EXTRACELLULAR DNA IN HEAD AND NECK BIOFILMS

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Abstract

Extracellular DNA (eDNA) is a ubiquitous component of the extracellular matrix of microbial biofilms. It has a number of functions that include a role as an adhesin during biofilm attachment, and facilitating matrix stability in mature biofilms. Increasingly, deoxyribonuclease (DNase) enzymes have been shown to reduce the colonisation of many microbial biofilms, both bacterial and fungal. Biofilms are estimated to be responsible for around 60% of bacterial infections, including many chronic diseases. The aim of this work was to determine the role of eDNA in chronic mixed-species biofilm infections, and to explore the potential of DNase enzymes for biofilm control. This included three major areas of research, focusing on chronic rhinosinusitis, tracheoesophageal speech valves (TESVs), and dental plaque. An important aspect was to test the efficacy of a novel bacterial nuclease, NucB, isolated from a seaweed-associated strain of *Bacillus licheniformis*, against microbial biofilms.

The colonisation of speech valves by micro-organisms was studied using scanning electron microscopy (SEM). In keeping with previous observations, these biofilms were co-aggregations of fungal and bacterial species. Using confocal laser scanning microscopy, eDNA was observed in the biofilm matrix. Extracellular DNA was extracted and quantified from TESV biofilms. All six biofilms studied had detectable nucleic acids, as measured by NanoDrop spectrophotometry. The eDNA was apparently heavily degraded, and produced smears by agarose gel electrophoresis. Nevertheless, eDNA appeared to be providing biofilm stability since micro-organisms were liberated from the surface of the valves following treatment with NucB in over 60% of the TESVs tested.

To assess the role of eDNA in biofilms associated with chronic rhinosinusitis, ‘obstructive mucin’ and sinus mucosa biopsy samples collected during functional endoscopic sinus surgery at the Freeman Hospital, Newcastle, were analysed for the presence of biofilms and biofilm-forming micro-organisms. An average of 3.75 bacterial species per patient were cultured from obstructive mucin. The most commonly isolated micro-organisms were *Staphylococcus aureus*, coagulase-negative staphylococci and α-haemolytic streptococci. Micro-organisms were not detected by transmission electron microscopy of the obstructive mucin and this material appeared to originate through a host inflammatory response. However, bacteria were visualised on the surface of sinus mucosa using a peptide nucleic acid fluorescence *in situ* hybridization (PNA-FISH) universal bacteria probe. Twenty-four bacterial isolates were

assessed for their ability to form biofilms in a microtitre plate model. All micro-organisms tested formed biofilms, and 14 of 22 were susceptible to NucB. In total, 15 of 24 microbial species produced eDNA that was detectable by agarose gel electrophoresis. By SEM, cellular colonisation was lower in treated samples and, in the case of *Streptococcus constellatus* FH20 stringy, matrix-like material was not present after DNase treatment.

The role of eDNA in matrix stability and initial biofilm attachment was also studied in oral bacteria. *Streptococcus gordonii* DL1, *Streptococcus mutans* GS-5, *Fusobacterium nucleatum* 25586 and *Actinomyces oris* MG1 were examined using DNase treatment, CLSM, and eDNA extraction. Of these species, all except *S. gordonii* appeared to rely on high molecular weight (HMW) eDNA for biofilm attachment and biofilm stability. Although *S. gordonii* did not produce detectable HMW eDNA, nucleic acids were detectable by NanoDrop spectrophotometry. Furthermore, this species produces an extracellular nuclease which may degrade the HMW eDNA in the conditions used to culture biofilms. Interestingly, four *S. mutans* strains differed in their sensitivity to DNase treatment. Oral biofilms were also modelled in a BioFlux microfluidics device using flowing human saliva. Mixed-species biofilms and single species biofilms of *S. mutans* UA159 and *S. gordonii* DL1 were cultured using this technique, to determine whether this model would allow more realistic experiments for DNase testing.

Finally, the extracellular nuclease, SsnA, of *S. gordonii* DL1 was characterised. A nuclease-deficient mutant did not produce extracellular nuclease activity on DNase Test agar or during a Forster Resonance Energy Transfer (FRET) assay. Nuclease activity was cell-wall-associated as predicted from the predicted amino acid sequence of SsnA. Allelic exchange mutagenesis determined that *ssnA* expression was regulated by CcpA in response to repressing sugars. However, in planktonic culture non-repressing carbon sources also inhibited enzyme activity during a FRET assay. Further experiments using acidic buffers replicated the inhibition of SsnA without the presence of sugars. SsnA was purified as a GST-tagged fusion protein in an *Escherichia coli* protein expression system, and had anti-biofilm activity against *S. mutans* GS-5. However, this species is strongly acidogenic and therefore it is hypothesised that although SsnA may be a competition biofilm factor, acid production by *S. mutans* may reduce its efficacy *in vivo*.

In conclusion, this thesis has provided strong evidence for the role of eDNA in facilitating biofilm formation and mature biofilm stability of clinically relevant

biofilms. Nucleic acids were present in biofilms associated with a chronic infection, medical implant biofouling and dental plaque. A variety of DNase enzymes (NucB, DNase I, and SsnA) were capable of reducing biofilm colonisation. Given the adhesive function of eDNA in biofilm matrices it is proposed that DNase enzymes may be beneficial for controlling healthcare-related biofilms.

Abbreviations

µM	Micromolar
AFS	Allergic fungal sinusitis
APS	Ammonium persulfate
AtlA	Autolysin A
bpDNase I	Bovine pancreas deoxyribonuclease I
BSA	Bovine serum albumin
CA	Cell wall anchor motif
Ca	Calcium
CBDA	Calgary Biofilm Detection Assay
CcpA	Catabolite control protein A
CCR	Carbon catabolite repression
CF	Cystic fibrosis
CFS	Cell free saliva
CFU	Colony forming units
chlm	Chloramphenicol
CLSM	Confocal laser scanning microscopy
ConA	Concanavalin A
CRS	Chronic rhinosinusitis
CSP	Competence-stimulating peptide
CV	Crystal violet
CVC	Central venous catheter
DAPI	4',6-diamidino-2-phenylindole
DDAO	7-hydroxy-9H-(1,3-dichloro-9,9-dimethylacridin-2-one)
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dsDNA	Double stranded deoxyribonucleic acid
DTT	Dithiothreitol
ECRS	Eosinophillic chronic rhinosinusitis
eDNA	Extracellular deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
EMRS	Eosinophillic mucin rhinosinusitis
ENT	Ear, nose, and throat

EPS	Extracellular polymeric substances
FAA	Fastidious anaerobe agar
FAM	Fluorescein amidite
FCS	Fetal calf serum
FESS	Functional endoscopic sinus surgery
FISH	Fluorescence *in situ* hybridization
FITC	Fluorescein isothiocyanate
FRET	Förster resonance energy transfer
GbpC	Glucan-binding protein C
gDNA	Genomic DNA
GSH	Glutathione
GST	Glutathione S-transferase
H_2O_2	Hydrogen peroxide
hBD-3	Human β-defensin-3
HCl	Hydrochloric acid
HMW	High molecular weight
iDNA	Intracellular deoxyribonucleic acid
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LB	Luria Bertani
LMW	Low molecular weight
MALDI-TOF	Matrix assisted laser desorption/ionization time-of-flight
MALDI-TOF/TOF	Matrix assisted laser desorption/ionization time-of-flight/time of flight
MalR	Maltose repressor protein
Mg	Magnesium
MI	Michigan State
MNase	Micrococcal nuclease
NCBI	National Center for Biotechnology Information
NET	Neutrophil extracellular trap
NHS	National Health Service
NucB	Nuclease B
NUPPA	Newcastle University protein and proteome analysis
OB	Oligonucleotide/oligosaccharide binding
OD	Optical density
ORF	Open reading frame

PAc	Cell-surface protein antigen
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PI	Propidium iodide
PMF	Peptide mass fingerprinting
PNA	Peptide nucleic acid
PNAG	Poly-N-acetylglucosamine
PQS	*Pseudomonas* quinolone signal
PVC	Polyvinylchloride
qPCR	Quantitative polymerase chain reaction
QS	Quorum sensing
rhDNase I	Recombinant human DNase I
RNA	Ribonucleic acid
RNase	Ribonuclease
rRNA	Ribosomal ribonucleic acid
RPM	Revolutions per minute
RTF	Reduced transport fluid
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Scanning electron microscopy
SP	Signal peptide
SpnA	*Streptococcus pyogenes* nuclease A
ssDNA	Single stranded deoxyribonucleic acid
SsnA	*Streptococcus suis* nuclease A
SsnA	Streptococcal secreted nuclease A
TCA	Trichloroacetic acid
TEM	Transmission electron microscopy
TEMED	Tetramethylethylenediamine
TESV	Tracheoesophageal speech valves
USA	United States of America
UV	Ultraviolet
VGS	Viridans group streptococci
WGA	Wheat germ agglutinin
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

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Chapter 1: Introduction

Bacteria are often thought of as planktonic life forms, devoid of senses and very much alone in life. This concept exists because microbiology was born via the study of micro-organisms in test tube pure culture. However, it has become increasingly apparent that our perception of micro-organisms, and this life-cycle, is not applicable to most of the environments that they reside in. For the most part, micro-organisms must survive in challenging circumstances and therefore live as co-aggregations. Antonie van Leewenhoek in the 17th century was the first to observe collections of micro-organisms that he termed 'scruf' on the surface of his teeth. This term was lost, and in 1978 Costerton *et al.*, (1978) coined the term 'biofilm' for a collection of sessile micro-organisms that are enclosed in an extracellular matrix. However, it is important to note intermediate researchers like Arthur Henrici who also discovered that most aquatic bacteria live on surfaces rather than as planktonic organisms (Henrici, 1933). Microbiologists now believe that the biofilm is the most common way that micro-organisms exist. This life-system is vastly different to planktonic micro-organisms, as it facilitates a community that has different niches, and provides a robust barrier to external insults via the extracellular matrix.

These highly specialised and complex biological systems have been identified in 3.2-billion-year-old rocks in Pilbara Craton, Australia (Rasmussen, 2000). Ancient biofilms have also been preserved in sediments that were likely in a marine hydrothermal environment in modern day South Africa, 3.3-3.5 billon-years-ago (Westall *et al.*, 2001). Interpreting microbial signatures in these ancient rocks is not easy but by studying them it may be possible to apply the same techniques in the search for extra-terrestrial life on Mars (Westall *et al.*, 2003). Earth in the Paleoarchaean eon of 3.2-3.5 billion-years-ago would bear little resemblance to modern Earth. During this period micro-organisms would have produced energy by anoxygenic photosynthesis due to a lack of atmospheric oxygen and organic matter (Nisbet and Fowler, 2011). It is interesting that at this stage, biofilms were capable of surviving on planet Earth. Clearly, this early appearance in nature exhibits how integral biofilm production is to prokaryotic organisms. Furthermore, it suggests that the robustness of biofilms, to external insults and poor ecological conditions, increases microbial survival and allows them to reside in all manner of environments. Given the environmental constraints of Archaen Earth (high ultraviolet (UV) exposure and temperature extremes) it might have been beneficial to evolve synergistic social interactions. In addition, the extracellular

matrix would have provided a desirable barrier to poor external conditions. Therefore, biofilms apparently increased the fitness of singular micro-organisms that were surviving 3.5 billion-years-ago.

Prokaryotic biofilm formation continues to be incredibly versatile on modern Earth, allowing micro-organisms to adapt to a wide range of environments (Costerton *et al.*, 1987). An example of extreme biofilm formation exists amongst the archaeal *Sulfolobus* spp., that grow in geothermically active areas at temperatures of 70-85°C and pH of 2-3 (Koerdt *et al.*, 2010). Microbial biofilms are also capable of surviving and growing near deep-sea sulphide vents, in temperatures as low as 2°C or as warm as 100°C+ (Schrenk *et al.*, 2003). Three and a half billion years of evolutionary adaptation has led to microbial biofilms that are incredibly tenacious. It appears that the biofilm mode of life is the most successful biological system on Earth. Knowledge of primitive Earth, and microbial biofilm survival in extreme environments can give the basic understanding of this system, primarily the reasons for its evolution and the incredibly tenacity of biofilms.

1.1 Biofilm formation and life-cycle

There are a sequence of changes as a singular micro-organism becomes attached to a surface, and begins forming a biofilm. This process is complex, and shares complexity with the life cycles of multicellular organisms. *Myxococcus xanthus* swarms are an example of multicellular behaviour exhibited by a collection of prokaryotes, and biofilms have been likened to this biological system (Monds and O'Toole, 2009). Biofilm formation has been most thoroughly researched in model organisms such as Gram-positive *Bacillus subtilis* (Vlamakis *et al.*, 2013) and Gram-negative *Pseudomonas aeruginosa* (Sauer *et al.*, 2002). Essentially there are five stages to biofilm formation and maturation (Figure 1.1): (i) initial reversible attachment, (ii) irreversible attachment, (iii) biofilm maturation with an extracellular matrix, (iv) continued maturation with distinct cell phenotypes, and (v) biofilm dispersal.

Initial adhesion is the first major step in biofilm formation and relies firstly on physio-chemical forces. Lifshitz-Van der Waals forces are the most significant (generally) attractive forces, occurring due to an interaction between oscillating dipoles on surface molecules. This attractive force is negatively impacted by electrostatic forces on the bacterial cell surface and substratum, which cause repulsion. Ultimately, when bacterial cells are close enough to a surface (<5 nm) the attractive force is stronger than

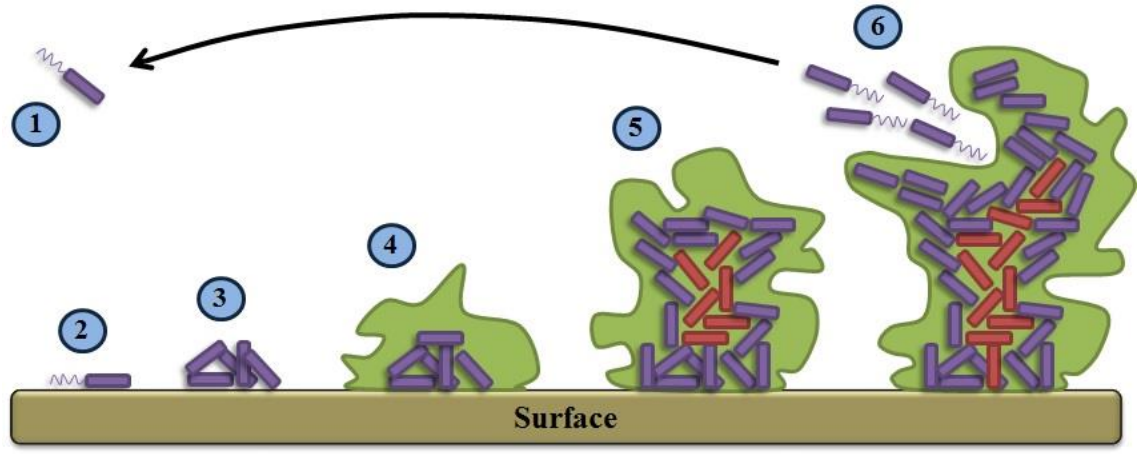


Figure 1.1 The life-cycle of a mono-species biofilm. Biofilm formation occurs over several stages, and is usually initiated by environmental cues. 1) The initial attachment of a micro-organism can be active if the species is motile and/or produces adhesions that are actively regulated, or passive if the species lacks motility. 2) Microbes are at first reversibly adhered to a surface, usually facilitated by a conditioning film that promotes adhesion through Lifshitz-Van der Waals and electrostatic forces. 3) Cells differentiate into a non-motile phenotype and become irreversibly attached by adhesion/receptor interactions to a surface, before producing an extracellular matrix (4). 5) As the biofilm ages micro-organisms take on functionally distinct roles (as outlined by red coloured cells), although the cells are genetically identical. The last step in biofilm maturation is the shedding of free-floating cells from the biofilm (6). This process can be actively promoted by matrix-degrading enzymes, or caused by environmental forces.

the repulsive force and adhesion is initiated. Although greatly simplified, this forms the basis of the Derjaguin, Landau, Verwey, and Overbeek (DLVO) theory of bacterial adhesion (Hermansson, 1999). However, this theory does not fully describe the complexity of microbial attachment to a surface, where other forces such as gravity, sheer stresses, Brownian motion, acid-base interactions, and hydrophobic interactions also facilitate or reduce adhesion. Molecules on the surface of micro-organisms, and those on the attachment surface impact the adhesion process by altering the balance of physio-chemical forces. Bacterial adhesion is complex and for the purpose of this thesis, the macromolecule eDNA, and the effect it has on bacterial attachment is the major point of consideration (discussed in section 1.4.1). Once strongly adhered to a surface, by intermolecular forces, bacteria can become irreversibly bound by the interaction between specific receptor and adhesin proteins. Micro-organism attachment is often facilitated by a conditioning film of molecules that builds up on clean surfaces once they are exposed to a liquid or gas. Initial attachment is thought to be initiated once microbes sense environmental conditions that would favour biofilm formation. For instance, environmental conditions such as temperature, glucose, and pH alter total biofilm extent in clinical isolates of *Burkholderia pseudomallei* (Ramli *et al.,* 2012). It is likely that the optimal conditions for biofilm formation are species, and even strain, specific. With motile species, like *P. aeruginosa*, it is logical that biofilm formation may be an active process. Biofilm maturation to microcolonies in *P. aeruginosa* PA14 on polyvinylchloride (PVC) plastic requires flagella and type IV pili mediated motility (O'Toole and Kolter, 1998). However, this model of *P. aeruginosa* biofilm formation is challenged by the results of Heydorn *et al*., (2002) where *P. aeruginosa* PA01 motility-deficient mutants formed more microcolonies than wild-type. Therefore, it appears that biofilm formation is strain as well as species dependant.

After irreversible attachment, micro-organisms begin to synthesise and excrete an extracellular matrix which ultimately surrounds the cells. As with many aspects of biofilm formation, matrix production could be either an active or a passive process. Cell to cell signalling appears to play a role in *P. aeruginosa* matrix production (Davies, 1998), although the key regulators are still unknown (Branda *et al.*, 2005). In *Vibrio cholerae* it appears that quorum sensing again has a role in matrix production. For this species, increased cell density leads to HapR-mediated down-regulation of matrix production (Zhu and Mekalanos, 2003). Therefore, in this micro-organism low cell density leads to extracellular matrix synthesis. Individual components of the biofilm matrix can be released in a variety of mechanisms. For instance, extracellular DNA, can

be produced via excretion mediated by DNA-containing membrane-bound vesicles (Renelli *et al.*, 2004), or lytic release (Paganelli *et al.*, 2013). Therefore, not only is the regulation of matrix production incredibly complex, but also the excretion or synthesis of individual components.

Another important aspect of biofilm development is the detachment of cells in mature biofilms. This phase of the biofilm life-cycle allows cells to colonise new environments and escape competitors or harsh environmental conditions. Furthermore, it has a major role in disease transmission. Biofilm detachment can be either an active or passive process. Additionally, there are three major modes as outlined by Kaplan *et al*., (2010): erosion, seeding and sloughing. Erosion is a process of constant cell release during biofilm formation, whereas seeding and sloughing release large clumps of cells in mature biofilms. Sloughing occurs from the exterior of the biofilm, and seeding from internal cavities. Of particular interest is the production of extracellular enzymes by biofilm-forming micro-organisms that target the biofilm matrix, thereby releasing cells. These enzymes are interesting because they could be used to disperse deleterious biofilms. Examples of naturally produced biofilm detaching enzymes are dispersin B (Kaplan *et al.*, 2003), NucB (Nijland *et al.*, 2010) and alginate lyase (Boyd and Chakrabarty, 1994).

The oral biofilm (dental plaque) (discussed again in section 1.8.2), and its formation, provides an incredibly useful model for studying the biofilm life-cycle. There are well-known steps in dental plaque formation, which begins with molecules adhering to the oral surface. These molecules build up and are collectively known as the acquired salivary pellicle (Lendenmann *et al.*, 2000). The acquired salivary pellicle is a collection of glycoproteins, from human saliva, that bond to tooth enamel forming a film coating. Following this process, micro-organisms adhere to the pellicle, often passively, via weak physio-chemical forces that reside on the microbial and pellicle surfaces. Weak attachment is later replaced by irreversible attachment via adhesin-receptor binding with strong attachment forces (Whittaker *et al.*, 1996). Thereafter, secondary and late colonisers adhere to cell-surface receptors of already attached micro-organisms (Kolenbrander *et al.*, 2006). This process of microbial succession is incredibly complex with multiple interactions, antagonistic and synergistic, occurring between a diverse range of oral bacteria. There are still many interactions to be studied, and many proteins that may have important roles in shaping dental plaque microbial composition. Firstly, understanding these interactions could reveal novel proteins for controlling dental

plaque. Furthermore, as the oral biofilm provides a detailed model of biofilm formation as a whole, by studying it our knowledge of other biofilm systems will increase.

1.2 Biofilm resistance and chronic diseases

Biofilms are suggested to be less susceptible to phagocytes, viral attack, UV radiation, shear stresses, dehydration, biocides, and antibiotics than planktonic micro-organisms (Wolcott and Ehrlich, 2008). For instance, the biofilm matrix may block UV light in *P. aeruginosa* RM4440, as shown using a model that replicates an alginate matrix typical of *Pseudomonas* spp. biofilms (Elasri and Miller, 1999). Resistance, to antibiotics or biocides is due to many factors: 1) restricted penetration of antimicrobials, 2) slow growth of bacteria, 3) resistant phenotypes (persisters), and 4) altered chemical micro-environments (Lewis, 2001; Stewart and Costerton, 2001; Hall-Stoodley *et al.*, 2004). Micro-organisms living within a biofilm can exhibit extremely complex and co-ordinated behaviour. Cell signalling and quorum sensing allows the microbes to sense and respond to the local environment, thereby increasing survival chances (Hall-Stoodley and Stoodley, 2009). High levels of resistance are therefore related to the matrix "wall", bacterial behaviour, and the heterogeneous communities within the biofilm. Complexity means the biofilm is extraordinarily resistant to deleterious agents and conditions.

Bacterial biofilms often form on living tissues or on the inert surfaces of indwelling medical devices (reviewed by Costerton *et al.*, 1999). Of the bacterial infections treated by physicians, some 60% are thought to be biofilm related (Costerton *et al.*, 1999). A common example of this would be dental plaque, a biofilm that is kept in check by the mechanical action of a toothbrush. Dentistry and improved oral hygiene is an example where modern medicine has been able to effectively treat a biofilm disease. However, for the most part, where conventional medicine treats planktonic bacteria satisfactorily, traditional therapy can have little effect on biofilms. For example, biofilms are substantially less susceptible to antibiotics than free-floating bacteria (Gilbert *et al.*, 1997). Therefore, it is not surprising that chronic diseases arise from biofilm infections. Some examples of biofilm issues in the head and neck region are shown in Figure 1.2.

Biofilm infections and their link to a disease pathogenesis can be hard to establish. For example the sinuses can be colonized by microbial biofilms in healthy patients, as well as those suffering from chronic rhinosinusitis (CRS) (Bezerra *et al.*, 2011). Furthermore, diagnosis of a biofilm infection is often related to the sampling technique

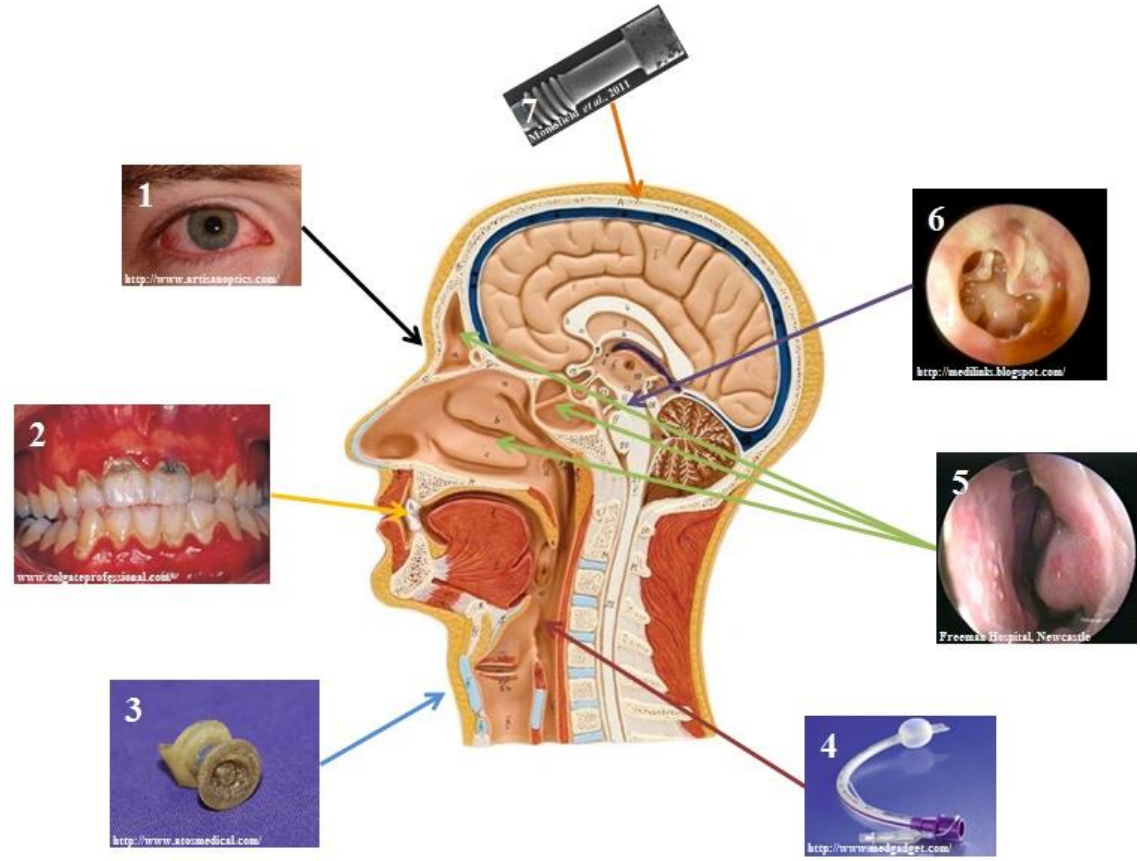


Figure 1.2 Clinical complications caused by microbial biofilms in the head and neck region of humans. Anti-clockwise from 1) contact lens associated keratitis, 2) dental plaque, 3) tracheoesophageal speech valve biofouling, 4) ventilator-associated biofouling, 5) chronic otis media, 6) chronic rhinosinusitis, and 7) bone adhered hearing aid biofouling.

used. For instance, biofilm sampling through culture-dependent analysis can lead to culture-negative results (Hall-Stoodley *et al.*, 2006), which is likely due to the inadequacies of culture media in sustaining biofilm micro-organisms. The diagnosis criteria for biofilm infections has been added to and modified over the last 10 years to include several features. Parsek and Singh (2003) suggest that the infection must be surface-associated, exhibit cell aggregates living within a matrix, be localised to one region of the host, and be recalcitrant to antimicrobial treatment. Additionally, given the reduced ability of culture media to support biofilm micro-organism, biofilm diagnosis must include multiple diagnostic techniques (Fux *et al.*, 2003). For example, in chronic otitis media detection of microbial DNA by PCR and fluorescence *in situ* hybridization microscopy gave higher microbial detection rates (80-90%) than culture-dependent analysis (Hall-Stoodley *et al.*, 2006). In keeping with their extensive research, Hall-Stoodley and Stoodley (2009) suggest diagnosis criteria should also include evidence of reduced host immune system efficacy in contact with microbial aggregates and take into account a negative culture result with obvious infection symptoms. Diagnosis should include the biofilm matrix, although it is briefly highlighted in the 'Parsek-Singh criteria' mentioned above. Components of the matrix vary widely but it may be that common components, such as extracellular DNA (eDNA), could provide a reliable marker for biofilm colonisation. Furthermore, the extracellular matrix is an important component in biofilm formation and resistance. The biofilm matrix is discussed in greater detail in the next section.

1.3 The biofilm matrix

Biofilm micro-organisms account for approximately one tenth of the total dry mass of a biofilm (Flemming and Wingender, 2010), although this is likely to vary greatly depending on the species present. Ninety percent of a biofilm is the extracellular matrix of macromolecules and water that are predominately produced by the micro-organisms themselves. The extracellular matrix has also been called the glycocalyx, a slime, and extracellular polymeric substances (EPS). In the past the extracellular matrix was thought to consist mostly of polysaccharides, and the term EPS, meaning exopolysaccharides, often relates to this older concept. The role of polysaccharides in the *P. aeruginosa* biofilm matrix, principally alginate, Psl and Pel, has been heavily researched (Ryder *et al.*, 2007). However, although the extracellular matrix is a plastic trait, and will change depending on many factors, it appears that many other substances

contribute to the extracellular matrix. This slime is composed of polysaccharides, lipids, proteins, nucleic acids, water, and other small compounds (Flemming and Wingender, 2010). Furthermore, in *P. aeruginosa* PA01 polysaccharides are only a minor component of the biofilm matrix in relation to proteins and DNA (Matsukawa and Greenberg, 2004).

There is substantial difficulty in obtaining reliable biochemical profiles of biofilm matrix samples. It can be hard to separate the extracellular matrix from cells, and there can be substantial differences in yields of the different components depending on the technique used. Ultimately, it is currently prohibitive to study all the elements of a single species biofilm matrix, and researchers tend to focus on one component. Through this approach we now understand the matrix components to have at least fourteen functions, as outlined by Flemming and Wingender (2010). These functions include providing a protective barrier, acting as a nutrient reservoir, retaining water, and exchanging genetic information. In particular, many of the components, proteins, nucleic acids, and polysaccharides, can be involved in biofilm adhesion and retaining cohesion in mature biofilms. Of these macromolecules, DNA appears to be the most universal component of biofilm matrices that facilitates biofilm stability and initial attachment.

1.4 Extracellular DNA and its proposed functions

Extracellular DNA ‘slime’ was observed in micro-organisms in the mid-1950s (Catlin, 1956; Catlin and Cunningham, 1958). Four micro-organisms were found to produce eDNA that was compositionally similar to intracellular DNA. Furthermore, eDNA accumulation in the biofilm matrix of *Staphylococcus aureus* was increased in a broth of pH 6 with low calcium content (Catlin and Cunningham, 1958). This was because the extracellular DNase that controls *S. aureus* biofilm formation was inhibited in these conditions. More recently, DNA was found to be important in cell flocculation for a marine bacterium from the genus *Rhodovulum* (Watanabe *et al.*, 1998). However, after a publication by Whitchurch *et al*., (2002) showed that DNA in the biofilm matrix of *P. aeruginosa* was important during biofilm formation, attention became focussed on eDNA in biofilms with a structural function. Subsequently, there has been extensive research into the role of eDNA in microbial biofilms. The exact role of eDNA remains uncertain but it seems probable it has a number of functions including a means of exchanging genetic information (Molin and Tolker-Nielsen, 2003), a nutrient source

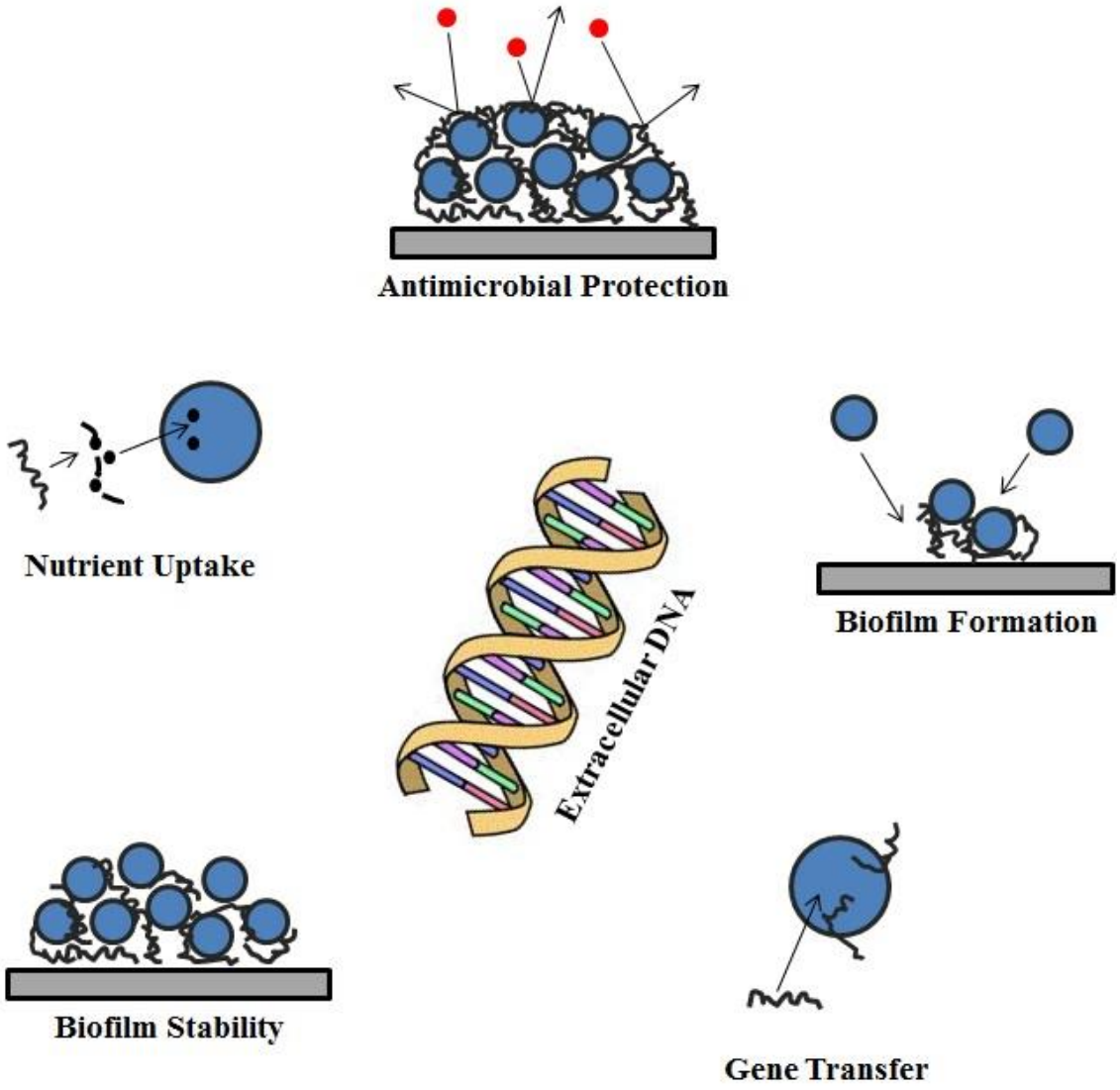


Figure 1.3 Microbial uses of extracellular DNA. The extracellular matrices of many biofilm-forming micro-organisms contain nucleic acids. Furthermore, the environment can also harbour DNA that persists long enough to be actively used by micro-organisms in a number of ways including, horizontal gene transfer, nutrient uptake, binding of antimicrobials, induction of antibiotic resistance genes, biofilm adhesion and maintain matrix stability in mature biofilms.

(Finkel and Kolter, 2001), a scaffold for matrix stability (Dominiak *et al.*, 2011), use as an adhesin (Vilain *et al.*, 2009) and antimicrobial protection (Jones *et al.*, 2013) (Figure 1.3). Despite the uncertainty, eDNA, which was once viewed as simply a by-product of cell lysis, now appears integral to the extracellular matrix and biofilm organisation in many micro-organisms.

The amount of eDNA present within the biofilm matrix differs greatly between microbial species. In comparison to the protein and polysaccharide components of *P. aeruginosa* biofilm matrices eDNA is 5 and 18-fold more abundant respectively in a static microtiter biofilm model (Matsukawa and Greenberg, 2004). Extracellular DNA is 17% of the total DNA extracted from *P. aeruginosa* biofilms (Steinberger and Holden, 2005). In this study four micro-organisms were examined and there was substantial variation in the total amount of eDNA extracted, with *Pseudomonas putida* producing eDNA so that it was a third of the total DNA extracted. The amount of eDNA also differed between the four biofilms tested by Tang *et al*., (2013), with *Reinheimera* sp. Strain F8 producing the most at 33 µg/mL, a modest 15 µg/mL produced by *Pseudomonas* sp. FW, and only minor amounts in *Microbacterium* sp. FW3 (1.5 µg/mL), and *Serratia* sp. FW2 (0.07 µg/mL). In activated sludge eDNA is found in surprisingly high concentrations (300 mg/mL) within some microcolonies (Dominiak *et al.*, 2011). Total eDNA extracted differs greatly between the method employed. Wu and Xi (2009) evaluated seven eDNA extraction methods, finding that chemical and enzymatic treatments yielded the highest amounts of DNA from biofilm matrices. Furthermore, they found that *Acinetobacter* sp. strain AC811 produced 15 µg/mL eDNA, *Escherichia coli* 9 µg/mL, *P. aeruginosa* 7 µg/mL and *S. aureus* 12 µg/mL. It is difficult to compare eDNA concentrations between studies due to differences in biofilm growth, biomass quantification and DNA quantification.

It is possible that micro-organisms release eDNA, or use environmental DNA as a template for gene uptake. The release of eDNA is discussed in detail in section 1.4.3. Natural DNA release and DNA uptake (competence) has been demonstrated for bacteria such as *Streptococcus pneumoniae* (Steinmoen *et al.*, 2002) and *Neisseria gonorrhoeae* (Hamilton *et al.*, 2005). For *S. pneumoniae* cell lysis, of a subpopulation of cells, appears to be induced by competence stimulating peptides (CSPs) and this leads to DNA release (Moscoso and Claverys, 2004). Mutants lacking the CSP sensor gene, *comD*, did not produce exogenous DNA. Furthermore, cell lysis is suggested to play a role as mutants lacking the major autolytic amidase, LytA, or an autolytic lysozyme, LytC, released less DNA (Steinmoen *et al.*, 2002; Moscoso and Claverys, 2004).

Streptococci have evolved to naturally exchange genetic information, and eDNA is integral to this process. For instance, *Streptococcus mutans* may obtain transforming DNA from neighbouring species, such as *Streptococcus gordonii*, via bacteriocin production (Kreth *et al.*, 2005). In this example, the bacteriocin, mutacin IV, probably led to cell lysis of *S. gordonii* and uptake of a pVA838 shuttle vector by *S. mutans*. However, CSP-induced DNA release has also been suggested to promote biofilm stability in *S. mutans* and *S. pneumoniae* (Moscoso and Claverys, 2004; Petersen *et al.*, 2005; Perry *et al.*, 2009). DNA release and uptake may have important consequences for bacteria. For example, antibiotic resistance genes have been found within river sediment eDNA in Northern China, and bacteria can naturally uptake these genes (Mao *et al.*, 2013). Extracellular DNA uptake by *S. mutans* may also allow adaptation to the oral cavity by creating phenotypic changes that assist persistence (Narisawa *et al.*, 2011). Within biofilms the release and uptake of DNA may share similarities to eukaryotic sexual reproduction, as it could allow the dissemination of genes that lead to greater survival, as hypothesised by Spoering and Gilmore (2006).

Besides providing a means of transferring antibiotic resistance genes (Mao *et al.*, 2013), eDNA can also reduce the activity of antimicrobials directly. Extracellular DNA produced by *Haemophilus influenzae* is capable of binding the human β-defensin-3 (hBD-3) antimicrobial peptide and reducing its efficacy against biofilms (Jones *et al.*, 2013). The activity of hBD-3 could be restored by incubating biofilms in the presence of DNase I. Exogenous DNA can have a negative effect on bacterial survival by causing cell lysis due to removal of metal cations from the cell surface (Mulcahy *et al*., 2008). However, it does appear that the anionic ability of DNA can also protect bacteria from antimicrobial killing. Recently, Chiang *et al.*, (2013) discovered that exogenous DNA that is artificially added to *P. aeruginosa* biofilms decreases the efficacy of aminoglycosides. This reduction in antibiotic efficacy has been linked to cation chelation, principally Mg^{2+} (Mulcahy *et al.*, 2008). In *P. aeruginosa* a lack of available Mg^{2+} induces the PhoPQ and AmrAB regulators that activate the antimicrobial peptide resistance operon in this species. The role of metal cations, eDNA, and antimicrobial resistance in *P. aeruginosa* has been reviewed extensively by Lewenza *et al*., (2013).

1.4.1 Extracellular DNA increases biofilm stability and attachment

Extracellular DNA appears to play a key role in facilitating the initial attachment of some micro-organisms to surfaces. The presence of eDNA does not affect Lifshitz-Van

der Waals forces (Das *et al.*, 2010). However, eDNA on the cell surface increases bacterial cell surface hydrophobicity, and enhances acid-base interactions (Das *et al.*, 2010; Das *et al.*, 2011). DNA is essentially hydrophilic as the sugar-phosphate backbone is polar, and the hydrophobic nucleobases are shielded inside the macromolecule. The bacterial cell wall contains both polar and non-polar molecules, and the interaction of DNA with the cell wall leads to increased hydrophobicity possibly by it interacting with polar sites like peptidoglycans, leaving hydrophobic sites free. Hydrophobic bacteria aggregate to one-another and to hydrophobic surfaces, although eDNA has also been shown to increase binding to hydrophilic surfaces such as glass (Das *et al.*, 2010). Acid-base interactions are repulsive in the absence of eDNA in *Streptococcus mutans* LT11, *P. aeruginosa* PA01, and *Staphylococcus epidermidis* 1457 (Das *et al.*, 2011). Electron transfers between eDNA, bacterial cells, and substrata appeared to facilitate bond formation for these micro-organisms. However, there was a point at which bacteria absorbed too much eDNA and it became deleterious for cell attachment (Das *et al.*, 2011). It is important to note, that bacteria that do not produce eDNA will still adhere to surfaces because of the effect of Lifshitz-Van der Waals forces and other interactions (see section 1.1). The metal chelating properties of DNA (Mulcahy *et al.*, 2008) may also impact on biofilm formation. Recently, the binding of Ca^{2+} to eDNA was shown to increase cationic binding between bacterial cells via eDNA (Das *et al.*, 2014). Extracellular DNA can intercalate with pyocyanin, a metabolite produced by *P. aeruginosa*, in a process that increases cell-cell aggregation (Das *et al.*, 2013). A pyocyanin-deficient mutant, Δ*phz*A-G, was unable to bind eDNA which led to decreased hydrophobicity. This example suggests that eDNA interacts with other molecules in order to exert a positive effect on cell adhesion.

There are many examples of eDNA promoting the initial attachment of biofilm-forming micro-organisms. *Bacillus cereus* ATCC 14579 cells become covered in eDNA during the exponential growth phase, and this facilitates biofilm formation (Vilain *et al.*, 2009). Cell lysis and eDNA release is an important step in *S. aureus* biofilm formation (Mann *et al.*, 2009). Harmsen *et al.*, (2010) showed that eDNA was important in the initial biofilm formation of 41 *Listeria monocytogenes* isolates by treating biofilms with DNase I. Furthermore, genomic DNA could restore biofilm adhesion in eDNA-free cultures but only in the presence of the peptidoglycan building block N-acetylglucosamine. This again indicates that eDNA often interacts with other molecules when facilitating cell-cell or cell-surface interactions. Exogenous addition of DNA cannot fully restore biofilm formation after DNase I treatment in many microbial

species, which is again indicative of other molecules probably contributing to eDNA-associated biofilm formation (Harmsen *et al.*, 2010; Lappann *et al.*, 2010; Das *et al.*, 2011). However, exogenous DNA (320 ng/mL) added to mature *C. albicans* biofilms increased biomass by up to 60% (Martins *et al.*, 2010). Addition of chromosomal meningococcal DNA to *Neisseria meningitidis* biofilms restored attachment in DNase I treated cultures but not to the levels seen in untreated biofilms (Lappann *et al.*, 2010). Exogenous DNA seeding (pneumococcal and salmon sperm DNA) also had a positive effect on *S. pneumoniae* biofilm extent in mutants lacking phage and bacterial lysins (Carrolo *et al.*, 2010). Clearly, eDNA facilitates biofilm attachment in many microbial species but this process is complex and likely requires other molecules to function correctly.

Extracellular DNA has also been shown to contribute to mature biofilm stability, although it is often suggested that this is a lesser role for eDNA than in initial attachment. For example, DNase treatment of *Rhodococcus ruber* C208 biofilms reduced biofilm formation by up to 25% but had no effect on mature biofilms (Gilan and Sivan, 2013). In this species RNase treatment had a more substantial effect than DNase treatment. Biofilm stability is most heavily influenced by eDNA in early biofilms, and eDNA contributes less to stability as biofilms mature. *P. aeruginosa* biofilms are only susceptible to DNase treatment up to 60 hours after inoculation (Whitchurch *et al.*, 2002). Again, *Vibrio cholerae* biofilm colonisation was reduced at 24 hours but not 72 hours by DNase treatment (Seper *et al.*, 2011). Many studies incubate biofilms for between 20-48 hours before extracting eDNA or testing dispersal with DNase enzymes. It is unknown if this truly represents mature biofilm formation. However, there are many examples of eDNA promoting stability in these early biofilms (Fredheim *et al.*, 2009; Tetz *et al.*, 2009; Harmsen *et al.*, 2010; Nijland *et al.*, 2010; Steichen *et al.*, 2011; Muscariello *et al.*, 2013). Pulmozyme®, a human DNase enzyme, reduced pneumococcal biofilm extent in 6-day-old biofilms in a dose-dependent manner (Hall-Stoodley *et al.*, 2008). This suggests that eDNA contributes to biofilm stability in some mature biofilms but it is unknown if eDNA exerts much of an influence in biofilms that have been forming over longer periods (weeks or months). It may be that other molecules become more influential during biofilm maturation as eDNA degrades either through environmental or microbial processes.

The amount of eDNA produced by different strains of the same species or by different species varies extensively and the concentration does not always correlate with DNase sensitivity (Shields *et al.*, 2013). Harmsen *et al.*, (2010) showed that high

molecular weight (HMW) DNA exerted more of an influence on biofilm formation than low molecular weight (LMW) DNA. A nuclease-deficient mutant of *S. aureus* produced more HMW DNA compared to wild type, and biofilm formation was enhanced in the mutant (Kiedrowski *et al.*, 2011). Interestingly, LMW (500 bp or less) DNA inhibited *Caulobacter crescentus* biofilm formation (Berne *et al.*, 2010). In this species biofilm formation was not inhibited by HMW genomic DNA (gDNA). Extracellular DNA extracted from biofilm matrices often migrates at a similar rate to intracellular DNA (apparent size ~30 kbp) on agarose gels (Kiedrowski *et al.*, 2011; Shields *et al.*, 2013). However, two *Streptococcus anginosus* strains isolated from chronically infected sinuses did not produce HMW eDNA but were susceptible to DNase treatment, indicating that LMW, or degraded DNA can influence biofilm stability (Shields *et al.*, 2013).

It appears that eDNA facilitates initial cell attachment to surfaces and can promote cell aggregation in early biofilms. The eDNA macromolecule is perhaps the only conserved component of biofilm matrices, as its primary structure is relatively unchanged between microbial species. There are areas of research that merit further investigation, including the contribution of eDNA to mature biofilm stability, and the interaction of other molecules with eDNA during cell-cell or cell-surface binding.

1.4.2 Origins of microbial eDNA

Clearly, eDNA appears to be an integral component of the extracellular matrix in many microbial biofilms. The release of eDNA and the source (e.g. plasmid DNA, cellular DNA, etc) has been well studied in recent years. Extracellular DNA in *P. aeruginosa* biofilms had an identical sequence to gDNA (Steinberger and Holden, 2005; Allesen-Holm *et al.*, 2006). More recently, six genes of *L. monocytogenes* spread evenly across the genome were amplified from eDNA, again suggesting eDNA was essentially equivalent to gDNA prior to release into the extracellular milieu (Harmsen *et al*., 2010). Extracellular DNA was also found to be identical to gDNA in the fungal species *Aspergillus fumigatus* by random amplification of polymorphic DNA (RAPD) PCR (Rajendran *et al.*, 2013). Again, *Staphylococcus epidermidis* extracellular and intracellular DNA appeared to be homologous when studied (Qin *et al.*, 2007). However, when eDNA produced in a multi-species biofilm of *P. aeruginosa*, *Pseudomonas putida*, *Rhodococcus erythropolis*, and *Variovorax paradoxuswas* was analysed it appeared distinct from cellular or total DNA (Steinberger and Holden,

2005). Furthermore, there were sequence differences in eDNA and gDNA produced by an aquatic bacterium (strain F8) (Böckelmann *et al.*, 2006). The above studies indicate that the structure and origin of eDNA in the biofilm matrix of micro-organisms differs between different biofilms. Further research is required as the sequence of eDNA may be an important feature for its particular function in a microbial biofilm, and this may be one reason for the observed differences in the contribution of eDNA to biofilm stability between microbial species.

1.4.3 Extracellular DNA release mechanisms

The release of eDNA is variable but it appears that most species produce it during cell lysis. Cell lysis can be triggered by quorum sensing (QS) molecules, autolysins, metabolites or bacteriocins. In Gram-positive micro-organisms autolysins appear to play a major role in facilitating eDNA release. This hypothesis has been postulated because some biofilm-forming micro-organisms produce less eDNA when genes linked to cell lysis are disrupted. For example, eDNA production in *S. epidermidis* is reliant on the autolysin, AtlE (Qin *et al.*, 2007). A mutant lacking the *atlE* gene released only 10% of the eDNA that was produced by the isogenic wild type. In *S. aureus* cell lysis initiated by the *cidA* murein hydrolase regulator is linked to eDNA production (Rice *et al.*, 2007; Mann *et al.*, 2009). Furthermore, the recently identified major autolysin of *Enterococcus faecium*, $AtlA_{Efm}$, leads to eDNA release through cell lysis and this in turn causes increased biofilm formation (Paganelli *et al.*, 2013). Inactivation of the major autolysin of *S. gordonii*, *atlS*, resulted in decreased eDNA production (Liu and Burne, 2011). Poor biofilm formation of the *atlS*-deficient mutant was partially restored by exogenous addition of eDNA. Extracellular DNA production in *S. gordonii* is also dependant on hydrogen peroxide (H_2O_2) release (Itzek *et al.*, 2011). It has been hypothesised that high levels of H_2O_2 (1-2 mM) causes DNA damage that leads to eDNA release (22-35 ng/mL), possibly by cell lysis. It is thought that eDNA release through cell lysis only kills a subpopulation of cells as cultures continue to grow as measured by A_{600}. Interestingly, concentrations of H_2O_2 greater than 5 mM lead to eDNA production arrest through total killing of *S. gordonii* cells.

