus mitis*	Blood culture	St. Olavs hospital	6.000	0.750	0.750	8.000	2.000	6.000	64.000	>256
SM29	*Streptococcus mitis*	Blood culture	St. Olavs hospital	0.047	0.380	0.380	0.032	0.032	1.500	32.000	0.047
SO30	*Streptococcus oralis*	Blood culture	St. Olavs hospital	0.094	0.500	0.125	0.250	0.032	8.000	24.000	0.047
SM31	*Streptococcus mitis*	Blood culture	St. Olavs hospital	0.047	0.500	1.000	0.047	0.047	1.500	0.250	0.064
SO32	*Streptococcus oralis*	Blood culture	St. Olavs hospital	0.023	0.750	0.094	0.125	0.094	12.000	0.250	0.125
SSg33	*Streptococcus sanguis*	Blood culture	St. Olavs hospital	0.190	1.000	0.750	0.250	0.125	6.000	24.000	0.047
SO34	*Streptococcus oralis*	Blood culture	St. Olavs hospital	0.047	1.000	0.380	0.190	0.125	32.000	16.000	0.125
SG35	*Streptococcus gordonii*	Blood culture	St. Olavs hospital	0.016	0.750	0.500	0.032	0.032	6.000	0.250	0.032
SO36	*Streptococcus oralis*	Blood culture	St. Olavs hospital	0.190	0.500	0.750	0.250	0.125	4.000	4.000	0.125
SO37	*Streptococcus sanguis*	Blood culture	St. Olavs hospital								
SSv38	*Streptococcus salivarius*	Blood culture	St. Olavs hospital	0.064	0.750	0.250	0.032	0.032	8.000	0.250	0.094
SSg39	*Streptococcus sanguis*	Blood culture	St. Olavs hospital	0.125	0.750	1.000	0.125	0.094	6.000	0.190	0.047
SO40	*Streptococcus oralis*	Blood culture	St. Olavs hospital	0.047	0.750	0.190	0.047	0.047	2.000	0.250	0.047
SSg41	*Streptococcus sanguis*	Blood culture	Haukeland universitetssykehus	0.047	0.190	0.250	0.094	0.032	1.500	32.000	0.016
SM42	*Streptococcus mitis*	Blood culture	Haukeland universitetssykehus	0.064	0.250	1.500	0.047	0.094	3.000	0.250	4.000
SA43	*Streptococcus anginosus*	Blood culture	Haukeland universitetssykehus	0.125	1.500	0.064	0.380	0.250	8.000	0.380	0.094