In other *Streptococcus* species eDNA release can be promoted by CSPs, which are characteristic of QS molecules (Steinmoen *et al.*, 2002; Moscoso and Claverys, 2004; Petersen *et al.*, 2004; Perry *et al.*, 2009). *Streptococcus pneumoniae* eDNA release is promoted by CSPs that trigger the major autolysin, LytA, and the autolytic lysozyme,

LytC (Moscoso and Claverys, 2004). However, a non-autolytic pathway involving lysogenic phages has also been shown to facilitate eDNA release that improves biofilm formation in *S. pneumoniae* R36A and R36AP (Carrolo *et al.*, 2010). Quorum sensing also facilitates eDNA production in *P. aeruginosa* (Allesen-Holm *et al.*, 2006). Cell lysis of subpopulations of cells is triggered by the QS molecules acylated homoserine lactones and *Pseudomonas* quinolone signal (PQS) (Allesen-Holm *et al.*, 2006). Mutants deficient in these QS molecules produced biofilms that contained less eDNA, as visualised with confocal laser scanning microscopy (CLSM) and the DNA stain DDAO. Hydrogen peroxide liberation generated through phenazine production is also linked to cell lysis and eDNA release in *P. aeruginosa* (Das and Manefield, 2013). Phenazines are controlled by PQS molecules, and therefore it appears that QS is again causing eDNA release in this mechanism. Interestingly, it has been suggested that a basal level of eDNA production is controlled by a mechanism independent of QS in *P. aeruginosa* (Allesen-Holm *et al.*, 2006).

Non-lytic mechanisms of eDNA release have also been demonstrated. For example, *P. aeruginosa* produces membrane bound vesicles that contain DNA (Renelli *et al.*, 2004). Earlier observations of eDNA release in *S. gordonii* suggested that conditions of high H_2O_2 (1 mM and 10 mM) were causing eDNA excretion without the presence of any obvious cell lysis, as visualized by FISH ribosomal microscopy, and an apparent lack of autolysis when placed in an autolysis buffer, as measured by A_{600} (Kreth *et al.*, 2009). It is unknown if this mechanism acts independently of the AtlS-dependant pathway suggested by Liu and Burne (2011). Early biofilms of *Enterococcus faecalis* produce two distinct types of eDNA that both originate without any obvious cell lysis, as determined by biochemical assays (Barnes *et al.*, 2012). In this recent paper, eDNA was visualised using scanning electron microscopy (SEM) and anti-dsDNA conjugated to immunogold particles. This technique led to fascinating images that show eDNA localised near the septum of *E. faecalis* cells, which is suggestive of excretion, rather than cell lysis. Furthermore, the eDNA appeared to be intertwined with another unidentified matrix component. As *E. faecalis* biofilms mature eDNA release becomes controlled by a fratricidal mechanism that involves cell lysis (Thomas *et al.*, 2009). A subpopulation of cells deficient in QS are lysed by gelatinase (GelE) produced by the majority of cells. Due to QS-deficiency the subpopulation does not produce a gelatinase immunity protein, SprE. The target of GelE was shown to be the major autolysin, AtlA, as knockout mutants produced over 10-fold less eDNA than the wild-type. Both AtlA and GelE mutants formed less extensive biofilms than wild type. Clearly, in *E. faecalis*

eDNA release mechanisms change during the biofilm life cycle from lysis-independent to lysis-dependant methods. Interestingly, the absence of AtlA (Atn) in *E. faecalis* does not stop the release of eDNA but slows it, again suggestive of multiple release pathways (Guiton *et al.*, 2009). In early biofilms, with low cell density, it may be beneficial to produce eDNA via a non-lytic mechanism.

Extracellular DNA may also be produced via environmental factors, such as microbial competition. *Streptococcus mutans* bacteriocin-mediated cell lysis of *S. gordonii* leads to the release of eDNA (Kreth *et al.*, 2005). Although this DNA was hypothesised to be used during horizontal gene transfer it is also possible that *S. mutans* acquires it for biofilm formation. Biofilm stability in *S. mutans* can be reliant on eDNA (Perry *et al.*, 2009). In mixed species biofilms DNA release may result from autolysin production from one species that subsequently lyses other species. This would be a synergistic interaction if eDNA then contributed to biofilm stability. Harsh environmental conditions, acidic pH or high temperatures, could also lead to cell lysis and DNA release. Again, increased levels of DNA in biofilm matrices could have beneficial consequences for micro-organisms surviving in extreme conditions.

1.4.4 Inflammatory effects of extracellular DNA

Extracellular DNA produced by *P. aeruginosa* has been implicated in the inflammatory response of the host immune system in cystic fibrosis (Lipford *et al.*, 1998). These findings suggest that eDNA is not immunologically inert. This immune response is triggered by non-methylated CpG (cytosine-guanine) dinucleotide motifs in bacterial DNA binding to Toll-like receptors (Hemmi *et al.*, 2000). Non-methylated CpG motifs exist in a much lower frequency in mammalian DNA, and thus the host immune system is alerted to foreign bacterial DNA. However, host derived eDNA can elicit an auto-immune response in the chronic disease systemic lupus erythematosus (Su and Pisetsky, 2009). Bacterial CpG DNA activates macrophages, lymphocytes, NK cells and dendritic cells to produce cytokines, initiating immune responses (Krieg *et al.*, 1995; Krieg, 2002). Respiratory epithelial cells are also stimulated by CpG motifs (Platz *et al.*, 2004). Biofilm infections are often epithelial associated attachments so this immune reaction might be triggered. It may partly explain why some chronic biofilm infections, like CRS, are associated with inflammation. This is a potentially damaging consequence of producing eDNA for biofilms, as it will lead to increased bacterial cell

death. However, the combined beneficial aspects of eDNA production (discussed above) must out-weigh this deleterious effect for this trait to have evolved.

Recently, DNase I was found to reduce the proinflammatory effect of *P. aeruginosa* biofilms through degradation of matrix eDNA (Fuxman Bass *et al.*, 2010). Furthermore, eDNA-deficient *P. aeruginosa* strains stimulated a lower release of cytokines by neutrophils. Dornase alfa, a human DNase I, has an anti-inflammatory effect in severe cases of cystic fibrosis (Konstan and Ratjen, 2012) which may be partly due to degrading bacterial eDNA. However, treatment with DNase enzymes is not without caution as it may reduce the efficacy of host immune system extracellular traps that employ eDNA to adhere micro-organisms. In plants, exogenous DNase I reduces the innate immunity of root-tip defences in a system that shares homology to mammalian cells (Hawes *et al.*, 2011).

1.5 Microbial extracellular DNases and eDNA turnover

As eDNA is abundant in the local environment, micro-organisms have evolved to produce extracellular deoxyribonuclease enzymes. DNase enzymes catalyse the hydrolysis of the phosphodiester bond that links the phosphate of one nucleotide with the sugar 3' carbon of the next nucleotide in the chain. The cleavage of nucleotides can either occur within the DNA strand (endonuclease) or at the ends of single stranded DNA (exonuclease).

In mammals, DNase I is an endonuclease that is universally produced by pancreatic cells. It has been heavily studied, and it understood to be involved in several processes, including, digesting DNA for nutrition (Lu *et al.*, 2003), eDNA waste management (Samejima and Earnshaw, 2005), and clearing DNA during apoptosis (Oliveri *et al.*, 2001). The activity of DNase I is dependent on the presence of both Ca^{2+} and Mg^{2+}. In particular, Ca^{2+} binds tightly to DNase I and stabilises its active conformation. Without Ca^{2+}, DNase I activity is negligible (Price, 1975). DNase I activity is highest at physiological pH (7.3-7.4), and is inhibited by sodium dodecyl sulphate (SDS), ethylene glycol tetraacetic acid (EGTA) (Price, 1975), actin (Lazarides and Lindberg, 1974), and many other substances. Importantly, the activity, function, and structure of microbial DNase enzymes is incredibly varied. As a result, it is not possible to infer a great deal from DNase enzymes, unless a novel one shares close homology to already characterised nuclease enzymes. However, DNase I because it has been extensively studied does provide a basis of DNase enzyme knowledge.

Many species of micro-organisms produce DNase enzymes that are released from the cell, or are bound to the cell-wall-surface. As extracellular DNase production is genus or species specific, it has long been used as a way to diagnostically determine a cultured isolate. For instance, DNase testing (e.g. DNase test agar) is often used to distinguish *S. aureus* from coagulase-negative staphylococci. Furthermore, in the 1950s Weckman and Catlin (1957) suggested that DNase tests could be a useful characteristic to study bacterial taxonomy. These authors also found that the supernatant fluid of *S. aureus* cultures contained highest DNase activity, suggesting that they were studying non-cell-wall-bound nuclease enzymes. This interesting finding occurred because the DNase test, which determined the viscosity of DNA after incubation with culture fluids, allowed the use of broth cultures, supernatants, and cell pellets. There are many different tests for DNase activity, with the common procedure being the growth of micro-organisms on DNase test agar. This test is quick, but cannot provide detailed information such as enzyme location in relation to the cell. The FRET nuclease activity assay developed by Kiedrowski *et al*., (2011) to study *S. aureus* Nuc1 is another approach to studying extracellular DNases. This assay uses a short qPCR probe with a fluorophore attached to the 5' end, and a fluorescence quencher on the 3' end. Once the probe is cleaved by a DNase enzyme, quenching of fluorescence ceases and light emission can be quantified with a microplate reader. Other DNase tests include *in gel* zymography, and agarose gel electrophoresis after DNA digestion (Shak *et al.*, 1990).

Extracellular DNase enzymes are produced by a diverse range of micro-organisms. Fungi, such as *Cryptococcus neoformans*, can produce extracellular nucleases, although this trait is genus specific (Cazin *et al.*, 1969). A wide-range of periodontal bacteria have been tested, and 27 out of 34 produced DNase activity (Palmer *et al.*, 2012). Clinical isolates of anaerobic micro-organisms, including *Fusobacterium* spp., very commonly produce extracellular DNase enzymes (Porschen and Sonntag, 1974). Again, extracellular nuclease activity was often genus specific amongst anaerobic micro-organisms. Many species of streptococci also possess extracellular DNases, including *S. pneumoniae* (Hasegawa *et al.*, 2010), *Streptococcus pyogenes* (Zhu *et al.*, 2013), and *Streptococcus suis* (Fontaine *et al.*, 2004). Extracellular DNase production also appears to be a key characteristic of *S. aureus* (Tang *et al.*, 2011) and *P. aeruginosa* (Mulcahy *et al.*, 2010). Ultimately, it appears as though this trait of micro-organisms is very common.

Extracellular DNA is a ubiquitous macromolecule, and its ubiquity is the likely reason for the sheer diversity of micro-organisms that produce one, or more DNase

Table 1.1 Extracellular bacterial nucleases and their proposed functions.

Micro-organism	Protein	Size (aa[a])	Conserved Domains	Proposed Function	References
Streptococcus gordonii	SsnA	779	MnuA domain, OBF (1)	Unknown	This thesis
Streptococcus suis	SsnA	1041	EEP domain, OBF (3)	Virulence	(Fontaine *et al.*, 2004)
Streptococcus pyogenes	Spd1	252	Non-specific nuclease domain	Virulence	(Korczynska *et al.*, 2012)
	SpnA	910	EEP domain, OBF (3)	Virulence	(Hasegawa *et al.*, 2010; Chang *et al.*, 2011)
	Sda1	390	Non-specific nuclease domain	NET evasion	(Buchanan *et al.*, 2006)
Streptococcus pneumoniae	EndA	248	Non-specific nuclease domain	NET evasion	(Midon *et al.*, 2011; Zhu *et al.*, 2013)
Streptococcus agalactiae	NucA	261	Non-specific nuclease domain	NET evasion	(Derré-Bobillot *et al.*, 2013)
Staphylococcus aureus	Nuc1	215	SNc domain	NET Evasion and biofilm formation	(Berends *et al.*, 2010; Kiedrowski *et al.*, 2011; Beenken *et al.*, 2012)
	Nuc2	177	SNc domain	Biofilm formation and virulence	(Beenken *et al.*, 2012; Kiedrowski *et al.*, 2014)
Bacillus licheniformis	NucB	142	NucA/NucB domain	Biofilm formation	(Nijland *et al.*, 2010)

Vibrio cholerae	Dns	231	EndA domain	NET evasion, transformation and biofilm formation	(Blokesch and Schoolnik, 2008; Seper *et al.*, 2011; Seper *et al.*, 2013)
	Xds	869	EEP domain, OBF (1)	NET evasion and biofilm formation	(Seper *et al.*, 2011; Seper *et al.*, 2013)
Shewanella oneidensis	ExeM	871	EEP domain, OBF (1)	Biofilm formation	(Heun *et al.*, 2012)
	ExeS	948	MnuA domain, OBF (1)	Biofilm formation	(Heun *et al.*, 2012)
	EndA	258	EndA domain	eDNA digestion	(Heun *et al.*, 2012)
Pseudomonas aeruginosa	EddB	779	MnuA domain, OBF (1)	eDNA digestion	(Mulcahy *et al.*, 2010)
Neisseria gonorrhoeae	Nuc	233	SNc domain	Biofilm formation	(Steichen *et al.*, 2011)
Mycoplasma genitalium	MG186	250	SNc domain	eDNA digestion	(Li *et al.*, 2010)

[a]Amino acid residues

MnuA, membrane-associated nuclease of *Mycoplasma pulmonis* homologue; OBF, oligonucleotide/oligosaccharide fold homologue; EEP, exonuclease-endonuclease-phosphatase catalytic domain homologue; NucA/NucB, deoxyribonuclease NucA/NucB homologue; EndA, endonuclease I homologue; SNc, staphylococcal nuclease homologue;

enzymes. There are many proposed functions for these enzymes, and it is possible that each DNase could have a number of roles. A selection of microbial DNases with their proposed functions is listed in Table 1.1. Many of the extracellular nucleases that are produced by pathogenic bacteria have been linked to virulence. A key factor that increases the virulence of DNase producing micro-organisms is the ability to degrade neutrophil extracellular traps (NETs). These networks of DNA and some globular protein domains are produced by neutrophils, and entrap bacteria (Brinkmann *et al.*, 2004). It is therefore highly desirable for a pathogenic micro-organism to be able destroy NETs, thereby reducing their antimicrobial potential. This trait has been studied in a number of micro-organisms. When *nuc*-deficient strains of *S. aureus* USA 300 LAC were cultured in the presence of NETs they were significantly more likely to be entrapped by the DNA matrix than wild-type *S. aureus* (Berends *et al.*, 2010). *Streptococcus agalactiae* NucA can degrade NETs, but substitution of histidine^{148} by alanine impaired this function as it abolishes enzyme activity by altering the enzyme active site (Derré-Bobillot *et al.*, 2013). Cell death caused by the host immune system during disease also releases large amounts of DNA into the extracellular milieu. Extracellular DNA can be found in the lungs of cystic fibrosis patients (Shak *et al.*, 1990). It is unknown if DNase production helps disseminate micro-organisms through viscous mucus that contains DNA but biofilm dispersal has been suggested as a mechanism to promote pathogen transmission (Hall-Stoodley and Stoodley, 2005).

Interestingly, *Shewanella oneidensis* produces three extracellular nucleases, ExeM, ExeS, and EndA, with each having a distinct function. Deletion of EndA removed the ability of *S. oneidensis* to use eDNA as a source of phosphorus (Heun *et al.*, 2012). ExeS and ExeM are involved in biofilm formation, as deletion of either gene affects biofilm formation (Gödeke *et al.*, 2011). When stained with DDAO, eDNA can be visualized surrounding dense aggregations of cells in the *exeM*-deficient *S. oneidensis* biofilm. Other species of bacteria that can produce two or more DNase enzymes that are proposed to have differing roles include *S. pyogenes* (Sumby *et al.*, 2005; Buchanan *et al.*, 2006; Hasegawa *et al.*, 2010; Chang *et al.*, 2011; Korczynska *et al.*, 2012), and *Vibrio cholerae* (Seper *et al.*, 2011; Seper *et al.*, 2013). In *V. cholerae* the extracellular nuclease, Dns, is involved with transformation (Blokesch and Schoolnik, 2008). As cell densities and the quorum-sensing regulator HapR increase Dns is repressed, leading to higher transformation frequencies. However, the other *V. cholerae* extracellular DNase, Xds has been linked to biofilm modulation through the degradation of DNA (Seper *et*

al., 2011). More recently, the same authors have proposed that Xds and Dns are involved in facilitating NET escape (Seper *et al.*, 2013).

Extracellular DNA may be an important bacterial nutrient in nutrient poor environments. Although DNA is an unstable molecule (Lindahl, 1993), it can persist for long periods of time. Recently, mitochondrial DNA was sequenced from a femur belonging to a Denisovan hominin that lived 400,000 years ago (Meyer *et al.,* 2013). Therefore, eDNA is a nutrient source that bacteria can rely on, and DNase production can allow utilization of this substrate. Deoxyribonucleic acid consumption by bacteria was first observed in *E. coli* (Finkel and Kolter, 2001) and has since been shown in *Shewanella* spp. (Pinchuk *et al.*, 2008; Heun *et al.*, 2012) and *Helicobacter pylori* (Liechti and Goldberg, 2013). The Archaean species, *Haloferax volcanii*, may use intracellular DNA as a phosphorus storage polymer, and it is hypothesised by Zerulla *et al*., (2014) that DNA may have evolved for this function. It is unknown whether micro-organisms ingest biofilm matrix eDNA during periods of starvation.

If extracellular DNases are required for nutrient metabolism then they may be influenced by transcriptional regulators, in relation to available carbon sources. The major regulator of carbon source utilisation in streptococci, such as *S. gordonii*, is the carbon catabolite regulator, carbon catabolite protein A (CcpA) (Dong *et al.,* 2004). This protein down-regulates genes encoding systems for utilization of less favourable carbon sources when more energetically favourable sources are available. There is evidence that CcpA also has a role in regulation of extracellular DNases. For instance, the gene of the cell-bound DNase of *S. suis*, SsnA, contains a catabolite responsive element (CRE) that is bound by CcpA, repressing *ssnA* during the stationary phase of cell growth (Willenborg *et al.*, 2014). In *S. pyogenes* MGAS5005, the extracellular DNase, Spd, is regulated by CcpA (Shelburne *et al.*, 2008). Gene transcription of a *ccpA*-deficient strain of *S. pyogenes* was compared with strain MGAS5005 by quantitative real time PCR. In times of nutrient limitation *spd* transcription was higher in the wild-type strain than the *ccpA* mutant, indicating that in this model CcpA is an activator of Spd in glucose-limited media, such as saliva. CcpA appears to regulate extracellular DNases in different environmental conditions, which may influence a number of DNase functions, including: (i) transformation, (ii) nutrient scavenging, (iii) virulence (e.g. NET degradation), (iv) host biofilm control, (v) competitor biofilm dispersal, and (vi) protection against the deleterious effects of eDNA. It is not yet known whether CcpA regulates extracellular DNases in oral streptococci.

The role of extracellular DNases during transformation and nutrient acquisition may be linked, and it has been suggested that competence evolved to meet the nutritional demands of bacteria. Extracellular dsDNA binds to cell-wall proteins, such as ComB in *B. subtilis* (DNA uptake reviewed in detail by Dubnau, 1999). Internalization of DNA relies on it being modified from dsDNA to ssDNA through the activity of a specific nuclease, such as EndA in *S. pneumoniae* (Lacks and Neuberger, 1975). DNA transporter proteins in the cell-wall facilitate ssDNA uptake, with the energy required coming from ATP. This process is therefore reliant on specific, cell-bound nucleases, such as EndA to convert dsDNA to ssDNA. It is possible that the strand released by nuclease activity could remain in the periplasm in Gram-negative bacteria, to be utilized as a nutrient source. However, it is likely that half the DNA is lost to the surrounding medium and it therefore represents a wasteful pathway for obtaining nutrition from DNA and more likely has the singular function of gene uptake. Non-specific nucleases, including NucB in *B. subtilis*, which are released into the extracellular milieu may be more plausible candidates for DNase enzymes involved in DNA metabolism. In this manner, non-specific nucleases may degrade dsDNA into a size that is able to permeate the cell wall, or allow further enzyme activity, such as extracellular phosphatases that can remove phosphorus groups from DNA. This is a phenomenon that occurs in *Shewanella* spp. (Pinchuk *et al.*, 2008). The role of extracellular DNases in DNA uptake is likely to change depending on environmental pressures. Recently, *S. pneumoniae* EndA was shown to be secreted, and have anti-NET activity via a pathway that is independent of competence development (Zhu *et al.*, 2013). Extracellular DNA uptake is complex, and it would be interesting to study the impact that strain, species and environmental variability has on the DNA uptake machinery used.

Given the clear role of eDNA in promoting biofilm adhesion and maintaining mature biofilm stability there have been a number of studies examining the role of extracellular DNases in releasing cells from biofilms. As already discussed *S. oneidensis* produces three extracellular nucleases that alter biofilm formation (Gödeke *et al.*, 2011). Furthermore, biofilm extent is increased in *S. aureus* when *nuc1* and *nuc2* extracellular nuclease genes are deleted (Kiedrowski *et al.*, 2011; Beenken *et al.*, 2012). However, these *in vitro* experiments were not reproduced in a murine catheter model, where biofilm formation was decreased in *nuc1* and *nuc2* mutants compared to wild-type *S. aureus* (Beenken *et al.*, 2012). In addition, it has been proposed that extracellular nuclease release may be responsible for biofilm dissemination in *Bacillus licheniformis* (Nijland *et al.*, 2010). Recently, a *nucB*-deficient mutant of *B. licheniformis* was found

to produce a thicker biofilm during sporulation (Edward Mason, unpublished data). It was Akrigg and Mandelstam (1978) who first demonstrated that *B. subtilis* produces a DNase, NucB, during sporulation. It is thought that *B. subtilis* NucB degrades DNA released by mother cell lysis, at the latter stages of sporulation (Hosoya *et al.*, 2007), and this may share homology with *B. licheniformis* NucB. Although the effects of extracellular DNases against the host micro-organism have been studied it is so far unknown if these enzymes are used to compete against other micro-organisms in mixed-species biofilms. Many micro-organisms rely on eDNA for promoting biofilm formation, and it therefore seems plausible that bacteria such as *S. aureus* could reduce the colonisation of eDNA-producing bacteria.

Extracellular DNases may also protect against the deleterious effects of eDNA on microbes. For instance, eDNA can bind and sequester divalent metal cations, like Mg^{2+} and Ca^{2+}. Removal of these cations from the bacterial cell surface, by eDNA, can lead to cell lysis (Mulcahy *et al.*, 2010). Furthermore, bacterial DNA can alert the host immune system to the presence of foreign micro-organisms (Hemmi *et al.*, 2000). Therefore, invading bacteria could mask themselves by degrading their own eDNA. Lastly, eDNA inhibits biofilm development in *Salmonella enterica* (Wang *et al.*, 2013). Biofilm formation was restored when abiotic surfaces were treated with DNase I.

In conclusion, persistence of DNA in the environment has led to many possible functions for extracellular DNase enzymes. The most important roles of these enzymes are likely facilitating NET degradation, consumption of DNA as a nutrient source, and regulating biofilm formation. A neglected aspect of research is biofilm competition. For instance, many periodontal bacteria produce DNase enzymes (Palmer *et al.*, 2012), and this could re-shape the mixed-species biofilms that form in the oral cavity.

1.6 Biofilm removal with DNase enzymes

Given the importance of eDNA in allowing adhesion of many microbes to surfaces, and providing matrix support in mature biofilms, it is a logical approach to try and eradicate biofilms with DNase enzymes. Biofilm-forming micro-organisms produce many anti-biofilm molecules, like DNases, that can inhibit or disperse other microbial species (reviewed by Rendueles and Ghigo, 2012). These enzymes are perfectly adapted for the degradation of extracellular matrix components, including eDNA. Therefore, it may be possible to develop novel biofilm detachment antimicrobials by studying biofilm competition and enzyme release.

An example of a naturally produced matrix degrading enzyme is dispersin B, which is produced by *Aggregatibacter actinomycetemcomitans* (Kaplan *et al.*, 2003). This enzyme degrades poly-N-acetylglucosamine (PNAG), a polysaccharide component of some biofilm matrices, such as *A. actinomycetemcomitans*, and *S. epidermidis* (Kaplan *et al.*, 2003; Kaplan *et al.*, 2004). *Escherichia coli* is able to reduce the initial attachment of micro-organisms though the release of group II capsule polysaccharides (Valle *et al.*, 2006). Many more naturally produced anti-biofilm proteins exist but DNase enzymes show particular promise. One advantage of DNase removal of micro-organisms is that it can act both during initial formation, and once biofilms are mature, due to the importance of eDNA throughout the biofilm life-cycle. Furthermore, the structure of DNA is ubiquitously conserved throughout nature, and therefore DNase enzymes do not suffer from specificity issues. Molecules like dispersin B only degrade the biofilm matrices of organisms that contain PNAG. If eDNA exists in a biofilm matrix, a DNase enzyme will disperse it, as long as the eDNA is not protected by another mechanism.

Some biofilm-forming micro-organisms release extracellular nucleases that can inhibit or disperse other microbial species. For instance, a nuclease produced by *S. aureus*, Nuc1, reduces biofilms formed by *Haemophilus parasuis*, *Actinobacillus pleuropneumoniae*, and *P. aeruginosa* (Tang *et al.*, 2011). Recently, a marine strain of *B. licheniformis* isolated from the surface of seaweed was found to produce a microbial extracellular nuclease (Nijland *et al.*, 2010). This nuclease, NucB, is suggested to have evolved for the purpose of host biofilm dispersal but also disperses biofilms of *E. coli*, *Micrococcus luteus* and *B. subtilis*. Both Nuc1 and NucB may play important roles in mixed-species biofilm competition.

Several DNase enzymes have been used to study the role of eDNA in biofilm formation. At least 43 microbial species have been cultured as biofilms and subjected to DNase treatment, either during adhesion or after maturation (Table 1.2). Ultimately, 90% of these species were shown to be sensitive to DNase treatment (publication bias is an important factor), underlining the significant proportion of biofilm-forming micro-organisms that produce eDNA for biofilm stabilisation and attachment. Therefore, research has in some cases looked at the potential to apply DNase enzymes to treating biofilm healthcare issues. Interestingly, Pulmozyme®, a human recombinant DNase (rhDNase), has been used in the treatment of cystic fibrosis (CF) since the mid-1990s.

Table 1.2 Examples of microbial biofilms that have been treated with exogenous DNases.

Species	Observations	DNase Enzyme	References
Gram-positive bacteria			
Aggregatibacter actinomycetemcomitans	Biofilms grown on glass coverslips were dispersed by DNase treatment	DNase I	(Inoue *et al.*, 2003)
Bacillus cereus	Biofilm formation requires eDNA	DNase	(Vilain *et al.*, 2009)
Bacillus licheniformis	Dispersal of 26-h-old biofilms	NucB, DNase I	(Nijland *et al.*, 2010)
Bacillus subtilis	Mature biofilm dispersal	NucB	(Nijland *et al.*, 2010)
Enterococcus faecalis	Reduced accumulation of 26-h-old biofilms	DNase I	(Thomas *et al.*, 2008)
Lactobacillus plantarum	24-h-old biofilm reduced by 50% when treated	DNase I	(Muscariello *et al.*, 2013)
Listeria monocytogenes	Attachment of biofilm cells reduced, mature biofilm dispersed	DNase I	(Harmsen *et al.*, 2010)
Micrococcus luteus	Mature biofilm dispersal	NucB	(Nijland *et al.*, 2010)
Rhodococcus ruber	Inhibition of up to 25%, no dispersal	DNase I	(Gilan and Sivan, 2013)
Staphylococcus aureus	Decreased biofilm formation, and mature biofilm dispersal	NucB, DNase I, DNase1L2, Varidase, rhDNase I	(Nemoto *et al.*, 2000; Eckhart *et al.*, 2007; Rice *et al.*, 2007; Tetz *et al.*, 2009; Tetz and Tetz, 2010; Tang *et al.*, 2011; Kaplan *et al.*, 2012; Shields *et al.*, 2013; Swartjes *et al.*, 2013)

Staphylococcus epidermidis	Biofilm formation inhibited at 10 mg/mL	rhDNase I	(Kaplan *et al.*, 2012; Shields *et al.*, 2013)
Staphylococcus haemolyticus	All of the 53 biofilm-producing isolates were detached when treated	DNase I	(Fredheim *et al.*, 2009)
Streptococcus anginosus	Static biofilms dispersed after 18 h incubation	NucB	(Shields *et al.*, 2013)
Streptococcus constellatus	Biofilm extent reduced when treated	NucB	(Shields *et al.*, 2013)
Streptococcus intermedius	Biofilm formation reduced by 52% in presence of DNase	DNase I	(Petersen *et al.*, 2004)
Streptococcus mutans	16-h-old biofilm biomass was reduced by 20-30%	DNase I	(Perry *et al.*, 2009)
Streptococcus pneumoniae	6-h-old, 18-h-old, 6-day-old biofilms dispersed	NucB, DNase I, Pulmozyme®	(Moscoso *et al.*, 2006; Hall-Stoodley *et al.*, 2008; Shields *et al.*, 2013)
Streptococcus pyogenes	Dispersal of 24-h-old biofilms	DNase I	(Tetz *et al.*, 2009)
Streptococcus salivarius	18-h-old biofilms dispersed	NucB	(Shields *et al.*, 2013)
Gram-negative bacteria			
Actinetobacter baumanii	DNase treatment resulted in decrease biofilm biomass	bpDNase I	(Tetz *et al.*, 2009)
Actinobacillus pleuropneumoniae	Up to 50% dispersal of static biofilms cultured for 36 h	NUC1, DNase I, MNase	(Tang *et al.*, 2011)

Bdellovibrio bacteriovorus	Biofilm detached when treated, also inhibited	DNase I	(Medina and Kadouri, 2009)
Bordetella bronchiseptica	Static, flowing and mouse respiratory tract biofilms reduced when treated	DNase I	(Conover *et al.*, 2011)
Bordetella pertussis	Static, flowing and mouse respiratory tract biofilms reduced when treated	DNase I	(Conover *et al.*, 2011)
Burkholderia cenocepacia	Treatment led to increased biofilm thickness	Pulmozyme®	(Novotny *et al.*, 2013)
Campylobacter jejuni	Decrease in 2-day-old biofilms	DNase I	(Svensson *et al.*, 2009)
Comamonas denitrificans	Inhibited initial biofilm attachment	DNase I	(Andersson *et al.*, 2009)
Escherichia coli	24-h-old biofilm dispersal by up to 70%, decreased tolerance to antibiotics	NucB, bpDNase I	(Tetz *et al.*, 2009; Nijland *et al.*, 2010)
Fusobacterium nucleatum	Biofilms were not disersed in the model used	DNase I	(Ali Mohammed *et al.*, 2013)
Haemophilus influenzae	Biofilm biomass reduced in 24 and 6 h biofilms	bpDNase I	(Izano *et al.*, 2009; Tetz *et al.*, 2009)
Haemophilus parasuis	Up to 40% dispersal of static biofilms cultured for 36 h	NUC1, DNase I, MNase	(Tang *et al.*, 2011)
Klebsiella pneumoniae	Treatment reduced biofilm biomass at 24 h	DNase I	(Tetz *et al.*, 2009)
Myxococcus xanthus	Efficacy of DNase treatment was increased when combined with SDS, or sonication	DNase I	(Hu *et al.*, 2012)
Neisseria gonorrhoeae	Mature biofilm dispersal	Nuc	(Steichen *et al.*, 2011)
Neisseria meningitidis	Biofilm formation was inhibited in 31 out of 50	DNase I	(Lappann *et al.*, 2010)

	meninogococcal strains tested		
Porphyromonas gingivalis	Biofilms were not disersed in the model used	DNase I	(Ali Mohammed *et al.*, 2013)
Pseudomonas aeruginosa	Biofilms younger than 60 h are reduced	Varidase, DNase I, DNase1L2, lsDNase, Nuc1	(Whitchurch *et al.*, 2002; Nemoto *et al.*, 2003; Eckhart *et al.*, 2007; Parks *et al.*, 2009; Tang *et al.*, 2011; Brown *et al.*, 2012; Swartjes *et al.*, 2013)
Rhodovulum sp.[a]	Flocculated cells dispersed by DNase treatment	bpDNase I	(Watanabe *et al.*, 1998)
Salmonella enterica	Initial biofilm formation (6 h to 48 h) increased in presence of DNase	DNase I	(Wang *et al.*, 2013)
Shewenlla oneidensis	Biofilms cultured in static and flowing models were disrupted by treatment	DNase I	(Gödeke *et al.*, 2011)
Vibrio cholerae	Treatment reduced colonisation at 24 h, but not 72 h	DNase I	(Seper *et al.*, 2011)
Gram-variable bacteria			
Gardnerella vaginalis	Biofilm formation was inhibited by 80%, and mature biofilms dispersed by 50%	bpDNase I	(Hymes *et al.*, 2013)
Fungi			
Aspergillus fumigatus	24-h-old biofilms were destabilized when treated	bpDNase I	(Rajendran *et al.*, 2013)
Candida albicans	Biofilm formation reduced	bpDNase I	(Martins *et al.*, 2010; Pammi *et al.*,

			2013)
Mixed-species biofilms			
C. albicans and *S. epidermidis*	Dual-species biofilms were more susceptible to DNase treatment than mono-species	DNase I	(Pammi *et al.*, 2013)
TESV Biofilms	Biofouling micro-organisms were liberated from the surface of valves	NucB	(Shakir *et al.*, 2012)
Biofilms in activated sludge	DNase treatment resulted in dissolution of microbial flocs	DNase I	(Dominiak *et al.*, 2011)

[a]Flocculated cells were treated, not cultured as a biofilm.

bpDNase I, bovine pancreatic DNase I; rhDNase I, recombinant human DNase I; DNase I, exact origins unknown; DNase1L2, DNase 1-like 2 produced by epidermal keratinocytes; Varidase, streptokinase and streptodornase (streptococcal DNase) solution; MNase, micrococcal nuclease; NucB, extracellular nuclease of *B. licheniformis*; Nuc, extracellular nuclease of *N. gonorrhoeae*; NUC1, staphylococcal nuclease; lsDNase, *Lucilia sericata* nuclease; Pulmozyme®, proprietary name for dornase alfa, a human recombinant DNase I used in cystic fibrosis treatment.

Cystic fibrosis is characterized by airways obstruction caused by thick mucus secretions. These secretions contain DNA, which contributes to the viscosity of the mucus. As a result, inhalation of Pulmozyme® by CF sufferers can lead to increased lung function and reduced antibiotic demand (Frederiksen *et al.*, 2006). However, recent research suggests that *Burkholderia cenocepacia* biofilms mature to be thicker in the presence of Pulmozyme® (Novotny *et al.*, 2013). It is unknown what implications this has for Pulmozyme® treatment as the majority of DNA in CF lung mucus is human in origin (Lethem *et al.*, 1990). Therefore, the fact that Pulmozyme® is a human DNase may mean it is better suited to the degradation of human DNA. Recombinant human DNase I has shown efficacy against some biofilm-forming microbes (Hall-Stoodley *et al.*, 2008; Kaplan *et al.*, 2012).

Another example of DNase treatment that pre-dates the Whitchurch *et al*., (2002) paper is the use of Varidase® to treat chronic wound infections. The efficacy of Varidase® is based on the combination of a DNase (streptodornase) and a plasminogen activator (streptokinase). Due to the presence of a DNase enzyme, Varidase® has been shown to reduce biofilm colonisation of *S. aureus* and *P. aeruginosa in vitro* (Nemoto *et al.*, 2000; Nemoto *et al.*, 2003). Varidase® has shown efficacy in wound healing (Poulsen *et al.*, 1983) but its quality and composition (streptodornase and streptokinase are a small proportion of total protein content) has been questioned. It is no longer widely available, and it appears to have limited efficacy in wound treatment (Steed, 2004). The use of a Blow fly, *Lucilia sericata*, nuclease is another example of DNase treatment potential in wound healing (Brown *et al.*, 2012). Blow fly larvae are used in wound debridement, and therefore it is a logical approach to test the extracellular enzymes it produces against microbial biofilms. Established biofilms of a *P. aeruginosa* wound isolate were dispersed by *L. sericata* DNase (Brown *et al.*, 2012). Further validation, for instance in an *in vivo* model, is required before it is used a treatment aid.

A potentially important characteristic of DNase enzymes is their ability to act synergistically with antibiotics. Within a biofilm, microbial cells are significantly more resistant to antimicrobial agents (Stewart and Costerton, 2001). Therefore, the release of cells by a DNase could increase the efficacy of antibiotics. Furthermore, eDNA binds positively charged antimicrobials such as aminoglycosides, which reduces their diffusion through the extracellular matrix (Chiang *et al.*, 2013). DNase enzymes have been shown to increase antimicrobial efficacy in a number of biofilm systems, including bacteria and fungi (Tetz *et al.*, 2009; Kaplan *et al.*, 2012; Martins *et al.*, 2012; Ratner and Hymes, 2013).

There are a number of studies that have shown DNase activity against microbial biofilms. Of the forty-three species studied to date only *Fusobacterium nucleatum*, *Porphyromonas gingivalis*, *Burkholderia cenocepacia*, and *Salmonella enterica* have exhibited no change in extent or thicker biofilm biomass when treated with DNase (Ali Mohammed *et al.*, 2013; Novotny *et al.*, 2013; Wang *et al.*, 2013). It is unknown how publication bias, towards positive results, has influenced the number of DNase-resistant microbial biofilms reported. Increasingly, research is also studying the role of eDNA in *in vivo* biofilms and mixed-species models, as there has been a tendency to study in mono-species models where biofilms form on abiotic surfaces. Recently, DNase I was shown to have increased efficacy against *Candida albicans* and *S. epidermidis* biofilms when grown as a dual species biofilm in a mouse subcutaneous catheter mouse model (Pammi *et al.*, 2013). Furthermore, DNase treatment has disseminated biofilms in activated sludge (Dominiak *et al.*, 2011). In another *in vivo* model, of the mouse respiratory tract, *Bordetella* spp. nasal biofilms have been shown to be sensitive to DNase treatment (Conover *et al.*, 2011). These promising studies show the potential of nuclease enzymes in complex biofilms, and hopefully will be expanded in the next 5 years.

Although many DNase enzymes exist and have been shown to have efficacy against microbial biofilms, NucB from *B. licheniformis* EI-34-6 (Nijland *et al.*, 2010) has a number of potential benefits compared with mammalian enzymes. Human recombinant DNases require glycosylation to be active, thermally stable, and have resistance to proteolysis, therefore meaning that they cannot be produced in bacterial protein expression systems (Fujihara *et al.*, 2008). In contrast, NucB can be produced in large quantities, using a method that has been optimized (Rajarajan *et al.*, 2013). Producing pure DNase enzymes is important, as shown with Varidase®, where a lack of purity led to treatment concerns. NucB has been produced to a point where it is 95% pure (Prof. Alastair Hawkins, unpublished data). Importantly, optimal conditions (pH 8, 5 mM Mn^{2+} cations, 37°C) for NucB enzyme activity have been quantified, which will improve efficacy against microbial biofilms. Differential scanning calorimetry analysis has shown that NucB is thermally stable to a temperature of approximately 57°C (Prof. Alastair Hawkins, unpublished data). Interestingly, NucB is capable of re-folding once cooled from 80°C to 25°C. Nuclease activity, using a salmon sperm DNA substrate, was ca. 40% once cooled, compared to the original non-heated NucB. Although activity was lower once heated, in contrast, DNase I does not appear capable of any re-folding once heated to its melting point of 67.4°C in pure water (Chan *et al.*, 1996). Recently, NucB

was included in a micronucleus toxicity assay (Cyprotex, UK) in CHO-K1 (Chinese hamster ovary) cells and found to not be deleterious to cell viability at a concentration used for biofilm dispersal (5 µg/mL) (Unpublished work). Lastly, NucB has likely evolved for the purpose of biofilm dispersal, and this may explain the greater efficacy it has against *B. licheniformis* biofilms than DNase I (Nijland *et al.*, 2010). Dispersal of microbial biofilms with NucB was also incredibly rapid, taking only 2 minutes. As antimicrobial resistance is a growing concern, new anti-biofilm agents like NucB are becoming increasingly important. With research, NucB could become important in tackling medically relevant biofilm issues.

1.7 Medical device associated biofilm infections

Artificial devices used to improve human quality of life were the first to be recognised as being colonised by microbial biofilms (Hall-Stoodley *et al.*, 2004). Biofilm growth on an artificial device is widely referred to as biofouling. Many medical devices are known to be colonised by microbial biofilms, and these include hip replacements, prosthetic joints, catheters, heart valves, and pacemakers (reviewed by Donlan, 2001). Any device that is in contact with the human body has the potential to become colonised. However, medical devices are an indispensable element of modern healthcare, particularly through managing critically ill patients. For instance, the central venous catheter (CVC) is used for a combined 15 million days per year in the USA alone (von Eiff *et al.*, 2005). Obviously, the microbial biofilms that form on these devices have the potential to create disease. They also deteriorate devices quicker than would occur if they were biofilm-free. Micro-organisms become irreversibly bound to medical devices ensuring that replacement, with subsequent costs and increased staff workload, is the only option to stop infection. Therefore, microbial biofouling on artificial medical devices is a major issue in healthcare today.

Many of the infections are nosocomial in origin but can originate from the skin of patients, poor cleaning of devices and other environmental sources (Donlan, 2001). Due to the prevalence of these biofilms they have been termed as chronic polymer-associated infections. Commonly these infections are caused by staphylococci (principally *S. aureus* and *S. epidermidis*), *P. aeruginosa*, *E. coli*, *C. albicans* and *Klebsiella pneumoniae*. However, many more micro-organisms have been isolated from medical devices. To date, despite numerous efforts to reduce biofilm colonisation of artificial

medical devices (reviewed by Francolini and Donelli, 2010) there still remains a great deal of progress to be made.

It is currently unknown whether eDNA contributes to medical-device-associated biofilm stability or attachment. Furthermore, DNase treatment, although a potentially important therapeutic step in treating these infections, has to our knowledge never been tested against medical device biofilms. Nuclease treatment could be of benefit because it could either inhibit biofilm formation, or be applied as a cleaning solution for biofilm dispersal.

1.7.1 Tracheoesophageal speech valve biofouling

Since the introduction of the silicone rubber tracheoesophageal speech valve (TESV) (Figure 1.4), developed by Blom and Singer (Singer and Blom; Singer *et al.*, 1981) in the 1970s, voice rehabilitation in patients who have had a total laryngectomy has been revolutionised. Several different prostheses exist but all are made from silicone rubber and work in a similar way; allowing air into the oesophagus for phonation whilst preventing food from entering the respiratory tract via the neopharynx. The TESV is placed in a tracheoesophageal fistula that is created during surgery. However, because these devices are placed in a non-sterile environment they quickly-become colonized by micro-organisms. *Candida albicans*, a common opportunistic pathogen, is often involved with TESV biofouling (Bauters *et al.*, 2002). Microbial colonization results in the need to remove TESVs from patients every 3-6 months (Hancock *et al.*, 2013; Kress *et al.*, 2013). Silicone deterioration due to microbial biofilms leads to TESV leakage (Balm *et al.*, 2011), tracheal irritation (Acton *et al.*, 2008), and increased difficulty in producing speech (Elving *et al.*, 2003). Two distinct versions of the TESV exist. The non-indwelling voice prosthesis can be removed by the patient, whereas the indwelling type has to be removed by a speech therapist or clinician. Indwelling TESVs have a longer lifespan and have become the favoured method, particularly the system manufactured by Provox, in European countries (Balm *et al.*, 2011). Although the indwelling system requires a hospital visit the replacement of the TESV is a quick, minor procedure that can be completed in an outpatient setting. Non-indwelling devices are easier to use, and can be regularly patient-maintained, but are more likely to become colonized by micro-organisms through handling. At the Freeman Hospital, Newcastle, TESVs are purchased from Blom-Singer, and both indwelling and non-indwelling devices are used by patients.

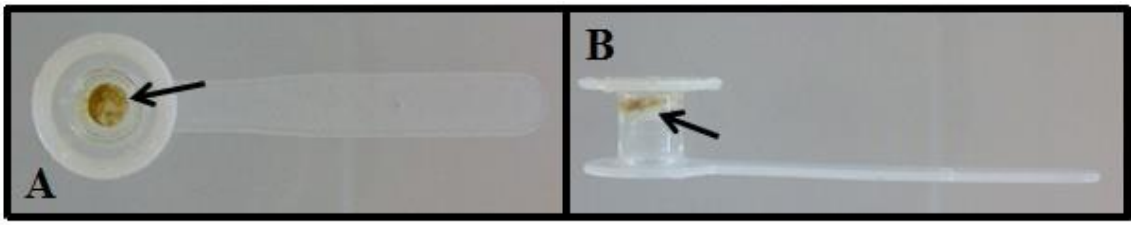


Figure 1.4 A heavily fouled tracheoesophageal speech valve. The TESV was viewed from the posterior (A) and lateral sides (B). Black arrows indicate a dense biofilm that has formed on the silicone surface.

Patients who have previously undergone radiotherapy experience a shorter lifespan of valves of up to one month less on average (Eerenstein *et al.*, 1999). Irradiated users produce less saliva and carry more *Candida* spp. in the oropharynx, which may explain quicker device replacement (Martin *et al.*, 1981). The production of less saliva may aid bio-deterioration as it has been demonstrated that saliva reduces the bond of microbes to silicone surfaces (Busscher *et al.*, 1997). Many patients who require voice prostheses will have received radiotherapy for the cancer of the larynx or hypopharynx that required a total laryngectomy. There are many complications to consider with TESV patient use.

It is unlikely that a single bacterial or fungal species would reside on a TESV. However, *C. albicans* is often considered the key colonizer of voice prostheses (Eerenstein *et al.*, 1999). Other *Candida* spp., like *Candida glabrata*, are also isolated from TESVs (Bauters *et al.*, 2002). It is also clear that commensal bacteria, which reside on the skin, or the oral cavity, are often present on speech valves. These micro-organisms include staphylococci, oral streptococci, and lactobacilli (Buijssen *et al.*, 2012). For example, *Lactobacillus* spp., were found on 97% of explanted TESVs by Buijssen *et al.*, (2007). Bacteria have increased importance as they are known to increase the adherence of *C. albicans* to surfaces (Holmes *et al.*, 1995; Holmes *et al.*, 1996). Interestingly, the adhesion of *C. albicans*, *Candida krusei* and *Candida tropicalis* to silicone rubber was less when bacteria were present (Millsap *et al.*, 2001). However, FISH analysis of TESV biofilm architecture has shown interactions between streptococci, lactobacilli and fungi (Buijssen *et al.*, 2012). *Micrococcus luteus* and *Klebsiella oxytoca* speech valve isolates attached to *C. albicans* cells *in vivo* (Kania *et al.*, 2010). It is unknown if the species composition of TESV biofilms differs between indwelling and non-indwelling devices.

Currently there are no methods for cleaning indwelling TESVs, and non-indwelling devices, although cleanable, still require replacement every 3-5 months (Van Den Hoogen *et al.*, 1996). Many previous attempts have been made to control the development of biofilms on voice prostheses: use of antibiotics and antifungal agents, adjusting the surface properties of the silicone rubber, probiotic nutrition, synthetic saliva, and biosurfactants (reviewed by Rodrigues *et al.*, 2007). Although TESVs are often colonised by fungi, and the use of antifungals like amphotericin B increases speech valve lifespan (Mahieu *et al.*, 1986), they are not routinely used because of microbial resistance worries. Consumption of buttermilk appears to reduce biofilm formation on TESVs (Busscher *et al.*, 1998). However, its use is limited as it requires 2

litres of buttermilk to be ingested daily. Biosurfactants isolated from *Lactococcus lactis* and *Streptococcus thermophilus* have been shown to decrease adherence of micro-organisms to silicone rubber in an artificial throat model (Rodrigues *et al.*, 2004). Biosurfactants are used by bacteria, like *Streptococcus mitis*, to compete with other biofilm producers, to stop them adhering nearby (van Hoogmoed *et al.*, 2000). Further novel techniques for inhibiting or dispersing TESV biofilms are desirable.

The colonization of TESVs by microbial biofilms provides an interesting model for biofilm research. Patients regularly replace the devices so they are relatively easy to obtain. Furthermore, they are colonized by important opportunistic pathogens and commensal micro-organisms. For the study of eDNA, TESV biofouling could prove particularly useful. The contribution of eDNA to mixed-species biofilms has been under-researched to date. A number of species associated with TESV biofouling have been shown to produce eDNA. For instance, extracellular DNA is present in *C. albicans* SC5134 biofilm matrixes and treatment with bovine DNase I decreases biofilm biomass (Martins *et al.*, 2010). Further research by Martins *et al*., (Martins *et al.*, 2012) showed increased efficacy of the antifungals, amphotericin B, and caspofungin, against *C. albicans* when biofilms were treated with DNase I. Other biofilm-forming species associated with TESV biofouling that are susceptible to DNase treatment include, *Staphylococcus* spp., streptococci, and lactobacilli (Qin *et al.*, 2007; Perry *et al.*, 2009; Kaplan *et al.*, 2012; Muscariello *et al.*, 2013). These are promising results although it is unknown how significantly eDNA contributes to a TESV biofilm. Multi-species biofilms in activated sludge have been dispersed with DNase treatment (Dominiak *et al.*, 2011), as have dual-species biofilms of *C. albicans* and *S. epidermidis* (Pammi *et al.*, 2013) but DNase treatment of TESVs could provide additional proof for the importance of eDNA in polymicrobial biofilms.

Biofilm formation is detrimental to TESVs and this has an impact on patients and healthcare services. Therefore, if proven to have efficacy, DNase treatment of TESVs could improve their use. Nuclease enzymes could be applied in two ways; either directly coating the voice prosthesis, or adding to a cleaning fluid. Many patients use voice prostheses that can be independently cleaned but whilst a cleaning product may be successful patients may not always comply with a cleaning routine. Furthermore, patients are unable to clean indwelling voice prostheses. A coating that included a nuclease, such as NucB, could prove a more powerful method of reducing TESV biofilm colonisation. Recently, Swartjes *et al*., (2013) showed that coating polymethylmethacrylate with DNase I can reduce adhesion of *S. aureus* and *P.*

aeruginosa by over 90%. However, enzyme activity began to reduce after 8 hours, and therefore further research is required to improve DNase coating efficacy over longer periods.

1.8 Chronic biofilm infections on living tissues

The list of infections known to be caused by persistent microbial biofilms is vast: otitis media, CRS, periodontitis, native valve endocarditis, cystic fibrosis, pneumonia, bacterial prostatitis, muscoskeletal infections, biliary tract infections and many more (reviewed by Costerton *et al.*, 1999). Biofilms are up to 1,000-fold more resistant to antibiotics than planktonic infections (Gilbert *et al.*, 1997). Furthermore, when planktonic bacteria disseminate from the biofilm, for instance from dialysis catheters, the infection can spread around the body, causing bacteremia and other secondary problems (Allon, 2004). The human immune system will very rarely resolve biofilm infections, even in healthy individuals (Stewart and Costerton, 2001). With no universal cure these diseases are a 21st century medical challenge.

It is unknown what contribution eDNA makes to biofilm stability in clinically relevant biofilms. Given that eDNA is a ubiquitous biofilm attachment and stability macromolecule, it may be that DNase enzyme treatment could have a positive influence on many biofilm infections. An infectious disease with an established biofilm link is discussed next.

1.8.1 Chronic rhinosinusitis

Of the many human infections associated with biofilms, CRS is one of the most common, resulting in 18 to 22 million visits to general practitioners in the USA per year (Benninger *et al.*, 2003). Furthermore, CRS affects approximately 10% of the adult European population (Hastan *et al.*, 2011). Rhinosinusitis is an inflammation of the mucous membrane that lines the paranasal sinuses. The disease is divided into several categories, largely based on the length of time a patient has experienced symptoms of rhinosinusitis. Acute and recurrent acute rhinosinusitis last up to four weeks, and may consist of many episodes (between disease-free states) in the latter case. The transition to CRS is known as subacute rhinosinusitis and is defined as rhinosinusitis that has lasted four to twelve weeks. Rhinosinusitis is deemed ‘chronic’ if it has lasted twelve

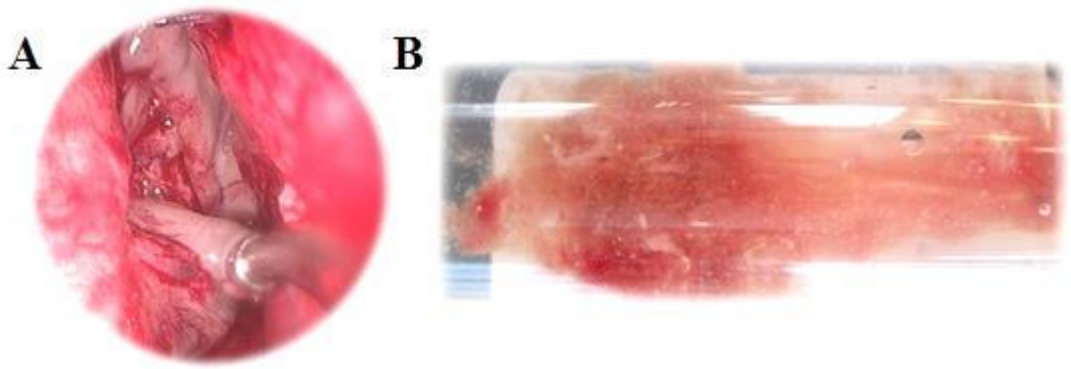


Figure 1.5 Functional endoscopic sinus surgery. Chronic rhinosinusitis mucus is aspirated during FESS (A) and placed in a test tube filled with reduced transport fluid (B).The aspirate is a tenacious, thick, bloody mucus, although the appearance varies widely between patients.

consecutive weeks or longer. The symptoms patients experience are similar, and as a result the disease can be hard to classify, which is why classification is defined chronologically. In addition, the location of the infection can be based in one or more of the paranasal sinuses. There are several paranasal sinuses, including, maxillary, frontal, ethmoidal, and sphenoidal sinuses.

Treatment of the infection begins with medical therapy and only in severe cases is surgical therapy recommended (Ragab *et al.*, 2004). Wood and Douglas (2010) advocate 3 week medical treatment with a corticosteroid (prednisone) and an antibiotic (e.g. doxycycline). After this period, patients use a sinus douche to administer saline, and also use a corticosteroid spray. Ultimately, if symptoms still persist after this regime, surgery is instructed. Although antibiotics are favoured by clinicians there is little evidence for their efficacy (Lim *et al*., 2010). Macrolides, such as roxithromycin, may be beneficial because of their anti-inflammatory effect (Wallwork *et al.*, 2006). Furthermore, the use of the antifungal amphotericin B improved symptoms in 75% of patients tested (Ponikau *et al.*, 2002). Nasal lavage with amphotericin B has been shown to have no treatment efficacy (Ebbens *et al.*, 2006). Chronic rhinosinusitis is challenging to treat because it caused by a number of factors (discussed in the next paragraph). Surgical therapy, primarily the non-invasive procedure, functional endoscopic sinus surgery (FESS) (Figure 1.5A), is carried out in hard to treat cases, to open up the airways and relieve some symptoms. However, the efficacy of this treatment is unproven (Khalil and Nunez, 2006). An example of the material removed during FESS is shown in Figure 1.5B.

In all cases CRS is an inflammatory disease. However, the cause of the inflammation can be due to many factors, and it is likely that CRS is an overarching name for a number of different diseases with similar symptoms. For instance, the development of CRS has been linked to smoking (Hastan *et al.*, 2011), allergies (Perez-Novo *et al.*, 2005), viruses (Benninger *et al.*, 2003), fungi (Lanza *et al.*, 2006), bacteria (Benninger *et al.*, 2003), staphylococcal super antigens (Seiberling *et al.*, 2005), and underlying systemic diseases (Benninger *et al.*, 2003). Increasingly over the last decade bacterial and fungal biofilms have been recognised as a major cause of CRS, with increasing evidence from several authors (see Table 1.3). It is important to note that microbial biofilms are not the sole cause of CRS, but it appears they are a significant factor in the pathogenesis of CRS. Furthermore, the presence of biofilms is independent of other clinical factors like allergies (Zhang *et al.*, 2011).

Table 1.3 Bacterial biofilms on clinical mucosal specimens removed from sufferers and non-sufferers of chronic rhinosinusitis.

Reference	Imaging Method	CRS Mucosa	Control Mucosa
(Bezerra *et al.*, 2011)	SEM	24/33 (73%)	13/27 (48%)
(Healy *et al.*, 2008)	FISH-epifluorescent microscopy	9/11 (82%)	2/3 (67%)
(Hochstim *et al.*, 2010)	Hematoxylin-eosin staining	15/24 (63%)	1/10 (10%)
(Foreman *et al.*, 2009)	FISH-CLSM	36/50 (72%)	0/10 (0%)
(Psaltis *et al.*, 2007)	CLSM	17/38 (45%)	0/9 (0%)
(Ramadan *et al.*, 2005)	SEM	5/5 (100%)	ND[a]
(Sanclement *et al.*, 2005)	SEM	24/30 (80%)	0/4 (0%)
(Sanderson *et al.*, 2006)	FISH-epifluorescent microscopy	14/18 (78%)	2/5(40%)

[a]ND, not determined.

SEM, scanning electron microscopy; FISH, fluorescence *in situ* hybridization; CLSM, confocal laser scanning microscopy.

Evidence of microbial biofilms on the surface of sinus mucosa is produced through microscopic analysis of mucosal biopsies. A range of techniques have been used to visualise micro-organisms in chronically infected sinuses, including, FISH-CLSM (Foreman *et al.*, 2009), hematoxylin-eosin staining (Hochstim *et al.*, 2010), and SEM (Sanclement *et al.*, 2005). They are present on the sinus mucosa of CRS patients in 45-100% of cases (Table 1.3) (Foreman *et al.*, 2012). The most convincing visual studies of biofilms on CRS patient mucosa is via the use of FISH stains, as these are selective for prokaryotic or eukaryotic organisms. Bacteria appear as punctate dots against large eukaryotic cells (Shields *et al.*, 2013). Further evidence for the biofilm pathogenesis of CRS was revealed through testing isolated micro-organisms for their ability to form biofilms *in vitro*. Prince *et al*., (2008) cultured sinonasal aspirates in a Calgary Biofilm Detection Assay (CBDA) and 45 out of 157 were positive for biofilm formation. Again, biofilm formation was linked to surgical interventions. The CBDA has also been used more recently, with biofilm formation shown in 21% of patient samples (Zhang *et al.*, 2011). Lastly, the simple microplate crystal violet assay has also been used to show biofilm formation in bacteria isolated from the paranasal sinuses of CRS patients (Bendouah *et al.*, 2006). It is important to consider that the threshold set for biofilm formation will have a strong influence on the amount of biofilm-forming micro-organisms reported and there is no gold standard approach for this.

Although micro-organisms are implicated in the pathogenesis of CRS, the bacterial flora of healthy and diseased paranasal sinuses are largely similar (Araujo *et al.*, 2007). There is a high prevalence of coagulase-negative staphylococci, *Streptococcus* spp., and *S. aureus* in both groups. More recently, culture-independent techniques have been used to study the microflora of CRS (Stressmann *et al.*, 2011; Feazel *et al.*, 2012; Boase *et al.*, 2013). Although molecular methods have greater detection sensitivity, culture-dependent and culture-independent techniques appear to demonstrate similar micro-organisms in CRS disease (Feazel *et al.*, 2012). It is unclear if specific pathogens are involved in disease manifestation. Pathogenic species like *S. aureus*, *M. catarrhalis*, *P. aeruginosa*, and *S. pneumoniae* can be more prevalent in CRS patient sinuses (Boase *et al.*, 2013). However, it seems increasingly likely that a biofilm phenotype contributes to the symptoms of CRS, and it is unknown how biofilm formation begins. Although it is important to note that biofilms are found on the surface of healthy sinus mucosa (Table 1.3).

There are still a number of aspects of CRS microbiology that are unclear, including the prevalence of anaerobic micro-organisms (Ramadan *et al.*, 2002) and the role of

fungi in facilitating disease (Ebbens *et al.*, 2009). Fungi are either cultured in high incidence (Ponikau *et al.*, 1999), or detected in few cases (Araujo *et al.*, 2007). The fungal pathogenesis of CRS is distinct to biofilm-associated CRS, and probably affects a minor proportion of sufferers of the disease. Similarly, anaerobic micro-organism carriage is either found to be in a high number of cases (Brook, 1989) or very low (Doyle and Woodham, 1991). Culture technique will likely bias results because laboratories close to sample collection will facilitate greater survival of anaerobes. The oxygen content of sinuses can drop when blocked with extensive amounts of mucin (Carenfelt and Lundberg, 1977). Therefore, in extreme cases, anaerobic micro-organisms may be more common.

As previously discussed, biofilm-forming micro-organisms exhibit greater levels of antibiotic resistance, which may explain the lack of efficacy in treating CRS with antibiotics in some cases (Lim *et al.*, 2010; Fokkens *et al.*, 2012). Interestingly, biofilms are often observed in patients who have previously undergone FESS, which is suggestive of the difficulty in eliminating biofilm infections (Bendouah *et al.*, 2006; Zhang *et al.*, 2011) Novel ideas for eradicating microbial biofilms are required to improve the management of CRS. Extracellular DNA is a common characteristic of the biofilm matrices of micro-organisms. Therefore, targeting the eDNA molecule and reducing biofilm stability may increase treatment efficacy. Bacteria that are CRS-associated and have been dispersed or inhibited with DNase I include *S. pneumoniae* (Hall-Stoodley *et al.*, 2008), *Neisseria* spp. (Lappann *et al.*, 2010), *P. aeruginosa* (Whitchurch *et al.*, 2002), *S. aureus* (Kaplan *et al.*, 2012), and *E. coli* (Tetz and Tetz, 2010). In addition, *Bordetella* spp. have been dispersed from the upper respiratory tract of mice (Conover *et al.*, 2011), highlighting the efficacy of delivering a DNase solution to a nasal biofilm. However, it is unknown how recently isolated bacteria from a clinical condition will respond to DNase treatment. It is also unknown if eDNA is important in CRS-associated biofilm-forming micro-organisms. At this early stage it would be useful to culture isolates in uncomplicated models to test their sensitivity to DNase enzymes.

No optimal therapy for CRS has yet been discovered. Medical therapy with antibiotics has limited efficacy (Lim *et al.*, 2010), as does FESS (Khalil and Nunez, 2006). Nasal irrigation with saline solutions does have some benefits, although it is less effective than topical steroids (Harvey *et al.*, 2007). Adding a chemical to a solution that can be delivered to the sinus that subsequently dissolves the biofilm is a concept that could improve treatment. One previous attempt of this using sterile water, citric acid and caprylyl sulfobetaine (zwitterionic surfactant) applied at pressure to biofilm

forming isolates from CRS was successful at causing biofilm reduction (Desrosiers *et al.,* 2007). There was a 99.9% reduction of *P. aeruginosa* biofilm mass. Given this research was an *in vitro* study this cannot yet be applied in a clinical environment but it does suggest that this concept may work *in vivo*. Addition of the novel bacterial nuclease, NucB, to a saline solution could work in much the same way. Administered prior to surgery to soften or even dissolve the biofilm it could allow for less intrusive surgery. Remaining planktonic bacteria may be more susceptible to conventional antibiotics. However, before this application the efficacy of nuclease treatment needs to be researched in clinical isolates from CRS patient sinuses. An improvement in clinical practice would undoubtedly save health services considerable amounts of time and money. Furthermore, it would improve the quality of life for up to 10% of the adult European population.

1.8.2 Human oral health and biofilms

The biofilms that develop on teeth and on oral mucosal surfaces are complex communities. For example, culture-independent methods have shown that 700 microbial species can colonise the human mouth (Aas *et al.,* 2005). As outlined in section 1.1, the formation of the oral biofilm occurs through a sequence of events. Ultimately, the process has enough homology with other biofilm systems that the oral biofilm, or dental plaque, provides a model for the overall understanding of biofilms. Importantly, although the mouth contains many beneficial micro-organisms, there are circumstances whereby pathogenic microbes can thrive. A key component of a disease-causing oral biofilm is the biofilm matrix. Therefore, by understanding the role of eDNA in facilitating biofilm attachment or biofilm stability it may be easier to target disease-causing biofilms.

Some oral bacteria have been shown to have greater importance in the development of dental plaque, both in healthy and diseased mouths. For instance, streptococci, including *S. gordonii*, are well characterised as initial colonizers of the tooth surface. *Streptococcus* spp. constitute 60-80% of the oral biofilm microflora in the early stages (4-8 hours) of biofilm formation, as analysed by 16S rRNA PCR amplification (Diaz *et al.*, 2006). The reason for their abundance at this early time point is the wide plethora of surface adhesins that streptococci use to attach to surfaces (reviewed by Nobbs *et al.*, 2009). *Actinomyces* spp. are also common in early dental plaque formation, and have been shown to interact with streptococci in a way that leads to increased biofilm

formation (Palmer *et al.*, 2001). Recently, *Actinomyces naeslundii* 2 was renamed to *Actinomyces oris* (Henssge *et al.*, 2009). *Actinomyces oris* is important because *Fusobacterium nucleatum* binds to this species. During *in vitro* flow cell experiments using saliva, *F. nucleatum* only grew if *A. oris* was also present (Periasamy *et al.*, 2009). Therefore, *A. oris* and *Strepotococcus* spp. are responsible for providing a basis for the co-aggregation of many oral bacteria, as *F. nucleatum* can bind to early and late colonizers (Kolenbrander *et al.*, 2006). Due to this ability, *F. nucleatum* is known as a bridging micro-organism in the succession of oral biofilm formation. Another important aspect of *F. nucleatum* colonisation is its putative role as a pathogenic species in periodontitis. *Fusobacterium nucleatum* is a poor inducer of immune response (Signat *et al.*, 2011) but is a dominant organism in the periodontium (Bolstad *et al.*, 1996). It seems likely that whilst *F. nucleatum* is a commensal micro-organism its ability to bind pathogenic species like *Porphyromonas gingivalis* or *Prevotella intermedia* could facilitate the onset of a disease-causing biofilm. Streptococci and *F. nucleatum* also bind to each other in co-aggregations. These dynamics were analysed by Al-Ahmad *et al*., (2007) by employing specific FISH probes and CLSM. A key *Streptococcus* spp. is *S. mutans*, a micro-organism strongly linked to the causation of dental caries (van Houte, 1994). Clearly, oral bacteria are able to form many interactions that can have positive or deleterious impacts on human health. Often oral disease is related to environmental conditions. With dental caries, increased dietary sugar leads to an acidic pH that selects for acidogenic micro-organisms, such as *S. mutans*. These changes also modify the extracellular matrix, which impacts on species composition and cariogenicity (Paes Leme *et al.*, 2006). Although it is impossible to define a healthy microflora it is important to consider the roles of commensal species, such as *S. gordonii*, in facilitating a disease-causing biofilm.

Oral micro-organisms have been shown to produce eDNA, including *S. gordonii*, *S. sanguinis* (Kreth *et al.*, 2009), *S .mutans* (Perry *et al.*, 2009), *F. nucleatum* and *Porphyromonas gingivalis* (Ali Mohammed *et al.*, 2013). Although eDNA did not contribute to biofilm stability in *F. nucleatum* and *Porphyromonas gingivalis* (Ali Mohammed *et al.*, 2013), it promoted stability in *S. mutans* (Perry *et al.*, 2009). These are the limited examples of experiments being designed to test the contribution of eDNA to oral bacteria biofilm matrices. *Candida albicans*, is often associated with oral health issues, such as denture stomatitis (Kulak-Ozkan *et al.*, 2002), and has been shown to require eDNA for biofilm stability (Martins *et al.*, 2010). Further research is required to determine the significance of eDNA in oral biofilms, starting with simple

single-species models and ultimately moving towards mixed-species microcosms grown from human saliva. Modelling natural biofilms such as dental plaque presents a significant challenge for oral microbiologists. Currently many research laboratories employ a static model system in microtiter wells which is useful for studying early biofilm formation. However, continuous-flow systems that circulate fresh nutrients allow for the development of mature biofilms in an environment that is more akin to the natural habitat of the mouth (e.g. hydrodynamic influences). Recently, a high-throughput model system, the BioFlux (Fluxion), has been shown to replicate the species composition of the oral biofilm whilst culturing in flowing human saliva (Nance *et al.*, 2013). This system would represent a unique opportunity to test DNase enzymes against oral biofilms in a laboratory environment.

Another interesting facet of eDNA production in the oral biofilm is microbial competition. For instance, *S. mutans* acquires transforming DNA from *S. gordonii* by causing cell lysis with a bacteriocin (Kreth *et al.*, 2005). However, many oral bacteria also produce extracellular DNase enzymes, including *S. gordonii* (Palmer *et al.*, 2012). Therefore, micro-organisms like *S. mutans* that rely on DNA either for matrix stability or horizontal gene transfer could be negatively impacted by DNase-producing bacteria. This is a concept that has not been reported to date but may be an important factor in oral biofilm microbial competition.

Excellent oral hygiene is an effective way of maintaining a healthy mouth and removing oral disease causing bacteria (van der Weijden and Hioe, 2005; van der Weijden and Slot, 2011). However, the NHS spends £1.23 billion per year treating dental diseases (Audit Commission for Local Authorities and the National Health Service in England and Wales, 2002), many of which are caused by oral bacteria. As has been shown, there is considerable evidence for DNase enzymes having an effect on forming or pre-formed biofilms. Therefore, there is much needed research to determine the *in vitro* effect of deoxyribonuclease enzymes, like NucB or DNase I, versus oral biofilms, to resolve their potential value in oral hygiene products. Obviously, studies on natural dental plaque formed *in situ*, and ultimately *in vivo* studies in humans, will be required to establish the potential of NucB or other DNases for the control of oral biofilms.

1.9 Aims and objectives

Before 2010 there had been minimal research into the role of eDNA in biofilm-forming micro-organisms recently isolated from clinical diseases. Furthermore, the potential for DNase enzymes to treat clinical issues caused by biofilms was as yet untested. Therefore, the primary aim of this thesis was to assess the efficacy of NucB in two ENT biofilm-associated problems. Subsequently, research was focused on oral biofilms, eDNA and microbial nucleases. The objectives were as follows:

1. To characterise the role of eDNA in TESV biofilms
    - Biofilms were visualised in detail using CLSM and SEM. Next, eDNA was extracted and quantified from TESV biofilms. Lastly, TESVs were treated with NucB to study the biofilm dispersal potential of the enzyme.
2. To study the efficacy of NucB against biofilm-forming CRS isolates
    - Micro-organisms were isolated from material aspirated during FESS. These isolates were then assessed for biofilm formation, and subsequently treated with NucB. Microscopic analysis of sinus mucosa and sinus mucin was also undertaken. Furthermore, eDNA was extracted and quantified from isolate biofilms.
3. To measure eDNA and DNase efficacy in selected oral bacterial biofilms
    - Four oral bacteria, *S. gordonii*, *A. oris*, *S. mutans* and *Fusobacterium nucleatum* were cultured as biofilms and treated with DNase I. Extracellular DNA in the biofilm matrix was also quantified. The efficacy of NucB and DNase I was compared versus *S. mutans* GS-5. Lastly, oral biofilms were modelled in a microfluidics device during a research visit to the University of Michigan, MI, USA.
4. To characterise the extracellular DNase produced by *S. gordonii*
    - The gene encoding extracellular nuclease production was identified by bioinformatics research. Given the reliance of *S. mutans* GS-5 on eDNA, the interaction between *S. gordonii* nuclease activity and this species was studied. The nuclease, SsnA, was cloned into an *E. coli* protein expression system and purified. DNase activity of SsnA was then assessed using *in gel* zymography and biofilm assays. The regulation of *ssnA* by carbon sources, and inhibition of SsnA by acid was also studied.

Chapter 2: Materials and Methods

2.1 Reagents and equipment

2.1.1 *Reagents*

Reagents are listed in the text, with manufacturers, and if required are abbreviated after first mention.

2.1.2 *Equipment*

Table 2.1 Equipment used in this study

Application	Device	Manufacturer
Gradient PCR	T100 PTC-200	Bio-Rad MJ Research
PCR	GeneAmp PCR System 9700	Applied Biosystems
Centrifugation	J2-21 SK10	Beckman Sigma Centrifuges
Bench-top Centrifugation	MiniSpin	Eppendorf
Orbital Shaker		Jencons
Static Incubator	30/37°C	Laboratory Thermal Equipment
Anaerobic Incubator	Bug Box Plus	Ruskin
Microfluidics Biofilm Culture	Bioflux™	Fluxion
Sample Disruption	TissueLyser LT	Qiagen
Microplate Reader	Synergy HT	Biotek
Determination of Nucleic Acid Absorption	ND-1000	NanoDrop
Determination of Optical Densities	UV2	Unicam
Confocal Laser Scanning Microscopy	TCS SP2	Leica
Transmission Electron Microscopy	CM100	Philips
Scanning Electron Microscopy	Stereoscan 240	Cambridge

DNA Electrophoresis	Gel Tank	Apollo Instrumentation
	Power Pac 300	Bio-Rad
Agarose Gel Imaging	G:Box	Syngene
Protein Electrophoresis	Gel Tank	ATTO
	Power Pack	Consort

2.2 Ethical approval

Ethical approval (no. 10/H0904/44) was gained for the chronic rhinosinusitis (CRS) study by the National Research Ethics Service Committee (North East – Sunderland). Each patient had to give their consent before enrolment to the study.

For the tracheoesophageal speech valve (TESV) project no ethical approval was required as the study was anonymous and did not use human tissue samples.

2.3 Sample collection

2.3.1 *Tracheoesophageal speech valves*

Tracheoesophageal speech valves were obtained from patients at the Freeman Hospital, Newcastle-upon-Tyne. Patients regularly change their voice prostheses due to leakage of the valve. After removal at the hospital's Speech and Language Therapy Department the valves were placed in phosphate buffered saline (KH_2PO_4 20mM, Na_2HPO_4 20mM, NaCl 0.15 mM, pH 7.1) (PBS) and transferred to the Centre for Oral Health Research, Newcastle University. Tracheoesophageal speech valves were stored at 4°C for up to three days before analysis.

2.3.2 *Chronic rhinosinusitis obstructive mucin and sinus mucosa*

A total of 20 patients undergoing functional endoscopic sinus surgery (FESS) for the treatment of CRS at the Freeman Hospital, were recruited to this study. All patients met the CRS diagnosis criteria published by the Chronic Rhinosinusitis Task Force (Benninger *et al.*, 2003). Patients were recruited to the study only if obstructive mucin was observed during the surgical procedure. During FESS, 'obstructive mucin' that was dislodged surgically was collected with mucous traps (Sigma Aldrich) and immediately placed into sterile reduced transport fluid (RTF) (Syed and Loesche, 1972). Specimens

were transferred to the Centre for Oral Health Research and stored at 4°C. All samples were processed within 24 h. Mucosal biopsy specimens were fixed in 10% formalin directly after surgery, and stored at 4°C for up to one month.

2.3.3 *Saliva*

Whole stimulated saliva was collected from six healthy individuals who were asked not to eat 2 h prior to collection. Saliva was collected on ice, aliquoted and kept at -20°C until use as the inoculum in mixed species biofilm experiments

2.4 Microbiological methods

2.4.1 *Bacterial strains and culture techniques*

2.4.1.1 *Culture of tracheoesophageal speech valve isolates*

Micro-organisms liberated from the surface of used TESVs (see 2.10.1) were cultured on Sabouraud Dextrose Agar (Oxoid) and Nutrient Agar (Oxoid). Plates were incubated aerobically at 37°C and checked for growth every 24 h for at least 7 days. Sabouraud Dextrose Agar was used as a medium on which to grow fungi, including *Candida* spp., that often foul TESVs. Nutrient Agar was used as a non-selective medium for the culture of both bacteria and fungi.

2.4.1.2 *Isolation and culture of chronic rhinosinusitis micro-organisms*

A variety of growth media were employed for the isolation and routine culture of CRS isolates. Blood agar contained 37 g/L Brain Heart Infusion (Oxoid), 5 g/L Yeast Extract (Merck), and 15 g/L Agar (Merck). After sterilization, 5% (v/v) Defibrinated Horse Blood (TCS Biosciences) was added. Chocolate agar was prepared using the same recipe except that, after the addition of horse blood, the medium was heated to 70°C for 10 min. Fastidious Anaerobe Agar (FAA) was purchased from LabM and Sabouraud Dextrose Agar was from Oxoid.

For isolation of micro-organisms, a portion of sinus aspirate from each patient was homogenized in sterile phosphate buffered saline (PBS) and inoculated onto blood agar, chocolate agar, FAA and two plates of Sabouraud Dextrose Agar. Blood and chocolate

agar plates were incubated in 5% CO_2 at 37°C. Pre-reduced FAA plates were incubated at 37°C anaerobically (Ruskinn, Bugbox Plus) in a gas mix consisting of 10% CO_2, 10% H_2 and 80% N_2. The Sabouraud Dextrose Agar plates were incubated aerobically, one at 37°C and another at 30°C. Plates were examined every 24-48 h for at least seven days. Individual colonies were picked and sub-cultured three times to obtain pure isolates. Strains were stored at -80°C in BHY medium (brain heart infusion 37 g/L and yeast extract 5 g/L) diluted to 50% strength by the addition of glycerol (Sigma Aldrich).

2.4.1.3 *Routine culture of oral bacteria*

Streptococcus gordonii DL1, *Actinomyces oris* MG1 and *Streptococcus mutans* GS-5 were routinely cultured in THYE medium containing 30 g/L Bacto™ Todd Hewitt Broth (Becton Dickinson and Co.) and 5 g/L yeast extract at 37°C anaerobically (10% CO_2, 10% H_2 and 80% N_2). *Fusobacterium nucleatum* subsp. *nucleatum* ATCC 25586 was grown in BHIG comprising 37 g/L brain heart infusion, 5 g/L yeast extract, and 2.5 g/L L-glutamic acid (Sigma Aldrich) anaerobically at 37°C.

2.4.1.4 *Culture of* Escherichia coli

Strains of *Escherichia coli* (see table 2.2) were used for cloning and overexpression of proteins. The strains were grown in Luria Bertani (LB) Broth (Melford Laboratories Ltd.) at 35°C, whilst shaking at 200 RPM in aerobic conditions. *Escherichia coli* were also grown aerobically on LB agar (LB broth plus 15 g/L agar) at 37°C. When required, the appropriate antibiotics were added (all Sigma Aldrich); ampicillin (50 μg/mL), kanamycin (50 μg/mL), erythromycin (400 μg/mL) and chloramphenicol (33 μg/mL).

2.4.1.5 *Biofilm formation in microtiter plates*

For biofilm assays, microbial stock cultures (5 μL) were added to triplicate wells of sterile polystyrene microtiter-well plates containing growth media (200 μL). Plates were wrapped in parafilm and incubated without shaking aerobically or anaerobically at 37°C for various time periods. Following overnight growth (20 h), non-adherent planktonic cells were removed and transferred to a clean 96-well plate (Greiner Bio-One) and the OD_{600} was read in a microplate reader (BioTek Synergy HT) to quantify growth in the

planktonic phase. Biofilm extent was quantified using the crystal violet assay (see section 2.10.2).

2.4.2 *Strains used in this study*

Table 2.2 Strains used in this study

Strain	Description	Source or Reference
E. coli strains		
TOP10	F– *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara leu*) 7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G	Invitrogen
GEXssnA	amp^r, cam^r, expresses SsnA-GST	This thesis (NSJ)[a]
DH5α	F– Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (rK–, mK+) *pho*A *sup*E44 λ– *thi*-1 *gyr*A96 *rel*A1	Invitrogen
BL21(DE3)pLysS	F- *ompT hsdS$_B$* ($_{rB}$-$_{mB}$-) *gal* *dcm* (DE3) pLysS (cam^r, amp^r)	G. Scholefield, CBCB, UK
B. subtilis strains		
ATCC6633	Produces subtilin	ATCC
NZ8900	pNZ8901	(Nijland *et al.*, 2010)
S. gordonii strains		
DL1	Wild type	Newcastle Dental Hospital Isolate
ccpA::*ermAM*	Δ*ccpA*, erm^r	This thesis
malR::*ermAM*	Δ*malR*, erm^r	This thesis
ssnA::*ermAM*	Δ*ssnA*, erm^r	This thesis (NSJ)[a]
ssnA::*aphA3*	Δ*ssnA*, kan^r	This thesis (NSJ)[a]

Other oral bacteria		
A. oris MG1	Wild type	J. Cisar, NIDCR, USA
F. nucleatum 22586	Wild type	ATCC
S. mutans GS-5	Wild type	Newcastle Dental Hospital Isolate
S. mutans NG8	Wild type	L. C. Dutton, Bristol University, UK
S. mutans UA140	Wild type	Unknown
S. mutans UA159	Wild type	ATCC
CRS Isolates		
C. propinquum FH1	Isolated from patient 8	This thesis
C. pseudodiphtheriticum FH2	Isolated from patient 6	This thesis
M. catarrhalis FH3	Isolated from patient 3	This thesis
M. catarrhalis FH4	Isolated from patient 6	This thesis
S. aureus FH5	Isolated from patient 3	This thesis
S. aureus FH6	Isolated from patient 5	This thesis
S. aureus FH7	Isolated from patient 7	This thesis
S. epidermidis FH8	Isolated from patient 1	This thesis
S. epidermidis FH10	Isolated from patient 4	This thesis
S. epidermidis FH11	Isolated from patient 5	This thesis
S. lugdunensis FH12	Isolated from patient 6	This thesis
S. lugdunensis FH13	Isolated from patient 8	This thesis
S. lugdunensis FH14	Isolated from patient 15	This thesis
S. warneri FH15	Isolated from patient 7	This thesis
S. warneri FH17	Isolated from patient 14	This thesis
S. anginosus FH18	Isolated from patient 3	This thesis
S. anginosus FH19	Isolated from patient 17	This thesis
S. constellatus FH20	Isolated from patient 4	This thesis
S. constellatus FH21	Isolated from patient 7	This thesis
S. intermedius FH22	Isolated from patient 4	This thesis
S. pneumoniae FH26	Isolated from patient 10	This thesis
S. salivarius FH27	Isolated from patient 1	This thesis
S. salivarius FH28	Isolated from patient 4	This thesis

S. salivarius FH29	Isolated from patient 17	This thesis

[a]Unpublished strain constructed by Nick Jakubovics as part of this project.

2.4.3 *Identification of micro-organisms*

2.4.3.1 *Routine microbiological identification*

Whilst culturing micro-organisms, they were often checked for the presence of contaminant microbes using standard microbiological techniques. These included the Gram stain, the catalase test, and checking cell morphology using light microscopy. The appearance of colonies on agar was also used as a general guide for culture of the correct microbe.

2.4.3.2 *Microbiology of chronic rhinosinusitis*

All isolates were initially characterized by Gram staining, inspection of colony morphology, testing for catalase production, haemolysis, DNase production and ability to grow aerobically or anaerobically. The majority of clinical isolates were further identified to species level using a Matrix Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF) Mass Spectrometer (Bruker, Microflex). Isolates were streaked onto blood agar, incubated under 5% CO_2 or in the absence of oxygen, at 37°C for 24 h and transferred to the Pathology Department, Freeman Hospital, Newcastle upon Tyne, for identification.

In cases where MALDI-TOF analysis yielded ambiguous results, for example the majority of α-haemolytic streptococci, bacterial identification was confirmed by analysis of the 16S rRNA gene. The gene was amplified using the oligonucleotide primers 0063F and 1387R (Marchesi *et al.*, 1998) in a ReddyMix™ reaction. PCR reactions were run using a GeneAmp PCR System 9700 (Applied Biosystems). PCR products were checked on a 1% agarose gel, and fragments of the expected size were sequenced by MWG Eurofins. Forward and reverse sequences were aligned and sequence matched using the Ribosomal Database Project website (http://rdp.cme.msu.edu/).

2.4.4 *Growth of bacterial populations*

Bacterial growth was calculated by measuring the turbidity of a bacterial population, growing in culture medium. Using a spectrophotometer the absorbance at 600 nm of 1 mL of suspension was determined, using unconditioned medium to measure background absorbance.

This method of optical density measuring was used to calculate the growth rate of organisms. Isolates were cultured overnight, subcultured the following day into pre-warmed media, and grown for a period of 6-8 h, with optical density measurements taken every hour. To determine the doubling time the number of generations (g) was calculated using the following equation:

$$g = (Log_{10}N_t - Log_{10}N_0)/Log_{10}^2$$

$N_t = A_{600}$ at time exiting the exponential growth phase

$N_0 = A_{600}$ at time beginning the exponential growth phase

After g was calculated, the value was divided by the number of min the exponential growth phase lasted, thereby giving a doubling time.

2.4.5 *DNase agar test for extracellular nuclease activity*

DNase agar is a nutrient rich medium that contains DNA. If an organism produces an extracellular enzyme that hydrolyses DNA it is possible to observe this activity by staining the agar. All chronic rhinosinusitis isolates, oral bacteria and mutants of *S. gordonii* DL1 were tested for nuclease activity in this way. A single thick streak of each bacterium was plated onto DNase Test Agar (Oxoid). Plates were incubated aerobically or anaerobically at 37°C for between 24-96 h. Once colonies had grown, plates were flooded with 4 mL of 0.1% (w/v) toluidine blue (Sigma Aldrich) or 4 mL of 1N HCl, to highlight nuclease production. HCl precipitates the DNA, creating an opaque medium except for areas of nuclease activity, whereas toluidine blue appears blue in the presence of intact DNA and pink when complexed with nucleotides.

2.5 Molecular biology methods

2.5.1 *Polymerase chain reaction*

DNA was typically amplified in 50 µL reactions, using ReddyMix™ (ABgene) or Expand High Fidelity PCR System (Roche). ReddyMix™ was used to amplify short fragments of DNA, or when diagnostically checking the size of amplified DNA. For reactions that required more efficient PCR, with fewer base errors, the Expand High Fidelity PCR System was used as it contains the Tgo DNA polymerase (from *Thermococcus gorgonarius*) with proofreading activity. The general protocols for both kits are shown in Table 2.3 and 2.4, although annealing temperatures were optimised depending on the primers used.

Table 2.3 ReddyMix™ reaction mix and thermal cycling

50 µL Reaction Mix	**Thermal Cycler Protocol**		
Template – 1 µL Primers (10 µM) – 5 µL each ReddyMix™ – 25 µL dH2O – 14 µL	Initial Denaturation	94°C / 2 min	1x
	Denaturation Annealing Elongation	94°C / 10 s 55°C / 30 s 68°C / 1 min[a]	35 cycles
	Final Elongation	68°C / 7 min	1x

[a]1 minute per 1000 base pairs.

Table 2.4 Expand high fidelity PCR system reaction mix and thermal cycling

50 µL Reaction Mix	**Thermal Cycler Protocol**		
Template – 1 µL Primers (10 µM) – 5 µL each Nucleotide Mix – 1 µL Reaction Buffer[a] – 10 µL Taq Enzyme – 0.5 µL dH_2O – 27.5 µL	Initial Denaturation	94°C / 2 min	1x
	Denaturation Annealing Elongation	94°C / 15 s 56°C / 30 s 72°C / 2 min	10 cycles
	Denaturation Annealing Elongation	94°C / 15 s 56°C / 30 s 72°C / 2 min +5 s[b]	20 cycles
	Final Elongation	72°C / 7 min	1x

[a]When required, $MgCl_2$ concentration altered to 2.5 or 3.5 mM by adding $MgCl_2$ from a 25 mM stock solution and lowering dH_2O.

[b]increased by 5 s each cycle.

2.5.2 *Agarose gel electrophoresis*

DNA was separated and visualised on 1 % agarose gels. Molecular Biology Grade Agarose (Melford) was placed in 1x TAE buffer (40 mM Tris, 20 mM glacial acetic acid (Fisher Scientific), 1 mM ethylenediaminetetraacetic acid (EDTA) (Sigma Aldrich), pH 8.0) and dissolved by boiling. After the solution had cooled for 20 min, ethidium bromide (2 μg/mL) was added. The agarose solution was then poured into gel trays, with gel combs, and allowed to solidify. Set gels were placed into a gel tank and immersed in 1x TAE buffer. Samples containing DNA were mixed with 5x DNA loading dye (1:5) (Bioline) if needed. For estimation of DNA molecular weight, samples were compared against 5 μL of either HyperLadder™ 1 kbp (Bioline) or HyperLadder™ 100 bp. Gels were run at 120 V for up to 1 hour. DNA bands were visualised with an ultraviolet source (G:Box, Syngene).

2.5.3 *Purification of PCR products from PCR reactions or agarose gels*

PCR reactions that yielded single bands on agarose gels were purified from the PCR reaction mix using the StrataPrep PCR purification kit (Agilent Technologies), according to the manufacturer's protocol. This method of DNA purification uses a DNA binding solution, and a fiber matrix that binds DNA before it is eluted with elution buffer (10 mM Tris pH 8.5).

When one or more band was visible on an agarose gel, the band of interest was removed from the gel before further downstream applications. Gels were placed on a UV-table and appropriate bands were excised using a scalpel blade and then purified from the agarose gel using the QIAquick gel extraction kit (Qiagen). Agarose gel slices were weighed, and dissolved by heating at 50°C in a high-salt buffer. Nucleic acids were then absorbed onto a silica-membrane, impurities removed by washing, and eluted with elution buffer.

2.5.4 *Colony PCR*

Recombinant *Escherichia coli* colonies were screened by PCR for correct DNA inserts. Single colonies were picked from agar plates with a pipette tip and placed into a PCR reaction mix. DNA was released from within cells by heating at 95°C for 10 min, before standard thermal cycling was started.

When releasing DNA from *Streptococcus gordonii* colonies GeneReleaser® (Cambio) was used to facilitate DNA release from this Gram positive organism. GeneReleaser® uses a polmer matrix, and a range of incubation temperatures to release DNA and segregate PCR inhibitors released during cell lysis. To 1 µL of cells, 20 µL of GeneReleaser® was added before following the manufacturer's thermal cycler protocol. The PCR reaction mix was combined with the lysis solution and DNA amplified on a thermal cycler.

2.5.5 *Nanodrop spectrophotometry*

The NanoDrop ND-1000 (NanoDrop) spectrophotometer was used for determining the concentration of DNA in samples. Before measuring samples, elution buffer was placed on the NanoDrop stage to obtain a blank value of light absorbance. DNA samples were then loaded in 2 µL volumes onto the NanoDrop stage. This method estimates nucleic acid content based on an equation which subtracts readings that are likely to be protein. The ratio of 260 to 280 nm absorbance was used as an estimate of DNA puritiy (a ratio of 1.8-2.0 was considered acceptable).

2.5.6 *DNA extraction from whole cells*

Bacteria were cultured in BHY broth, and harvested by centrifugation at 4,000 *g*. Cells were resuspended in 150 µl spheroplasting buffer (26% (w/v) raffinose (Sigma Aldrich), 10 mM $MgCl_2$ (BDH), 20 mM Tris-HCl, pH 6.8) supplemented with 37.5 µg lysozyme (Sigma Aldrich) and 50 U mutanolysin (Sigma Aldrich), and incubated at 37°C for 30 min. Following incubation, DNA was extracted using the MasterPure™ Gram Positive DNA Purification Kit (Epicentre® Biotechnologies) in accordance with the manufacturer's instructions. Included during the extraction process was a bead beating (TissueLyser LT) step of 50 Hz, for 5 min, using 25 mg of acid washed glass beads (0.1 mm). After extraction, DNA was suspended in 25 µl elution buffer (10 mM Tris pH 8.5).

2.5.7 *Measuring DNA concentration with PicoGreen*

When required, plasmid and extracellular DNA concentrations were measured using the Quant-iT™ PicoGreen® dsDNA kit (Invitrogen). Measurements were made by following

the manufacturer's protocol. Briefly, DNA samples were diluted (1:20) in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5). A standard curve was made by diluting a Lambda DNA standard (2 μg/mL) in TE buffer in a range from 1000 ng/mL to 0 ng/mL. The PicoGreen® dye was diluted 200 times in TE buffer, and an equal volume was added to DNA samples or dsDNA standards. Samples were transferred to a 96-well microplate in 200 μL volumes. Fluorescence was measured using a Synergy HT microplate reader (excitation: 485 nm, emission: 528 nm). Unknown DNA concentrations were calculated from the DNA standard curve.

2.5.8 *PCR primer design*

Primers were designed from DNA sequences using Primer3Plus (Untergasser *et al.*, 2007) taking care to design them with optimal length (18-22 bp), guanine:cytosine content (40-60 %), and melting temperature (between 50-60°C). Primers were also designed in a way that minimised primer pairing and secondary structures. Oligonucleotide primers were ordered and synthesised by Eurogentec.

2.5.9 *Restriction digest*

DNA and plasmids were digested by specific enzymes during DNA cloning and for diagnostically checking plasmids for correct insertion of genes of interest. Appropriate restriction enzymes were selected using the NEBcutter V2.0 (Vincze *et al.*, 2003) or by manually scrutinising sequences. Typically digest reactions were made with the appropriate reaction buffer, and 1 U of enzyme for every 1 μg of DNA included. For diagnostic tests 500 ng of DNA was digested, while 1-2 μg of DNA was digested for cloning. Reaction mixes were incubated for 1 h at 37°C, and then heat inactivated (e.g. 80°C for 20 min) if appropriate. Digests were then checked on agarose gels, along with uncut DNA or plasmid.

Table 2.5 Restriction enzymes used during this study

Restriction Enzyme	Recognition Site[a]	NEBuffer[b]
*Asc*I	5' GG▾CGCGCC 3' 3' CCGCGC▴GG 5'	4 (100 %)
*Eco*RI	5' G▾AATTC 3'	*Eco*RI (100 %)

	3’ CTTAA▲G 5’	
*Hin*dIII	5’ A▼AGCTT 3’ 3’ TTCGA▲A 5’	4 (50 %)
*Xho*I	5’ C▼TCGAG 3’ 3’ GAGCT▲C 5’	4 (100 %), BSA
*Xba*I	5’ T▼CTAGA 3’ 3’ AGATC▲T 5’	4 (100 %), BSA

[a]Triangles indicate sites of digestion

[b]Buffer used, according to the New England BioLabs nomenclature. Percentage in brackets indicates the activity of each restriction enzyme in the buffer used. Lastly, bovine serum albumin (BSA) was included if required.

2.5.10 Escherichia coli *cloning*

Genes of interest were cloned into the pCR®2.1 vector, supplied by Invitrogen, using the TA Cloning® Kit (Invitrogen) and following the manufacturer’s guidelines. Briefly, PCR products were amplified with *Taq* polymerase, which adds a single 3’deoxyadenosine (A) overhang to the end of PCR products. Products were inserted into the pCR®2.1 vector by incubating for 20 h at 14°C in the presence of Topoisomerase I, following manufacturers guidelines. Competent *E. coli* TOP10 cells were transformed according to the manufacturer’s handbook. Transformations always included pUC19 as a positive control. The pCR®2.1 vector contains the *lacZα* gene, which allows for blue-white colony screening for PCR inserts. *E. coli* TOP10 has a mutant *lacZ* gene (lacZΔM15), and when expressed with *lacZα* produces β-galactosidase. This enzyme cleaves 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) (Melford) that is spread onto agar plates (40 µL of a 40 mg/mL stock solution) resulting in blue colonies. Successful ligation creates *E. coli* cells lacking in β-galactosidase, by disrupting the *lacZα* gene, thus resulting in white colonies. After transformation, white colonies were selected and analysed by colony PCR and gene sequencing.

2.5.11 *DNA sequencing*

Samples of DNA were sent to MWG Eurofins for DNA sequencing. Sample template concentration was measured using a NanoDrop spectrophotometer (ND-1000) and the concentration was adjusted to the amount required (plasmid DNA, 50-100 ng/µL; PCR

products, 10 ng/µL). Templates (15 µL) were sent with sequencing primers at a concentration of 10 pmol/µL in a total volume of 15 µL.

2.5.12 *Primers and plasmids*

Table 2.6 Primers used during this study

Primer	5'-3' Sequence	Source or Reference
Sequencing		
M13 Forward (-20)	GTAAAACGACGGCCAG	Invitrogen
M13 Reverse	CAGGAAACAGCTATGAC	Invitrogen
0063F	CAGGCCTAACACATGCAAGTC	(Marchesi *et al.*, 1998)
1387R	GGGCGGWGTGTACAAGGC	(Marchesi *et al.*, 1998)
gbpC5'	GAGAAAGCACTTTGGTTTCAA	(Sato *et al.*, 2002)
gbpEx3'	ATTGCCATGTAAGTCAACAGATGAG	(Sato *et al.*, 2002)
gbpC3'	GTCCCAACCTCTTTTTATAGA	(Sato *et al.*, 2002)
PA5'	GCGCAACAGCTACATACACT	(Sato *et al.*, 2002)
PAS3'	CCGCACGGATTTTACCA	(Sato *et al.*, 2002)
PAL3'	ATAATTCGTTGAACCGGCA	(Sato *et al.*, 2002)
ermseqF	TGAAACACGCCAAAGTAAACAA	This thesis (NSJ)
ermseqR	TTTGGTTGAGTACTTTTTCACTCGT	This thesis (NSJ)
Mutagenesis		
ccpAF1	AACCCCTTTGACTGGTAGGG	This thesis
ccpAR1	CCATAGAAACCCCTGCTTCA	This thesis
ccpAF2	GCAGGGGTTTCTATGGGGCGCGCCG GACTAGCTGATTGCG	This thesis
ccpAR2	GTCTCTTGGCGGCATGTAAG	This thesis
ermAMF1	TGCAGGCGCGCCGAATTATTTCCTC CCGTTAA	This thesis
ermAMR1	TGCAGGCGCGCCGGAGTGATTACAT GAACAAA	This thesis
malRF1	AATTGGCTGCCATTCGTTAC	This thesis

malRR1	GTTCATGTAATCACTCCTTACAGTTG AGGGCGAAACC	This thesis
malRF2	ACGGGAGGAAATAATTCTGGCATCT GGTGTTTGTGAT	This thesis
malRR2	ACCATCAGAGGCTGTCGGTA	This thesis
ermAMF2	GAATTATTTCCTCCCGTTAA	This thesis (NSJ)
ermAMR2	GGAGTGATTACATGAACAAA	This thesis (NSJ)
SsnA Purification		
ssnA_Pf7	GACTGGATCCGAAGAGACGGAAAA TTCTTC	This thesis (NSJ)
ssnA_Pr7	CGATGAATTCACTCTTTTTGTTTTCA CCTGA	This thesis (NSJ)

Table 2.7 Plasmids used during this study

Plasmid[a]	**Description**	**Size (bp)**	**Source or Reference**
pUC 19	amp^r promoter, amp^r, *lacZα*, pUC ori	2686	Invitrogen
pCR®2.1	*lacZα*, f1 ori, kan^r, amp^r, ori	3930	Invitrogen
pCR®2.1-*ccpA*	*ccpA* flanking regions insert	5010	This thesis
pCR®2.1-*ccpA*::*ermAM*	*ccpA* flanking regions insert, *ermAM* insert	5762	This thesis
pVA838	erm^r, cam^r	9065	(Macrina *et al.*, 1982)
pGEX-KT	pMB1 ori, *lacIq*, *lacZ*, tac promoter, GST, glycine kinker, thrombin cleavage site, BamHI/MCS/EcoRI, amp^r	4978	(Hakes and Dixon, 1992)
pGEXssnA	SsnA-GST	7128	This thesis (NSJ)

[a]see Appendix D for vector maps.