SO44	*Streptococcus oralis*	Blood culture	Haukeland universitetssykehus	1.000	1.500	> 32,000	3.000	0.750	24.000	24.000	3.000
SMu45	*Streptococcus mutans*	Blood culture	Haukeland universitetssykehus	0.032	0.750	0.064	0.047	0.047	3.000	0.500	0.064
SM46	*Streptococcus mitis*	Blood culture	Haukeland universitetssykehus	0.047	1.000	0.047	0.125	0.094	12.000	12.000	0.094
SO47	*Streptococcus oralis*	Blood culture	Haukeland universitetssykehus	0.125	0.500	0.125	0.125	0.047	32.000	32.000	0.047
SSg48	*Streptococcus sanguis*	Blood culture	Haukeland universitetssykehus	0.032	1.000	0.500	0.064	0.032	32.000	0.250	0.094
SO49	*Streptococcus oralis*	Blood culture	Haukeland universitetssykehus	1.500	0.750	32.000	4.000	1.000	16.000	32.000	1.000
SI50	*Streptococcus intermedius*	Blood culture	Haukeland universitetssykehus	0.064	1.000	0.032	0.125	0.094	16.000	0.094	0.064
SSv51	*Streptococcus salivarius*	Blood culture	UNN	0.023	1.000	0.750	0.047	0.023	12.000	96.000	0.064
SO52	*Streptococcus oralis*	Blood culture	UNN	0.064	1.000	0.190	0.190	0.064	24.000	32.000	0.094
SM53	*Streptococcus mitis*	Blood culture	UNN	0.064	1.000	0.250	0.190	0.094	32.000	0.190	0.094
SM54	*Streptococcus mitis*	Blood culture	UNN	0.125	0.750	0.190	0.380	0.125	8.000	0.250	4.000
SM55	*Streptococcus mitis*	Blood culture	UNN	0.094	0.380	>32	0.190	0.125	6.000	0.190	0.094
SM56	*Streptococcus mitis*	Blood culture	UNN	0.032	0.750	1.500	0.047	0.160	2.000	0.250	6.000
SA57	*Streptococcus anginosus*	Blood culture	UNN	0.047	0.750	0.190	0.125	0.094	8.000	0.094	0.064
SSv58	*Streptococcus salivarius*	Blood culture	UNN	0.125	1.000	0.380	0.047	0.064	12.000	0.250	0.064
SM59	*Streptococcus mitis*	Blood culture	UNN	0.023	1.000	0.190	0.032	0.016	3.000	48.000	4.000
SM60	*Streptococcus mitis*	Blood culture	UNN	0.094	0.750	0.250	0.094	0.047	4.000	0.190	0.023
SO61	*Streptococcus oralis*	Blood culture	Ullevål universitetssykehus	0.094	1.000	0.190	0.380	0.250	96.000	0.250	0.064
SO62	*Streptococcus oralis*	Blood culture	Ullevål universitetssykehus	0.047	1.500	0.190	0.190	0.125	24.000	2.000	6.000
SSg63	*Streptococcus sanguis*	Blood culture	Ullevål universitetssykehus	0.500	0.500	8.000	0.500	0.190	6.000	0.125	0.023
SV64	*Streptococcus vestbularis*	Blood culture	Ullevål universitetssykehus	1.500	0.750	2.000	0.500	0.380	12.000	0.380	0.094
SG65	*Streptococcus gordonii*	Blood culture	Ullevål universitetssykehus	0.012	1.000	0.500	0.016	0.016	6.000	0.380	0.047
SO66	*Streptococcus oralis*	Blood culture	Ullevål universitetssykehus	0.064	0.500	0.190	0.047	0.023	8.000	0.250	0.047
SO67	*Streptococcus oralis*	Blood culture	Ullevål universitetssykehus	0.125	0.750	0.064	0.250	0.064	12.000	64.000	0.047
SM68	*Streptococcus mitis*	Blood culture	Ullevål universitetssykehus	0.380	0.750	0.250	0.380	0.094	2.000	0.380	0.125
SO69	*Streptococcus oralis*	Blood culture	Ullevål universitetssykehus	1.500	1.000	>32	4.000	1.500	32.000	96.000	6.000
SM70	*Streptococcus mitis*	Blood culture	Ullevål universitetssykehus	4.000	0.750	0.094	3.000	1.000	1.500	0.190	3.000
SC71	Green streptococcus	Blood culture	Sørlandet sykehus	0.016	0.380	0.380	0.032	0.016	4.000	24.000	0.047
GS72	Green streptococcus	Blood culture	Sørlandet sykehus								
SM73	Green streptococcus	Blood culture	Sørlandet sykehus	0.190	0.750	0.190	0.380	0.125	8.000	0.380	4.000
SM74	Green streptococcus	Blood culture	Sørlandet sykehus	0.380	1.500	0.250	0.500	0.190	24.000	2.000	0.064
GS75	Green streptococcus	Blood culture	Sørlandet sykehus	0.250	0.190	12.000	0.125	0.094	8.000	0.250	3.000
GS76	Green streptococcus	Blood culture	Sørlandet sykehus								
SM77	Green streptococcus	Blood culture	Sørlandet sykehus	0.190	0.750	0.190	0.750	0.380	24.000	0.190	0.064
	Green streptococcus	Blood culture	Sørlandet sykehus								
SA79	Green streptococcus	Blood culture	Sørlandet sykehus	0.064	1.500	0.500	0.380	0.190	12.000	0.380	0.064
SM80	*Streptococcus mitis*	Blood culture	UNN	0.047	1.500	1.000	0.190	0.094	24.000	6.000	0.094
SM81	Green streptococcus	Blood culture	Sørlandet sykehus	0.190	1.500	0.380	0.190	0.047	6.000	48.000	0.032
SM82	Green streptococcus	Blood culture	Sørlandet sykehus	0.032	0.750	0.125	0.047	0.016	16.000	0.250	0.125

SSv83	*Streptococcus salivarius*	Blood culture	UNN	0.094	0.750	0.250	0.032	0.047	3.000	0.190	0.047
SM84	Green streptococcus	Blood culture	Sørlandet sykehus	0.032	0.750	0.125	0.094	0.047	1.500	0.125	0.047
SM85	Green streptococcus	Blood culture	Sørlandet sykehus	0.094	0.750	0.094	0.250	0.047	12.000	0.380	0.064
SM86	Green streptococcus	Blood culture	Sørlandet sykehus	0.190	1.000	0.190	0.094	0.094	6.000	0.190	0.940
SM87	Green streptococcus	Blood culture	Sørlandet sykehus	0.125	0.750	0.190	0.094	0.094	2.000	0.125	0.125
SA88	*Streptococcus anginosus*	Blood culture	UNN								
SM89	*Streptococcus mitis*	Blood culture	UNN	0.190	1.500	0.094	0.190	0.094	24.000	0.190	0.064
SO90	*Streptococcus mitis*	Blood culture	UNN								
SM91	Green streptococcus	Blood culture	UNN	0.064	0.750	0.125	0.125	0.064	24.000	0.190	0.064
SM92	*Streptococcus mitis*	Blood culture	UNN	0.064	1.500	0.125	0.190	0.094	24.000	0.250	0.064
SA93	*Streptococcus anginosus*	Blood culture	UNN	0.047	0.750	0.032	0.038	0.250	16.000	0.250	0.094
SM94	*Streptococcus mitis*	Blood culture	UNN								
SB95	*Streptococcus bovis*	Blood culture	UNN	0.125	3.000	0.094	0.125	0.190	3.000	256.000	0.094
SM96	*Streptococcus mitis*	Blood culture	UNN	0.500	0.500	>32	1.000	0.380	1.500	0.125	24.000
SA97	*Streptococcus anginosus*	Blood culture	UNN	0.094	1.500	0.016	0.190	0.190	6.000	0.380	0.125
SM98	*Streptococcus mitis*	Blood culture	UNN	0.125	1.000	6.000	0.250	0.094	2.000	0.190	4.000
SC99	*Streptococcus constellatus*	Blood culture	UNN	0.094	1.500	0.002	0.500	0.250	3.000	16.000	0.023
SM100	*Streptococcus mitis*	Blood culture	UNN	0.190	1.500	0.125	0.380	0.125	32.000	0.250	0.064