2.6 Microscopy

2.6.1 *Confocal laser scanning microscopy*

2.6.1.1 *LIVE/DEAD analysis of microbial biofilms*

The biofilm architecture of oral bacteria and CRS isolates, with a particular focus on extracellular DNA was visualized by confocal laser scanning microscopy (CLSM) using biofilms cultured on glass surfaces. Sterile 13 mm diameter glass coverslips were placed in wells of a six-well tissue culture plate containing 3 mL growth medium. Wells were inoculated with 50 µL of stock bacterial cultures and incubated statically in air at 37°C for 18-72 h. Coverslips were removed, rinsed three times with PBS, and inverted onto a rubber O-ring (10 mm diameter) that had been placed on a microscope slide and filled with LIVE/DEAD® *BacLight*™ stain (Molecular Probes). LIVE/DEAD *BacLight*™ contains two fluorescent stains, SYTO® 9 and propidium iodide (PI), that allow for the monitoring of bacterial cell viability (Boulos *et al.*, 1999). Both of these fluorescent dyes bind to nucleic acids but SYTO® 9 has specificity for chromosomal DNA, whereas PI will stain both intracellular and extracellular DNA. Furthermore, PI displaces SYTO® 9 if it enters a compromised cell membrane. These characteristics allow the kit to be useful for visualising eDNA, as well as monitoring cell viability. Biofilms were examined using a Leica TCS SP2 confocal microscope with an argon/neon laser for visualisation of SYTO® 9 (excitation 485 nm, emission 519 nm), and PI (excitation 536 nm, emission 617 nm).

2.6.1.2 *Extracellular DNA staining of voice prosthesis biofilms*

Voice prosthesis biofilms were visualised using a CLSM dual stain technique developed previously (Kania *et al.*, 2010) with the addition of 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen). Used TESVs were washed three times with PBS to remove planktonic cells. Samples were then immersed in 2 mL of PBS to which 10 µL of PI (1 mg/mL) (Sigma Aldrich) were added; this was incubated in darkness, at room temperature, for 5 min on a moving plate after which excess stain was rinsed off. The valve was then incubated with 50 µg/mL Concanavalin A Alexa Fluor® 488 (Invitrogen) for 5 min at room temperature. Finally, the biofilm was stained with 2 µg/mL DAPI at room temperature, in darkness, for 15 min. Excess stain was removed and valves were mounted on vacuum grease in 6 well tissue culture microtiter plates (Greiner Bio-One). PBS was added to wells until samples were fully immersed in

liquid. Biofilms were examined using a Leica TCS SP2 with a diode/argon/helium-neon laser for visualisation of ConA (excitation 485 nm, emission 519 nm), PI (excitation 536 nm, emission 617 nm) and DAPI (excitation 358 nm, emission 461 nm).

2.6.1.3 *Visualization of bacteria on the surface of sinus mucosa*

Fluorescence *in situ* hybridisation (FISH) was performed using a peptide nucleic acid (PNA) probe corresponding to the well-characterised EUB338 probe (Amann *et al.*, 1990). The probe was synthesized as a fluorescein amidite (FAM) conjugate by Panagene. Mucosal biopsy specimens were fixed in 10% formalin directly after surgery, and stored at 4°C for up to one month. For PNA-FISH analysis, specimens were transferred to 50% (v/v) ethanol and incubated for 16 h at -20°C. Biopsy material was transferred to 1 mL permeabilization buffer (10 mg/mL lysozyme in PBS) and incubated at 37°C for 30 min. Samples were immersed in 1 mL pre-warmed wash buffer (10 mM Tris-HCl pH 9.0, 1 mM EDTA) for 30 min at 55°C. Pre-warmed hybridization buffer (25 mM Tris-HCl pH 9.0, 100 mM NaCl, 0.5% SDS, 30% formamide) containing 150 pmol/mL of the PNA probe was added to samples and incubated in darkness for 90 min at 55°C. Unbound PNA probe was removed by incubating in pre-warmed wash buffer for 30 min at 55°C. Eukaryotic cells were counterstained by immersing the specimens in 1 mL PBS with DAPI in darkness at 20°C for 15 min. Samples were glue-mounted onto a plastic surface and immersed in 2 mL PBS. Visualisation of surface bacteria and eukaryotic cells was performed using a Leica TCS SP2 microscope with an argon/neon laser for imaging of FAM conjugates (excitation 495 nm, emission 520 nm), and DAPI (excitation 358 nm, emission 461 nm).

2.6.1.4 *Microfluidic oral biofilm modelling*

Biofilms were grown in a BioFlux (Fluxion) microfluidics device using a similar protocol used by Nance *et al.*, (2013). Biofilms were cultured from pooled human saliva, *S. mutans* UA159, and *S. gordonii* DL1. During single-species experiments *S. mutans* was grown in presence of 2% sucrose plus cell free saliva (CFS), and *S. gordonii* with 1% THYE plus CFS. Saliva biofilms were cultured in CFS only. After micro-organisms were cultured for 20 h they were stained with LIVE/DEAD® BacLight™ and visualised using a Leica SPE CLSM. Afterwards images were rendered using Imaris (BitPlane) imaging software so that a 3D representation of biofilm

architecture was made. Image analysis was performed on a dedicated image processing computer in the Bio-imaging Unit at Newcastle University.

2.6.2 *Scanning electron microscopy*

Scanning electron microscopy was used to study biofilm formation on TESVs and single-species biofilms of chronic rhinosinusitis isolates grown on coverslips. Both types of samples were fixed in 2% (v/v) glutaraldehyde at 4°C for 16 h. Specimens were then rinsed twice in PBS and dehydrated through a series of ethanol washes as follows: 25% ethanol 30 min, 50% ethanol 30 min, 75% ethanol 30 min, and two washes for 1 h in 100% ethanol. Samples were dried in a critical point dryer (Bal-tec), mounted on aluminium stubs and sputter coated with gold at Electron Microscopy Research Services, Newcastle University. Biofilms were visualised using a scanning electron microscope (Cambridge Stereoscan 240).

2.6.3 *Transmission electron microscopy of chronic rhinosinusitis obstructive mucin*

Samples of obstructive material removed from patients during functional endoscopic sinus surgery were cut into ~1 mm^3 pieces and placed into 2% glutaraldehyde, at 4°C, immediately after surgery. These samples were dehydrated through a series of ethanol washes, embedded and sectioned at Electron Microscopy Research Services, Newcastle University. Sections were analysed in a transmission electron microscope (Philips, CM100).

2.7 Quantification of extracellular DNA within biofilm matrices

To quantify eDNA in monospecies biofilms, bacteria (Table 2.8) were cultured in 6-well microtiter plates. To each well, 3 mL of medium was added and plates were incubated at 37°C for 72 h. Spent medium was replaced with fresh medium every 24 h. Following incubation, the supernatant was removed and two wells were used to determine the extent of the biofilms by staining with crystal violet. Briefly, 1 mL of 0.5% crystal violet was added and incubated at 20°C for 15 min, wells were washed three times with PBS, and residual stain was dissolved in 1 mL of 7% (v/v) glacial acetic acid. The A_{570} was read in a BioTek Synergy HT microplate reader. The

remaining four wells were used to quantify eDNA, using a modification of the method of Kreth *et al*., (2009). Wells were immersed in 1.5 mL of PBS and biofilms were gently harvested using a cell scraper. The biofilms from the four wells were combined, vortexed for 20 sec, and incubated at 37°C for 1 h with 5 µg mL^{-1} Proteinase K (Sigma Aldrich). Cells were separated from the supernatant, containing eDNA, by centrifugation at 16,000 *g* for 2 min. Extracellular DNA in the supernatant was extracted with phenol:chloroform:isoamyl alcohol (25:24:1) (Fisher Scientific). Samples were centrifuged at 16,000 *g* for 5 min, and the aqueous phase was collected. DNA was precipitated by isopropanol, pelleted by centrifugation at 16,000 *g* for 10 min, air dried, and re-suspended in 50 µL of 10 mM Tris pH 8.5. Intracellular DNA was purified using the MasterPure™ DNA Purification Kit. The concentration of DNA was determined using a NanoDrop-1000 spectrophotometer, or via PicoGreen® dsDNA quantification. DNA was also visualized by agarose gel electrophoresis. Each extraction was performed three times independently.

Table 2.8 Strains tested for eDNA production with culture techniques

Strain	Media	Growth Conditions[a]
S. gordonii DL1	THYE	Candle jar
A. oris MG1	THYE	Candle jar
F. nucleatum 22586	BHIG	Anaerobic
S. mutans GS-5	THYE	Candle jar
C. propinquum FH1	BHY	Aerobic
C. pseudodiphtheriticum FH2	BHY	Aerobic
M. catarrhalis FH3	BHY	Aerobic
M. catarrhalis FH4	BHY	Aerobic
S. aureus FH5	BHY	Aerobic
S. aureus FH6	BHY	Aerobic
S. aureus FH7	BHY	Aerobic
S. epidermidis FH8	BHY	Aerobic
S. epidermidis FH10	BHY	Aerobic
S. epidermidis FH11	BHY	Aerobic
S. lugdunensis FH12	BHY	Aerobic
S. lugdunensis FH13	BHY	Aerobic
S. lugdunensis FH14	BHY	Aerobic

S. warneri FH15	BHY	Aerobic
S. warneri FH17	BHY	Aerobic
S. anginosus FH18	BHY	Aerobic
S. anginosus FH19	BHY	Aerobic
S. constellatus FH20	BHY	Aerobic
S. constellatus FH21	BHY	Aerobic
S. intermedius FH22	BHY	Aerobic
S. pneumoniae FH26	BHY	Aerobic
S. salivarius FH27	BHY	Aerobic
S. salivarius FH28	BHY	Aerobic
S. salivarius FH29	BHY	Aerobic

[a]All strains were incubated statically at 37°C.

2.8 Biochemical techniques

2.8.1 *Protein gel electrophoresis*

Separation of proteins was achieved using 12 % SDS polyacrylamide gels (SDS-PAGE). First, a 12 % acrylamide resolving gel was prepared by mixing 8 mL 30 % acrylamide (29:1 acrylamide:bisacrylamide) (Sigma Aldrich), 5 mL 1.5 M Tri-HCl pH 8.8, 200 µL 10 % SDS (w/v) (Sigma Aldrich), 6.6 mL distilled water, 200 µL 10 % freshly prepared ammonium presulfate (APS) (w/v) (Sigma Aldrich) and 20 µL of the polymerisation agent tetramethylethylenediamine (TEMED) (Sigma Aldrich). This solution was gently mixed and poured between two sealed glass plates, until 2-3 cm from the top of the plates. Distilled water was overlaid to remove any bubbles, and the resolving gel was allowed to set, after which excess water was removed. The stacking gel was prepared by mixing together the reagents listed in Table 2.9, poured on top of the resolving gel and allowed to set once a comb of appropriate sized wells had been placed between the two glass plates. The glass plates were placed in a gel tank (ATTO) and submerged in 1x running buffer (0.2 M glycine (Melford), 0.025 M Tris, 0.1 % SDS, pH 8.3) before protein samples were added.

During gel preparation proteins were diluted 1:1 in a denaturing buffer, Laemmli 2x concentrate (Sigma Aldrich), and heated at 100°C for 10 min. Once gels had set, and proteins were denatured, the gel comb was removed and protein samples were added to wells. A protein standard ladder for determining the mass of proteins was always

included. Gels were run at 180 V for 1-2 h before being transferred to Coomassie Brilliant Blue R-250 staining solution (70 mL/L acetic acid, 400 mL/L methanol, 530 mL/L dH_20 and 2.5 g/L Coomassie Brilliant Blue R-250 (BioRad)) for 2-3 h. Protein bands were visualized by de-staining the gel for 2-3 h in a solution containing 100 mL/L glacial acetic acid, 500 mL/L methanol (Fisher Scientific) and 400 mL/L dH_2O.

Table 2.9 Composition of SDS-PAGE gels

Resolving Gel	Stacking Gel
H_2O 6.6mL	H_2O 7.25 mL
30% Acrylamide Mix 8.0 mL	30% Acrylamide Mix 1.25 mL
1.5M Tris (pH 8.8) 5.0 mL	1M Tris (pH 6.8) 1.39 mL
10% SDS 200 µL	10% SDS 100 µL
10% APS 200 µL	10% APS 100 µL
TEMED 20 µL	TEMED 20 µL

2.8.2 *Determination of protein concentration*

Protein concentrations were determined either via the Bradford assay, or by densitometry. The Bradford assay uses a dye, Coomassie Brilliant Blue G-250 (Bio-Rad), which binds to basic and aromatic amino acid residues, causing a change in the absorption maximum of the dye from 465 to 595 nm (Bradford, 1976). A protein standard curve was generated using concentrations of bovine serum albumin (BSA) (Thermo Scientific) ranging from 0.05 mg/mL to 0.5 mg/mL. Ten µL of each standard were added in triplicate to wells of a sterile polystyrene 96-well plate. Protein samples were diluted and added to the plate in 10 µL volumes. Next, 200 µL of the Bradford reagent (Bio-Rad), diluted 1:5 with dH_2O, were added to all samples and standards. After 20 min, to allow the colour change to develop, the absorbance of each well was measured at 560 nm using a Synergy HT (BioTek) plate reader. Protein concentrations were calculated from the standard curve generated.

Interference with the Bradford dye caused by detergents or highly concentrated buffers can lead to an overestimation of protein concentration. Therefore, in certain circumstances, protein concentration was estimated using BSA standards on SDS-PAGE gels. Gels with BSA standards ranging from 62.5 µg/mL to 1000 µg/mL and sample proteins were stained with Coomassie Brilliant Blue, and then scanned using a G:Box (Syngene) gel viewer. GeneTools (Syngene) software was used to calculate

protein concentration from the BSA standard curve, whilst also subtracting any background interference.

2.8.3 *Purification of* B. licheniformis *NucB*

NucB was produced by a collaborator (Nithya Rajarajan) using a previously described method (Nijland *et al.*, 2010). Bacterial strain *Bacillus subtilis* NZ8900 containing plasmid pNZ8901 was inoculated into 5 mL sterile Luria Bertani broth containing 5 mg l^{-1} chloramphenicol and cultured aerobically at 37°C for 18 h. The culture was adjusted to an optical density (OD_{600}) of 1.0, and 100 µL were transferred to 10 mL of sterile LB containing chloramphenicol and incubated aerobically at 37°C for 3 h until OD_{600} ~ 1.0 was reached. At this point, 5% v/v cell free supernatant of an overnight *B. subtilis* ATCC6633 culture was added to provide the subtilin required to induce NucB production, and this was incubated for a further 2.5 h. Cells were removed by centrifugation at 6000 *g* for 20 min and the supernatant was sterilized by passing through a 0.2 µm syringe filter (Pall Corporation). NucB was purified from the supernatant using trichloroacetic acid (TCA) precipitation followed by Superose™ 12 gel filtration. Proteins in the active fractions were further concentrated by TCA precipitation again and analysed by SDS-PAGE. The concentration of protein was estimated by densitometry analysis, in comparison with standards of known concentration (BSA). NucB was stored at 4°C for up to 3 months.

2.8.4 *Production of* S. gordonii *DL1 SsnA*

2.8.4.1 *Construction of SsnA-GST vector*

Previously, a system was designed to express SsnA from pGEX-KT, a vector that includes an N-terminal GST tag for affinity chromatography purification. The *ssnA* gene, minus the C-terminal LPxTG cell anchor motif and N-terminal secretion signal domain, was amplified using primers ssnA_Pf7 and ssnA_Pr7, creating a product of ~2150 bp. This product was cloned into pGEX-KT and transformed into *E. coli* DH5α. The vector was transferred to *E. coli* BL21(DE3)pLysS.

2.8.4.2 *Overexpression of* S. gordonii *DL1 SsnA*

Escherichia coli BL21(DE3)pLysSpGEXssnA was cultured overnight at 30°C in an orbital shaker (150 RPM) in 5 mL of YT-G (8 g/L tryptone, 5 g/L yeast extract, 2.5 g/L NaCl and 10 g/L glucose) containing ampicillin (100 μg/mL) and chloramphenicol (33 μg/mL).The next day, 2.5 mL of cells were subcultured to YT-G containing ampicillin and chloramphenicol and incubated at 30°C with 200 RPM shaking, until an OD of 0.6-1.0 was reached. At this point, 1 mL of the culture was taken and incubated as an uninduced control. To induce SsnA expression in the remaining culture isopropyl β-D-1-thiogalactopyranoside (IPTG) (Sigma Aldrich) was added to a final concentration of 1 mM, and cells were incubated at 30°C with 200 RPM shaking for a further 4 h. Every hour, a 1 mL sample was removed for analysis of protein expression. After 4 h, cells were harvested by centrifugation at 4,000 *g* for 20 min.

2.8.4.3 *Lysis of* E. coli *pGEX-ssnA*

Cleared lysates were generated from *E. coli* cell pellets that had been frozen overnight at -20°C. Each 50 mL cell pellet was thawed on ice for 15 min and resuspended in 5 mL of lysis buffer that contained PBS, protease inhibitors (Roche), Benzonase (3 U per mL of culture) (Novagen), 1 mM dithiothreitol (DTT) (Sigma Aldrich), 1 mM EDTA, 1 % Triton® X-100 (Sigma Aldrich), and 1 mg/mL lysozyme . The pellet plus lysis buffer was incubated on an end-over-end shaker at room temperature (20-25°C) for 30 min. After this period, 1% Triton® X-100 was added, and the lysate was centrifuged at 15,000 *g* for 30 min at 4°C. The supernatant was poured into a container and stored at 4°C.

2.8.4.4 *Purification of GST-Tagged SsnA*

E. coli cell lysates were separated by affinity chromatography using a glutathione (GSH) resin to capture the GST-tagged SsnA protein. Briefly, 1 mL of 50 % glutathione superflow (Qiagen) was poured into an empty polypropylene column (Qiagen). The settled resin was the equilibrated with 5 mL of PBS-EW, a buffer that contained PBS, 1 mM DTT, and 1 mM EDTA. Cleared lysates were run through the resin (collecting the flow-through) and then the column was washed twice with 2.5 mL of PBS-EW. After washing, 500 μL of elution buffer (50 mM Tris pH 8, 0.1 M NaCl, 50 mM L- reduced glutathione (GSH) (Sigma Aldrich), 0.1 % Triton® X-100 and 1 mM DTT) were incubated with the resin containing SsnA-GST for 15 min at room temperature (20-

25°C) and the elute collected thereafter. The elution step was repeated twice, until all SsnA-GST had been collected. Fractions were stored at -20°C, and analysed by SDS-PAGE.

2.8.4.5 *Thrombin cleavage of SsnA-GST*

For some experiments the N-terminal 26 kDa GST protein tag was cleaved with thrombin (Sigma). Thrombin cleaves between arginine and glycine at a specific cleavage site (Leu-Val-Pro-Arg-Gly-Ser). Sequence analysis using NEBcutter V2.0 (Vincze *et al.*, 2003) confirmed there were no other thrombin cleavage sites within the recombinant protein. Thrombin cleavage was optimised using various units of enzyme, times of incubation and temperatures of reaction. Thereafter reactions were performed at room temperature, for 24 h, with 1 U of thrombin for every 0.025 mg of SsnA.

2.8.4.6 *Buffer exchange of SsnA-GST*

After affinity chromatography, SsnA was exchanged from elution buffer to a storage buffer (10 mM Tris-HCl pH7.5, 10 mM $CaCl_2$ (Sigma Aldrich), 10 mM $MgCl_2$ and 50% glycerol) using Slide-A-Lyzer® dialysis cassettes (Thermo Scientific). After dialysis, following the manufacturer's protocol, the SsnA sample was aliquoted (100 µL) and stored at -80°C.

For thrombin cleavage experiments SsnA was exchanged from elution buffer to TNC buffer (20 mM Tris-HCl (pH 7.5), 50 mM NaCl, and 1 mM $CaCl_2$) for optimal cleavage of the GST-tag.

2.8.4.7 *Peptide mass fingerprinting*

To confirm the protein identity of the SsnA-GST preparation, samples were sent to the Newcastle University Protein and Proteome Analysis (NUPPA) centre for peptide mass fingerprinting (PMF). Six SsnA-GST samples (3 µg per well) were run on a SDS-PAGE gel. During this process, new buffers were made, gloves were worn, and new containers were used, to keep protein contamination to a minimum. After gel electrophoresis, the gel was transported to the NUPPA facility. At the NUPPA facility, the SsnA-GST band was removed from the gel, digested with trypsin, and the resulting peptide masses detected using a matrix-assisted laser desorption/ionization time of flight/time of flight

(MALDI TOF/TOF) mass spectrophotometer. Peptide masses of the 'unknown' protein were then compared against the known protein sequences of *S. gordonii* DL1, and statistically analysed to find the closest protein match.

2.8.5 *Gel zymography for determining nuclease activity*

Zymography is an electrophoretic technique that detects the degradation of a substrate by an enzyme, in this case the degradation of double stranded DNA in the presence of nuclease enzymes. Sample enzymes were prepared in Lammelli's buffer without boiling, as this may have interfered with the refolding of the enzyme after electrophoresis. SDS-PAGE gels were prepared with 100 μg/mL Salmon Sperm DNA (Sigma Aldrich) included in the resolving gel. Following electrophoresis, the gel was incubated in an enzyme reactivation buffer (40 mM Tris-HCl, 5 mM $CaCl_2$, 5 mM $MgCl_2$ and 3% delipidated milk powder (Premier International Foods)) for 20 h at 37°C. The gel was stained with ethidium bromide (0.5 μg/mL) for 30 min and washed 3 times in distilled water for 15 min each time. Areas of enzyme digestion appeared as dark spaces, where staining had not occurred, when viewed using a ultraviolet light source (G:BOX).

2.8.6 *Förster resonance energy transfer analysis of nuclease activity*

DNase activity was measured quantitatively using a fluorescence-based assay. Assays for nuclease activity often measure the release of oligonucleotides following DNA digestion, by calculating the change in optical density at 260 nm. Förster Resonance Energy Transfer (FRET) is distance-dependant energy transfer between two chromophores, from a donor chromophore to an acceptor chromophore. In this state, no photon is emitted. By using a qPCR PrimeTime™ probe (Integrated DNA Technologies), in a method previously developed (Kiedrowski *et al.*, 2011), it is possible to measure nuclease activity when the enzyme cleaves the phosphodiester bond between two nucleotides. Cleavage causes the donor and acceptor chromophores to no longer be in close proximity, allowing emission of fluorescence.

The FRET substrate was a short sequence (5' CCCCGGATCCACCCC 3') of single-stranded oligonucleotide, modified at the 5' end with a Hex fluorophore and at the 3' end with a Black Hole Quencher 2. FRET substrate was diluted to 2 μM in a buffer consisting of 20 mM Tris-HCl (pH 8) and 10 mM $CaCl_2$. Fluorescence was measured

by mixing 25 µL of diluted FRET substrate with 25 µL of a nuclease source in a well of a 384-well microtiter plate (Greiner Bio-One), and measuring the rate of fluorescence change (excitation 530 nm / emission 590 nm) at 30°C over 40 min using the Synergy HT (BioTek) microplate reader. Background nuclease activity was determined by mixing FRET substrate with the buffer of the nuclease source.

2.8.7 *Nuclease activity of planktonic cultures*

The FRET assay was also developed so that it could be used to determine the nuclease activity of bacterial cultures. Bacteria were cultured overnight at 37°C, the following day the optical density of cultures (OD_{600}) was read, and cultures were diluted to OD_{600} = 1. Cultures were then separated into a pellet and supernatant by centrifuging at 3,000 *g* for 10 min. The pellet was washed twice with PBS, to remove loosely bound exonucleases, and re-suspended in PBS to an OD_{600} of 1. The FRET assay (see above) was then performed using 25 µL of sample cells or supernatant.

S. gordonii DL1 cells were also re-suspended in acidic buffers, to investigate the effect of pH on SsnA. Overnight cells were prepared as before and re-suspended in a pH 4.5 buffer (77.1 g/L ammonium acetate, 70 mL/L glacial acetic acid), or a pH 5.5 buffer [96.3 mL of solution I (13.61 g/L KH_2PO_4 (BDH)) and 3.6 mL of solution II (35.81 g/L Na_2HPO_4)] and PBS (pH 7.1). FRET readings were taken at 0 h, 1 h and 2 h. Cells were incubated at room temperature (20-25°C). Whilst taking FRET measurements, cells were always washed in PBS and then re-suspended in PBS (as acid may affect the FRET assay). After 2 h, cells previously incubated in acidic buffers were transferred to PBS pH 7.1, so that any change in nuclease activity could be measured over the next two h. Cells were split in two, with one half being placed in PBS plus chloramphenicol (12.5 µg/mL). Chloramphenicol inhibits *de novo* synthesis of proteins (Gale and Folkes, 1953), and so any nuclease activity return would be due to re-folding of the enzyme.

2.9 Regulation of SsnA

2.9.1 *CcpA and MalR promoter binding sites*

Regulatory sequence motifs contained within the *ssnA* promoter region were identified using RegPrecise (http://regprecise.lbl.gov/RegPrecise/) (Novichkov *et al.*, 2010), a database of transcription factors. Contained within the *ssnA* promoter were consensus

binding sites for the transcription factors catabolite control protein A (CcpA) and the maltose repressor, MalR. To determine the role of these transcription factors in *ssnA* regulation they were chosen as targets for gene knockouts in *S. gordonii* DL1.

2.9.2 *Mutagenesis of* S. gordonii *DL1*

2.9.2.1 *Genetic manipulation*

The CcpA and MalR genes were replaced with the *ermAM* erythromycin resistance determinant by allelic exchange mutagenesis in *S. gordonii* DL1. Primer sequences are given in Table 2.6.

For *ccpA* mutagenesis the gene was replaced with *ermAM* via cloning in a pCR®2.1 vector. Primers ccpAF1 and ccpAR1 were used to amplify a 507-bp fragment consisting of a 61-bp 5' region of *ccpA* and 446-bp upstream. A 573-bp fragment compromising a 234-bp 3' region of *ccpA* and a 339-bp downstream region, was amplified using primers ccpAF2 (containing an *Asc*I (New England Biolabs) restriction site) and ccpAR2. Following this, a long PCR, using primers ccpAF1 and ccpAR2, was employed to amplify and combine both short fragments together into a 1080-bp product. This product included the up and downstream regions of the *ccpA* gene and a central *Asc*I restriction site. *Asc*I was chosen since pCR®2.1 and the *ccpA* flanking regions did not contain this restriction site. Next, the 1080-bp fragment was ligated into pCR®2.1, and transformed into *E. coli* TOP10, creating pCR®2.1-*ccpA*. A 752-bp region containing the *ermAM* erythromycin determinant was amplified from the streptococcal vector pVA838 (Macrina *et al.*, 1982) using primers ermAMF1 and ermAMR1, that both contained *Asc*I restriction sites. The *ermAM* product and the pCR®2.1-*ccpA* vector were digested with *Asc*I. Digested *ermAM* was then ligated into pCR®2.1-*ccpA*, and transformed into *E. coli* TOP10, creating pCR®2.1-*ccpA*::*ermAM*. The 1832-bp *ccpA*:*ermAM* insert was removed from the vector using the restriction enzyme EcoRI (New England Biolabs), run on an agarose gel, and then the correct fragment was purified from the gel before being used to transform *S. gordonii* DL1.

Mutagenesis of the *malR* gene was performed without a cloning vector. Using the primers malRF1 and malRR1 a 471-bp region including 58-bp of 5' *malR* sequence was amplified. A 421-bp downstream region was PCR amplified with malRF2 and malRR2, which contained 227-bp of 3' *malR* sequence. The malRR1 and malRF2 primers contained 17-bp complementary sequences for ermAMF2 and ermAMR2 primers. After

amplifying the erythromycin resistance determinant, *ermAM*, from pVA838 a PCR reaction was set-up that included the up and downstream *malR* products and *ermAM* in equal ratios. A long PCR was performed, with malRF1 and malRR2. The resulting 1644-bp product, containing the ermAM erythromycin resistance determinant, was then transformed into *S. gordonii* DL1.

2.9.2.2 *Transformation of* S. gordonii *DL1*

Prior to transformation, 10 mL of BHY broth were inoculated with *S. gordonii* DL1 and incubated at 37°C overnight. Whilst incubating, 2x 5 mL of BHY, plus 50 µL fetal calf serum (FCS) (Sigma Aldrich) (heat inactivated at 60^{o}C for 30 min), and 12.5 µL 40% glucose (BDH) were pre-warmed at 37°C. The next day, 50 µL of the *S. gordonii* DL1 overnight was subcultured into 5 mL of pre-warmed BHY/FCS/Glucose and incubated at 37°C, in a candle jar, until an OD_{600} of 0.3 was reached. Fifty µL were further subcultured into 5 mL fresh, pre-warmed BHY/FCS/Glucose and incubated for 60 min at 37°C in a candle jar. Aliquots (800 µL) of the culture were dispensed into 1.5 mL eppendorfs (Starlab), and DNA (up to 10 µg) was added and incubated at 37°C for a further 4 h. One hundred µL portions (neat or diluted) were plated onto selective agar and incubated for 48 h at 37°C. Transformation was confirmed by the presence of colonies on selective agars, colony PCR and DNA sequencing.

2.10 Dispersal of biofilm micro-organisms with DNase enzymes

2.10.1 *Dispersal of tracheoesophageal speech valve biofilm micro-organisms with NucB*

The efficacy of NucB at removing micro-organisms from tracheoesophageal speech valves (TESVs) was determined by treating halves of TESVs with NucB or PBS. First, TESVs were removed from the PBS they were stored in, placed on a sterile petri dish, and sliced in half along their longitudinal axes using a sterile scalpel blade. Each half was placed in separate wells of a sterile polystyrene 12-well microtiter plate (Greiner Bio-One), containing 3 mL sterile PBS, and washed 3 times with 3 mL sterile PBS. After washing, one half was submerged in 500 µL NucB (3 µg/mL) and 2.5 mL PBS, and the other half was placed in a well containing 3 mL PBS. Valve halves were incubated for 1 hour at 37°C.

After the incubation period, triplicate samples of the supernatant (50 µL), undiluted or diluted 10-fold in sterile PBS were spread evenly onto Sabouraud dextrose or nutrient agar using a sterile spreading loop. Plates were incubated aerobically at 37°C for 20 h. Colonies ≥ 2 mm in diameter were counted and these counts were used to determine the colony forming units (CFUs) released. Plates were only counted if they contained 10 to 400 colonies. Occasionally, cultured organisms captured were selected for isolation and identification using microscopy and Gram staining.

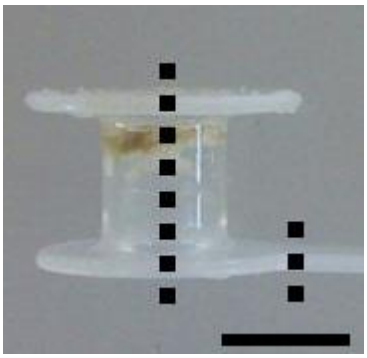


Figure 2.1 TESVs were cut along their longitudinal axes before being treated with NucB. The dotted line indicates the area where a scalpel blade was used to cut the TESV. Scale bar is 10 mm.

2.10.2 *Crystal violet biofilm assay*

For quantifying biofilm extent, both for determining growth of bacteria and treatment efficacy of nuclease enzymes, the crystal violet (CV) assay was used. Biofilm extent was quantified by staining with 100 µL 0.5 % (w/v) CV (per well). After incubating for 15 min at room temperature (20-25°C), wells were rinsed 3 times with PBS. Remaining CV was dissolved in 100 µL 7 % acetic acid (v/v) and the A_{570} was read using a microplate reader (Synergy HT). The absorbance value of a negative control (stained and washed un-inoculated wells) was subtracted from sample absorbance values. Each assay was repeated three times independently.

2.10.3 *Measuring the efficacy of DNase enzymes against biofilms formed on microtiter well surfaces*

To assess the efficacy of nuclease enzymes (bpDNase I, NucB, SsnA) at retarding or dispersing microbial biofilms, biofilms were cultured in microtiter plates, treated with enzyme (5 μg/mL) and biofilm extent was quantified with the crystal violet assay. When testing for biofilm inhibition, enzyme was included (with media and inoculae) during biofilm formation. For dispersal, enzyme was added after biofilm formation for 1 hour at 37°C. Biofilm extent was then measured with the CV assay. This was compared with control biofilms that had not been treated with nuclease enzymes.

2.11 Statistical analysis

Experiments were repeated in triplicates as standard. Graphs and tables were made using Microsoft Excel, and throughout results are given as the mean ± standard error. Statistical comparisons were made using IBM SPSS Statistics 19. Mean values of two groups were compared for significant difference using the two-sample *t*-test, after a test for normality had proved the distribution of sample values was normal. A *p*-value less that 0.05 was deemed an acceptable level of statistical significance.

Chapter 3: Tracheoesophageal Speech Valve Biofouling

3.1 Outline

The colonisation of medical implants by microbial biofilms is a major concern for healthcare services because it limits the lifespan of the devices and can cause infections such as sepsis (von Eiff *et al.*, 2005). Tracheoesophageal speech valves (TESV) are given to patients who have had a total laryngectomy as a way of rehabilitating speech. The TESV is the most commonly used method of alaryngeal speech owing to a number of advantages, primarily, natural sounding speech, and quick rehabilitation. In a recent study, Singer *et al*., (2013) measured speech intelligibility in over 200 patients using TESVs, esophageal speech, or electrolaryngeal devices, finding that TESVs gave the quickest rehabilitation of speech. Thousands of patients in the US use TESVs (Ramage *et al.*, 2006). However, the major disadvantage is the need to regularly replace valves, at a cost, when they become damaged over their lifespan by microbial biofilms. This study, the first of its kind, investigated the role of eDNA in providing stability in the polymicrobial biofilms that form on the surfaces of TESVs.

The average lifespan of TESVs is between 108 to 207 days (Hancock *et al.*, 2013; Kress *et al.*, 2013). Device design, patient characteristics and treatment program all change the lifespan of TESVs. The most common reason for replacement is leakage through the device, caused by *Candida* spp. biofouling (Balm *et al.*, 2011). Replacement is typically a quick procedure, requiring only a visit to an out-patient clinic. However, replacement every 3 to 6 months leads to substantial costs for healthcare services or patients. The Freeman Hospital, Newcastle (NHS) spent £44,000 in 2010 on TESVs, not including staff costs (Shakir *et al.*, 2012). Recently, it was estimated that the yearly cost for TESV use per patient is £530-£670, in the Newcastle area (Owen and Paleri, 2013). It is clear, that a reduction in microbial colonisation is needed to increase the lifespan of these devices for improved patient satisfaction and to minimise healthcare expenditure.

The ability to clean biofouling from TESVs depends on the device type, as there are a number on the market, both indwelling and non-indwelling (Provox and Blom-Singer produce the widest used TESVs). Several attempts have been made to increase the lifespan of TESVs (reviewed by Rodrigues *et al.*, 2007). These include: (i) probiotic diet, (ii) use of bio-surfactants, (iii) modification of the silicone surface, and (iv)

treatment with antifungal agents. However, biofilm colonisation could be controlled with DNases if TESV biofilms rely on eDNA for matrix support. This is a potentially novel technique for the cleaning of TESVs.

The biofilm that forms on the TESV surface is an aggregation of many fungal and bacterial species. *Candida albicans* is considered a key coloniser but other *Candida* spp., such as *Candida glabrata*, are often present (Bauters *et al.*, 2002). However, the location of the voice prosthesis, in the neck, connected to skin and close to the oral cavity leads to commensal bacteria colonising the TESV device. It is clear that staphylococci, oral streptococci and lactobacilli are often present in TESV biofilms (Buijssen *et al.*, 2012). Recently, lactobacilli were isolated from 33 of 34 explanted TESVs, and were shown to grow intertwined with *Candida* spp. (Buijssen *et al.*, 2007). *Candida albicans* binds to many bacterial species, including oral streptococci (Holmes *et al.*, 1996), and this has been shown to allow the colonisation of surfaces by Candida (Holmes *et al.*, 1995). FISH analysis of TESV biofilms has highlighted the important interaction of bacteria with fungi (Buijssen *et al.*, 2012). Therefore, whilst *Candida albicans* is often considered the key organism in TESV biofouling, bacteria cannot be overlooked in the colonisation process.

Exogenous DNA has been measured in *C. albicans* biofilms, and DNase treatment reduces mature biofilm extent of mono-species biofilms formed by strain SC5314 by up to 40% (Martins *et al.*, 2010). *Lactobacillus plantarum* LM3 biofilms were reduced by 51% when incubated with DNase I after growth for 24 h (Muscariello *et al.*, 2013). Futhermore, *Streptococcus mutans* UA159, an oral streptococcus, has 20% less biofilm extent when treated with DNase I than a control (Perry *et al.*, 2009). Lastly, *Staphylococcus* spp. are susceptible to DNase treatment (Qin *et al.*, 2007; Kaplan *et al.*, 2012; Shields *et al.*, 2013). Although these species are all relevant to the types of organisms that are known to colonise TESVs the biofilms were all grown *in vitro* as single species, and it remains to be seen how eDNA may contribute to a polymicrobial biofilm on a TESV. Recently, eDNA, and subsequent DNase degradation has been shown in multi-species activated sludge biofilms (Dominiak *et al.*, 2011).

The objectives of this study were to determine the eDNA content of TESV biofilms, both by microscopy and extraction, and then to treat TESV biofilms with NucB, a novel bacterial DNase. The primary aim was to establish if NucB has potential for dispersing

microbes from the surface of TESVs, thereby prolonging their lifespan. To our knowledge this is the first study to look at the contribution of eDNA to TESV biofilm stability, and one of few to determine eDNAs role in complex, clinical, polymicrobial biofilms.

3.2 Extracellular DNA in the extracellular matrix of TESV biofilms

The contribution of eDNA to the matrix stability and initial attachment of biofilm cells is well understood in single-species biofilms. However, it remains less well researched in mature polymicrobial biofilms. TESVs provide a good model for studying the reliance on eDNA of biofilm-forming micro-organisms in mature biofilms. Therefore, this study began by extracting eDNA from the biofilm matrix of used TESVs, and then visualizing the biofilms in more detail with SEM and CLSM.

3.2.1 Extraction of eDNA from the extracellular matrix

Six explanted TESV devices were collected and examined for the presence of eDNA. Biofilms were removed by careful scraping into an eppendorf, and eDNA was then extracted from the separated EPS fraction (see Materials and Methods 2.7). Finally, eDNA was measured using PicoGreen® dye, and visualized on a 1 % agarose gel (Figure 3.1). PicoGreen® spectrophotometry was chosen because this method of dsDNA quantification is more selective for dsDNA than determination of absorbance at 260 nm, where contaminants, nucleotides, RNA and single-stranded nucleic acids can interfere with measurements. The PicoGreen® dye emits fluorescence when bound to dsDNA and exhibits very little fluorescence when unbound. The total eDNA recovered from TESV biofilms varied between a minimum of 82 ng/mL (sample 6) and a maximum of 1200 ng/mL (sample 1) (Figure 3.1A). Importantly, all samples had detectable nucleic acids when analysed with the PicoGreen® stain. The large amount extracted from sample 1 was due to the large biomass of biofilms on this device. Therefore, eDNA concentration was obtained by calculating the amount of eDNA per µg of biomass. This measure showed sample 5 to have the highest concentration of eDNA, again, sample 6 had the lowest (Figure 3.1A). By running eDNA onto an agarose gel, the total abundance was visualised. Smears of nucleic acid were seen in samples 1, 4 and 5 (Figure 3.1B). These samples also had the highest total eDNA present, as measured by the PicoGreen® dye. There was no high molecular weight band of eDNA present, as seen in mono-species

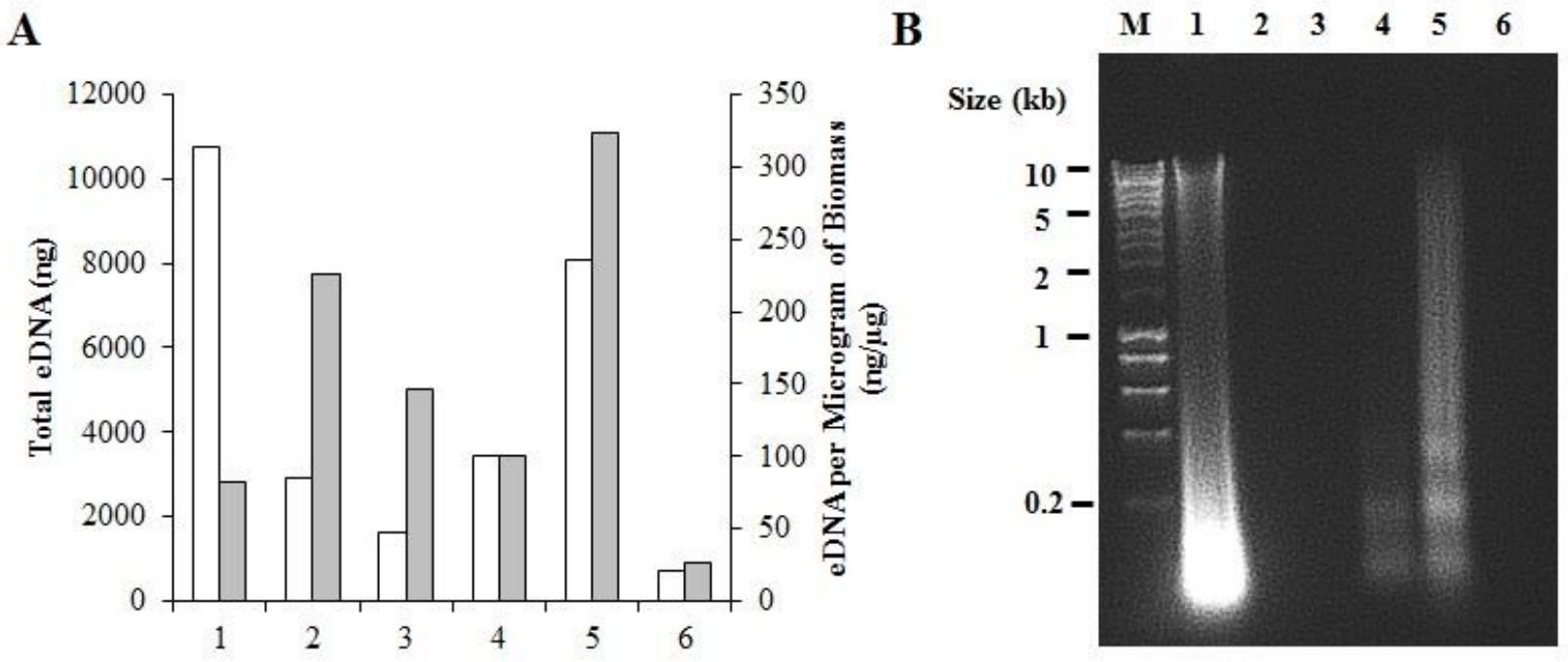


Figure 3.1 Extracellular DNA quantification from the biofilm matrices of six TESV biofilms. (A) Biofilms were removed by scraping, and the biomass weighed. Subsequently, eDNA was extracted from the EPS and measured using PicoGreen® dye so that the total eDNA present (total volume 9 mL) could be calculated (white bars), and the amount of eDNA per µg of biomass (grey bars). (B) Extracted eDNA was also visualised on a 1 % agarose gel, where M is the molecular weight marker, and 1-6 is the TESV biofilm samples.

chronic rhinosinusitis isolate biofilms (Chapter 4). Extracellular DNA migrated to a weight of between 200 bp to 10 kbp. This smear is indicative of DNA that has degraded, as might be expected from biofilms that were up to 3 months old. Samples 1, 4 and 5 contained lower molecular weight smears and bands, which may be RNA. This would not have been measured by PicoGreen® as this dye is selective for dsDNA. It may be possible to identify this material by treating "eDNA" extractions with DNase (see Chapter 5 Figure 5.1A) and RNase. For samples 4 and 5, there are distinct bands occurring at approximately 400 bp, 200 bp and a lower molecular weight. These bands resolve at similar weights in both samples.

3.2.2. Visualization of TESV biofilms and eDNA

Initially, one TESV was examined for the presence of microbial biofilms by scanning electron microscopy (SEM) (Figure 3.2) (see Materials and Methods 2.6.2). At lower magnifications, biofilms could clearly be seen on the flat outer surface of the TESV (Figure 3.2A). The silicone surface of the TESV was rough. It is unknown if this was due to deterioration as unused TESVs were not visualised. At higher magnification biofilms consisted of a dense network of *Candida* species, with both budding and filamentous cell types present (Figure 3.2B). Although biofilms were colonising the silicone surface, large areas were cell-free or contained only small aggregations of microbes (Figure 3.2C). Within the biofilms there was a network of bacterial cells, cocci and bacilli, with fungal cells also present (Figure 3.2D). Here, the biofilm was clearly polymicrobial and cells of different species are closely aggregated in a complex network-like structure. Extracellular matrix surrounded bacterial and fungal cells, although it should be noted that the sample had been dehydrated (critical for SEM) and therefore had lost its 3D architecture (Figure 3.2B and D).

To further investigate biofilm formation, and attempt to visualise eDNA in the EPS, TESVs were stained with PI and DAPI (DNA stains) to visualise micro-organisms and eDNA, and ConA-FITC (binding to α-D-mannosyl and α-D-glucosyl) to identify fungal cell walls (Figure 3.3) (see Materials and Methods 2.6.1.2). At lower magnification the size and thickness of the TESV biofilms was apparent (Figure 3.3A). There was little discernible structure, and the biofilm appeared to be an intertwined network of yeast hyphae and yeast cells. At higher magnifications, yeast cells were visualised at the single cell level (Figure 3.3C and D). These cells were either alive (blue) or dead (red

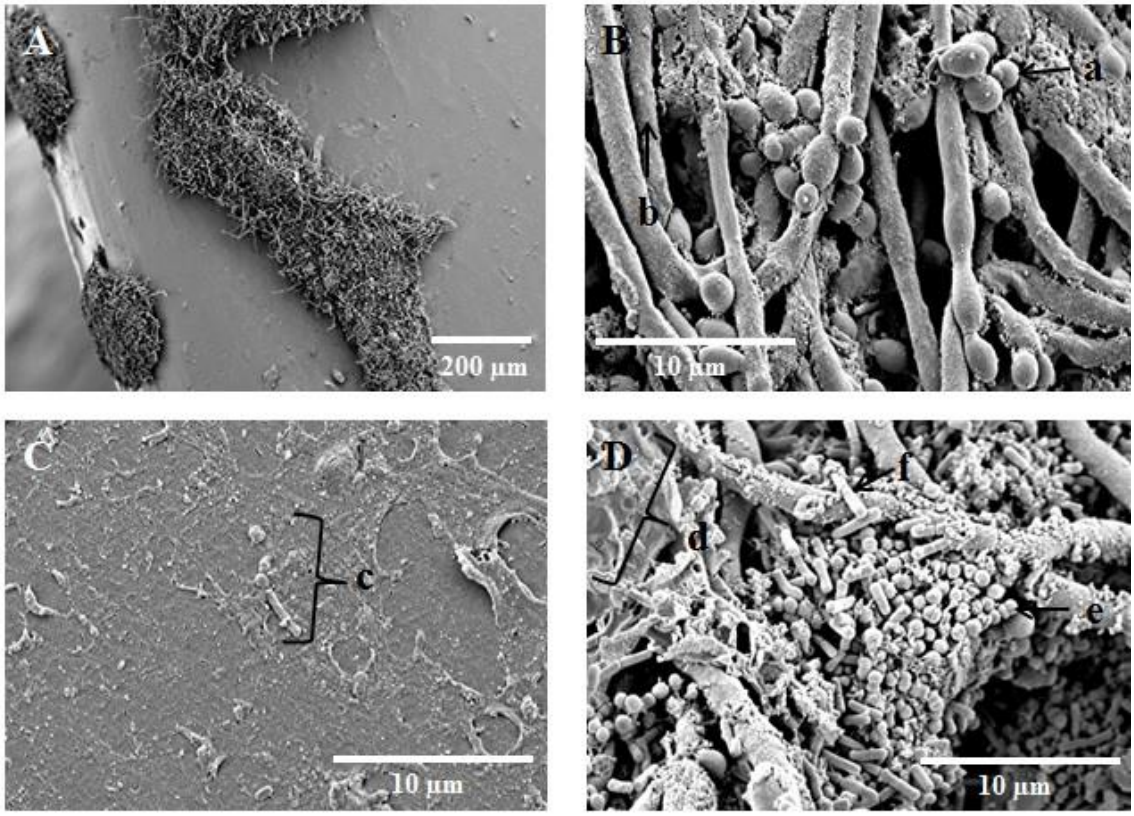


Figure 3.2 Scanning electron micrographs of a mature TESV biofilm. A TESV was fixed in glutaraldehyde, processed for SEM, and then viewed. (A) Colonies were visible to the naked eye and when viewed at high magnifications were clearly dominated by fungal hyphae. (B) Fungal colonization with both yeast cells (a) and fungal hyphae (b), a morphology indicative of *Candida* spp., known as pseudohyphae. (C) Large areas of the TESV contained very few cells (c) but a rough silicone surface. (D) In this micrograph there is a collection of cocci (e), bacilli (f), and fungal hyphae surrounded by an extracellular matrix (d).

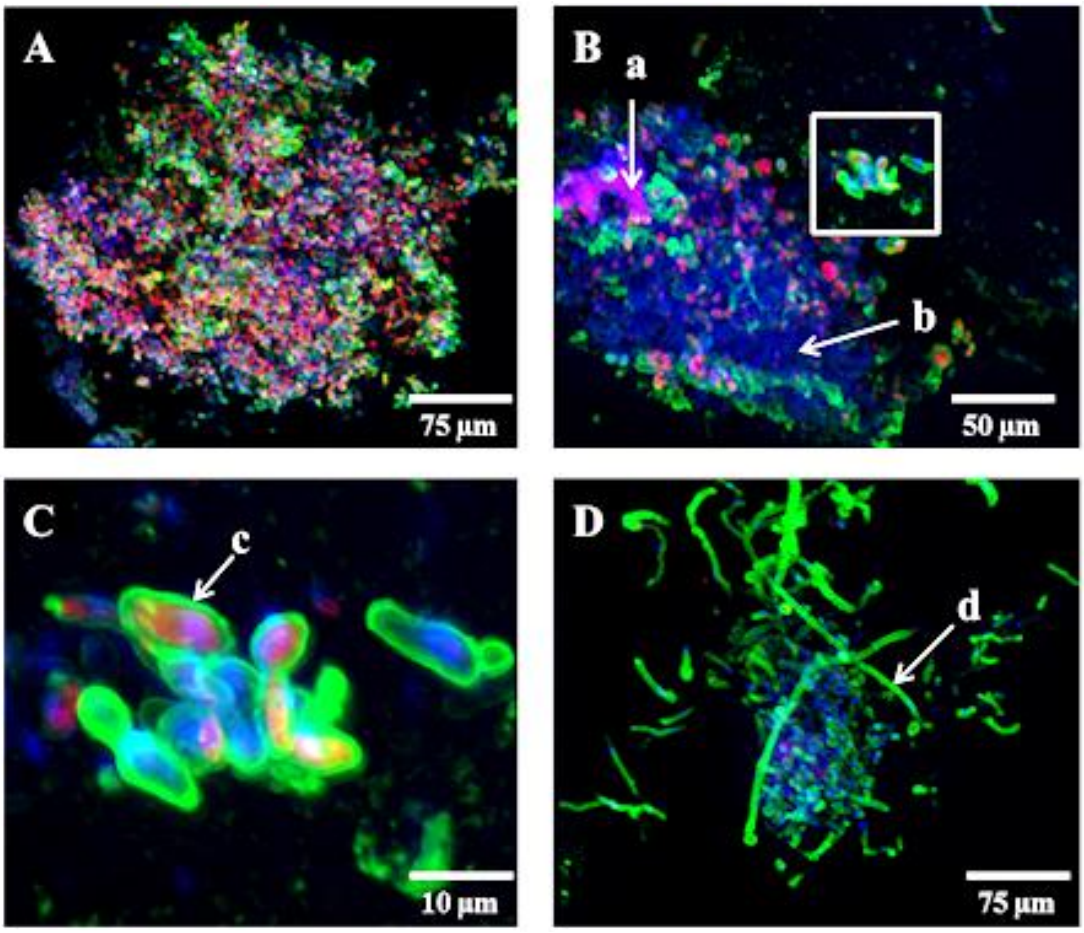


Figure 3.3 Biofilms on TESVs visualised with CLSM and triple-staining with fluorescent dyes. (A) Biofouling covered large areas and these biofilms were thick, with a network of fungal cells. (B) These biofilms contained areas of eDNA, apparent because of co-localisation of DAPI and PI (a) or very diffuse DAPI staining (b) in the extracellular matrix. (C) By increasing the magnification of an area (white box) fungal cells could be visualised at the single-cell level, where cells walls (c) were clearly stained by ConA-FITC. (D) Yeast hyphae (d) were also visible when stained with ConA-FITC. Red colour, PI; blue colour, DAPI; green colour, ConA-FITC.

and blue). Propidium iodide (red) only crosses the membrane of compromised cells, whereas DAPI (blue) can enter either live or dead cells. The nucleic acid stains PI and DAPI have been validated for bacterial micro-organisms. However, PI also enters damaged *Candida albicans* cell membranes (Jin *et al.*, 2005), and DAPI enters both prokaryotic and eukaryotic organisms. Therefore this staining approach was acceptable in these mixed-species biofilms. Extracellular DNA was visible as the co-localisation of red and blue dyes (Figure 3.3B). There were also large areas of diffuse blue staining. In these examples, the staining was not cellular, and appeared to be outside cells, which is indicative of eDNA. However, it is unknown why some eDNA would only stain with DAPI, and some with both DAPI and PI, this requires further investigation. Unlike with SEM, bacteria were hard to visualise using this technique. With CLSM only the outer layer of the biofilm was accurately imaged. It appeared that bacteria were more often found within the biofilm, and that fungal species tended to dominate the outer layers of the biofilm. This was evident with both CLSM and SEM.

3.3 Dispersal of TESV microbes with NucB

As shown by microscopy, the TESVs were heavily colonised by microbial biofilms. These biofilms also appear to contain eDNA. To determine whether eDNA contributes to biofilm stability, 20 TESVs were treated with NucB (3 µg/mL), a novel bacterial nuclease, to examine the efficacy of this enzyme at removing microbes from the silicone surface (see Materials and Methods 2.10.1). TESVs were cut in half and treated with either PBS (control) or with NucB (treatment) for 1 hour at 37°C. The solution that TESV valve halves were placed in was then diluted and plated onto growth media. Of the 20 TESVs treated, 3 were excluded from statistical analysis because one produced no growth, and when two were treated microbial colonies were too abundant to be accurately counted.

The rationale for the experimental design was that more micro-organisms may be liberated from the TESV half placed in a NucB solution, and that this could be counted on growth media. Nutrient agar was chosen as a non-selective agar, and Sabouraud agar was chosen to select for fungi (primarily *Candida* spp.), which are common on TESVs. Figure 3.4 highlights this method, and shows that when NucB was used (Figure 3.4A), more microbes grew on a nutrient agar plate, than when treated with PBS alone (Figure 3.4B). This suggests that increased numbers of microbes are liberated from TESV biofilms when NucB is present. Once the colony forming units (CFUs) were calculated

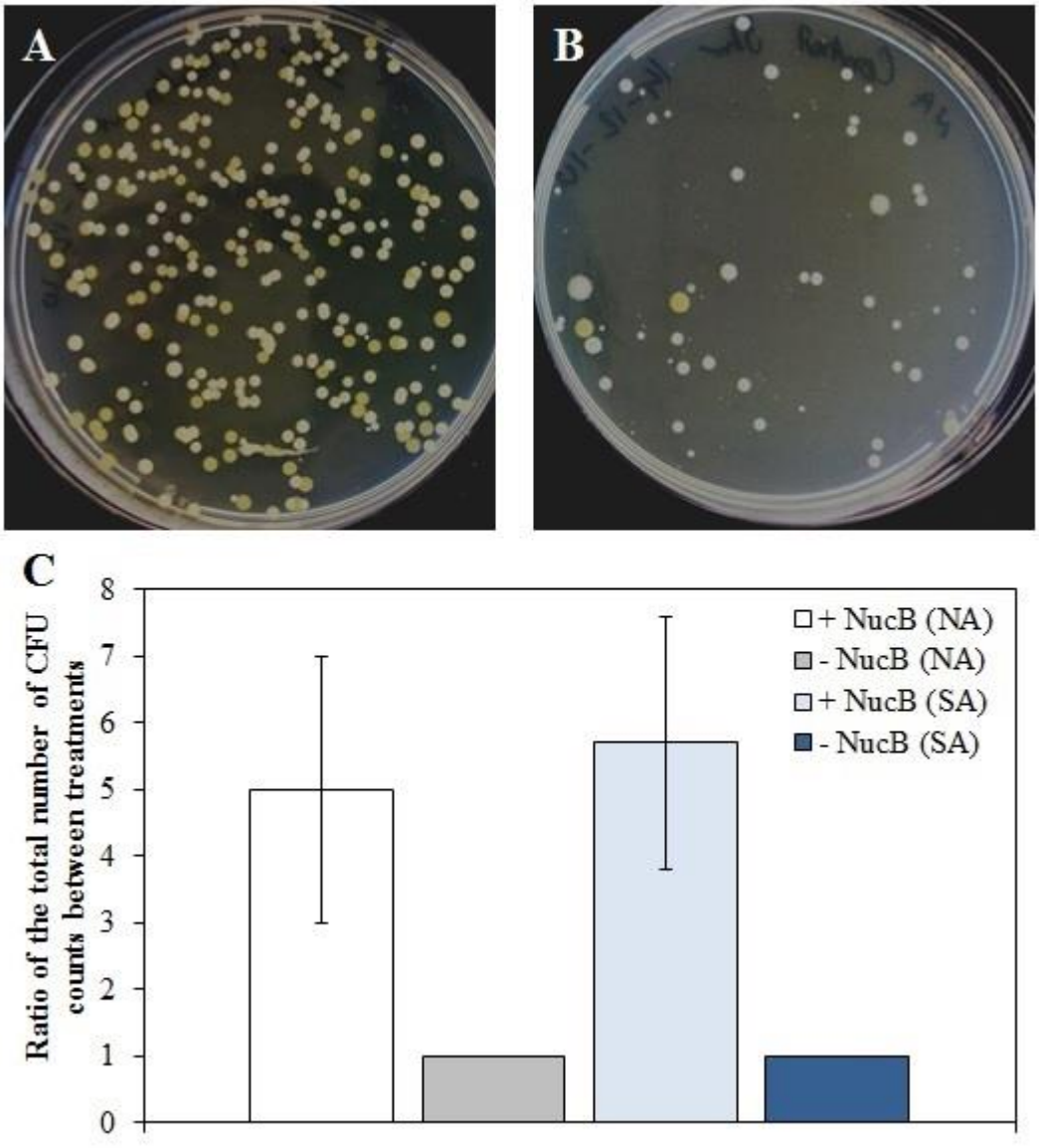


Figure 3.4 Liberation of micro-organisms from the surface of TESVs using a novel bacterial nuclease, NucB. TESV halves were placed into either a PBS control solution or a solution containing the NucB enzyme. Valves were incubated for 1 hour at 37°C, and then the solution was spread onto agar plates. This technique allowed the enumeration of the total number of microbes dispersed from TESVs and an example of this, on nutrient agar, shows that more micro-organisms were removed using a NucB solution (A), than the PBS control solution (B). A total of 17 TESVs were included in the analysis of colony forming units (CFUs) in treatment solutions after incubation, the ratio of CFUs between treatments on the two agars was calculated (C).

Table 3.1 Colony forming unit counts of micro-organisms released from TESVs biofilms, when using NucB or PBS, on nutrient agar.

TESV No.[a]	NucB CFU	PBS CFU	*p*-value[b]
1	10,230	2,760	**.0001**
2	55,740	78,120	*.0001*
3	2,520	600	**.0001**
4	21,960	21,840	.941
5	2,910	90	**.0001**
6	21,000	12,360	**.0001**
7	1,890	300	**.0001**
8	1,710	1,110	.145
10	27,960	24,300	**.0387**
11	10,080	3,930	**.0001**
12	6,420	1,470	**.0001**
14	9,690	8,850	.426
15	5,922	582	**.0001**
16	28,020	20,760	**.0001**
17	3,820	2,640	.058
19	2,400	200	**.0001**
20	15,400	15,040	.79

[a]TESV 9, 13, and 18 were excluded from analysis.

[b]*p*-values were calculated from a two-sample t-test comparing the means from 3 replicate counts. Bold *p*-values indicate NucB significantly releasing more micro-organisms, whereas an italic *p*-value shows a greater, significant release when using PBS.

Table 3.2 Colony forming unit counts of micro-organisms released from TESVs biofilms, when using NucB or PBS, on Sabouraud agar.

TESV No.[a]	NucB CFU	PBS CFU	*p*-value[b]
1	4,410	12,120	*.0001*
2	61,440	93,120	*.0001*
3	2,370	540	**.0005**
4	39,120	31,800	**.0038**
5	7,470	600	**.0001**
6	12,360	8,880	**.0021**
7	1,380	150	**.0001**
8	2,520	1,710	.108
10	14,880	5,910	**.0001**
11	5,790	3,510	**.0023**
12	2,670	90	**.0001**
14	7,530	4,050	**.0001**
15	6,900	480	**.0001**
16	30,480	21,600	**.0001**
17	5,838	4,122	**.0264**
19	2,360	140	**.0001**
20	15,720	8,960	**.0001**

[a]TESV 9, 13, and 18 were excluded from analysis.

[b]*p*-values were calculated from a two-sample t-test comparing the means from 3 replicate counts. Bold *p*-values indicate NucB significantly releasing more micro-organisms, whereas an italic *p*-value shows a greater, significant release when using PBS.

from both treatment solutions it was possible to conclude which treatment liberated more micro-organisms. When micro-organisms were captured on nutrient agar, more were cultured from TESV halves treated with NucB in 11 out of 17 samples (65%) (Table 3.1). Furthermore, the use of Sabouraud agar showed that *Candida* spp. were released in greater numbers when treated with NucB in 14 out of 17 samples (82%) (Table 3.2). The ratio of CFUs between PBS and NucB treatments showed that 5.7-fold (S.E. $^{+/-}$ 2.0) more microbes were released onto Sabouraud agar when NucB was used. There was 5.0-fold (S.E. $^{+/-}$ 1.9) more micro-organisms liberated onto nutrient agar when NucB was present in treatment solutions.

Micro-organisms that were captured on growth media were identified in order to determine if the microflora of these TESVs was similar to those previously studied. Many different species of micro-organisms were recovered from TESVs when placed in PBS or NucB solutions. This was evident by the variety of colony morphologies on nutrient and Sabouraud agar. Individual colonies were selected and re-plated three times to select for contaminant-free micro-organisms. More detailed analysis with Gram staining and light microscopy showed that Gram negative bacilli, yeast and Gram positive cocci were highly prevalent. Furthermore, microbial strains from two TESVs were identified by MALDI-TOF (at the Freeman Hospital, Newcastle). This method identified *Candida albicans* (both TESVs), *Corynebacterium striatum*, *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Enterococcus faecalis*, and *Pseudomonas aeruginosa*.

3.4 Discussion

This preliminary study aimed to determine the contribution of eDNA to the biofilm matrix stability of micro-organisms growing on TESVs. Biofouling has a significant impact on the lifespan of TESV devices and currently there is no optimal regime for increasing this lifespan. Furthermore, there has been little research into the abundance of eDNA in complex, mature, polymicrobial biofilms. Therefore, this project provided two novel aims.

Significant biofouling was observed in most of the TESV samples, and this was visible to the naked eye. The dominant micro-organisms on the TESV visualised by SEM, *Candida* spp., cocci and bacilli, fitted with the brief microbial identification of microbes from two TESVs. This analysis showed colonisation by *C. albicans*, staphylococci, *C. striatum*, *E. faecalis* and *P. aeruginosa*. These species all follow

previous research that has shown *Candida* spp. to be the dominant microflora, along with commensal bacterial species (Bauters *et al.*, 2002; Buijssen *et al.*, 2012). However, no lactobacilli were identified which have been found on 97% of TESVs previously (Buijssen *et al.*, 2007) but this was likely due to limited sampling, and/or the agar media utilised. Scanning electron microscopy and CLSM revealed that *Candida* spp. are the dominant micro-organisms on the outer layer of the TESV biofilm. During SEM visualisation bacteria were observed deeper into the biofilm. *Candida albicans* attaches to oral streptococci via specific cell surface proteins (Holmes *et al.*, 1996). These interactions may lead to biofilm enhancement, and therefore increased levels of anti-microbial resistance (Morales and Hogan, 2010). Fungal-bacterial interactions may be important during the colonisation of TESVs.

Nucleic acids, presiding in the biofilm matrix, were visualised during agarose gel electrophoresis as smears of DNA for 3 samples. These smears appear to show that the eDNA in TESV biofilms is degraded, and is different to the high molecular weight DNA found in single-species model biofilms, which has a migration similar to chromosomal DNA (see Chapter 4 and 5). DNA is an unstable molecule (Lindahl, 1993), and it is likely that the longevity of TESV biofilms (ca. 2-3 months) leads to a loss of DNA integrity and hydrolysis. It is feasible that microbial-produced DNase enzymes contribute to DNA degradation. How this impacts biofilm matrix stability over time is unknown. Previous research on the abundance of eDNA in mature, polymicrobial biofilms is limited but it has been measured in activated sludge biofilms (Dominiak *et al.*, 2011). Extracellular DNA may originate through cell lysis (Mann *et al.*, 2009), active release through quorum sensing (Spoering and Gilmore, 2006), or via excretion from membrane-bound vesicles (Renelli *et al.*, 2004). With such a mature biofilm (ca. 2-3 months) cell death and lysis is likely to be the most obvious explanation for the release of eDNA in this system. During CLSM there were live and dead microbial cells in TESV biofilms, although they were not quantified. There was also LMW material extracted from TESV biofilm matrices, that may be DNA, RNA or some other material. Sharp bands migrating to roughly 200 and 400 bp were visualised in two samples. It is interesting to note that eukaryotic chromatin DNA, with histones, migrates to approximately these molecular weights. This may indicate that human DNA is a component of TESV biofilm matrices. However, further research is required, with DNase, and RNase treatment of samples, to begin to understand this LMW material. Further analysis, with DNA sequencing, could reveal the origin of the eDNA. This

material is important, as it appears to be a significant proportion of the eDNA extracted from some TESV biofilm matrices.

Although eDNA is abundant in *C. albicans* SC5314 single-species biofilms cultured in RPMI medium (Martins *et al.*, 2010) it is unknown how competitive interactions between species may influence eDNA concentrations in multi-species biofilms. Therefore, extrapolating from single-species eDNA data could under-or-overestimate eDNA levels in polymicrobial biofilms. Ultimately, this study is the first to directly measure eDNA in a clinically relevant, mature, mixed-species biofilm.

Extracellular DNA was visualised in the biofilm matrix using a triple-stain technique developed for CLSM. This approach was similar to that used by Kania *et al*., (2010) but added DAPI to the previously used PI and ConA-FITC stains. Mannose containing macromolecules were stained by the lectin ConA, a technique previously used to stain the matrix of *Staphylococcus aureus* biofilms (Akiyama et al., 2002). However, fungal cell wall polysaccharides also contain mannose residues (Bowman and Free, 2006) and ConA-FITC provided clear images of fungi within TESV biofilms. DAPI was used to differentiate between living and dead cells, as live cells form a barrier to PI but not DAPI. However, DAPI (DNA stain) also allowed the visualisation of nucleic acids in the extracellular matrix, either as a diffuse staining or its co-localisation with PI. Although eDNA staining and CLSM is not without difficulties, this adds further evidence to eDNA being abundant in the matrices of biofilms on TESVs. The microscopy of biofilm matrix eDNA could have been improved by adding DNA to biofilms as a positive control, or by attempting to degrade eDNA with DNase enzymes as a negative control. Recently, immunogold labelled anti-dsDNA monoclonal antibodies and SEM has been used to show eDNA on the surface of *Enterococcus faecalis* cells during initial biofilm formation (Barnes *et al.*, 2012). This technique may allow a better detection of eDNA within TESV biofilms.

The major aim of this study was to determine whether TESV biofilms are susceptible to nuclease dispersion with the bacterial nuclease, NucB. NucB has shown clear potential against mono-species biofilms (Nijland *et al.*, 2010; Shields *et al.*, 2013) but this study also highlights its potential at dispersing mature biofilm-forming micro-organisms. Interestingly, *Candida albicans* were dispersed from the surface of TESVs treated in this study. This species of fungus can be a colonizer of TESVs on 40% of used valves (Bauters *et al.*, 2002), and is therefore an important organism in TESV biofouling. Furthermore, DNase I has dispersed *C. albicans* single-species biofilms by up to 40% (Martins *et al.*, 2010). Commensal bacteria, that are often associated with

TESV biofouling, including lactobacilli, streptococci, and staphylococci have been dispersed by DNase I in previous studies (Qin *et al.*, 2007; Perry *et al.*, 2009; Kaplan *et al.*, 2012; Muscariello *et al.*, 2013). From the data presented here, TESV biofilm-forming micro-organisms are susceptible to DNase treatment when present in natural mixed-species biofilms.

Many attempts have been made to increase the lifespan of TESV devices (reviewed by Rodrigues *et al.*, 2007). This is primarily motivated by the cost to healthcare services of replacing TESVs, which was estimated to be £44,000 at the Freeman Hospital, Newcastle, in 2010 (Shakir *et al.*, 2012). However, the colonisation of TESVs by micro-organisms also leads to problems for patient such as TESV leakage (Laccourreye *et al.*, 1997) and poor speech caused by reduced air flow (Elving *et al.*, 2003). Therefore, NucB could lead to major improvements in the maintenance of TESVs. However, the experimental design examined the dispersal of micro-organisms from the silicone surface of TESVs, which is not a true reflection of the removal of biofilms from this device. Firstly, the CFUs released were a small number of the total CFUs expected in mature biofilm. Also, this method does not give any indication of the number of dead cells released during NucB treatment; dead cells were visualised during CLSM. Ultimately, after treatment, biofilms were still visible to the naked eye on TESVs. This study provides preliminary evidence of the potential of NucB to remove micro-organisms from TESVs. Extracellular DNA can be more important in the early stages of biofilm formation (Whitchurch *et al.*, 2002). Therefore, cleaning of devices with a NucB solution from the beginning of use may provide the most potential to prolong the life span of TESVs. Another objective would be to tether the NucB protein to the silicone surface. The surface of TESVs have been coated with metals, or biosurfactants, in the past, to reduce microbial colonisation (Rodrigues *et al.*, 2007).

To conclude, this preliminary study has shown that eDNA is a measurable component of the biofilm matrices of TESVs, and that micro-organisms can be dispersed from the surface of these devices by NucB. This suggests that eDNA is acting as a biofilm stabiliser in mature, polymicrobial biofilms, and that NucB could have a potential role in improving the life span of TESV devices. Further work should concentrate on the potential reduction of biofilm formation on TESVs by NucB, and determine whether there are any micro-organisms that are more susceptible to NucB dispersion than others.

Chapter 4: Chronic Rhinosinusitis

4.1 Outline

In the previous chapter results indicated that extracellular DNA (eDNA) contributes to TESV biofilm matrix stability. This is important because it shows that clinically relevant polymicrobial biofilms, including fungal species, may be treatable with nuclease enzymes. Again working in collaboration with the Ear, Nose and Throat department of the Freeman Hospital, Newcastle, this study looked in greater detail at the contribution of eDNA to biofilm matrix stability, on this occasion against micro-organisms isolated from chronic rhinosinusitis (CRS).

Although many factors contribute to CRS it has become apparent over the last two decades that microbial biofilm infections are associated with many cases of CRS. Surface-attached microbial communities present a challenge for the treatment of many diseases, including CRS, because micro-organisms living within a biofilm are substantially more resistant to antimicrobials than planktonic cells (Gilbert *et al.*, 1997). This increased microbial resistance is readily apparent in CRS, because topical and systemic antibiotic treatments provide few significant positive outcomes (Fokkens *et al.*, 2012). Additionally, antibiotics administered during CRS management can be long-term (> 12 weeks), which may have a significant impact on increasing bacterial resistance, and negative side effects for patients. Ultimately, if the management of CRS is to improve, novel treatments are required to eliminate those cases caused by microbial biofilms.

The microflora of diseased sinuses is a key issue in developing novel therapeutic techniques. Micro-organisms that are most commonly isolated from CRS patients include coagulase-negative staphyloccoi, α-haemolytic streptococci, *Staphylococcus aureus*, *Corynebacterium* spp., *Prevotella* spp., *Peptostreptococcus* spp., and *Propionibacterium* spp (Doyle and Woodham, 1991; Brook, 2006; Araujo *et al.*, 2007). However, the microbiology of CRS is similar to non-diseased sinuses in healthy patients. Specific pathogens may not be responsible for the pathogenesis of CRS but rather the uncontrolled immune response to mixed-species biofilms. The majority of evidence for biofilm formation in diseased sinuses stems from microscopic analysis of sinus mucosa, which may act as a reservoir for pathogenic biofilms in 25-100% of CRS

patients (Foreman *et al.*, 2012). Biofilm formation in CRS isolates has been shown by culturing sinonasal aspirates in a Calgary Biofilm Detection Assay, with 28.6% of patient samples exhibiting biofilm formation (Prince *et al.*, 2008).

In view of the high prevalence of biofilms in CRS patients (see Table 1.3), with a diverse microflora, it appears that novel treatments should exploit a common characteristic of biofilms. As discussed previously, eDNA, a macromolecule often used by microbes to hold biofilms together, is one possible target. DNase I has dispersed or inhibited bacteria associated with CRS, including *Streptococcus pneumoniae* (Hall-Stoodley *et al.*, 2008), *Neisseria* spp. (Lappann *et al.*, 2010), *Pseudomonas aeruginosa (Whitchurch et al., 2002), S. aureus* (Kaplan *et al.*, 2012), and *Escherichia coli* (Tetz and Tetz, 2010). Extracellular DNA is also present in the biofilm matrices of *Staphylococcus epidermidis* (Qin *et al.*, 2007) and *Haemophilus influenzae* (Jurcisek and Bakaletz, 2007). Although it is unknown how these species might differ in their use of eDNA when isolated from disease, it does suggest that some CRS isolates may be dispersed by nuclease enzymes. Nasal irrigation with a solution containing a DNase would be the ultimate outcome but at this early stage more evidence is required, beginning with single-species biofilm models.

This study hypothesised that extracellular DNA may contribute to the biofilm matrix of CRS isolates. Furthermore, an important objective was to determine if NucB could disperse these isolates. To begin with, microscopic techniques were used to investigate the colonisation of patient sinuses with biofilm cells. This analysis looked at both the sinus mucosa, and obstructive mucin as a reservoir for biofilms. The microbiology of CRS was investigated by isolating bacteria on a number of media and identifying species with 16s rRNA sequencing or by MALDI-TOF mass spectrometry. Twenty-four isolates were selected and grown as biofilms using a static 96-well microtiter model. Extracellular DNA was extracted and quantified from the biofilm matrices of the 24 isolates. Lastly, NucB was incubated with mature CRS biofilms and any reduction of biofilm extent quantified.

4.2 Microscopic analysis of obstructive mucin and mucosal biopsies from CRS patients

Microscopic techniques have been used to visualise microbial biofilms associated with CRS. This section confirms the association of bacteria with the surface of sinus mucosa, as well as visualising the structure of the mucin that obstructs infected sinuses in some CRS patients.

4.2.1 *Transmission electron microscopy of obstructive mucin*

During functional endoscopic sinus surgery (FESS), thick, rubbery secretions were aspirated from the sinuses of CRS patients. This material could be coined 'obstructive mucin', as these secretions become so large that they fill entire sinus cavities. Functional endoscopic sinus surgery is the standard surgical treatment for CRS, and this procedure aims to remove as much mucin as possible from the paranasal sinuses (Bassiouni *et al.*, 2012). During this study, obstructive mucin was removed from the paranasal sinuses of CRS patients with or without polyps in the absence of other symptoms that would indicate eosinophilic chronic rhinosinusitis (ECRS). To begin with, it was thought that this material may be of bacterial origin and as such contain eDNA, therefore potentially responding to nuclease treatment. This material is extremely tenacious and removing it significantly extends the length of time required in the operating theatre, therefore if it were a biofilm eDNA dispersal may improve surgical treatment. To investigate the structure of the obstructive mucin, a portion of the material from two different CRS patients was cut into small pieces (~1 mm^3) immediately after surgery and fixed in glutaraldehyde. The mucin was embedded and sectioned for TEM analysis (see Materials and Methods 2.6.3). The material from the two patients appeared similar in structure, and consisted predominantly of an acellular matrix either with a striated appearance punctuated by occasional degraded host cells and cell debris (Figure 4.1A), or with little structure and many pockets of air space (Figure 4.1B). Erythrocytes were sometimes observed in the mucin matrix (not shown). However, areas containing large numbers of eosinophils were not observed in any field of view. Importantly, bacterial cells and fungi were not seen within the matrix of these samples. Therefore, on the basis of these analyses, the obstructive mucin was not a microbial biofilm.

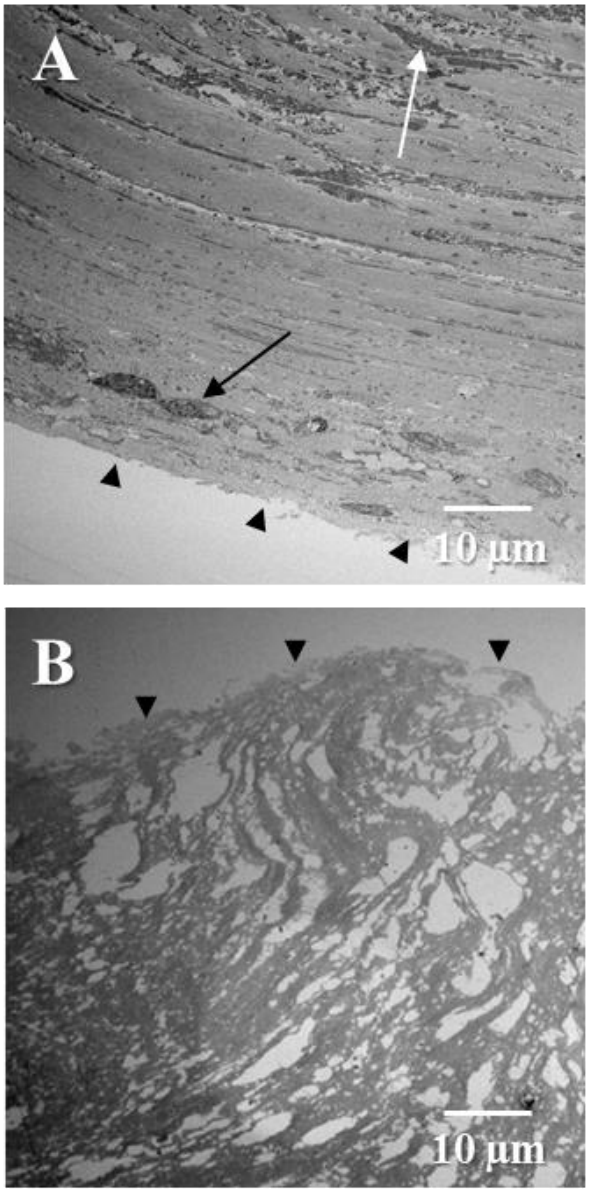


Figure 4.1 Transmission electron microscopy of obstructive mucin from CRS aspirates. In some cases (A), the mucin either formed a layered structure, with relatively intact cells towards the outer layers (black arrow) and more degraded cellular material further in (white arrow). (B) Alternatively, samples had little clear structure and the mucin was punctuated by pockets. The outermost layer of each sample is indicated by arrowheads.

4.2.2 *Surface-associated bacteria on sinus mucosa*

As discussed previously, many authors have discovered bacterial biofilms on the surface of sinus mucosa. So, whilst microbial cells were not visible in obstructive mucin, it couldn't be discounted that they were still in sinuses but localised to other areas. Therefore, mucosal biopsies were also collected from CRS patients, and analysed by confocal scanning laser microscopy (CSLM). Initially, propidium iodide and DAPI (plus cell membrane counterstaining with Alexa Fluor® 647 conjugate of wheat germ agglutinin (WGA)) were employed to stain bacteria. However, it was difficult to identify micro-organisms with confidence due to the lack of contrast between bacteria and host cells (Figure 4.2). Therefore, in subsequent samples bacteria were selectively stained by PNA-FISH with the EUB338 probe, and host cells were counterstained with DAPI (see Materials and Methods 2.6.1.3). PNA is an artificially synthesised analogue of DNA that employs a backbone of repeating N-(2-aminoethyl)-glycine units linked by peptide bonds (Nielsen *et al.*, 1991). This backbone contains no phosphate groups and therefore has no charge, unlike natural DNA. PNAs are not easily recognized by nucleases, are more stable over wider pHs and form stronger bonds to DNA, than DNA, as they contain no charge. Prior to the staining of sinus mucosa with the PNA-FISH probe, the technique was developed with *in vitro* cultured bacterial cells. This process included optimising the staining protocol and staining various bacteria associated with CRS (Figure 4.3). Six bacterial species, both Gram positive, and Gram negative, that are of relevance to CRS were tested. Although there was some variation in staining intensity all species were visualised with the FISH protocol. Optimisation of staining included different lengths of probe hybridization, varied strategies for cell wall permeabilization and altered fixation strategies. These techniques were compared via brightness of the resulting stain fluorescence during CLSM. Once the technique was optimised the probe was employed with CRS biopsy material. This method clearly showed microbial DNA on the mucosal surface of some mucosa specimens (Figure 4.4B). In addition to punctate staining, representing intact bacterial cells, patches of more diffuse staining were observed that may represent microbial eDNA. Some specimens were free of stainable bacteria (Figure 4.4A), indicating that sinus mucosa is not uniformly covered with bacteria. It is possible that bacteria that are difficult to permeabilize, like staphylococci (as shown in Figure 4.3), are residing on the sinus mucosa but were not stained by the PNA FISH probe.

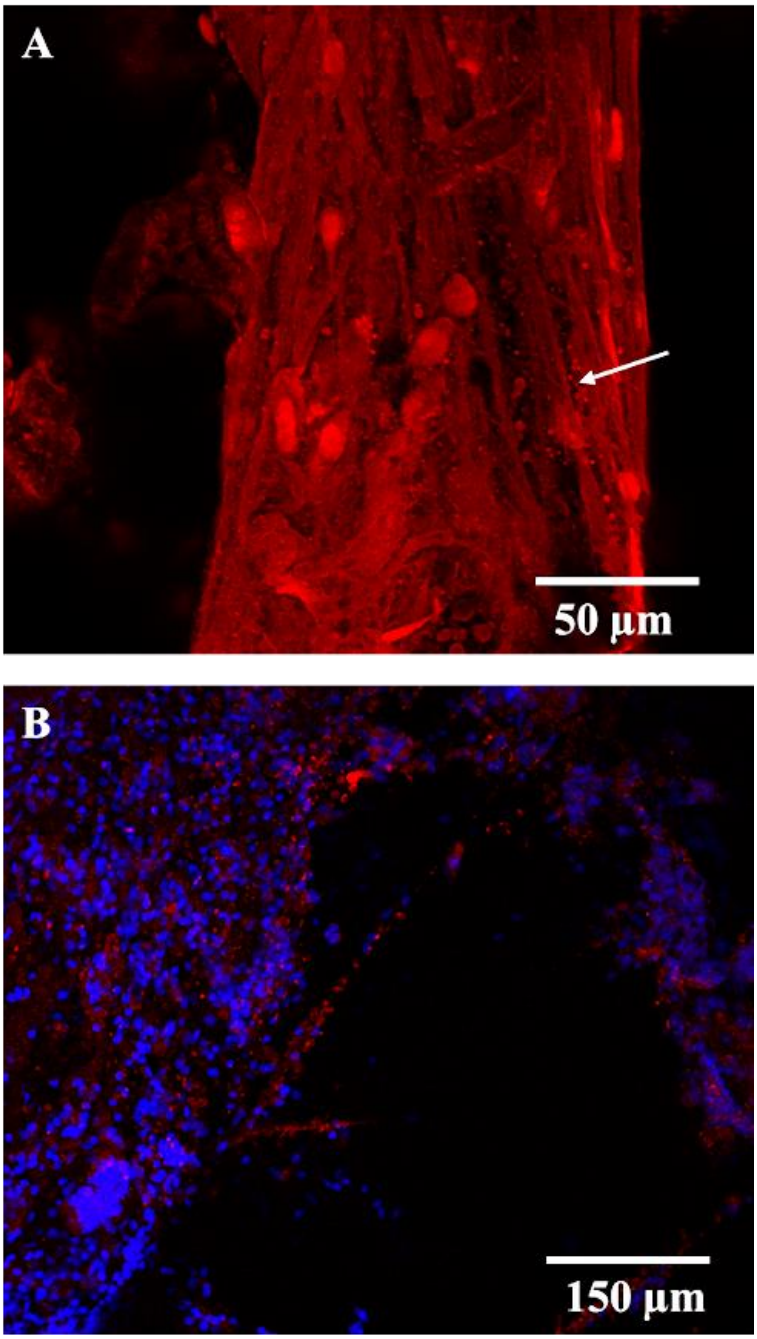


Figure 4.2 Formalin fixed sinus mucosa specimens visualized with fluorescent stains. (A) Propidium iodide non-specifically stained mucosal tissue, therefore making it hard to differentiate between host and bacterial cells. Bacterial cells may be present (white arrow), or this may be an artefact. Nine specimens were stained using this method. (B) The dual stain technique of DAPI and WGA yielded clearer images as background material was not stained. However, again there is no differentiation between host and bacterial cells.

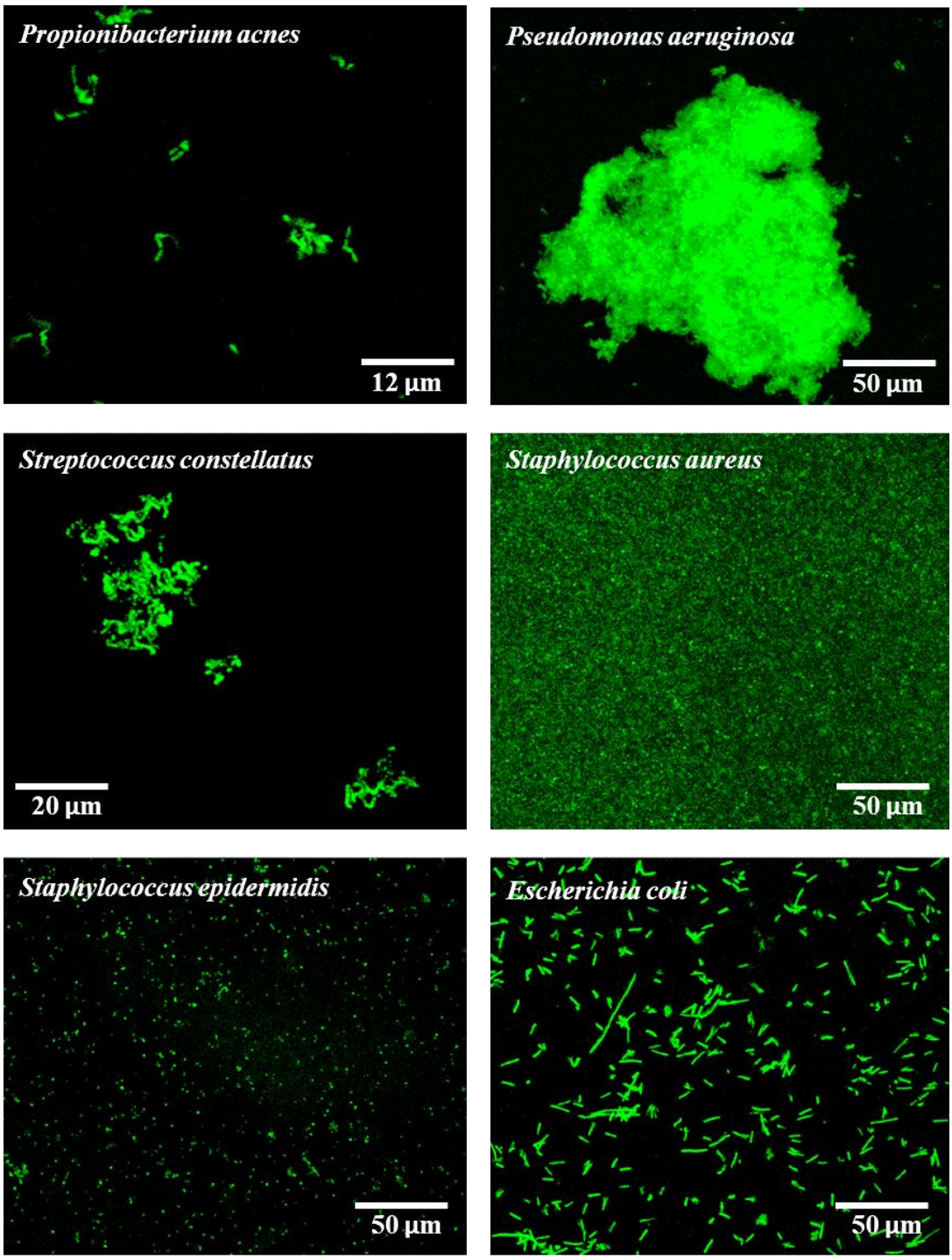


Figure 4.3 Determining the efficacy of a PNA-FISH universal bacterial probe at staining multiple species of bacteria. Several bacteria are found associated with sinuses, and so it was important to assess the staining potential of micro-organisms already isolated from diseased sinuses. All species tested were visible, although Gram positive bacteria, like *S. epidermidis*, did not stain as well as Gram negative organisms.

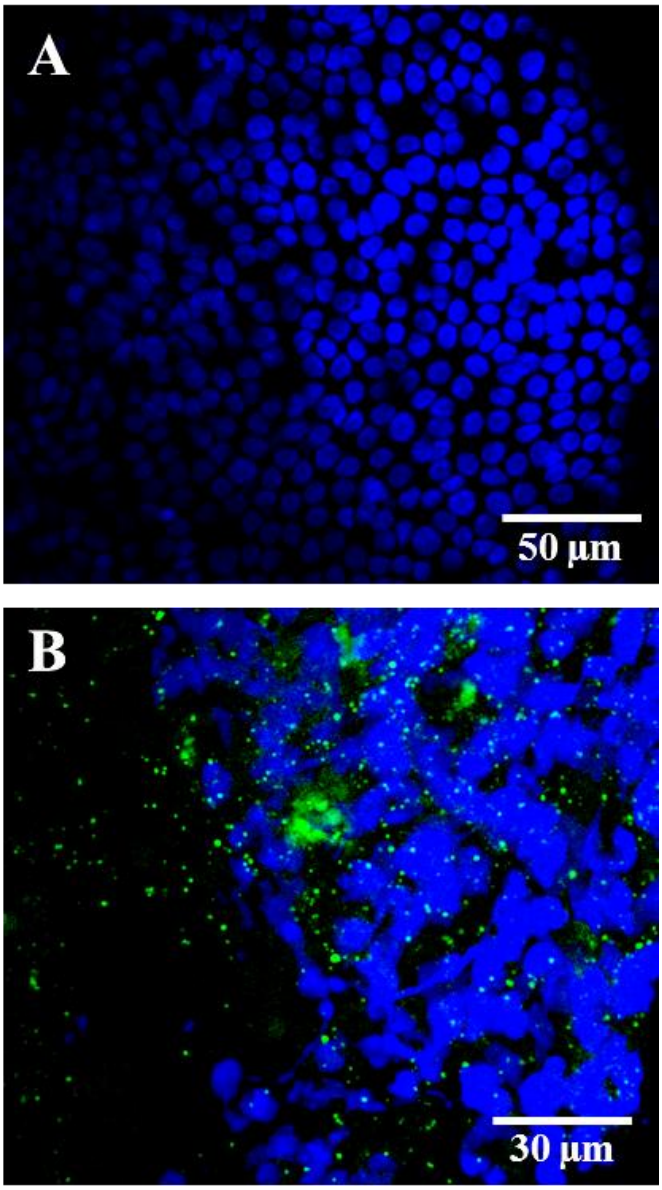


Figure 4.4 Confocal laser scanning microscopy of surface associated bacteria on mucosa removed from patients diagnosed with CRS. Bacteria (green) were visualized using a EUB338 PNA-FISH probe, and epithelial cells (blue) were counterstained with DAPI. In some fields, epithelial cells were observed in the absence of bacteria (A), and in other fields bacterial biofilm was evident (B). Nine mucosa specimens were stained using this technique.

4.3 Microbiology of chronic rhinosinusitis

Bacteria often reside in paranasal sinuses, diseased, or healthy, as shown by culture, molecular methods, and microscopy. In this section, sinuses were investigated for their microbiological content. Micro-organisms were also analysed for extracellular DNase activity, which is linked to virulence and may re-structure biofilms. Lastly, the biofilm-forming ability of selected bacteria was also determined.

4.3.1 *Isolation and identification of micro-organisms associated with CRS*

Obstructive mucin was collected during FESS by an ENT surgeon, placed in RTF and quickly transported to the laboratory. From here, the mucin was pulverized into a homogenous paste and used as an inoculum for agars. Specimens were placed onto chocolate, blood, fastidious anaerobe and Sabouraud agars, with all media supporting growth of micro-organisms, as shown in figure 4.5. These media where chosen from the standard operating procedure for the investigation of sinus aspirates, as outlined by the health protection agency (HPA, 2009). A diverse range of media and growth conditions was employed to aid the capture of as many micro-organisms as possible (see Materials and Methods 2.4.1.2). The average number of mucin aspirates collected from patients during surgery was 4 (fewest, 1; most, 16). One mucin sample per patient was homogenized and used as an inoculum. There was significant variation in aspirate texture, colour, size and firmness. Overall, 75 strains of bacteria were isolated from 20 patients, comprising a total of 16 different genera and 32 separate species (Table 4.1). Obstructive mucin contained between 2, to 6, bacterial species per patient. Micro-organisms were identified by a combination of biochemical and molecular methods (see Materials and Methods). The most prevalent organism associated with CRS aspirates was *Staphylococcus epidermidis*, which was isolated from 15 of 20 specimens (75%). *Staphylococcus aureus* and *Streptococcus* spp. were each isolated from 7 patients (35% of samples), *Corynebacterium* spp. were isolated from 6 patients (30%), and *Propionibacterium* spp. from 5 patients (25%). Other organisms that were less frequently isolated included *Haemophilus influenzae*, *Moraxella catarrhalis*, *Neisseria* spp., *Finegoldia magna*, and *Enterobacter aerogenes*. The majority of isolated bacteria were facultative anaerobes. However, both obligate aerobes (for example, *Neisseria* spp. and *M. catarrhalis*) and obligate anaerobes (*F. magna*, *Propionibacterium* spp.) were also isolated.

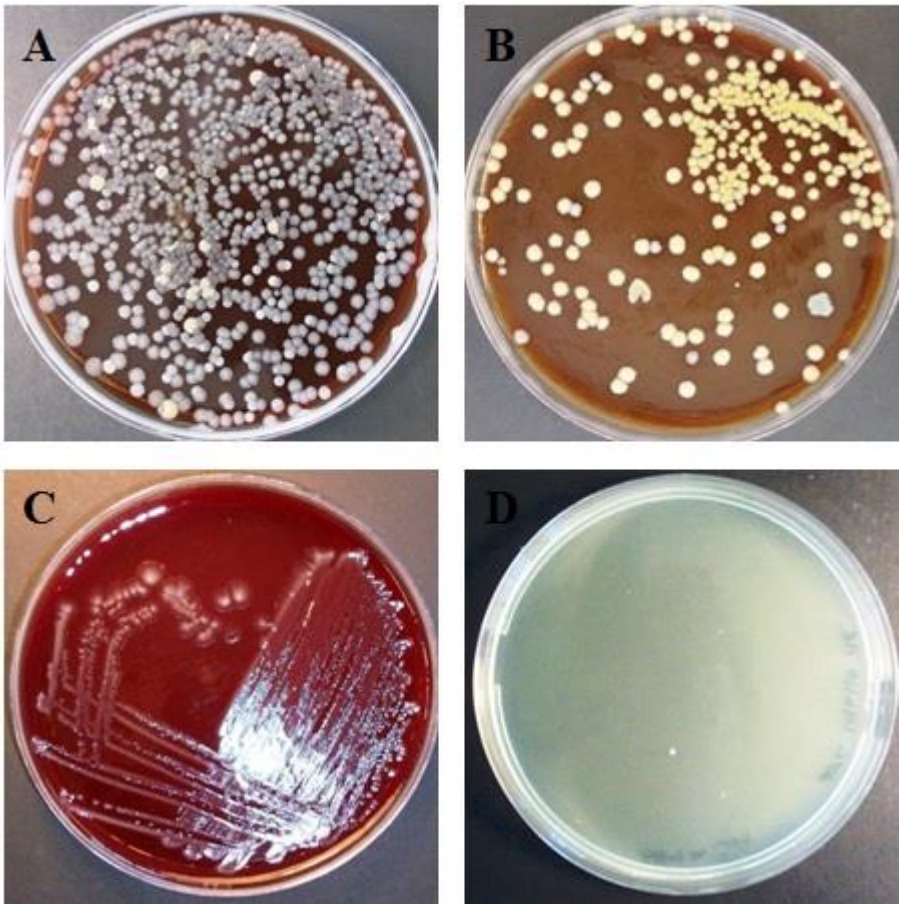


Figure 4.5 CRS micro-organisms were isolated on four media types. (A) Blood agar from patient 5, (B) chocolate agar from patient 7, (C) fastidious anaerobe agar from patient 3, and (D) Sabouraud agar from patient 4. Many colony morphologies are visible. Occasionally bacteria grew on Sabouraud agar if they were resistant to the low pH (5.6) of the medium.

Table 4.1 Bacteria isolated from CRS aspirates.