Figure 2S


S.Oralis_α-amylase
S.sanguinis_α-amylase
S.mitis_α-amylase
S.gordonii_α-amylase

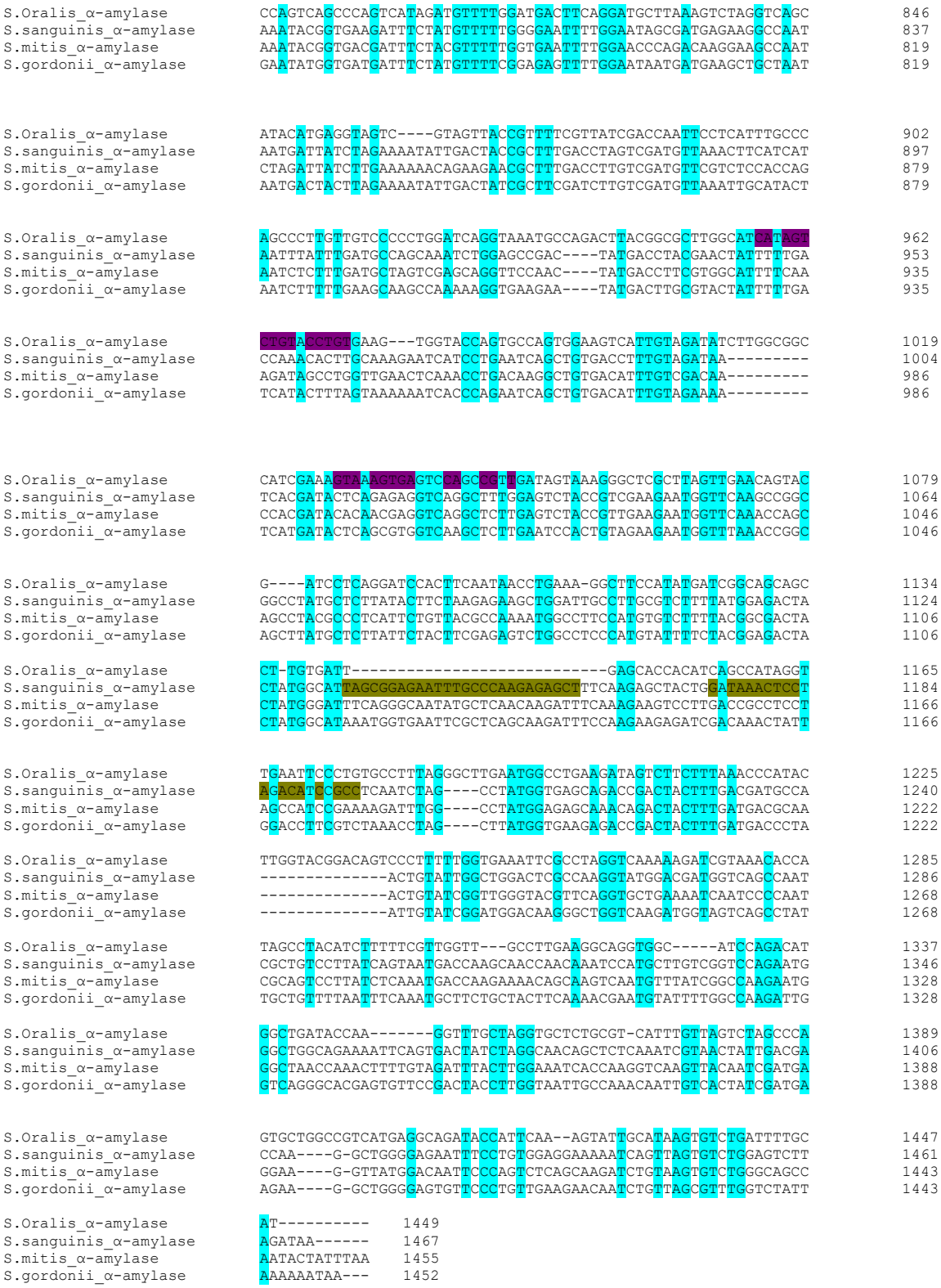


Figure 2S. The DNA sequences of *amyE genes used for strain specificity. The variable region of the gene was used to design species-specific primers and probes for the studied oral streptococci species.*