Patient	Microbial Species Present[a]	Total number of isolates
1	*Enterobacter aerogenes, Staphylococcus epidermidis, Streptococcus* sp., *Streptococcus pneumoniae, Streptococcus salivarius*	5
2	*Haemophilus influenzae, Micrococcus luteus, Staphylococcus epidermis, Staphylococcus hominis, Streptococcus pneumoniae*	5
3	*Moraxella catarrhalis,* ***Staphylococcus aureus***, ***Streptococcus anginosus***	3
4	*Klebsiella rhizophila, Staphylococcus epidermidis,* ***Streptococcus constellatus, Streptococcus intermedius, Streptococcus salivarius***	5
5	*Esherichia coli,* ***Staphylococcus aureus***, *Staphylococcus epidermidis*	3
6	*Corynebacterium pseudodiphtheriticum, Finegoldia magna, Klebsiella pneumoniae, Moraxella catarrhalis, Staphylococcus epidermidis, Staphylococcus lugdunensis*	6
7	***Staphylococcus aureus***, *Staphylococcus epidermidis, Staphylococcus warneri, Streptococcus constellatus*	4
8	*Corynebacterium propinquum, Staphylococcus epidermidis, Staphylococcus lugdunensis*	3
9	*E. aerogenes, Finegoldia magna, Propionibacterium* sp., *Streptococcus pneumoniae*	4
10	*Haemophilus influenzae, Moraxella catarrhalis, Staphylococcus epidermidis, Streptococcus pneumoniae*	4
11	*Staphylococcus epidermidis, Staphylococcus warneri*	2
12	*Citrobacter koseri, Staphylococcus epidermidis, Propionibacterium* sp., *Pseudomonas aeruginosa*	4
13	*Corynebacterium pseudodiphtheriticum, Propionibacterium granulosum,* ***Staphylococcus aureus***	3

14	*Staphylococcus epidermidis*, *Staphylococcus pasteuri*, *Staphylococcus warneri*	3
15	*Staphylococcus epidermidis*, ***Staphylococcus lugdunensis***, *Propionibacterium acnes*, *Propionibacterium granulosum*	4
16	*Corynebacterium propinquum*, *Neisseria meningitidis*, ***Staphylococcus aureus***	3
17	*Neisseria* sp., *Staphylococcus epidermidis*, ***Streptococcus anginosus***, *Streptococcus parasanguinis*, *Streptococcus salivarius*	5
18	***Staphylococcus aureus***, *Staphylococcus epidermidis*	2
19	*Corynebacterium pseudodiphtheriticum*, *Propionibacterium avidium*, ***Staphylococcus aureus***, *Staphylococcus epidermidis*	4
20	*Corynebacterium accolens*, *Corynebacterium pseudodiphtheriticum*, *Lactobacillus* sp.	3

[a]Strains highlighted in bold text produced extracellular deoxyribonuclease.

4.3.2 *Extracellular DNase activity of CRS isolates*

The production of extracellular DNase enzymes by clinical isolates was assessed using DNase test agar and staining with toluidine blue (see Materials and Methods 2.4.5). Hydrolysis of DNA produces nucleotides and these form a different complex with toluidine blue, resulting in pink, rather than blue agar. Extracellular DNase activity is of interest because of its role in virulence but also because it may re-structure microbial biofilms. All *S. aureus* isolates produced extracellular DNase, and other producers were *Streptococcus anginosus* group (*S. anginosus*/*S. constellatus*/*S. intermedius*) strains (80% of strains), *Staphylococcus lugdunensis* (33% of strains) and *Streptococcus salivarius* (33% of strains). Extracellular nuclease producers were isolated from 11 out of 20 (55%) patients. Examples of DNase activity are shown in Figure 4.6. In only two cases, more than one nuclease producing organism was isolated from the same patient sample.

4.3.3 *Biofilm formation of CRS isolates*

Twenty-four bacteria, isolated from obstructive mucin aspirates, were grown in 96-well microtiter plates to assay for biofilm formation (see Materials and Methods 2.4.1.5 and 2.10.2). Biofilm formation is a key aspect of this project because the hypothesis of NucB reducing biofilm extent hinges on microbial isolates producing biofilms *in vitro*. Representative strains of all species that produced extracellular DNase were selected for these studies, along with a similar number of non-producing organisms. Following incubation for 20 h in microtitre wells, all isolates had grown in the planktonic phase to OD_{600}>0.1 with the exception of three strains: *S. anginosus* FH19, *S. constellatus* FH21 and *S. pneumoniae* FH26 (Table 4.2). All 24 strains were also grown in planktonic culture (10 mL Falcon tubes) for 8 h, to determine their growth rates (doubling time in the logarithmic growth phase). There was significant variation in growth rates from the slowest, *S. constellatus* FH21 (not determined due to very little growth), to the fastest, *S. salivarius* FH27 (37 min). Generally isolates of the same species grew at similar rates, although *S. salivarius* FH28 had a doubling time almost 3 times slower than other *S. salivarius* isolates. Growth rates in planktonic culture did not always correlate with the planktonic growth measured in the microtiter assay. For example, *S. salivarius* FH27 had a doubling time of 37 minutes but only grew moderately well in the planktonic phase of the microtiter assay (OD_{600} = 0.20). This is because micro-

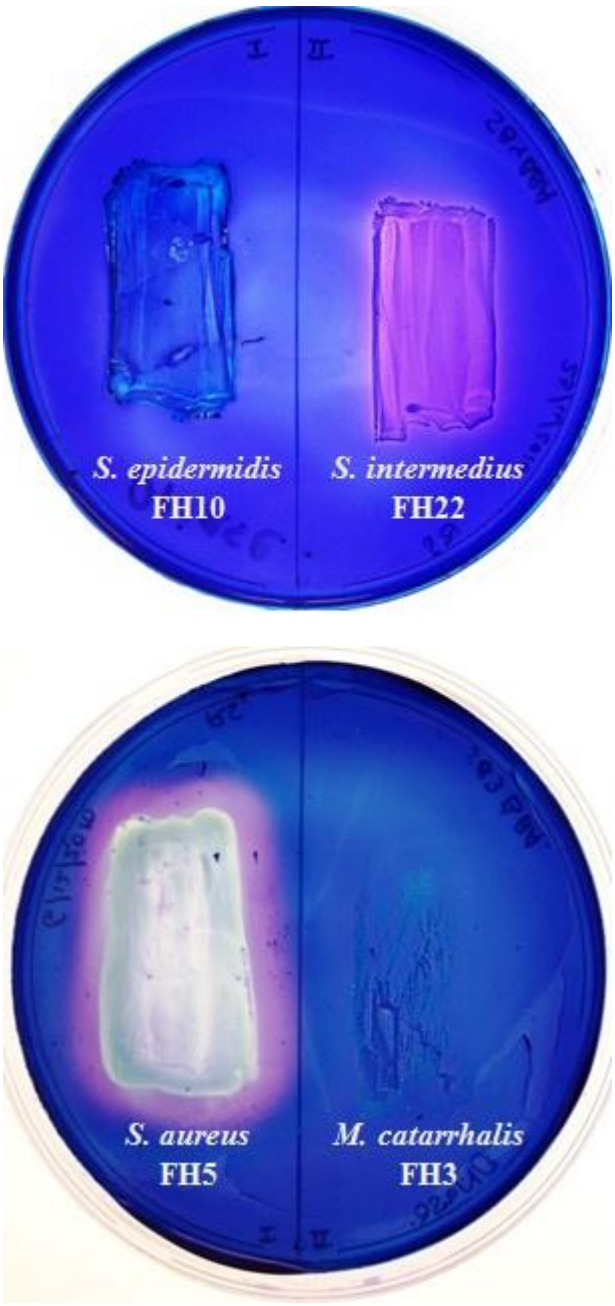


Figure 4.6 DNase agar test for extracellular DNase activity, using toluidine blue stain. This test provides a clear indication of nuclease production by isolates, visible as pink areas. *Streptococcus intermedius* produces a cell-associated nuclease, and therefore pink areas of DNA hydrolysis are in close proximity to the colony. In contrast, *S. aureus* extracellular DNase is released, and there is a zone of clearance of 5 mm around the colony. *Staphylococcus epidermidis* and *M. catarrhalis* do not produce exonucleases and as a result have no pink zone of DNA clearance.

Table 4.2 Biofilm formation and NucB sensitivity of selected isolates from CRS aspirates.

Strain[a]	Planktonic Growth OD_{600} Mean (S.E.)	Doubling Time Mean (S.E.)	Biofilm Growth OD_{570} Mean (S.E.)	Nuclease Production[b]	Remaining Biofilm after NucB Addition (%)	*p*-value
***Corynebacterium propinquum* FH1**	0.39 (0.06)	235.38 (11.67)	1.79 (0.78)	-	105	0.558
***Corynebacterium pseudodiptheriticum* FH2**	0.86 (0.24)	129.46 (2.35)	2.61 (0.43)	-	92	0.577
***Moraxella catarrhalis* FH3**	0.39 (0.07)	232.07 (6.46)	0.77 (0.21)	-	127	0.349
***Moraxella catarrhalis* FH4**	0.42 (0.14)	154.69 (5.05)	2.78 (0.21)	-	124	0.032
***Staphylococcus aureus* FH5**	0.40 (0.11)	61.93 (3.20)	1.84 (0.34)	+	77	0.003
***Staphylococcus aureus* FH6**	0.50 (0.08)	74.36 (0.02)	0.71 (0.12)	+	59	0.000
***Staphylococcus aureus* FH7**	0.79 (0.26)	60.55 (3.41)	1.23 (0.22)	+	40	0.000
***Staphylococcus epidermidis* FH8**	0.27 (0.09)	80.83 (5.78)	2.29 (0.41)	-	114	0.077
***Staphylococcus epidermidis* FH10**	0.48 (0.06)	90.49 (1.07)	1.59 (0.22)	-	67	0.001
***Staphylococcus epidermidis* FH11**	0.54 (0.13)	103.64 (4.17)	1.52 (0.24)	-	74	0.010
***Staphylococcus lugdunensis* FH12**	0.74 (0.03)	73.04 (3.64)	1.16 (0.23)	-	49	0.001
***Staphylococcus lugdunensis* FH13**	0.78 (0.05)	69.69 (0.23)	0.57 (0.10)	-	66	0.001
***Staphylococcus lugdunensis* FH14**	0.78 (0.13)	73.31 (15.60)	0.53 (0.05)	+	69	0.001
***Staphylococcus warneri* FH15**	0.59 (0.18)	72.05 (4.07)	0.89 (0.25)	-	126	0.005
***Staphylococcus warneri* FH17**	0.88 (0.16)	64.51 (3.02)	2.40 (0.55)	-	90	0.319
***Streptococcus anginosus* FH18**	0.16 (0.05)	54.00 (2.63)	1.16 (0.07)	+	34	0.000
***Streptococcus anginosus* FH19[c]**	0.07 (0.00)	89.94 (15.01)	0.22 (0.02)	+	59	0.015
***Streptococcus constellatus* FH20**	0.22 (0.04)	103.06 (27.51)	1.90 (0.39)	+	44	0.001
***Streptococcus constellatus* FH21[c]**	0.04 (0.03)	n/a	0.31 (0.05)	-	39	0.001
***Streptococcus intermedius* FH22**	0.19 (0.02)	66.79 (1.85)	3.07 (0.80)	+	46	0.000

***Streptococcus pneumoniae* FH26**	0.07 (0.03)	55.61 (1.06)	1.87 (0.31)	-	123	0.585
***Streptococcus salivarius* FH27**	0.20 (0.06)	37.34 (2.66)	0.99 (0.04)	-	92	0.240
***Streptococcus salivarius* FH28**	0.32 (0.04)	110.71 (28.52)	2.67 (0.96)	+	66	0.002
***Streptococcus salivarius* FH29**	0.23 (0.03)	39.36 (0.71)	1.08 (0.04)	-	96	0.692

[a]For details of genera, see Table 4.1.

[b]Production of nuclease was measured on DNase agar, and is indicated by a '+' sign.

[c]Isolates grew poorly in both the planktonic and biofilm phase.

organisms can double quickly but reach the stationary phase early due to growth limitations in the media used. All strains produced biofilms that were detectable by crystal violet staining. Crystal violet adheres to biofilms because it dissociates in aqueous solutions to CV+ and chloride (Cl-) ions, the CV+ ions then interact with negatively charged molecules in microbial biofilms, staining them purple. Therefore, it measures total biofilm extent, and is not a measure of total cell numbers. Generally, there was extensive variation in the extent of biofilm formation between different species and between different strains of the same species. For example, *M. catarrhalis* FH3 produced a very weak biofilm (A_{570} = 0.77), whereas *M. catarrhalis* FH4 formed extensive biofilms (A_{570} = 2.78). Of the strains tested, *Streptococcus anginosus* FH19 produced the least abundant biofilms (A_{570} = 0.22). The mean extent of biofilm formation by non-nuclease producers (A_{570}=1.51, S.E. 0.19, n=15) was not significantly different from that of nuclease producers (A_{570}=1.48, S.E. 0.32, n=9).

4.4 Analysis of the structural role of eDNA in CRS biofilms

All 24 isolates assayed produced biofilms. These biofilms were treated with NucB to determine its efficacy against CRS isolates and therefore how significant eDNA is in maintaining structural integrity. Later, certain biofilms were grown on glass coverslips and analysed by microscopy after treatment with NucB or with PBS.

4.4.1 *Efficacy of NucB against biofilm forming isolates*

Pre-formed biofilms were incubated for 1 h in the presence of the microbial DNase NucB (Table 4.2) (see Materials and Methods 2.10.3). Biofilms formed by 9 out of 9 (100%) nuclease producing strains were significantly reduced by NucB (T test comparing NucB treatment with buffer control, p<0.05, n=3). By contrast, only 5 out of 15 (33%) of the biofilms produced by non-nuclease producing bacteria were dispersed by NucB. In addition, 2 out of 15 (13%) non-nuclease producers had slightly increased levels of biofilm following incubation with NucB than without the enzyme. All *S. aureus* and *S. lugdunensis* isolates were significantly dispersed by NucB, which is of interest because these two species are closely related (Patel *et al.*, 2000). All *Streptococcus anginosus* group (*S. anginosus*/*S. constellatus*/*S. intermedius*) species were dispersed by NucB, and as a genus 6 out of 9 *Streptococcus* spp. were sensitive to DNase treatment. The *Corynebacterium* spp. (isolated in 30% of patients) treated were

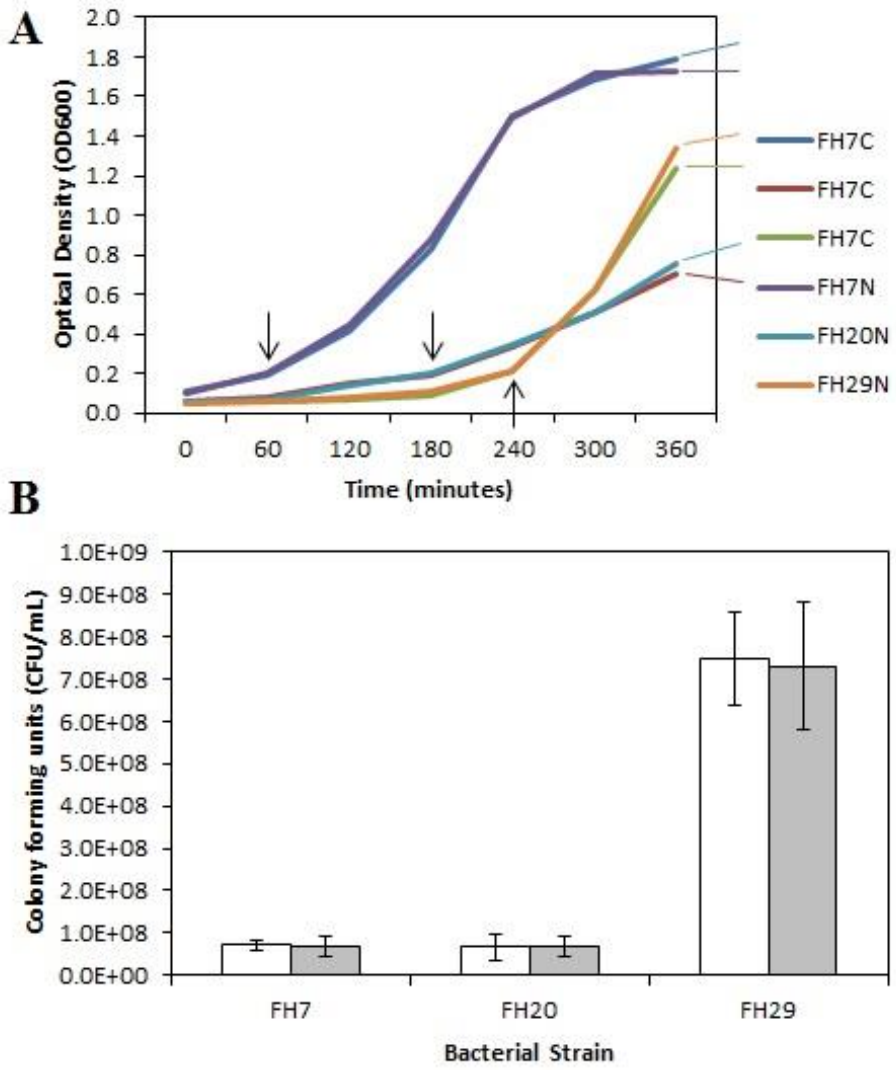


Figure 4.7 Effects of NucB on the planktonic cells of three CRS isolates. *S. aureus* FH7, *S. constellatus* FH20 and *S. salivarius* FH29 were grown in BHY over a period of 6 hours. (A) At an optical density of 0.2 (black arrows) isolates were challenged with 5 µg/mL NucB (N) or unchallenged (C) and culture was continued to determine the bacteriostatic effect of NucB. (B) Cells were also removed after an hour of incubation with NucB (grey bars) or without (white bars) and the total viable cells calculated via dilution series onto solid BHY media.

not reduced by NucB. *Streptococcus anginosus* FH18 was the most greatly dispersed micro-organism, with biofilm extent decreased by 66%. To assess whether NucB had detrimental effects on the cells themselves, three different isolates, *S. aureus* FH7, *S. constellatus* FH20 and *S. salivarius* FH29, were cultured to exponential phase in BHY broth and challenged with 5 μg ml^{-1} NucB. No effects were observed on the growth rate of cells following the challenge (Figure 4.7A). The number of viable cells in each culture continued to increase following NucB addition and 1 h after adding NucB there was no difference in the number of viable cells in cultures containing NucB compared with control cultures without the enzyme (Figure 4.7B). Overall, these data suggest that eDNA is an important component of the EPS for over 50% of the CRS isolates, including strains that produce extracellular DNase enzymes, and that addition of NucB dislodges cells without killing bacteria.

4.4.2 *Microscopic analysis of* in vitro *grown biofilms*

To obtain more detailed information about the effects of NucB, biofilms of selected organisms were cultured on glass coverslips and analysed by CLSM and SEM (see Materials and Methods 2.6.1.1 and 2.6.2). This work focussed on staphylococci and streptococci, since these were the genera most commonly isolated from CRS patients. The three species tested were *Staphylococcus aureus* FH7, *Streptococcus constellatus* FH20 and *Streptococcus intermedius* FH29. In the absence of NucB treatment, biofilms formed by *S. constellatus* FH20 were relatively thin and consisted primarily of a single cell layer that covered most of the surface (Figure 4.8). In places, clusters of cells projected from the surface to a depth of ~12 μm. Using LIVE/DEAD® BacLight™ stain, both live cells (green) and dead cells (red) were observed in biofilms; there was however a larger proportion of dead cells than live cells. Biofilms that had been treated with NucB were clearly less extensive than the untreated controls, and consisted of sparsely distributed single cells or very small aggregates of <10 cells (Figure 4.8B). Biofilm formation by *S. aureus* FH7 on the glass surface was also thin, with clusters of cells projecting from the surface (to ~8 μm) (Figure 4.8). *Staphylococcus aureus* FH7 survived better than *S. constellatus* FH20 during biofilm formation and specimen processing, as shown by the higher number of live cells (green). Again, slides that were incubated with NucB had far fewer cells attached to them than the PBS treated control biofilm (Figure 4.8D). To obtain higher resolution images, similar biofilms were

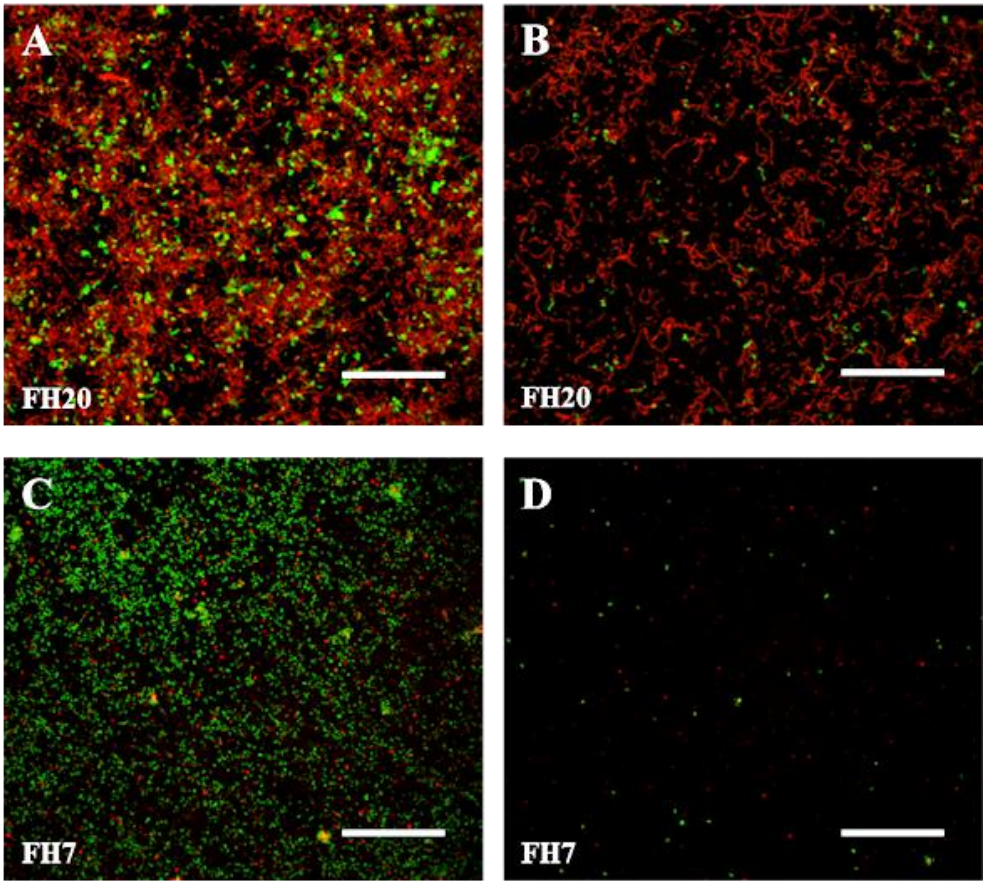


Figure 4.8 Confocal laser scanning microscopy of *S. constellatus* FH20 and *S. aureus* FH7 biofilms with or without NucB treatment. Biofilms were cultured on glass surfaces and visualised with CLSM using LIVE/DEAD® BacLight™ stain, which stains dead cells red and live cells green. (A and C) Biofilms treated with buffer alone, and (B and D) biofilms treated with NucB. Scale bar (white) is 50 µm.

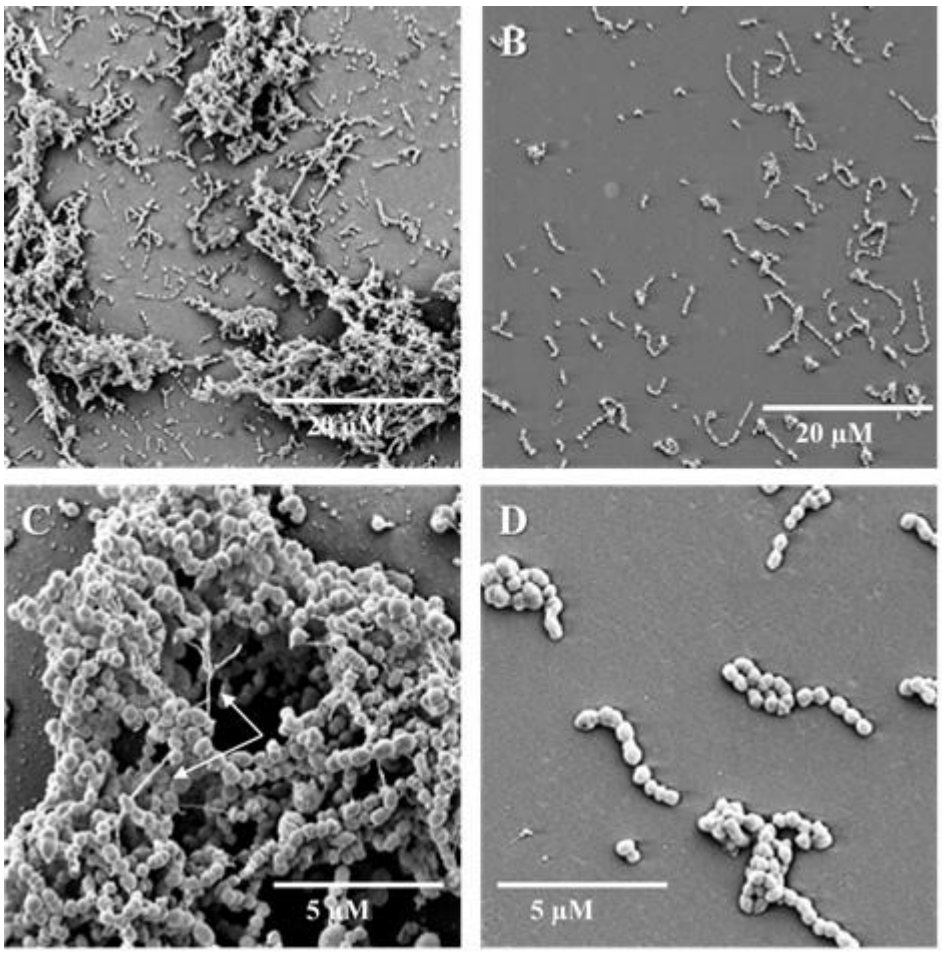
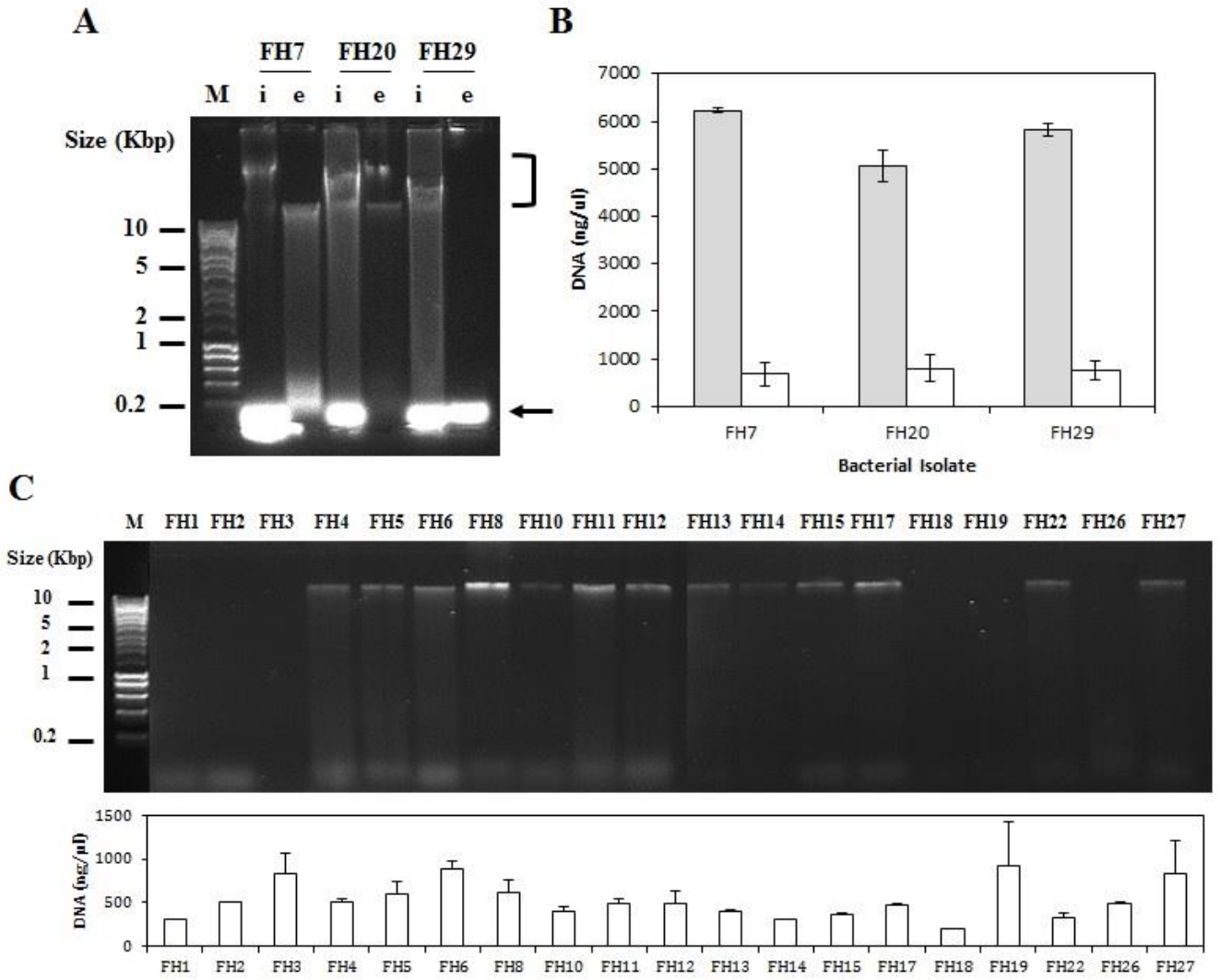


Figure 4.9 Scanning electron microscopy of *Streptococcus constellatus* FH20 biofilms treated with NucB or buffer control. Biofilms were visualised with SEM after treatment for 1 h with buffer (A) or with NucB (B). At higher magnification, extracellular material (white arrow) was observed in the absence of NucB treatment (C), but was not seen in NucB-treated biofilms (D). Images are representative of 3 replicates cultured on separate days.

analysed by SEM (Figure 4.9). Again, in the absence of NucB, cell aggregates were evident and a relatively large proportion of the surface was covered by micro-organisms (Figure 4.9A). By contrast, NucB-treated biofilms almost exclusively contained isolated cells or small clusters of cells (Figure 4.9B). In addition, extracellular material was apparent in untreated biofilms under high resolution SEM (Figure 4.9C), that was not seen in biofilms incubated with NucB (Figure 4.9D). Biofilms formed on glass surfaces by *S. aureus* FH7 or *S. intermedius* FH22 were also visualised by SEM (data not shown). As with *S. constellatus* FH20, biofilms that were treated with NucB contained far less biomass than those incubated in buffer alone. Extracellular polymers were also observed in biofilms of these two species.

4.5 Quantification of eDNA in model biofilms

The eDNA content of biofilms formed by CRS micro-organisms was characterised in 22 out of the 24 isolates selected for further analysis (see Materials and Methods 2.7). To quantify levels of eDNA in model biofilms, eDNA and intracellular DNA (iDNA) was extracted from biofilm cultures of *S. aureus* FH7, *S. constellatus* FH20 and *S. salivarius* FH29. The eDNA was analysed by agarose gel electrophoresis (Figure 4.10A). Sharp bands migrating at an apparent size of approximately 30 kbp were observed in eDNA fractions of *S. aureus* FH7 and *S. constellatus* FH20. However, no high molecular weight eDNA bands were seen in *S. salivarius* FH29. Intracellular DNA from all three organisms appeared as a smear of high molecular weight fragments, probably due to binding of chromosomal DNA to cell wall fragments. The high molecular weight eDNA bands were of similar weight to the chromosomal DNA that was extracted. In addition to the high molecular weight fragments, small fragments of DNA or RNA were seen at the bottom of the gel. Further controls, by treating the extracted "eDNA" with RNase and DNase could allow the identification of this LMW material. Nucleic acids in each fraction were quantified using the Nanodrop spectrophotometer (Figure 4.10B). For each strain, eDNA represented approximately 5-10 % of the total DNA present in the biofilm. To account for the possibility that samples may have contained RNA in addition to DNA, nucleic acids were also quantified using PicoGreen dye, which is strongly selective for double stranded DNA. When measured using this technique DNA concentrations were up to 15-fold lower, indicating the potentially significant proportion of ssDNA and RNA that contributes to the eDNA nucleic acid concentration measured by NanoDrop spectrophotometry. The NanoDrop


A
FH7
FH20
FH29
M
i
e
i
e
i
e
Size (Kbp)
10
5
2
1
0.2
B
DNA (ng/ul)
7000
6000
5000
4000
3000
2000
1000
0
FH7
FH20
FH29
Bacterial Isolate
C
M
FH1
FH2
FH3
FH4
FH5
FH6
FH8
FH10
FH11
FH12
FH13
FH14
FH15
FH17
FH18
FH19
FH22
FH26
FH27
Size (Kbp)
10
5
2
1
0.2
DNA (ng/µl)
1500
1000
500
0
FH1
FH2
FH3
FH4
FH5
FH6
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FH11
FH12
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FH14
FH15
FH17
FH18
FH19
FH22
FH26
FH27

Figure 4.10 The visualization and quantification of eDNA from CRS isolates. (A) Intracellular DNA (i) or eDNA (e) was purified from bacterial biofilms of *S. aureus* FH7, *S. constellatus* FH20 or *S. salivarius* FH29, and analysed by agarose gel electrophoresis. High molecular weight chromosomal DNA is indicated by a black bracket; low molecular DNA or RNA is highlighted at the bottom of the gel by a black arrow. M; size marker. (B) The concentration of DNA in the intracellular (grey bars) and extracellular (white bars) fractions from bacterial biofilms was measured by NanoDrop spectrophotometry. Bars represent means of three independent extracts, and SEs are indicated. (C) Extracellular DNA concentration in biofilms was also visualised for another 19 isolates, with distinct chromosomal DNA bands migrating at an apparent size of 30 kbp; the total DNA concentration was measured by NanoDrop spectrophotometry, bars represent the average of three replicates and error bars are S.E.

spectrophotometer measures all molecules that absorb or scatter light at the wavelength of interest (260 nm) and these could include ssDNA, nucleotides, RNA, contaminants and molecules that absorb UV light. *Staphylococcus aureus* FH7 chromosomal DNA extractions contained substantial amounts of protein. No significant differences were observed between the total amount of eDNA in S. salivarius FH29 biofilms (760 ng/µL) and eDNA in biofilms formed by the other two strains (740 ng/µL). Therefore, despite the lack of a clear band by agarose gel electrophoresis, it appears that eDNA was present in *S. salivarius* FH29 biofilms.

To assess whether eDNA was present in biofilms formed by other CRS isolates, biofilms of each strain were cultured in 6-well polystyrene microtiter plates and eDNA was purified as described in the Materials and Methods chapter. Only two strains were omitted from this analysis: *S. constellatus* FH21 grew very poorly in biofilms and it was not possible to extract eDNA, and *S. salivarius* FH28 was prone to contamination and, after several attempts, it was decided not to pursue DNA purification from this strain. By agarose gel electrophoresis, sharp bands corresponding to high molecular weight eDNA products were observed in all *Staphylococcus* spp., *S. constellatus* FH20 and in *S. intermedius* FH22 (Figure 4.10C). By contrast, similar bands were not detected from *Corynebacterium* spp., *S. anginosus* or *S. pneumoniae*. Only *M. catarrhalis* and *S. salivarius* had inter-strain differences in the production of eDNA. Thus, eDNA was not visualised in *M. catarrhalis* FH3 biofilm extracts, whereas eDNA was clearly present in *M. catarrhalis* FH4 (Figure 4.10C). Similarly *S. salivarius* FH29 did not produce a band of eDNA on an agarose gel, whereas a sharp band was seen in *S. salivarius* FH27. The concentrations of eDNA in samples were determined using the Nanodrop spectrophotometer. Concentrations of eDNA in the extracts ranged from 206 ng/µL to 917 ng/µL (Figure 4.10C). Interestingly, there appeared to be little correlation between the concentration of eDNA and the presence or absence of a band on the agarose gel. For example, *M. catarrhalis* FH3 produced one of the highest concentrations of eDNA, but no band on the gel. Conversely, the eDNA concentration from *S. intermedius* FH22 was just 325 ng/µL even though this strain clearly produced a band of eDNA on a gel. The production of an extracellular nuclease did not correlate with the presence of a band of eDNA on a gel. All strains of *S. aureus* (nuclease-positive) and *S. epidermidis* (nuclease-negative) produced clear bands of eDNA, for example. With the exception of *S. anginosus* FH18 and *S. anginosus* FH19, all strains that failed to produce a clear band of eDNA on an agarose gel were insensitive to NucB treatment.

4.6 Discussion

Despite continued research, the pathophysiology of CRS is relatively poorly understood. CRS is one of the most common upper respiratory tract diseases, affecting approximately 10% of the adult European population (Hastan *et al.*, 2011). Therefore, it impacts significantly on society, with high cost to healthcare services, and reduced quality of life in sufferers. There is increasing evidence, although still unclear, that micro-organisms are a major factor in the development of CRS (Foreman *et al.*, 2012). The major objectives of this study were to characterize the microbial bioburden of sinuses in CRS patients with obstructive mucin, to determine whether isolated micro-organisms produced biofilms and to assess the efficacy of a novel bacterial nuclease, NucB, for biofilm control.

As with many aspects of CRS, the excess mucus production during sinus inflammation that can lead to blocking of sinuses, is not well characterised. Mucus hypersecreation in the respiratory tract can be related to allergic disorders, as first outlined in 1952 (Hinson *et al.*, 1952). With CRS, this has long be associated with allergic fungal sinusitis (AFS) and eosinophilic mucin rhinosinusitis (EMRS), the two of which may be subcategories of a collective pathological condition eosinophilic chronic rhinosinusitis (ECRS) (Ferguson, 2000). At the Freeman Hospital, Newcastle, CRS patient sinuses often contain thick mucus that is removed during FESS. This material was analysed by TEM and it appeared to contain few degraded eosinophils and therefore appears to be different to ECRS mucin (Ponikau *et al.*, 1999). Furthermore, no fungi were observed during TEM or culture from mucin. The fungal association with CRS is complicated and has long been debated (Ebbens *et al.*, 2009). Culture analysis has either yielded very high incidences of fungal carriage (Ponikau *et al.*, 1999), or very low fungal isolation (Araujo *et al.*, 2007). Whatever the role of fungi in CRS, in the cases presented here none were of a detectable fungal origin. Bacteria were not visualised in obstructive mucin, although these can be hard to view using TEM, as sections are thin. This result conflicts with the culture analysis of mucin aspirates, where 91% were culture positive for bacteria. TEM sections most often contained material that had no distinct structure, or cells, either eukaryotic or prokaryotic. The up-regulation of MUC5AC and MUC5B mucin genes has been identified in CRS patients, genes that are expressed in low-levels in healthy sinus epithelium (Kim *et al.*, 2004). Overproduction of mucus proteins may be the major pathological process creating the mucin observed in this study.

Whilst obstructive mucin did not appear to be a reservoir of micro-organisms, bacterial biofilms were observed on the surface of sinus mucosa. The detection of bacteria employed a PNA-FISH probe, a technique that has increased effectiveness over standard FISH (Pereira *et al.*, 2008). Although this approach is highly sensitive, bacteria can remain undetected if DNA copy numbers are low (Boase *et al.*, 2013). Nevertheless, the biofilms that were visualized were similar to those demonstrated previously (Sanderson *et al.*, 2006). Additionally, positive bacteria culture was observed in 20 out of 20 patients, suggesting a significant bacterial carriage in the sinuses examined. Occasionally micro-organisms were not detectable on mucosal specimens during microscopic analysis.

The microbial population of CRS in this study was dominated by *S. aureus*, coagulase-negative staphylococci and α-haemolytic streptococci. Other studies, using culture-dependent techniques have isolated similar bacteria in CRS patients (Brook, 2006; Araujo *et al.*, 2007). Potentially pathogenic bacteria identified include *P. aeruginosa*, *H. influenzae*, *S. aureus*, *M. catarrhalis*, *Neisseria* spp., *S. pneumoniae*, and Corynebacteria. It remains unclear if specific pathogens are responsible for the disease pathogenesis of CRS. Previously, bacterial micro-organisms were isolated from only 4 out of 14 healthy maxillary sinuses (Abou-Hamad *et al.*, 2009). A recent study however, identified organisms in all 6 healthy control specimens (Boase *et al.*, 2013). There appears to be increased abundance of micro-organisms and certain pathogenic species including, *S. aureus*, *M. catarrhalis*, *P. aeruginosa* and *S. pneumoniae* in CRS patient sinuses (Boase *et al.*, 2013).

Interestingly, obligate anaerobes (*Propionibacterium* spp. or *F. magna*) were cultured during this study. There remains a debate over the role of anaerobic bacteria in CRS pathogenesis (Ramadan *et al.*, 2002). Anaerobes have been isolated in as few as 0% of cases (59 patients) (Doyle and Woodham, 1991), and as many as 88% (Brook, 1989). A difference even exists between rates isolated from the the maxillary sinus, either 6% or 88% (Brook, 1989; Biel *et al.*, 1998). This suggests that it is not sinus topography that is creating differences between studies. In this study, bacteria were isolated from three paranasal sinuses, maxillary, sphenoid and ethmoid. Anaerobic carriage is most likely to be relatively low (Biel *et al.*, 1998; Ramadan *et al.*, 2002; Araujo *et al.*, 2007) as the sinuses are air-filled cavities, although O_2 concentrations can drop to zero during mucin blockage (Carenfelt and Lundberg, 1977), facilitating the growth of facultative anaerobes and anaerobes, as found during this study.

Culture-independent techniques are becoming more accessible, and have been used to study the microflora of CRS (Stressmann *et al.*, 2011; Feazel *et al.*, 2012; Boase *et al.*, 2013). Molecular methods may have increased detection sensitivity, as culturing relies on micro-organisms adapting to a new environment, without any of the complex interactions that occur between species. However, culture-dependent and culture-independent techniques appear to isolate very similar bacteria in CRS patients (Feazel *et al.*, 2012). The bacterial microflora of CRS patient sinuses in this study is in agreement with the previous literature.

Biofilm formation in the paranasal sinuses is likely to be a key factor in the pathogenesis of CRS, and therefore determining if strains have the capacity to form biofilms is important. The *in vitro* model employed in this study suggested that all of the bacteria tested were capable of biofilm formation. This is unsurprising as it has been hypothesised that at least 99% of bacteria can exhibit the biofilm phenotype (Costerton *et al.*, 1987). However, in a previous study of the biofilm-forming capacity of *P. aeruginosa*, *S. aureus*, and coagulase-negative staphylococci isolated from CRS patients biofilm formation was apparent in 22 of the 31 isolates studied (Bendouah *et al.*, 2006). Again, bacteria were cultured in 96-well plates and stained with CV. The isolates were recovered from patients one-year after FESS, whereas in this study bacteria were removed during FESS and it is unknown how this will affect biofilm-forming capacity. Furthermore, the definition of biofilm formation (absorbance reading) will have an effect on how many isolates are considered biofilm formers. Another important study used a different model to assess biofilm formation by CRS bacteria, by inoculating a modified Calgary biofilm device with sinonasal swabs (Prince *et al.*, 2008). Interestingly, cultures were obtained from CRS patients with evidence of mucus secretion, and so may have had symptoms similar to patients in this study. Biofilm formation was compared against a *P. aeruginosa* PA01 positive control, and two mutants, *sad-31* and *sad-36*, which are non-biofilm forming. Biofilm growth higher than the *sad-31* mutant was set as the threshold for biofilm formation. However, of the 157 patient swabs only 45 (28.6%) exhibited any biofilm formation in the Calgary model. The higher incidence of biofilm-forming capacity in the bacteria isolated during this investigation is likely related to the freshness of isolates, the model system used, and the growth conditions employed. Biofilm formation differs between growth media, such that nutrient-poor or nutrient-rich media can contribute to higher biofilm extent (Dewanti and Wong, 1995; Stepanović *et al.*, 2004). Prince *et al.*, (2008) assayed for biofilm formation aerobically, with Luria-Bertani broth, which will underestimate the

capacity of anaerobic bacteria to form biofilms from sinonasal swabs. As shown by this study, and others, anaerobes make up a significant proportion of the microflora of CRS patient sinuses. Our model did not test the capacity of obligate anaerobes to form biofilms.

More than half of the isolates tested were significantly dispersed when treated with NucB. Staphylococci are often associated with CRS, and the majority of strains tested were dispersed. *Staphylococcus aureus* has been found to be sensitive to DNase I in previous studies (Tetz and Tetz, 2010; Kaplan *et al.*, 2012), indicating a clear role for eDNA in creating biofilm stability in this species. However, *M. catarrhalis*, a species of bacteria that in recent study was cultured only from CRS patients, and not healthy sinuses (Boase *et al.*, 2013), was not dispersed by nuclease treatment. *Moraxella catarrhalis* FH4 biofilm extent was slightly but significantly increased by the NucB enzyme. Although eDNA is known to promote biofilm stability in most bacteria, in *Caulobacter crescentus* biofilms, the addition of 20 µg/mL DNase I during biofilm culture increased biofilm formation (Berne *et al.*, 2010). Therefore, as in *C. crescentus*, eDNA may have an inhibitory effect on the *M. catarrhalis* biofilms studied here. Both *Corynebacterium* spp. remained similar, to control biofilms, in biofilm extent after NucB treatment. Species and strain variability in eDNA production and nuclease sensitivity is an interesting finding, and could be explored further by genome sequencing of the bacteria. This could demonstrate the genetic differences that may be responsible for changes in nuclease sensitivity, a point briefly explored in Chapter 5.

Confocal laser scanning microscopy showed very clear dispersal of both *S. aureus* FH7, and *S. constellatus* FH20. Although the CLSM images were not subjected to quantitative analysis, this technique generally showed clearer dispersal of biofilms than the 96-well polystyrene plate model. During CLSM biofilms were cultured on glass, rather than polystyrene, which may have led to differences in eDNA dependence. Furthermore, the CLSM technique visualises cells, whereas the CV assay stains both cells and matrix, which could have led to differences in apparent dispersal efficacy. *Staphylococcus aureus* UAMS-1 48 hour mature biofilms cultured in a flow-cell model have been effectively dispersed by treating with 0.5 U/mL DNase I for 24 hours, as examined by CLSM (Mann *et al.*, 2009). In this model, results indicated that higher inoculum biofilms of *S. aureus* UAMS-1 were more susceptible to DNase I treatment, highlighting the role different conditions have in nuclease effect. Generally, in our study, biofilms were cultured starting from a high inoculum, which may partly explain the significant disruptive effect of NucB in over half the isolates tested. Scanning

electron microscopy of *S. constellatus* FH20 biofilms again indicated a dispersal effect of the NucB enzyme. Additionally, control biofilms contained a stringy, extracellular matrix-like material which was missing in treated biofilms. Matrix polymers have been visualized in biofilms of *Candida albicans* GDH 2346, cultured on PVC catheter disks with or without shaking (Hawser *et al.*, 1998). Here, cells are clearly linked by strands of extracellular matrix, as seen with *S. constellatus* FH20. It has been hypothesised that matrix material contributes to the resistance of microbial biofilms to antimicrobials (Fux *et al.*, 2005). The removal of this material by NucB is a positive sign, and may aid the efficacy of antibiotics, as noted in other biofilm-forming species, where DNase I had a synergistic effect with several antibiotics (Tetz *et al.*, 2009). This remains an untested theory, and requires further study.

An important aspect of the study was to examine the abundance of extracellular DNase producers in the microflora of CRS. DNase production is viewed as an important virulence marker as it allows organisms such as *Streptococcus pyogenes* or *S. aureus* to escape neutrophil extracellular trap (NET) killing (Buchanan *et al.*, 2006; Berends *et al.*, 2010). However, exonucleases may also influence the structure of biofilms, particularly in the context of the complex mixed-species biofilms that are likely to form in diseased sinuses. Biofilms are thicker in nuclease-deficient mutants of *S. aureus* and *Vibrio cholerae* than wild-type organisms of the same species (Kiedrowski *et al.*, 2011; Seper *et al.*, 2011). However, in a clinically relevant model of catheter biofilms, a clinical *S. aureus* isolate, UAMS-1, that was nuclease-deficient, produced a similar biofilm to the wild-type (Beenken *et al*., 2012). Therefore the total bioburden of biofilms in CRS patients may be affected by strain differences in nuclease production. Here, *S. lugdunensis, S. constellatus* and *S. salivarius* strains were either positive or negative for extracellular DNase production. This indicates that extracellular DNase production is regulated, that micro-organisms may have different nuclease phenotypes depending on environment, or that DNase function is lost by some strains, for instance due to environmental influences such as nutrient availability. The production of DNases is likely to be tightly regulated, for example, in response to stress or glucose (Kiedrowski *et al.*, 2011). The role of DNase production in shaping biofilm structure in clinical ecosystems requires more investigation.

Several of the species isolated produced extracellular DNases. The DNase agar test is often used to differentiate species of the same genus, as is often the case with *S. aureus* and coagulase-negative staphylococci. Interestingly, a *S. lugdunensis* isolate was DNase production positive. This species is similar to *S. aureus* and is often differentiated on the

basis of being DNase negative. However, here and in a previous study (Matthews *et al.*, 2011) this has been found to not always be the case, which may have implications for clinical diagnostics. Other differences existed between the reported DNase production of propionibacteria, and α-haemolytic streptococci and strains tested here (Hoeffler, 1977; Palmer *et al.*, 2012). Clearly, DNase activity is a variable trait of clinical isolates.

Twenty two isolates were analysed for the presence of eDNA in biofilm matrices. All isolates contained eDNA that was quantifiable by spectrophotometry. Of these isolates large eDNA fragments were visible on agarose gels in 15 of the strains. In some strains, such as *M. catarrhalis* FH3, NanoDrop spectrophotometry revealed high concentrations of eDNA that were not visible on agarose gels. Interestingly, this species was not nuclease sensitive and therefore low molecular weight DNA may not stabilise the biofilm of this isolate. The biofilm matrix could also contain inhibitors that retard DNase efficacy, or the LMW eDNA may be resistant to DNase hydrolysis. In *Pseudomonas fluorescens*, eDNA accumulation was altered by RNA release, because it diminished DNase activity (Catlin and Cunningham, 1958). It appears that RNA may be common in biofilm matrices of CRS isolates, and that this could impact DNase activity. This would require further research using RNase and DNase controls to degrade extracted biofilm matrix nucleic acids, to ascertain the identity of the LMW macromolecules with some confidence. All *S. aureus* isolates produced eDNA that was visible on agarose gels and biofilms that were significantly dispersed by NucB. This was also observed in all *S. lugdunensis* strains. These closely related organisms clearly rely heavily on eDNA to add support to the biofilm matrix. Six of the 8 DNase producing organisms' matrices contained DNA fragments that were visible on agarose gel. It is unknown if DNase production differs in these micro-organisms when cultured on agar, to growth in the static polystyrene model. For instance, periodontal bacteria produced extracellular DNase activity differently depending on the nuclease assay used (Palmer *et al.*, 2012). This may explain the intact HMW eDNA found in the biofilm matrices of some extracellular DNase producers, as DNase may not be produced in the microtiter biofilm assay. Both bacteria that were susceptible to nuclease treatment but did not produce a defined band of eDNA on agarose gels were *S. anginosus* strains. Either the large fragments of eDNA degraded during the investigation, or the size of DNA is not a determining factor in how much strength it adds to a biofilm matrix. Most eDNA is likely to be released during cell lysis, and is therefore of high molecular weight (of chromosomal origin) (Allesen-Holm *et al.*, 2006). *Streptococcus anginosus* may release smaller fragments of DNA, or its extracellular nuclease activity degrades

the chromosomal DNA that is released during cell lysis, without any impact on biofilm stability. These results clearly indicate that the majority of fresh clinical CRS isolates release eDNA into the biofilm matrix.

Although the pathogenesis of CRS is unclear there is a link to microbial biofilms aiding the inflammatory process behind CRS. Biofilms are resistant to standard antimicrobial treatments, as shown by the reduced efficacy of antibiotics in treating CRS (Lim *et al.*, 2010). There is a pressing need for novel therapeutic techniques. This investigation shows that CRS-associated bacteria produce biofilms, and that these biofilms are reduced in extent by the nuclease enzyme, NucB, *in vitro*. Additionally, to add further weight to this finding, CRS isolates produce eDNA that can be quantified, or visualized. However, this investigation is at an early stage, as biofilms were modelled using a simple approach. NucB has potential in controlling the biofilms formed by CRS isolates but further validation will rely on *in vivo* data. It is important to note that *Bordetella* spp. mono-species biofilms have been dispersed with DNase I in an *in vivo* mouse upper respiratory tract model (Conover *et al.*, 2011). The synergistic effect of NucB and antimicrobial agents is of particular interest as a 50% reduction in the biomass of biofilms in sinuses could be further increased with the aid of antibiotics. Ultimately, as with TESV biofouling, eDNA appears to play a critical role in creating biofilm stability in clinically relevant bacteria.

Chapter 5: Oral Biofilms

5.1 Outline

The results in Chapters 3 and 4 of this thesis have shown the clear role eDNA has in maintaining the stability of biofilms formed by some clinically relevant bacteria. This included freshly isolated bacteria from diseased sinuses, and 2-3 month mature biofilms on the surface of TESVs. Dental plaque is an oral biofilm that forms on the surface of teeth and, if not adequately controlled, leads to the development of dental caries or periodontitis. Like other biofilms, dental plaque consists of a robust layer of microbial cells embedded in a matrix of polymeric material. The matrix serves a number of important functions, for example enhancing adhesion of microbial cells to the tooth surface and serving as a barrier against external insults. At present, it is not clear to what extent eDNA contributes to the structural integrity of oral biofilms. This chapter aimed to determine the importance of eDNA for biofilms of four important oral bacteria, *Streptococcus gordonii*, *Actinomyces oris*, *Streptococcus mutans* and *Fusobacterium nucleatum*. Research was also expanded into the direction of mixed-species biofilm modelling.

The mechanisms of eDNA release by oral bacteria have been studied in *S. gordonii*, *Streptococcus sanguinis* and *S. mutans*. With *S. gordonii* and *S. sanguinis*, eDNA production through a non-lytic mechanism related to hydrogen peroxide (H_2O_2) release has been demonstrated (Kreth *et al.*, 2009). However, cell death as a result of high levels of H_2O_2 may be responsible for eDNA release in *S. gordonii* (Itzek *et al.*, 2011). Furthermore, a recent study by Liu and Burne (2011) described the important role of the AtlA autolysin of *S. gordonii* DL1, for the release of eDNA that leads to biofilm development. Biofilm formation in mutants lacking the AtlA autolysin could be partially restored with the addition of 50 ng/µL eDNA. Interestingly, eDNA production was 100-fold higher in the presence of oxygen. In *S. mutans*, competence-stimulating peptide (CSP)-induced cell death leads to eDNA release and increased biofilm formation (Perry *et al.*, 2009). Extracellular DNA has also been measured in the EPS of *F. nucleatum* and *Porphyromonas gingivalis* (Ali Mohammed *et al.*, 2013). Clearly, eDNA is produced by some oral bacteria, in both planktonic and biofilm culture.

Extracellular DNase activity is a common trait of oral bacteria (Palmer *et al.*, 2012). It is unknown how these enzymes may re-structure oral biofilm communities, particularly those that rely on eDNA for biofilm stability. Palmer *et al*., (2012) demonstrated that oral bacteria DNase activity depends on growth conditions, and it is unknown how this impacts biofilm formation on the mouth surface. However, Ali Mohammed *et al*., (2013) found that *F. nucleatum* and *P. gingivalis* are not dispersed by bovine DNase I, despite eDNA being found in the EPS of these two species. The biofilm-reducing effects of DNases on oral biofilms is an under-studied area of oral microbiology.

Each of the four species investigated in this study has a key role in the oral biofilm, and oral health. *Streptococcus* spp., including *S. gordonii*, are important in the early formation of dental plaque and are the dominant species in the early supragingival oral biofilm (Jenkinson and Lamont, 1997; Diaz *et al.*, 2006). Along with *A. oris*, *S. gordonii* are known to promote biofilm formation and facilitate the growth of other oral bacteria through attachment to cell surface proteins (Rickard *et al.*, 2003). Whilst *S. gordonii* and *A. oris* are viewed as oral commensals, *F. nucleatum* and *S. mutans* are considered deleterious as they contribute to periodontitis and dental caries, respectively (Loesche, 1986; Dzink *et al.*, 1988). Therefore, by selecting these organisms this study will provide an overview of eDNA dependence in both commensal and pathogenic oral bacteria.

Excellent oral hygiene is an effective way of maintaining a healthy mouth and removing oral disease causing bacteria (van der Weijden and Hioe, 2005; van der Weijden and Slot, 2011). However, improvements can still be made in this sector of healthcare, with the NHS spending £1.23 billion per year treating dental diseases (Audit Commission for Local Authorities and the National Health Service in England and Wales, 2002). With no great change to oral hygiene practice over the last 50 years there is a pressing need for novel treatment techniques. As shown previously, NucB has clear potential against many biofilm-forming bacteria. Therefore, the addition of NucB to toothpastes or mouthwashes could improve the efficacy of these products. Ultimately, before this happens it is important to investigate the role of eDNA in maintaining oral biofilm stability in simple models and to build on this research.

This study aimed to determine the role of eDNA in assisting the initial attachment of oral bacteria to a surface, and in maintaining biofilm stability. The investigation began

by extracting eDNA from the EPS of the four bacteria studied. Extracellular DNA was stained and viewed by CLSM. Oral biofilms were treated with DNases, both in a single-species and mixed-species model. *Streptococcus mutans* strain sensitivity to DNase treatment was studied, to further investigate the variability in eDNA dependence amongst strains of the same species of bacteria. Lastly, oral biofilms were cultured in a flowing, microfluidics model to assess the model's potential for future testing of DNase enzymes versus oral biofilms.

5.2 Extracellular DNA production by selected oral bacteria

To date there has been little research focused on determining the contribution of eDNA to the biofilm matrices of oral bacteria. In this study four oral bacteria were cultured as biofilms and eDNA was extracted, quantified and then visualised with CLSM.

5.2.1 *Extracellular DNA extraction from oral biofilm matrices*

To investigate eDNA production by oral bacteria, biofilms were cultured in 6-well microtiter dishes and eDNA was extracted (see Materials and Methods 2.7). Four wells were used for DNA extraction from biofilms, and the final two wells were stained with crystal violet (CV) to assess the growth of the biofilms. Extracellular DNA was only extracted from wells where bacteria had reached a biofilm state, set at a threshold of an absorbance value >0.1 when dissolved CV was measured at A_{570}. All biofilms grew to acceptable levels. The biofilm matrices of *S. mutans* GS-5, *A. oris* MG1 and *F. nucleatum* 25586 contained high-molecular weight DNA (Figure 5.1A). In these species eDNA resolved to a similar molecular weight as the iDNA extracted from cells. This is a similar finding to that in CRS isolates. With *F. nucleatum* 25586 and *S. mutans* GS-5 there were smaller nucleic acids visualized in the eDNA fraction. Enzymatic treatment of eDNA extracts from these two species with DNase I resulted in the absence of the high-molecular-weight band (Figure 5.1B), confirming that the band was due to DNA. However, DNase I did not degrade the lower molecular weight band of *F. nucleatum* 25586, indicating that this fraction was not dsDNA and was probably RNA and/or ssDNA. No DNA band was observed in eDNA extracts of *S. gordonii* by agarose gel electrophoresis (Figure 5.1A). Intracellular and extracellular DNA fractions were measured by NanoDrop spectrophotometry to calculate the proportion of total DNA

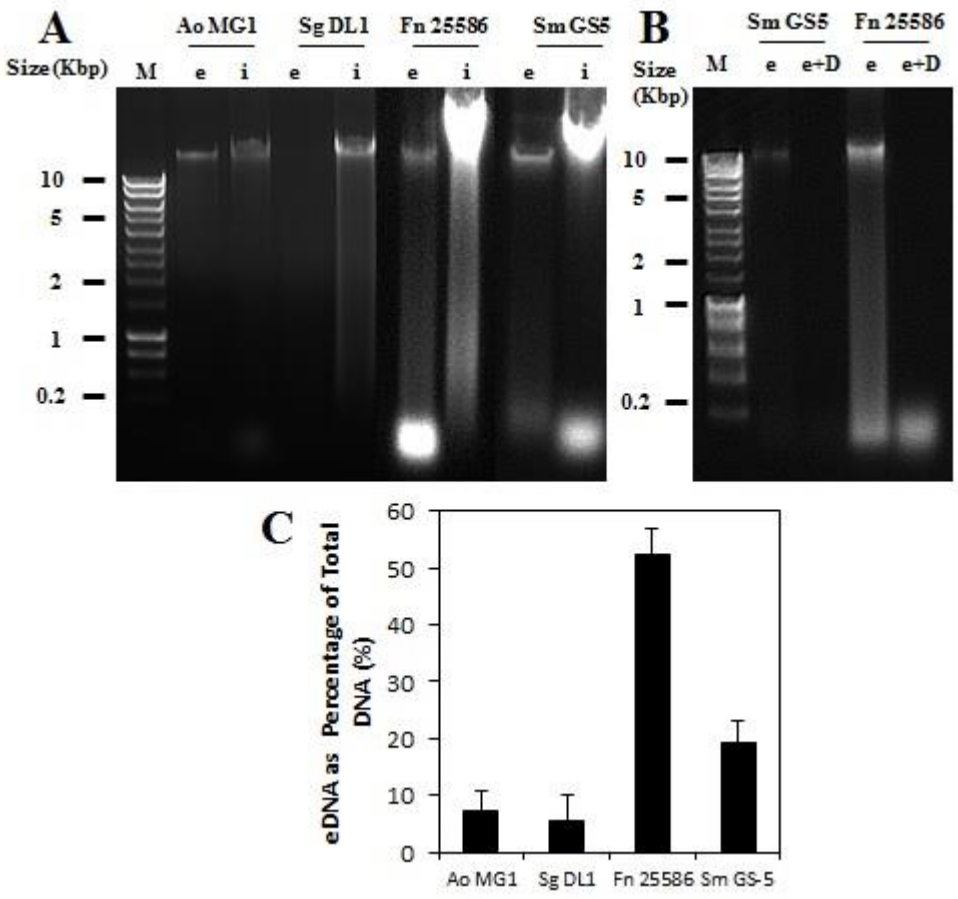

Figure 5.1 Extracellular DNA extractions from monospecies oral biofilms. Biofilms of oral bacteria were grown for 72 h and eDNA was quantified and visualized. (A) Agarose gel electrophoresis of extracted extracellular (e) and intracellular (i) DNA. (B) 500 µg/mL DNase I enzyme treatment (e+D) of eDNA fractions at 37°C for 2 h from *F. nucleatum* 25586 and *S. mutans* GS-5. (C) Proportion of the total DNA in biofilms that was eDNA. Extracellular and intracellular DNA was quantified by spectrophotometry, bars represent standard error from three replicates.

extracted from biofilms that eDNA contributed for each species (Figure 5.1C). *Fusobacterium nucleatum* 25586 eDNA was 52% of the total biofilm DNA. For this species iDNA was lower than other species (888 ng/µL versus 4399 ng/µL). This could be due to inefficient cell lysis during DNA extraction, or a genuine result. However, eDNA was present in higher amounts from *F. nucleatum* 25586 biofilm matrices (989 ng/µL) than for other species (e.g. *S. mutans* GS-5 654 ng/µL). There was a bright LMW (>200 bp) band of material for *F. nucleatum*, that may be ssDNA, RNA, or some other material. NanoDrop spectrophotometry likely overestimates dsDNA concentration when these other molecules are present, which may explain the high eDNA readings in *F. nucleatum*. For *S. mutans*, eDNA also made up a significant proportion (19%) of total DNA. *Actinomyces oris* MG1 and *S. gordonii* biofilm matrices contained similar amounts of eDNA, as measured by NanoDrop, 7% and 6% respectively. This was despite the lack of visible eDNA band for *S. gordonii* DL1 during agarose gel electrophoresis (Figure 5.1A).

5.2.2 *Extracellular DNA visualised by CLSM in biofilm matrices*

Biofilms of the four species were cultured in 6-well microtiter plates for 72 h and analysed by CLSM. Biofilms grew on glass coverslips, and were inverted into a staining liquid so that biofilm structure and EPS was maintained (see Materials and Methods 2.6.1.1). All biofilms consisted of thin single-cell layers with occasional aggregations that projected ~20 µm from the surface. The biofilms contained live cells (green), dead cells (punctate red), and some contained extracellular DNA (diffuse red). Yellow was observed in some images, possibly indicating co-localisation of live cells and eDNA. *A. oris* MG1 biofilms contained diffuse areas of red staining, which is consistent with eDNA (Figure 5.2B). This was the clearest example of eDNA across the four species analysed. Areas of eDNA appeared to be in close proximity to aggregations of dead *A. oris* MG1 cells, indicating a possible source of the DNA, from lysed cells. Extracellular DNA was less visible close to live *A. oris* MG1 cells and aggregations. In biofilms of *S. mutans* GS-5, patches of yellow staining were clearly visible, and some diffuse red staining was apparent on expanded images (Figure 5.2D). Yellow staining may be explained as the adherence of eDNA to the bacterial cell wall, therefore live cells appear both green, and red, thereby merging to give a yellow colour. It is possible that yellow staining is an artefact caused by the intercalation of both propidium iodide (PI) and SYTO® 9 with DNA inside a single cell that has a compromised cell membrane

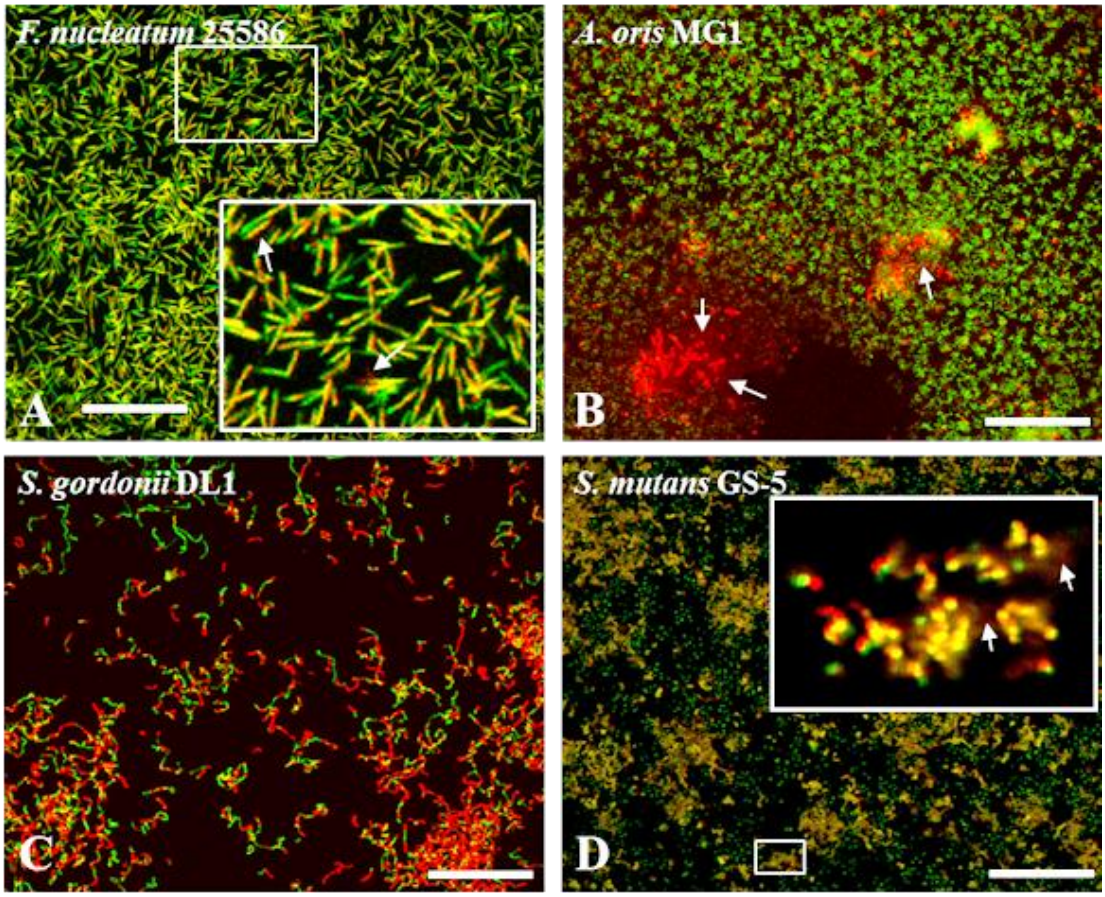

Figure 5.2 Confocal microscopy images of eDNA within oral biofilms. All species were cultured for 72 h and then incubated with LIVE/DEAD® *BacLight*™ biofilm stain for 15 minutes, before CLSM image acquisition. (A-D) Maximum projection CLSM images of *F. nucleatum* 25586, *A. oris* MG1, *S. gordonii* DL1, and *S. mutans* GS-5 respectively. White arrows highlight areas of eDNA. Scale bar is 50 µm.

permeable to PI. However, this is unlikely as PI has a higher affinity for DNA and displaces SYTO® 9 under normal circumstances, thereby allowing the differentiation of live and dead cells (Boulos *et al.*, 1999). Increased magnification on areas of *S. mutans* GS-5 cell aggregates showed diffuse red staining between biofilm cells. Here it is appears that eDNA may be adhering cells together that are in close proximity. Areas of extracellular red staining were also observed by close examination of *F. nucleatum* 25586 biofilms (Figure 5.2A). Biofilm cells of this species were organized in a regular manner. A number of *F. nucleatum* 25586 cells were yellow, again indicating the presence of eDNA on the cell surface of these bacteria. Very few cells appeared to be dead. When magnification was increased, areas of red staining close to *F. nucleatum* 25586 cells can be observed. Here, eDNA appeared to have been released from cells. This red staining was not cell shaped and therefore may be nucleic acids, or an artefact of some kind. Extracellular DNA was less apparent in micrographs of *S. gordonii* DL1 biofilms, where there were no diffuse patches of red staining (Figure 5.2C). However, some areas of red/green co-localization, giving a yellow appearance, were observed. *Streptococcus gordonii* DL1 biofilms often contained greater than 50% dead cells, this was in contrast to the other species where live cells were more apparent.

5.3 Sensitivity of oral biofilms to DNases

The four selected oral bacteria were grown in a static, 96-well microtiter plate model to assay DNase I sensitivity. Extracellular DNA was clearly quantifiable and visible in these biofilms and therefore it was important to determine its role in aiding biofilm adherence and stability.

5.3.1 *96-well plate model of DNase I sensitivity*

Extracellular DNA is required by some micro-organisms to attach cells to substrata, in the earliest phase of biofilm maturation (Whitchurch *et al.*, 2002). To test for the inhibitory effect of DNase I on biofilm settlement, 5 µg/mL DNase I was included with the inoculum and growth media, for each species, whilst culturing for 20 h in the biofilm model. Again, all biofilms grew to an acceptable level of A_{570} >0.1. *Streptococcus mutans* GS-5, *A. oris* MG1, and *F. nucleatum* 25586 biofilms were all significantly inhibited by DNase I (Figure 5.3A). Biofilm formation by *S. mutans* GS-5 and *F. nucleatum* 25586 was reduced by >70% when DNase I was present overnight. *A.*

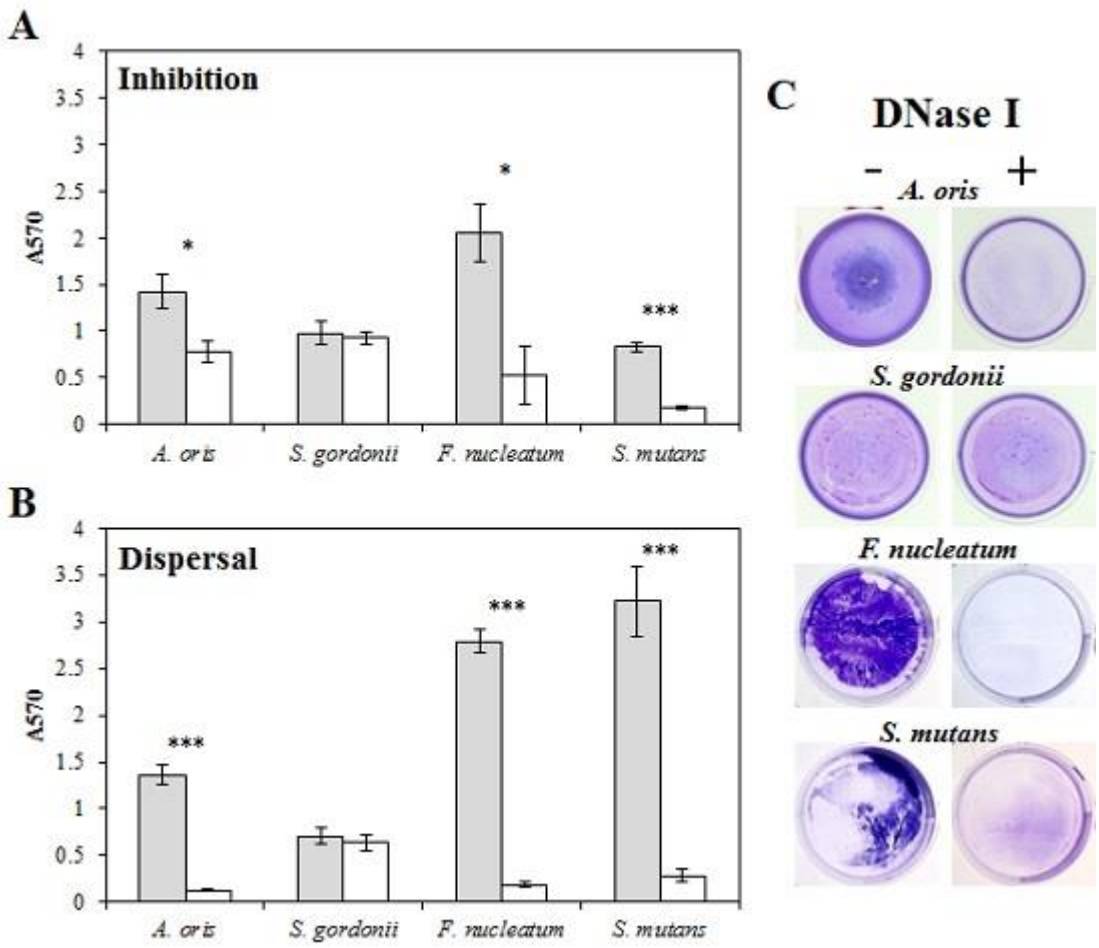

Figure 5.3 Effects of DNase I treatment on biofilms. Using a microtiter plate biofilm model, 5 µg/mL DNase I was included during biofilm formation for 20 h (A), or was added to preformed biofilms after 20 h (B). Biofilms were stained with crystal violet, and the biofilm biomass was quantified by measuring A_{570}. Shaded bars indicate controls, and white bars show values with NucB treatment. Bars represent mean data from three independent experiments and standard errors are shown. (C) Crystal violet-stained biofilms, grown in 6-well microtiter dishes (3 mL media, 50 µL inoculum, 37°C for 72 h), without or with 5 µg/mL DNase I treatment (C). Statistical significance was calculated with the two sample *t*-test.

oris was less sensitive but biofilms were still reduced, on average, by 46%. By contrast, DNase I did not significantly impair biofilm formation by *S. gordonii* DL1 (Figure 5.3A).

Mature biofilms (20 h) were treated with 5 µg/mL DNase I to assess the role of eDNA in maintaining biofilm integrity in the four oral bacteria. In this assay, *S. mutans* GS-5, *A. oris* MG1 and *F. nucleatum* 25586 were remarkably sensitive to DNase I (Figure 5.3B). Biofilm extent was significantly reduced by ≥90% for these micro-organisms, in comparison to PBS treated controls. Additionally, dispersal efficacy was higher than the inhibitory effect on biofilm formation in these three species. The DNase I enzyme did not disrupt preformed biofilms of *S. gordonii* DL1 (Figure 5.3B). Mature biofilms, grown in 6-well microtiter plates for 72 h, were treated with 5 µg/mL DNase I, or PBS, and stained with CV to give a visual representation of the impact of the enzyme on biofilm extent (Figure 5.3C). This assay again showed clear biofilm reduction for *S. mutans* GS-5, *A. oris* MG1 and *F. nucleatum* 25586 but no effect on *S. gordonii* DL1 biofilms.

Treatment of *A. oris* MG1 biofilms with heat-inactivated DNase I did not cause a reduction in biomass, indicating that enzyme activity was essential for biofilm removal (Claire Graham, unpublished work). To assess whether DNase I affected bacterial growth or viability, *A. oris* and *S. gordonii* biofilms were cultured in the presence or absence of 5 µg mL^{-1} DNase I, and the CFU/mL in biofilms and planktonic phases were quantified (Nick Jakubovics, unpublished work). In each case, DNase I did not affect the total CFU/mL present. However, incubation of *A. oris* in the presence of DNase I resulted in significantly more viable cells in the planktonic phase and significantly fewer cells in the biofilm compared with the control. Therefore, DNase I appeared to be acting specifically through inhibition of biofilm formation rather than by influencing cell viability *per se*.

5.3.2 *Efficacy of NucB and DNase I against* S. mutans *GS-5*

The bacterial enzyme NucB has a number of advantages for biofilm control compared with bovine DNase I. However, it is unknown if it has increased efficacy against oral biofilms. Therefore, to assess the activity of NucB against *S. mutans* GS-5 biofilms, preformed biofilms were treated with NucB at a range of concentrations (Figure 5.4A). DNase I was also used to treat *S. mutans* GS-5 biofilms at the same concentrations. Optimal buffers were used for both DNase I and NucB. For both enzymes a

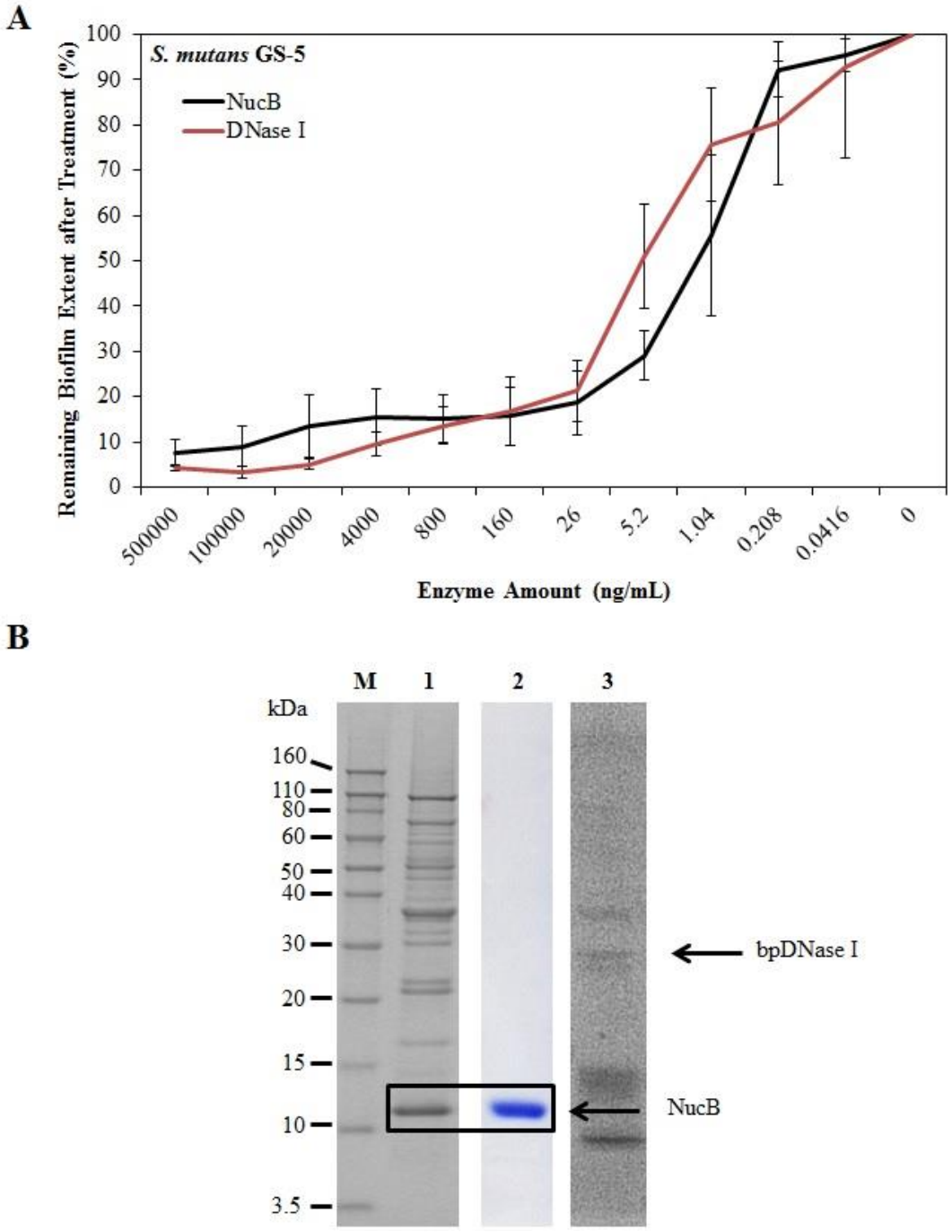

Figure 5.4 Dispersal of *S. mutans* GS-5 by NucB and DNase I. (A) A range of concentrations of NucB and DNase I were incubated with preformed (20 h) *S. mutans* GS-5 biofilms at 37°C for 1 h, and residual biofilms were quantified by crystal violet staining. Mean values from three independent assays are shown and error bars represent standard error of the mean. (B) Samples of DNase enzymes were visualised in roughly equal amounts (5 µg, according to concentrations given) using SDS-PAGE. Lanes: M) protein molecular weight marker; 1) NucB (12 kDa) overproduction from *Bacillus licheniformis* N8900 (Nithya Rajarajan, unpublished work); 2) Purified NucB (Alistair Hawkins); 3) bpDNase I (29 kDa) (Sigma Aldrich).

concentration-dependent reduction in biofilm biomass was observed. The EC_{50} (half maximal effective concentration) values were calculated (using SigmaPlot) to be 1.14 ng mL^{-1} for NucB and 4.18 ng mL^{-1} for DNase I. Therefore, NucB was effective at an approximately 3.7-fold lower concentration than DNase I. However, at a high concentration, there was little difference in enzyme activity. This suggests a threshold whereby there is sufficient enzyme activity to always disperse >80% *S. mutans* GS-5 for both enzymes.

Comparison of enzyme activity relies on the use of equal amounts of protein. Therefore, NucB and DNase I were visualised using SDS-PAGE with 5 µg of total protein (according to manufacturers or colleagues measurements) loaded onto gels (Figure 5.4B). Previously, NucB was compared with DNase I but no attempt was made to determine the purity of both enzymes (Nijland *et al.*, 2010). For the *S. mutans* GS-5 assay NucB of approximately 95 % purity was used (Lane 2, Figure 5.4B). However, the bpDNase I obtained from Sigma Aldrich is contaminated by a number of unknown proteins, with the purity <50% (Lane 3, Figure 5.4B). The differences in observed protein purity makes comparisons between anti-biofilm activity difficult. This is further complicated by the unknown enzyme activity (U/mg) of both protein preparations. In the Nijland *et al*., (2010) study, NucB of relatively low purity (Lane 1, Figure 5.4B) was compared against DNase I that was likely similar in purity to the preparation used during our experiments. These earlier observations which used low purity NucB and DNase I may give a better comparison of relative enzyme efficacy against biofilms. In this previous study NucB had higher efficacy against biofilms of *B. licheniformis* DSM13, than DNase I, similar to the results obtained in this thesis with *S. mutans* GS-5.

5.3.3 *Dispersal of mixed-species saliva biofilms with NucB*

To determine whether eDNA has a role in acting as an adhesin in oral biofilms mixed-species microcosms grown from human saliva were treated with NucB (see Materials and Methods 2.4.1.5, 2.10.2, and 2.10.3). For these experiments, 3 µg/mL NucB (for NucB purity see Lane 1, Figure 5.4B) was added to forming and pre-formed biofilms grown from six different natural human saliva samples. Firstly, these samples were grown in anaerobic conditions, in artificial saliva for 20 h, to culture microcosms that modelled the subgingival oral environment (Figure 5.5A/B). When assayed in these conditions biofilm formation was significantly inhibited in three out of six biofilms when NucB was included during biofilm development. Biofilm formation was reduced

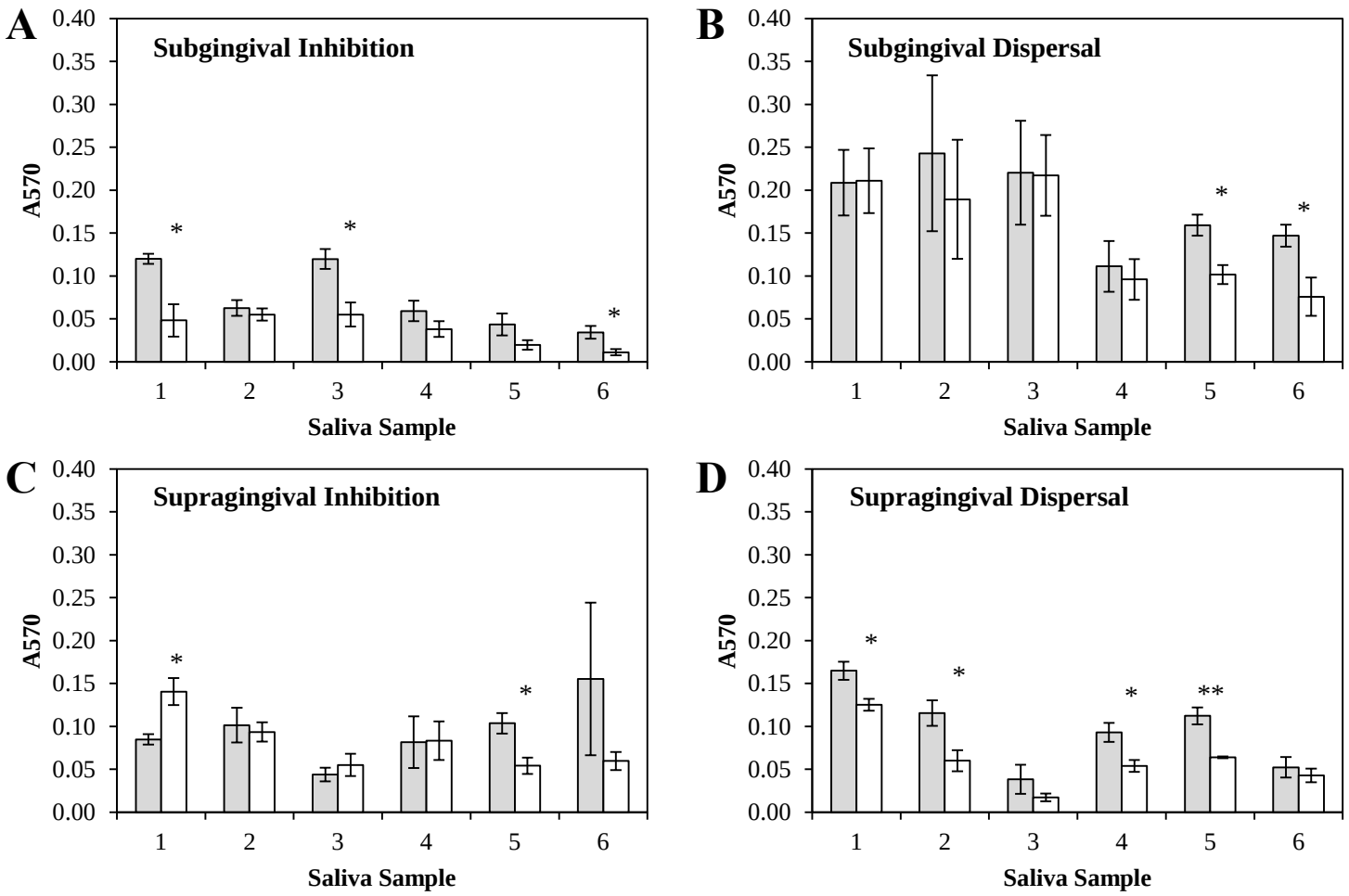
A
Subgingival Inhibition
B
Subgingival Dispersal
C
Supragingival Inhibition
D
Supragingival Dispersal
A570
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
1
2
3
4
5
6
Saliva Sample
*
**

Figure 5.5 Effects of NucB against microcosm biofilms. Artificial saliva medium was inoculated with untreated saliva from six different volunteers (numbered 1-6) and incubated anaerobically to model subgingival dental plaque, or aerobically in the presence of 2% sucrose for biofilms modelling supragingival dental plaque. NucB was included during biofilm formation to test inhibition, or after biofilm development to assess dispersal. Biofilms without NucB (shaded bars) or with NucB (white bars) were quantified by staining with crystal violet. Bars represent mean values from three independent experiments and standard errors are indicated. Statistical significance was calculated with the two sample *t*-test.

by up to 60% by NucB in this model. Furthermore, two of the six mature biofilms were significantly reduced by NucB treatment. To mimic supragingival dental plaque, human saliva was inoculated in artificial saliva media, with the addition of sucrose, and cultured aerobically for 20 h (Figure 5.5C/D). In this model one biofilm was inhibited by NucB, and another was significantly increased when treated with NucB in these conditions. Also, significant dispersal of preformed biofilms was observed in four of the six supragingival biofilms treated. Biofilm formation of the six microcosms was very variable in both growth conditions and at times biofilm extent was poor. However, 10 of the 24 biofilms tested were significantly reduced by NucB.

5.3.4 *Extracellular nuclease activity of single and mixed-species oral bacteria*

The extracellular DNase activity of the four oral bacteria and the human saliva samples was tested on DNase agar. Micro-organisms were grown for 48 h and then plates were flooded with 1 N HCl to precipitate DNA. Dark areas are indicative of extracellular nuclease production by isolates. Only *S. gordonii* DL1 of the four oral bacteria, produced DNase activity that was clearly visible on DNase agar (Figure 5.6A). Extracellular DNase production was also studied using a FRET assay (see Materials and Methods 2.8.7). This method can differentiate between membrane-bound and secreted nuclease activity. Furthermore, it analyses nuclease activity in a different culture state (planktonic). Oral bacteria have been shown to vary their extracellular DNase production depending on culture state (Palmer *et al.*, 2012). When the four micro-organisms were grown in planktonic cultures and nuclease production was assayed using the FRET protocol there was a small amount DNase activity from *F. nucleatum* 25586 (Figure 5.6B). It is unknown if this is within the detection limit of the assay. For both *S. mutans* GS-5 and *A. oris* MG1 extracellular DNase enzyme activity was not detected. The FRET assay also suggests that the DNase that *S. gordonii* DL1 produces is membrane bound, as 80% of the total nuclease activity was measured in this fraction (Figure 5.6B). It is important to consider that the FRET qPCR probe is ssDNA, and therefore any extracellular DNases that are specific for dsDNA hydrolysis would not be detected. All six human saliva samples tested exhibited extracellular DNase activity on DNase test agar (Figure 5.6C). However, it remains unknown which micro-organisms are contributing to this activity as several colony types were visible from each saliva culture. There was substantial variation in total DNase activity and type (cell bound or released) amongst these cultures. It would have been beneficial to include a negative

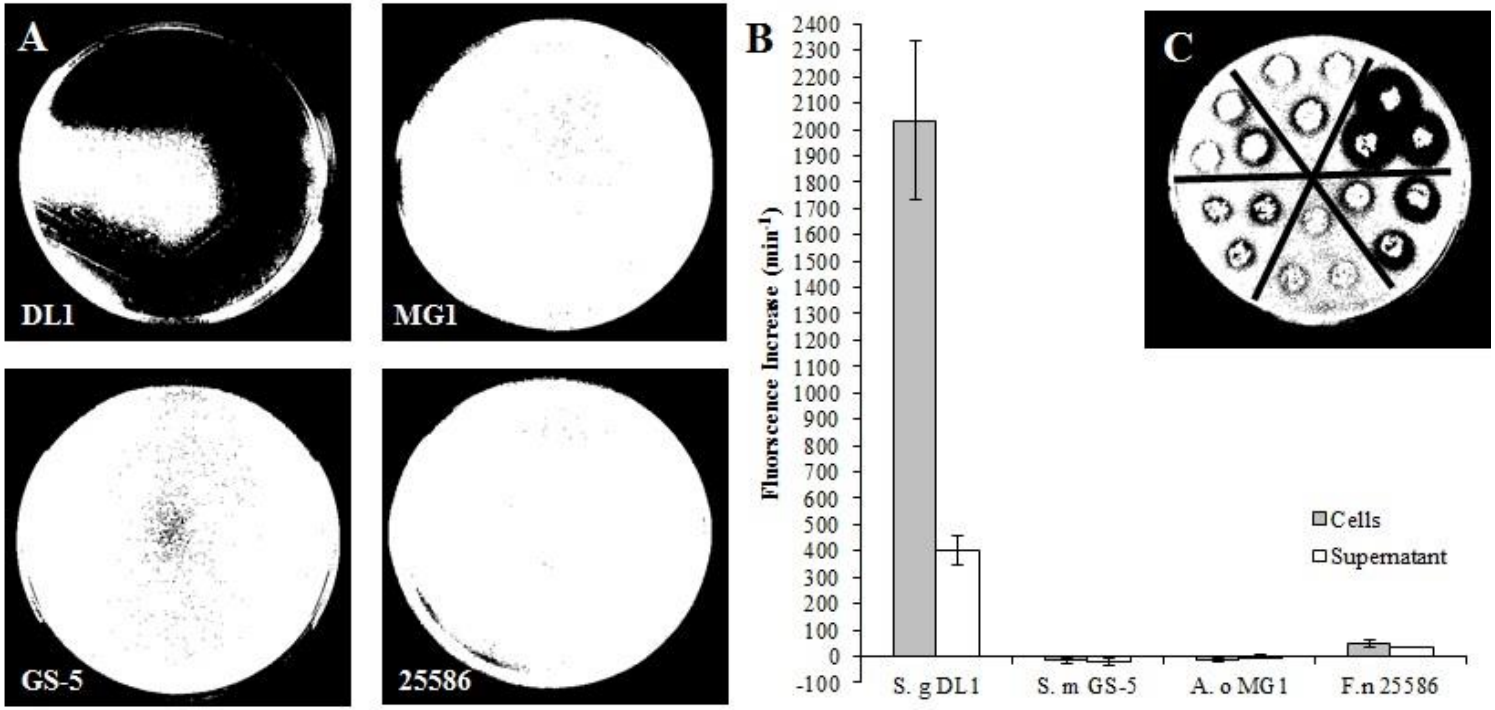

Figure 5.6 Extracellular DNase activity of *S. gordonii* DL1, *A. oris* MG1, *S. mutans* GS-5, *F. nucleatum* 25586 and human saliva cultures. (A) The four oral bacteria were cultured on DNase test agar and 1 N HCl was used to determine DNA degradation, with a dark area indicating DNase activity. (B) DNase activity was assayed for cells and supernatant of planktonic cultures of the four oral bacteria using FRET. (C) Six natural human saliva samples were plated on DNase test agar and assayed for extracellular nuclease activity. DNase test agar plates were visualised with a digital camera (G:Box, Syngene) and images were altered with Adobe Photoshop CS3 using posterization adjustment. Dark zones within agar plates are areas of DNA hydrolysis, whereas white zones are agar that contains intact DNA.

control, like *S. epidermidis*, or a positive control, like *S. aureus*, to make it clearer which colonies were producing DNase activity.

5.4 Variable efficacy of DNases versus *Streptococcus mutans* biofilms

Streptococcus mutans GS-5 is acutely sensitive to nuclease enzymes. However, as previously observed in CRS isolates, DNase sensitivity of microbial biofilms can differ between strains of the same species. To further investigate this phenomenon four *S. mutans* isolates were treated with NucB, during biofilm formation and after maturation.

5.4.1 *NucB activity in a static microplate model*

Four strains of *S. mutans* (GS-5, NG8, UA140 and UA159) were grown in 96-well microtiter plates for 20 h and NucB (3 µg/mL) was either included during biofilm formation or added for 1 h after biofilm formation. Biofilm extent was monitored using the CV assay. As shown previously with both DNase I and NucB, *S. mutans* GS-5 biofilm extent was inhibited or dispersed by >80% during nuclease treatment (Figure 5.7). However, the three other strains differed in their sensitivity to NucB (Figure 5.7). For *S. mutans* NG8 there was 80% less biofilm formation when NucB was included overnight, which was similar to *S. mutans* GS-5. This strain of S. *mutans* was not dispersed by NucB, unlike *S. mutans* GS-5. *Streptococcus mutans* UA140 and *S. mutans* UA159 biofilms were neither inhibited nor dispersed by NucB. Furthermore, although not significant, biofilm growth was higher in treated biofilms. These results demonstrate the variability in DNase sensitivity amongst strains of the same species of bacteria.

5.4.2 *Sequencing mutations in* S. mutans *GS-5*

When first isolated *S. mutans* GS-5 was strongly cariogenic but has since lost this cariogenicity. Mutations in *gbpC* and *pac* genes have been found in laboratory strains of *S. mutans* GS-5 (Sato *et al.*, 2002). These mutations have been hypothesized to be a reason for the lower cariogenicity associated with this strain of *S. mutans*. However, mutations in glucan-binding proteins, like GbpC, and surface adhesins, like PAc, may also lead to changes in biofilm formation (Lynch *et al.*, 2007). Sequence analysis of the *gbpC* and *pac* genes was developed using the primers in Table 2.6 (Materials and Methods). The 1752-bp *gbpC* gene was amplified with primers gbpC5’ and gbpC3’.

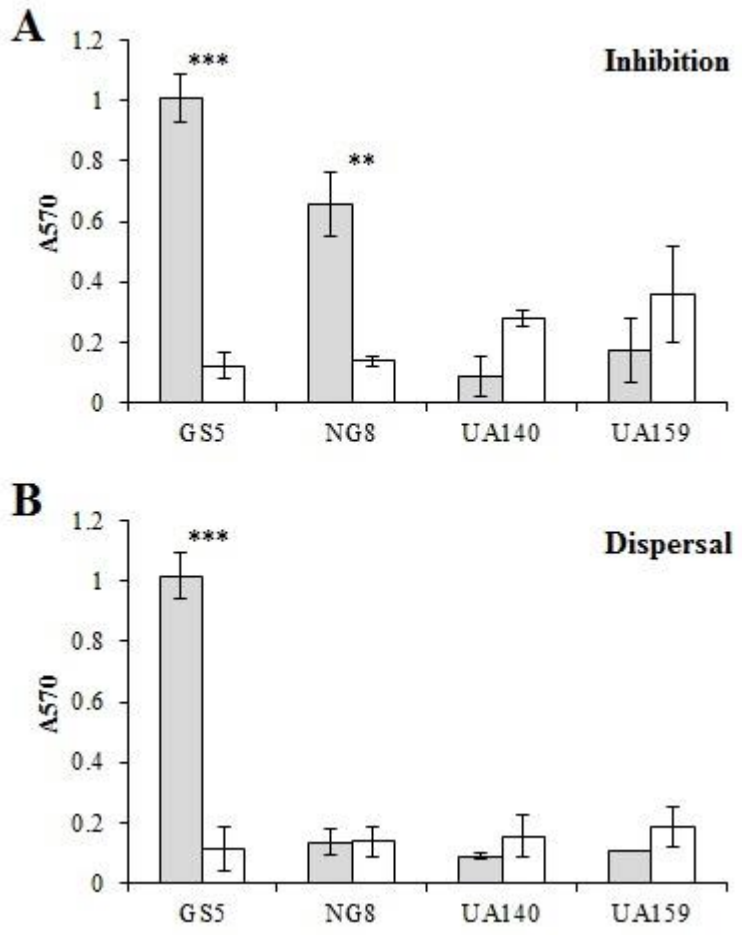

Figure 5.7 *Streptococcus mutans* strain sensitivity to NucB treatment. NucB (3 µg/mL) was added during biofilm formation to test for inhibition (A), or once biofilms had grown for 20 h to determine dispersal (B) in *S. mutans* strains GS-5, NG8, UA140 and UA159. Biofilms without NucB (shaded bars) or with NucB (white bars) were quantified by staining with crystal violet. Bars represent mean values from three independent experiments and standard errors are indicated. Statistical significance was calculated with the two sample *t*-test.

>gbpCF_gbpC5 -- 13..954 of sequence

ATTTGTATAAAAAGACATTATTTTTTAATTTATGAGTCTTATTCTTTGAAAAA
ATGTCTTTTTATTGTTATAATTAAAATTGTATTCTTATGAATTAATAATATAA
AGGATGGTTTTTATGAAATCGAAAACTGCTAAAATTACTTTGCTAAGCAGCC
TTGCTTTGGCGGCTTTTGGAGCAACGAATGTTTTTGCAGATGAAGCATCAAC
TCAATTAAATTCTGATACTGTTGCAGCACCTACTGCTGATACACAAGCATCA
GAACCGGCTGCAACAGAAAAAGAACAGTCTCCTGTTGTAACTGTTGTC**TAA**
AGTCACACACAAGGAAATACAACAACGACAACATCTCAAGTTACTTCTAAA
GAATTGGAAGATGCTAAGGCTAATGCTAATCAGGAAGGTTTAGAAGTCACT
GAAACTGAAACTCAAAAACAGCCTTCGGTAGAAGCTGCAGATGCAGATAAC
AAAGCACAGGCACAAACAATTAATACAGCGGTAGCTGATTATCAAAAGGCA
AAAGCTGAATTTCCTCAAAAACAAGAACAATATAATAAAGATTTTGAAAAG
TATCAGTCTGATGTCAAGGAGTATGAAGCTCAAAAGGCAGCTTACGAGCAA
TATAAAAAAGAAGTTGCACAGGGTTTGGCATCTGGGCGTGTTGAAAAAGCC
CAAGGACTTGTGTTTATTAATGAACCTGAGGCAAAACTTTCTATTGAGGGTG
TTAATCAGTACCTAACAAAAGAAGCACGTCAAAAACATGCAACTGAAGATA
TTCTTCAGCAATATAATACTGATAATTATACAGCTGCTGATTTTACCCAAGC
AAATCCATATGATCCAAAAGAAGATACTTGGTTCAAAATGAAAGTGGGAGA
TCAGATTTCAGTTACCTATGATAATATCGTTAATTCAAAATATAATGATAAA
AAGATTAGTAAGGT

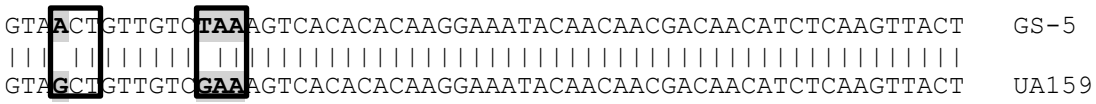

Figure 5.8 Sequencing mutations in *S. mutans* GS-5 *gbpC*. A substitution at the first nucleotide of codon 65 (underlined and boxed) results in a TAA termination codon in strain GS-5, while codon 65 is GAA, encoding glutamic acid in other strains. Furthermore, a GCT codon is ACT in our strain of *S. mutans* GS-5.

>PACR_PAL3 -- 13..1010 of sequence (reverse complement)

CCGCCGGTTAAACCAACAGCTCCAACTAAACCAACTTATGAAACAGAAAAG
CCATTAAAACCGGCACCAGTAGCTCCAAATTATGAAAAGGAGCCAACACCG
CCGACAAGGACACCGGATCAAGCAGAGCCAAACAAACCCCACACCGCCGA
CCTATGAAACAGAAAAGCCGTTGGAGCCAGCACCTGTTGAGCCAAGCTATG
AAGCAGAGCCAACACCGCCGACAAGGACACCGGATCAGGCAGAGCCAAAT
AAACCCACACCGCCGACCTATGAAACAGAAAAGCCGTTGGAGCCAGCACCT
GTTGAGCCAAGCTATGAAGCAGAGCCAACGCCACCGACACCAACACCAGAT
CAACCAGAACCAAACAAACCTGTTGAGCCAACTTATGAGGTTATTCCAACA
CCGCCGACTGATCCTGTTTATCAAGATCTTCCAACACCTCCATCTGTACCAA
CTGTTCATTTCCATTACTTTAAACTAGCTGTTCAGCCGCAGGTTAACAAAGA
AATTAGAAACAATAACGATATTAATATTGACAGAACTTTGGTGGCTAAACA
ATCTGTTGTTAAGTTCCAGCTGAAGACAGCAGATCTCCCTGCTGGACGTGAT
GAAACAACTTCCTTTGTCTTGGTAGATCCCCTGCCATCTGGTTATCAATTTA
ATCCTGAAGCTACAAAAGCTGCAAGCCCTGGCTTTGATGTCACTTATGATAA
TGCAACTAATACAGTCACCTTCAAGGCAACTGCAGCAACTTTGGCTACGTTT
AATGCTGATTTGACTAAGTCAGTGGCAACGATTTATCCAACAGTGGTCGGA
CAAGTTCTTAATGATGGCGCAACTTATAAGAATAATTTCACGCTCACAGTCA
ATGATGCTTATGGCATTAAATCCAATGTTGTTCGGGTGACAACTCCTGGTAA
ACCAAATGATCCAGATAATCCAAATAATAATTATATTAAACCAACT**AAA**GG
T**TAA**TAAAAACGAAAATGCGTGT

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TAAACCAACT***AAA***GGTTAATAAAAACGAAAATG  GS-5 (Our strain)
|||||||||| |||||||||||||||||||
TAAACCAACT-***AA***GGTTAATAAAAACGAAAATG  UA159/LJ23/NN2025
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Figure 5.9 Sequencing mutations in *S. mutans* GS-5 *pac*. In our strain of *S. mutans* GS-5 there is an insertion mutation in the *pac* gene (an extra adenine base) that causes a frameshift, resulting in a TAA (stop) codon (underlined and boxed). This mutation is not present in other strains of *S. mutans*.

Once amplified, the *gbpC* gene was visualised on an agarose gel, purified and sent for sequencing using various primers. A full length DNA sequence was generated using MEGA5.1 (Tamura *et al.*, 2011). Mutations in the *gbpC* gene, compared to other *S. mutans* strains, were discovered by searching the sequence. In our laboratory strain of *S. mutans* GS-5 a substitution at the first nucleotide of codon 65 (T for G), results in a TAA termination codon (Figure 5.8). For *S. mutans* UA159 codon 65 is GAA, encoding glutamic acid. There is also a missense mutation, changing a GCT codon (alanine) to an ACT codon (threonine). However, strains LJ23, 109cS and NN2025 also have the ACT codon. It seems likely that the most major mutation would be the change to the stop codon TAA. Therefore *S. mutans* GS-5 likely produces a truncated GbpC protein. Analysis of the *pac* gene identified an insertion mutation (extra A), which causes a frameshift and likely results in a truncated version of the PAc protein (Figure 5.9). This mutation was not observed in the *S. mutans* strains, UA159, LJ23 or NN2025 and therefore was likely picked up during laboratory culture. Again, this mutation may interfere with biofilm formation.

5.5 Microfluidic model of oral biofilms

Modelling natural biofilms such as dental plaque presents a significant challenge for oral microbiologists. Currently we are employing a static model system in microtiter wells which is useful for studying early biofilm formation. However, continuous-flow systems that circulate fresh nutrients allow for the development of mature biofilms in an environment that is more akin to the natural habitat of the mouth (e.g. hydrodynamic influences). Therefore, these biofilms more closely approximate natural biofilms. Recently the BioFlux system (Fluxion Biosciences) has been developed that consists of microfluidic channels, in which biofilms are formed under a flow of nutrients. One great advantage of the system is that tiny amounts of liquid are required, and it is therefore realistic to develop biofilms under flowing human saliva and use enzymes that are not easily accessible in high amounts. In addition, the presence of multiple (24) channels in a single microfluidic chip enables multiple replicates to be performed in a single run. Dr. Alexander Rickard's laboratory is one of the first in the world to establish the BioFlux system for modelling oral biofilms. This laboratory has ample experience with both complex biofilm models and confocal laser scanning microscopy. Therefore, after obtaining funding from the Society for General Microbiology (President's Fund for Research Visits) I spent 6 weeks in Dr. Rickard's laboratory learning this novel

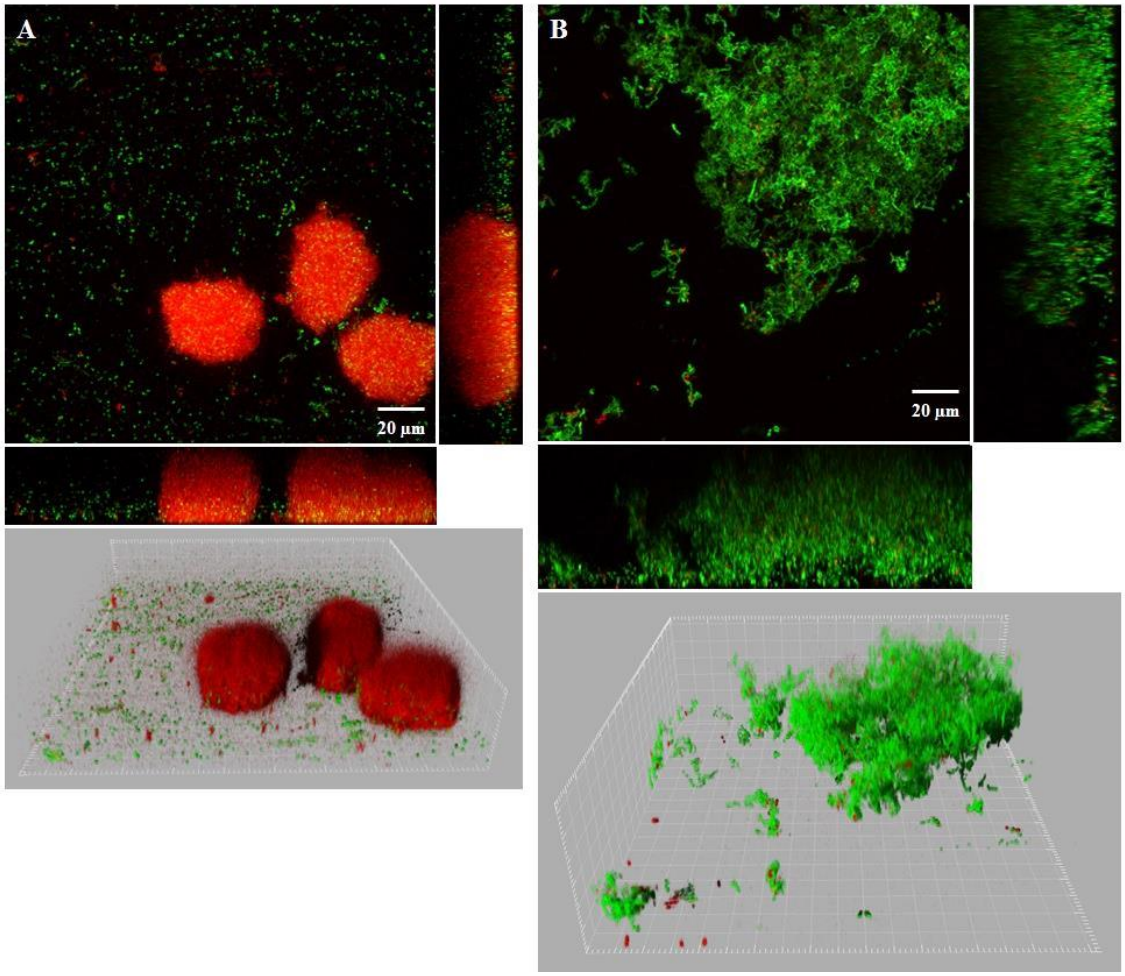

Figure 5.10 Single-species biofilms cultured in flowing human saliva and visualised with CLSM. BioFlux microfluidic plates were inoculated with *S. mutans* UA159 (A) and *S. gordonii* DL1 (B) and grown in filter-sterilized saliva for 20 h at 37°C. After culture, biofilms were stained with LIVE/DEAD® *BacLight*™ stain and observed using CLSM. Images were obtained using 3D imaging software (Imaris, Bitplane). *Streptococcus mutans* UA159 was grown in the presence of sucrose (2%) and *S. gordonii* DL1 filter-sterilized human saliva was supplemented with 1% THYE.

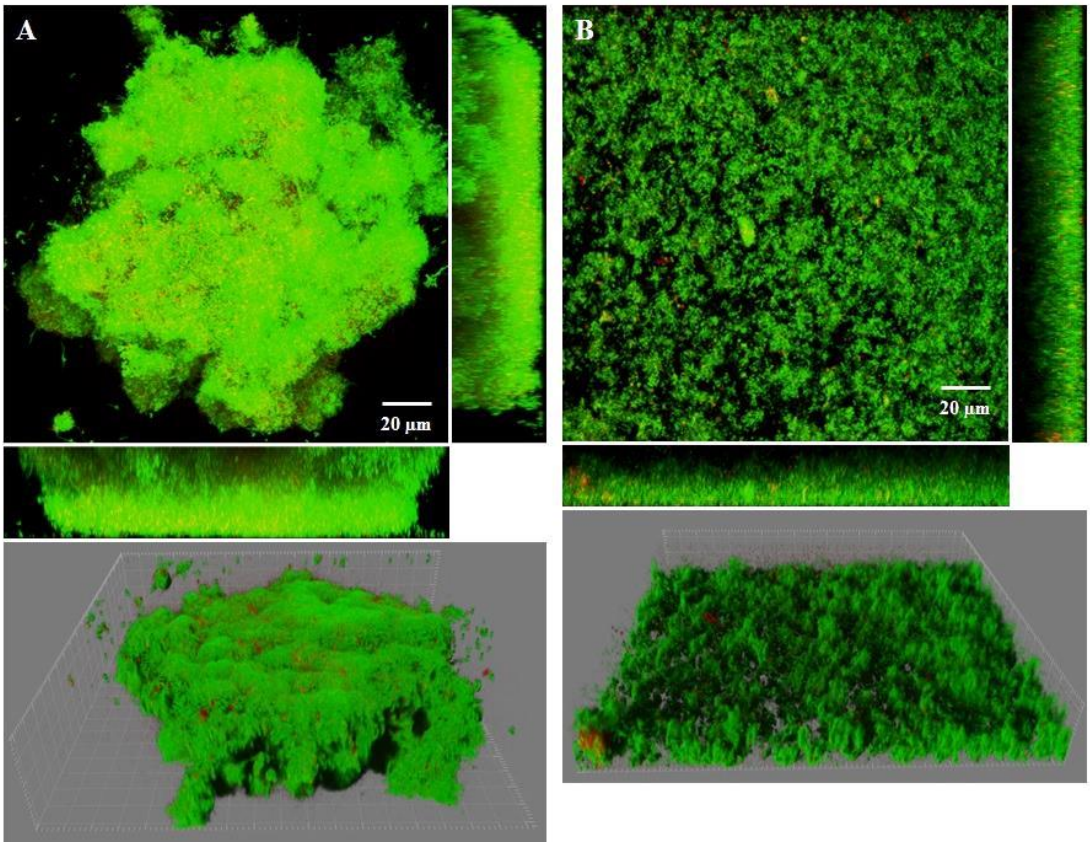

Figure 5.11 Oral mixed-species biofilms grown in flowing human saliva and visualised with CLSM. Human saliva was pooled from six individuals and used as the inoculum for BioFlux microfluidic experiments. Microfluidic plates were inoculated and saliva micro-organisms were fed with filter-sterilized human saliva for 20 h at 37°C. Biofilms were stained with LIVE/DEAD® *BacLight*™ stain and examined using CLSM and 3D imaging software (Imaris, Bitplane).

technique. The major aim was to gain an understanding of the BioFlux microfluidic system so that we may consider obtaining a similar system for the laboratory at Newcastle University.

BioFlux microfluidic plates were inoculated with *S. mutans* UA159, *S. gordonii* DL1, or pooled human saliva and grown in filter-sterilized saliva for 20 h at 37°C (see Materials and Methods 2.6.1.4). *Streptococcus mutans* UA159 was grown in the presence of sucrose (2%) and *S. gordonii* DL1 filter-sterilized human saliva was supplemented with 1% THYE. After culture, biofilms were stained with LIVE/DEAD® *BacLight*™ stain and observed using CLSM (Leica TCS SPE). Images were obtained using 3D imaging software (Imaris, Bitplane). Initially, single-species biofilms of *S. mutans* UA159 and *S. gordonii* DL1 were examined using the BioFlux system (Figure 5.10). *Streptococcus mutans* UA159 formed biofilms that consisted of a thin layer of micro-organisms and several clumps of larger aggregations of bacteria (Figure 5.10A). Interestingly, the aggregations were stained mostly red, giving the appearance of dead bacteria. The clumps were circular with a diameter and depth of 30 µm. With *S. gordonii* DL1, streptococci cells and chains were visible, forming larger aggregations than seen with *S. mutans* UA159 (Figure 5.10B). Biofilms projected from the microfluidic channel surface by to 40 µm. Dead cells (red) are visible but in a lower frequency than seen with *S. gordonii* biofilms grown in static models (Figure 5.2C). Pooled human saliva was also used an inoculum, to model dental plaque biofilms (Figure 5.11). The majority of biofilms again consisted of large aggregations of bacteria, with areas in-between aggregations that appeared sterile (Figure 5.11A). These biofilms projected from the surface by 40 µm, and were up to 120 µm wide. However, there was variation as shown in Figure 5.11B. Thin (20 µm) layers of saliva bacteria did on occasion cover the entire surface of the microfluidic channel.

5.6 Discussion

Although eDNA has been shown to promote bacterial attachment, and act as a matrix support in many biofilm forming species (reviewed by Jakubovics *et al.*, 2013) it has been poorly studied in oral bacteria. *Streptococcus gordonii* DL1, has been shown to produce eDNA but it is unknown how this relates to biofilm matrix stability. Therefore, this study was initiated to address the key question of whether eDNA is important in single and mixed-species oral biofilm formation.

High molecular weight eDNA accumulated in the biofilm matrices of *A. oris*, *F. nucleatum* and *S. mutans* during biofilm development. Interestingly, *F. nucleatum* produced significant quantities of eDNA in this model (989 ng/µL), whereas it has previously been shown to be a minor producer of eDNA (20 ng/µL) (Ali Mohammed *et al.*, 2013). Although a HMW band of nucleic acids was not visible on agarose gels for *S. gordonii*, nucleic acids were still measured by NanoDrop spectrometry. However, it has been shown that high molecular weight DNA (>11 kb) is needed if it is to act as a structural support in *S. aureus* biofilms (Izano *et al.*, 2008). This may explain the lack of efficacy of DNase I against *S. gordonii* biofilms. *Streptococcus gordonii* produces an extracellular DNase, and this may degrade the DNA that is released during biofilm growth. It is also possible that culture conditions reduced the release of HMW nucleic acids. Extracellular DNA release in biofilm culture of *S. gordonii* has been shown to be 100-fold higher when incubated in aerobic conditions, as opposed to anaerobic (Liu and Burne, 2011). It appears that oxygen or the metabolites produced during growth in these conditions increases eDNA release. Although DNA damage appears to be the cue for eDNA release in *S. gordonii* (Itzek *et al.*, 2011) in our laboratory growth in the presence of oxygen did not increase eDNA content in our strain of *S. gordonii* (Claire Graham, unpublished work). It seems likely that the culture conditions used in this study were not optimal for eDNA release but that eDNA release is also tightly regulated. These hypotheses require further investigation.

High molecular weight DNA was extracted from the biofilm matrices of *A. oris* and *S. mutans*. Furthermore, fluorescent staining of eDNA and CLSM gave clear evidence that nucleic acids were present in the biofilms of these species. These data suggest that eDNA is produced in these biofilms both on polystyrene and glass surfaces. For *A. oris*, there were clear areas of eDNA within the mono-species biofilm. These areas of propidium iodide staining (red), a DNA stain that cannot enter live cells, were close to aggregates of dead cells, highlighting a potential source of the eDNA in this species. *Streptococcus mutans* eDNA visualisation was more complex, as there appeared to be a DNA matrix attaching cells together but also potentially eDNA close to the cell surface of bacteria. When live cells (green) are encased in nucleic acids (red), they appear yellow, and this was often the case for *S. mutans* biofilms. Previously, DNA has been shown to increase *S. mutans* LT11 adherence to hydrophobic and hydrophilic surfaces (Das *et al.*, 2010). This suggested that *S. mutans* cell attachment is aided by eDNA during biofilm adherence. Extracellular DNA was visualised in *F. nucleatum* biofilms but it was less clear. Again cells may have had nucleic acids close to the cell surface.

This could be studied in greater detail using immunogold labelled anti-dsDNA monoclonal antibodies and SEM. Using this technique Barnes *et al.*, (2012) provided striking images of eDNA close to the cell surface of *Enterococcus faecalis* cells during initial biofilm formation. Very little eDNA was visualised in *S. gordonii* biofilms, which suggests that our staining technique is sensitive to high molecular weight eDNA.

Interestingly, when the eDNA extracted from the EPS of *F. nucleatum* and *S. mutans* was treated with DNase I for an hour, the high molecular weight band was digested but the low molecular weight fragments remained. This suggests that low molecular weight bands were RNA, although 23S/16S/5S rRNA bands were not clearly visible on agarose gels. RNase was not used during the extraction process for eDNA. DNase and RNase treatment of eDNA from the EPS of *Listeria monocytogenes* and *Bacillus cereus* biofilms has shown that low molecular weight RNA is present in eDNA extractions (Vilain *et al.*, 2009; Harmsen *et al.*, 2010). For *Pseudomonas fluorescens*, RNA was found to be an inhibitor of *P. fluorescens* DNase activity (Catlin and Cunningham, 1958), so it is interesting that it is found in oral biofilm matrices.

DNase I significantly reduced the formation and extent of biofilms in *A. oris*, *S. mutans* and *F. nucleatum*. However, *S. gordonii* biofilms remained intact after DNase I treatment, which may suggest that high molecular weight eDNA is most important when eDNA acts as a biofilm support. The DNase I sensitive nature of *F. nucleatum* 25586 biofilms is in contrast to a previous study, where the same strain was not sensitive to DNase I treatment (Ali Mohammed *et al.*, 2013). In this study only LMW DNA was visible on agarose gels, whereas our findings suggest HMW DNA is produced within *F. nucleatum* biofilm matrices. The differences in the molecular weight of DNA produced by *F. nucleatum* may explain the increased efficacy of DNase I in our study. It is also possible that culture conditions, particularly the length of incubation, which was 28 hours longer in the Ali Mohammed *et al.*, (2013) study, gave rise to different DNase I sensitivities. Also, our biofilms were treated with 5 µg/mL DNase I, which is far lower than the 125-1000 µg/mL concentrations that were used by Ali Mohammed *et al.*, (2013). It is unknown how high concentrations of DNase enzymes affect the activity against microbial biofilms. The variable nature of eDNA production and thus DNase sensitivity is a key point, and needs to be investigated, particularly if DNases are to be used in a therapeutic setting.

DNase I and NucB had a substantial effect on *S. mutans* GS-5 biofilms. However, NucB sensitivity of *S. mutans* biofilm forming strains was variable. *Streptococcus mutans* NG8 biofilm formation was inhibited by NucB but UA159 and UA140

monospecies biofilms were not sensitive to NucB. Although not significant, both UA140 and UA159 biofilms were slightly increased in extent when treated with NucB. A similar finding was demonstrated by *Moraxella catarrhalis* FH4, and *Staphylococcus warneri* FH15 biofilms in Chapter 4. Extracellular DNA can have a deleterious effect on biofilm formation, for instance in *Caulobacter crescentus* CB15, where DNase treatment increased biofilm attachment (Berne *et al.*, 2010). It is possible that in mature biofilms, undergoing nutrient limitation, that DNase treatment facilitates DNA uptake as a nutrient source, allowing an increase in bacterial growth. This is an interesting hypothesis that would require further study. These findings follow previous data that have shown marked differences between DNase sensitivity of biofilms formed by different species of bacteria or, sometimes, by different strains of the same organism (Lappann *et al.*, 2010; Shields *et al.*, 2013). Whilst the strains, NG8, UA140 and UA159 were not investigated in detail (eDNA extraction or microscopy) there does seem to be a genuine difference in NucB sensitivity. Certain *S. mutans* GS-5 laboratory strains carry mutations in the cell surface adhesins Antigen I/II (encoded by the *pac* gene) and Glucan-binding protein C (*gbpC* gene) (Sato *et al.*, 2002). The strain used in this study also carried these mutations. GbpC is the principal receptor for glucan, and loss of function leads to a decrease in biofilm extent (Lynch *et al.*, 2007). It may be that *S. mutans* GS-5 has replaced the function of GbpC and Antigen I/II with eDNA, although this remains an untested hypothesis. However, eDNA has already been shown to be important in bacterial adherence for *S. mutans* LT11 (Das *et al.*, 2010).

The efficacy of NucB and DNase I against *S. mutans* GS-5 was compared, however, the comparison of these two enzymes is made difficult by differences in enzyme purity. DNase I was found to contain multiple proteins, whereas NucB was at least 95 % pure. Previous research has shown the increased efficacy of NucB compared with DNase I, although both enzymes were relatively impure in these assays (Nijland *et al.*, 2010). Further investigations are required to determine if NucB has increased anti-biofilm activity over other DNase enzymes. It would be particularly useful to determine the amount of enzyme activity per mg of enzyme and this could be performed in the FRET assay. NucB had efficacy at dispersing or inhibiting mixed-species microcosms cultured from six human saliva samples. Extracellular DNA has been identified in multi-species biofilms and DNase enzymes have been used to reduce biofilm extent (Steinberger and Holden, 2005; Dominiak *et al.*, 2011; Shakir *et al.*, 2012). Interestingly, there was substantial variation in NucB activity amongst the six samples tested. This suggests that natural differences in the microbial composition of human saliva and dental plaque

leads to a change in biofilm structure and role of eDNA. There was also a change in NucB efficacy between biofilm formation and once biofilms had matured in some samples. It may be that the role of eDNA changes as biofilms mature. In *Pseudomonas aeruginosa*, eDNA helps the early formation of the biofilm, and then becomes less important in mature colonies (Whitchurch *et al.*, 2002).

Further testing is required to determine the anti-biofilm activity of NucB against mixed-species biofilms. Continuous-flow systems that circulate human saliva would allow for more realistic experiments of DNase biofilm dispersal. Recently, a microfluidic system, the BioFlux, has been shown to allow the culture of mixed-species biofilms that are similar in composition to supragingival plaque (Nance *et al.*, 2013). Saliva microcosms, single-species *S. gordonii* DL1, and single-species *S. mutans* UA159 biofilms were cultured using this device. In particular there was a vast difference in biofilm structure when comparing static single-species biofilms and those cultured in the microfluidics device. Statically cultured biofilms are relatively thin continuous layers of cells, whereas culture in the BioFlux leads to thicker biofilms that are non-continuous. They resemble the biofilms visualised by SEM on the surface of TESVs in Chapter 3. In the future it is hoped that the BioFlux system could be used to test DNase enzymes against oral biofilms grown in human saliva.

Across the four organisms tested, only *S. gordonii* showed clear extracellular DNase production. Many oral bacteria have been shown to produce extracellular nucleases (Palmer *et al.*, 2012). *Fusobacterium nucleatum* produced a small amount of nuclease activity when assayed using the FRET protocol. This organism has been shown to be an extracellular DNase producer but not when using the DNase test agar assay, which is similar to our findings (Palmer *et al.*, 2012). It is possible that during planktonic culture cell lysis is releasing an intracellular DNase that accounts for the nuclease activity seen in the FRET assay. The nuclease activity of *S. gordonii* was predominately in the cell fraction of planktonically cultured cells, which is suggestive of a cell bound nuclease (this nuclease is discussed in greater detail in Chapter 6). An interesting finding was that all six saliva cultures were DNase positive when assayed with DNase test agar. It is unknown which microbial species contributed to this but it appears that extracellular DNase activity is common amongst plaque forming bacteria. This assay would be improved with the use of positive and negative DNase-producing bacterial controls.

The results of this study show clear evidence that oral bacteria produce eDNA that is essential for providing structural support. Furthermore, mixed-species oral biofilms also rely on eDNA. There is significant variation in the total abundance of eDNA, DNase

sensitivity, and temporal changes in DNase sensitivity amongst different oral bacteria and strains. Studies on natural dental plaque formed *in situ*, and ultimately *in vivo* studies in humans, will be required to establish the potential of NucB or other DNases for the control of oral biofilms.

Chapter 6: Characterising *Streptococcus gordonii*, SsnA

6.1 Outline

Oral biofilms, single and multi-species, have been shown (Chapter 5) to contain micro-organisms that rely on eDNA for initial attachment of biofilm cells to a surface and for stability of the biofilm once it matures. An interesting finding was that *Streptococcus gordonii* DL1 produces an extracellular nuclease (Figure 5.6). Furthermore, in the conditions used, *S. gordonii* biofilms appeared to contain little eDNA and was not susceptible to treatment with exogenous nuclease. Therefore, it is possible that the extracellular DNase of *S. gordonii* is active within biofilms, and that *S. gordonii* utilises an eDNA-independent mechanism for biofilm formation. *Streptococcus gordonii* is an important early colonizer of the tooth surface, and interacts with a number of organisms during the accumulation of dental plaque. Therefore, any extracellular enzymes it produces could have an important ecological role in the oral cavity. This study aimed to identify the exonuclease of *S. gordonii* and characterise its activity.

Significant amounts of DNA fragments can persist in the environment, for instance in soil, where fresh eDNA may remain intact for a number of days (Nielsen *et al.*). Certain micro-organisms have evolved to produce extracellular deoxyribonucleases, to try and exploit this resource. The potential role of these enzymes is vast, including degrading neutrophil extracellular traps (NETs) (Berends *et al.*, 2010), facilitating biofilm re-structuring (Kiedrowski *et al.*, 2011; Steichen *et al.*, 2011), reducing transformation (Blokesch and Schoolnik, 2008), and utilising DNA as a nutrient source (Liechti and Goldberg, 2013). Intuitively, given the lack of nutrients in most of the environments micro-organisms reside in, scavenging DNA as a nutrient source would seem the most likely role of these enzymes (discussed in Introduction section 1.5). In ocean sediments, eDNA contributes to phosphorus cycling, with an estimated 0.45 gigatons of eDNA in the top 10 cm of deep sea sediments (Dell'Anno and Danovaro, 2005). This material is estimated to account for half of the phosphorus assimilated by bacteria in this ecosystem. Phosphorus uptake from eDNA in hyposaline environments is hypothesised to be important for the archaean species *Haloferax volcanii*, although it is unclear what role a putative thermonuclease, Hvo_1477, has in this eDNA processing (Chimileski *et al.*, 2014). In some cases, such as *Shewanella oneidensis* MR-1, microbes produce more than one extracellular nuclease, and different nucleases can have different functions

(Heun *et al.,* 2012). The roles of the nuclease produced by *S. gordonii* are currently unknown.

Many bacteria produce extracellular deoxyribonucleases with notable examples being *Staphylococcus aureus* (Kiedrowski *et al.,* 2011), and *Vibrio cholerae* (Seper *et al.,* 2013). Recently, Palmer and others (2012) screened periodontal bacteria and identified 27 different species that were capable of expressing extracellular DNase activity, including *S. gordonii*. Interestingly, secreted DNase activity was dependant on the culture state of the bacteria, and was either cell-adhered or truly extracellular. Many streptococci are capable of producing extracellular deoxyribonucleases. These enzymes are often linked to virulence (e.g. facilitating escape from NETs) in *Streptococcus pyogenes* (Hasegawa *et al.,* 2010), *Streptococcus pneumoniae* (Beiter *et al.,* 2006), and *Streptococcus agalactiae* (Derré-Bobillot *et al.,* 2013). Oral streptococci, of which *S. gordonii* is a member (Facklam, 2002), have been shown to produce extracellular DNases (Palmer *et al.,* 2012). It is unknown exactly what biological role the streptococci nucleases play in mixed-species biofilms.

The focus of many studies on extracellular DNase enzymes has been their roles in virulence, and the escape of micro-organisms from NETs. However, given that many streptococci, including oral species, exist primarily within mixed-species communities, it is possible that extracellular DNases are produced to reduce the colonisation of competitors. This seems likely since biofilms of several different oral bacteria including the cariogenic species *S. mutans* are dispersed by nuclease treatment (see Chapter 5). This aspect of extracellular DNase production, and microbial antagonism, is a neglected area of research. It has been shown previously that *S. gordonii* (and *S. sanguinis*) produces hydrogen peroxide (H_2O_2) when grown aerobically, that has a growth limiting effect on *S. mutans* (Kreth *et al*., 2008). This study highlights the competitive interactions that occur between these two species.

The major aim of this project was to characterise the extracellular nuclease of *S. gordonii,* SsnA, with a particular focus on anti-biofilm activity against *S. mutans*. The experimental approach involved expressing and purifying the enzyme. It was observed that carbon sources affected enzyme activity and therefore this study also aimed to investigate the regulation of SsnA production. Acid production after sugar fermentation, and its effect on SsnA enzyme activity was also studied. This project builds upon the

previous result chapters which have shown the reliance of eDNA in some biofilm-forming micro-organisms in a number of systems. With this investigation the research had a molecular biological focus, which adds an extra edge to the thesis as a whole.

Results in this chapter, and parts of Chapter 5, are being prepared for submission to *Molecular Microbiology*.

6.2 Identification of a cell surface nuclease (SsnA) of *Streptococcus gordonii*

The extracellular deoxyribonuclease activity of *S. gordonii* DL1 was shown in Chapter 5 (Figure 5.6). The nuclease producing phenotype of *S. gordonii* is likely to be a regular feature of *S. gordonii* strains as 10 strains have been shown to degrade DNA in a DNase test agar assay (Nick Jakubovics, unpublished data). The gene encoding for this protein was identified by screening the *S. gordonii* DL1 genome for DNase 1-like domains using NCBI Blast. Three genes were identified as having putative DNase 1-like domains. These three targets were then compared with other GenBank genes to find sequence homology to previously identified extracellular nucleases. A LPxTG cell wall surface protein (accession no. YP_001450928.1) (Figure 6.1A), with a putative DNase I-like domain was found to have the most extensive homology to other putative extracellular deoxyribonucleases. The 2337-bp gene, encoding a 779 amino acid (aa) protein showed greatest homology, 80% identity, to a putative 733-aa endonuclease (accession no. WP_003043336.1) of *Streptococcus anginosus*. This species of *Streptococcus* was found to produce an extracellular nuclease in Chapter 4 (Table 4.1). The protein also shared 76% identity over aa 17-709 with a putative endonuclease (accession no. WP_002923596.1) of *Streptococcus sanguinis*. A previously identified secreted nuclease of *Streptococcus suis* (Fontaine *et al.*, 2004) shared 44% identity with the putative *S. gordonii* extracellular DNase (accession no. YP_006081396.1). The gene encoding the *S. suis* nuclease was designated *ssnA* for *S. suis* secreted nuclease A. Given that *S. gordonii* and *S. suis* are both members of the *S. sanguinis* group of streptococci, the putative DNase gene of *S. gordonii* was also termed *ssnA* (and will be referred to as such from now on). However, in this case *ssnA* is defined as 'streptococcal secreted nuclease A' as this is a more appropriate term for the gene. Other proteins with sequence homology to *S. gordonii* SsnA included the *Gemella haemolysans* endonuclease (accession no. WP_004263991.1), 66% identity, and the *Bacillus cereus* endonuclease (accession no. WP_000279241.1), 41% identity from aa

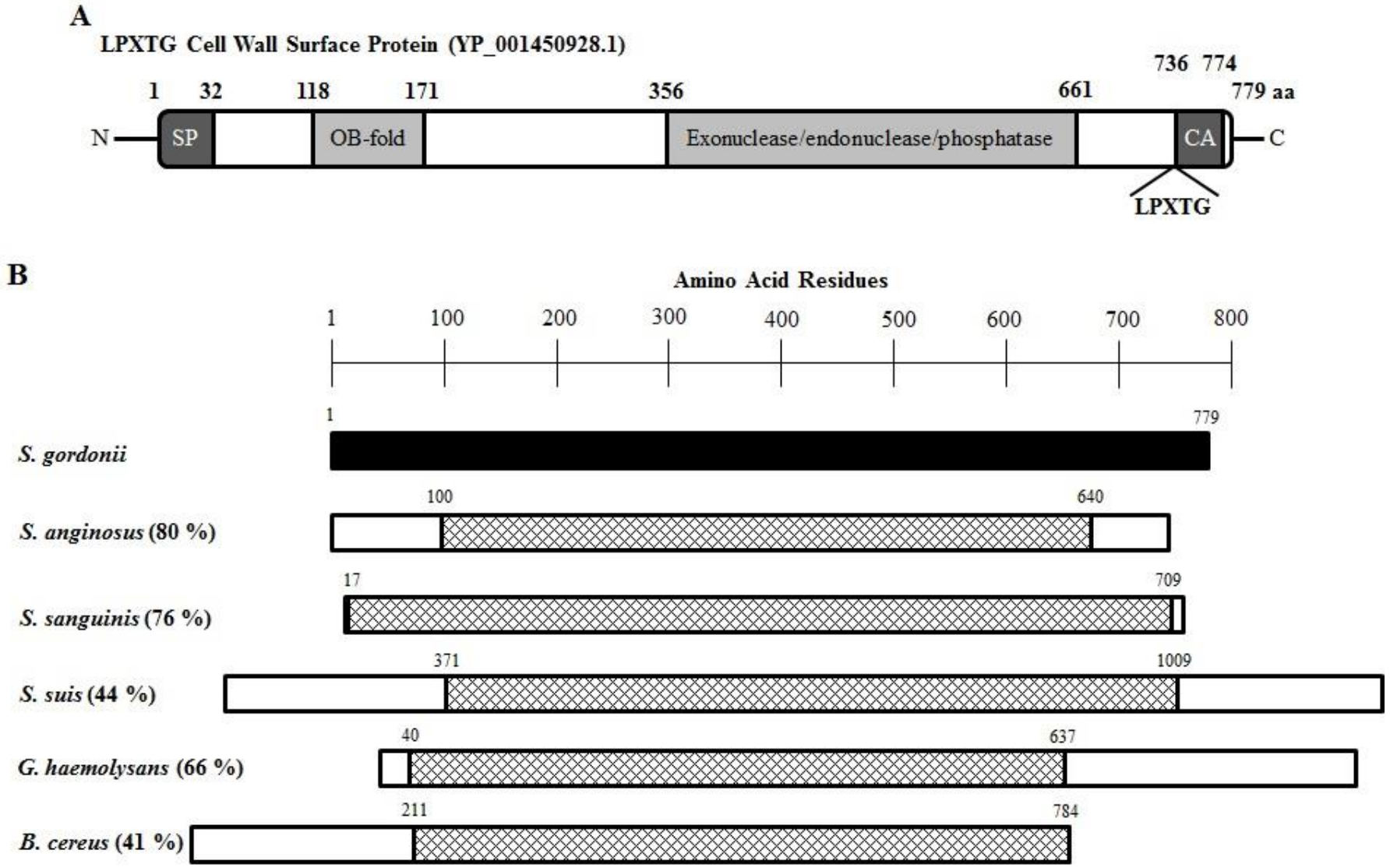
A
LPXTG Cell Wall Surface Protein (YP_001450928.1)
1
32
118
171
356
661
736
774
779 aa
N
SP
OB-fold
Exonuclease/endonuclease/phosphatase
CA
C
LPXTG
B
Amino Acid Residues
1
100
200
300
400
500
600
700
800
S. gordonii
1
779
S. anginosus (80 %)
100
640
S. sanguinis (76 %)
17
709
S. suis (44 %)
371
1009
G. haemolysans (66 %)
40
637
B. cereus (41 %)
211
784

Figure 6.1 Predicted conserved domains of SsnA and homology with nucleases from other species. (A) The 2337-bp open reading frame of the *S. gordonii* extracellular DNase encodes a protein of 779-aa. The SsnA precursor protein is predicted to contain a signal peptide (SP, position 1-32) at the N-terminal, and a cell wall anchor motif (CA, position 736-774) at the C-terminal end. SsnA also has a putative OB-fold domain (position 118-171) at the N-terminal end, and a C-terminal end that contains a putative exonuclease/endonuclease/phosphatase domain (position 356-661) (B) SsnA shares homology with a number of other bacterial produced nucleases, and selected examples are shown. Homology (hatched boxes) begins and ends within the amino acid residues indicated by the numbers above each box. Percentage identities are included after species names in brackets.

211-784. The regions that shared sequence homology with *S. gordonii* SsnA are shown in Figure 6.1B.

The SsnA protein was analysed for conserved domains using the NCBI Conserved Domain Database, SignalP-4.1 and CW-PRED (Litou *et al.*, 2008; Petersen *et al.*, 2011; Marchler-Bauer *et al.*, 2013). SsnA contains a putative oligonucleotide/oligosaccharide-binding fold (OB-fold) (similarity to *Bacillus subtilis* OB-fold) and predicted binding sites for Mg^{2+}, and phosphate. The C-terminal end of SsnA consists mostly of a putative exonuclease/endonuclease/phosphatase domain. These are all features that are expected with DNase-like enzymes. The amino acid sequence also contains an LPxTG-motif (LPATG) at the C-terminal end, which is a conserved sequence for Gram positive cell wall surface proteins. Cleavage of LPxTG between Thr and Gly by sortase A leads to covalent anchoring to the cell wall at the new Thr C-terminal end. All predicted domains with amino acid positions are shown in Figure 6.1A.

Prior to this project, the 2337-bp *ssnA* gene was knocked-out in *S. gordonii* DL1 to further confirm that SsnA was the protein encoding extracellular nuclease activity (Nick Jakubovics, unpublished work). Extracellular DNase deficiency was analysed using DNase Test agar (Figure 6.2A). By contrast with the wild-type progenitor, *Streptococcus gordonii* Δ*ssnA* did not produce any extracellular DNase activity. Furthermore, using a FRET assay nuclease activity was not detected in *S. gordonii* Δ*ssnA* conditioned media or cells (Figure 6.2B). *Streptococcus gordonii* DL1 extracellular deoxyribonuclease activity was compared with *Staphylococcus aureus* FH7 by the FRET assay. DNase activity of *S. gordonii* DL1 was 5-fold higher in the cell fraction (cellular and supernatant fractions were in identical volumes), which gives further indication that SsnA is a cell-bound protein. *Staphylococcus aureus* FH7 nuclease activity was highest in the conditioned media (3-fold), as was expected since the major extracellular DNases of this organism are not cell-bound. This positive control gives confidence in the ability of the FRET assay to determine nuclease activity in different fractions of a planktonic culture of micro-organisms.

6.3 Biofilm dispersal of *S. mutans* GS-5 with *S. gordonii* DL1

To determine how extracellular deoxyribonuclease production by *S. gordonii* affects *S. mutans* biofilm extent, *S. gordonii* DL1 cells and conditioned media were added to pre-formed (20 h) *S. mutans* GS-5 biofilms. *Streptococcus gordonii* DL1 cells reduced *S. mutans* GS-5 biofilm extent by 40%, when compared to the control where only PBS

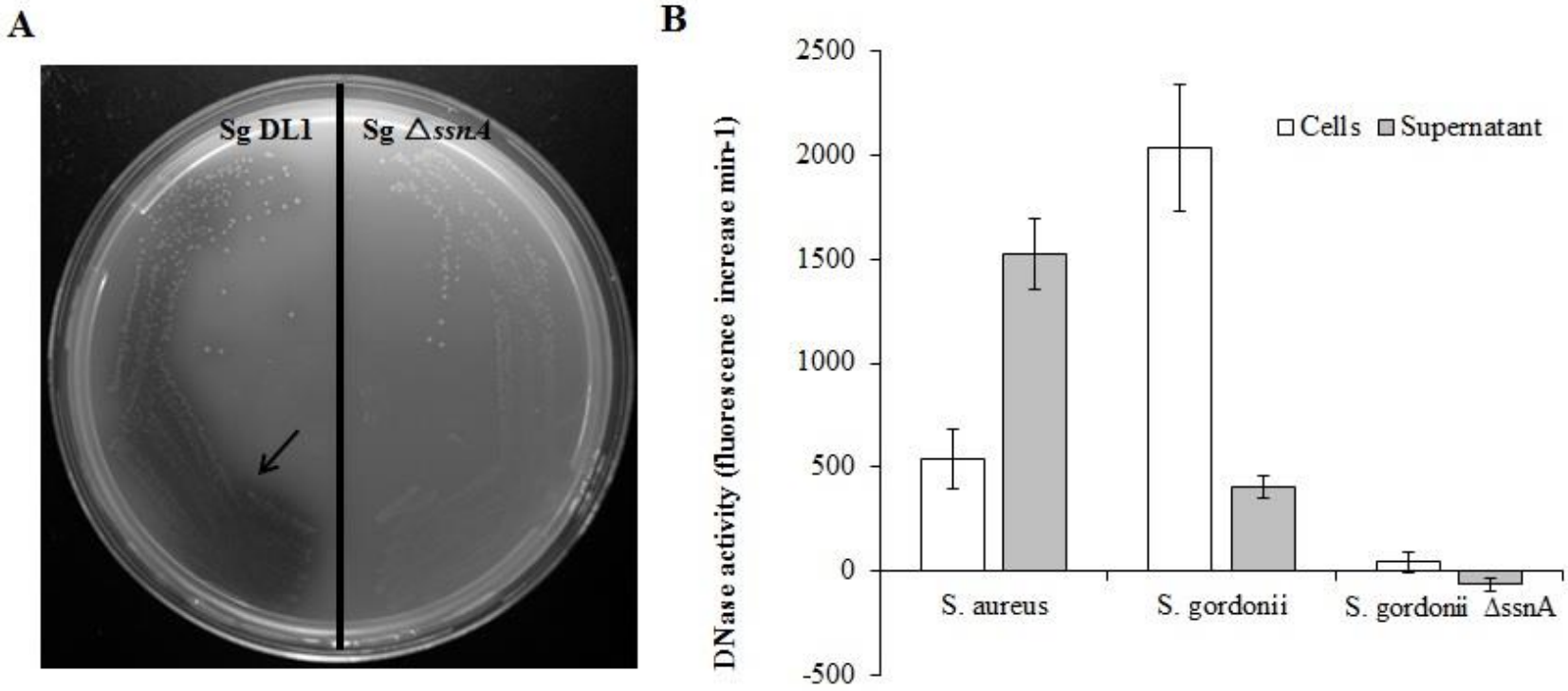
A
Sg DL1
Sg ΔssnA
B
DNase activity (fluorescence increase min-1)
2500
2000
1500
1000
500
0
-500
Cells
Supernatant
S. aureus
S. gordonii
S. gordonii ΔssnA

Figure 6.2 Targeted disruption of *ssnA*. An *ssnA*-deficient mutant was created by mutagenesis. This was to confirm that this gene is responsible for the extracellular nuclease produced by *S. gordonii* DL1. (A) *Streptococcus gordonii* DL1 and *S. gordonii* Δ*ssnA* were cultured on DNase Test agar to test for DNase activity. A zone of DNA degradation (dark halo) as indicated by the arrow, was apparent for *S. gordonii* DL1 after DNA was precipitated with 1N HCl. There was no zone of DNA removal for the *ssnA*-deficient mutant. This confirms *ssnA* as the gene responsible for extracellular nuclease activity. (B) The nuclease activity of *S. gordonii* DL1, *S. gordonii* ΔssnA and *S. aureus* FH7 was examined by the FRET assay. Again, no significant nuclease activity was quantified in the cell (white box) and supernatant (grey box) fractions for the *ssnA*-deficient mutant. Most extracellular deoxyribonuclease activity is produced by the cell fraction of *S. gordonii*, which fits with the predicted cell anchor motif within the *ssnA* sequence. *Staphylococcus aureus* extracellular nuclease activity is highest in the supernatant fraction, as expected. Standard error from the mean (3 replicates) is indicated by the error bars.

was included (Figure 6.3). Intact cells of *Streptococcus gordonii* Δ*ssnA* did not significantly disperse pre-formed *S. mutans* GS-5 biofilms. The nuclease activity of cultures was quantified using a FRET assay, confirming the presence of DNase in *S. gordonii* DL1 cell and supernatant fractions. Bovine DNase I (bpDNase I) (5 μg/mL) was also included as a control, as it has been previously shown to reduce *S. mutans* GS-5 biofilm extent by up to 90% (Chapter 5). This result was again repeated, highlighting the dependence of *S. mutans* GS-5 on eDNA in this system. Interestingly, both *S. gordonii* DL1 and *S. gordonii* Δ*ssnA* conditioned media had a dispersal effect. Supernatant from wild-type dispersed *S. mutans* by 40 %. There was a small amount of *S. mutans* dispersal by supernatant from the *ssnA*-deficient mutant though it was only 20 %. Conditioned media of *S. gordonii* DL1 contains nuclease activity but 5-fold less than cell fractions (Figure 6.2B). The *ssnA*-deficient mutant produces minimal nuclease activity. *Streptococcus gordonii* DL1/Δ*ssnA* were grown aerobically, conditions that likely lead to H_2O_2 production, explaining why conditioned media from both strains reduced *S. mutans* biofilm extent (although other factors are possible). As discussed earlier, *S. gordonii* produces H_2O_2 that has an antagonistic effect against *S. mutans* (Kreth *et al.*, 2008).

6.4 SsnA activity in response to sugar fermentation

6.4.1 Sugar regulation and the role of CcpA and MalR regulators

Carbon catabolite repression (CCR) is a regulatory mechanism in bacteria to ensure the selection of optimal carbon sources. Many genes are repressed or activated depending on the carbon source present. There is evidence that *S. aureus* Nuc1 is glucose regulated (Kiedrowski *et al.*, 2011; Beenken *et al.*, 2012). Therefore, it was hypothesised that expression of *ssnA* may be either up or down-regulated depending on the carbon source present. This would have an immediate impact on the ecology of the oral biofilm, particularly with regard to eDNA degradation. The upstream promoter region of the *ssnA* gene was screened for potential binding sites linked to CCR transcription factors using RegPrecise (Novichkov *et al.*, 2010). Interestingly, there was a consensus binding site in the *ssnA* promoter region for carbon catabolite protein A (CcpA), a protein involved in global transcriptional regulation in response to sugars like glucose (Figure 6.4). There was also a consensus binding site for MalR, a protein involved in maltosaccharide uptake and utilization (Figure 6.4). Therefore, the *ccpA* and *malR* genes

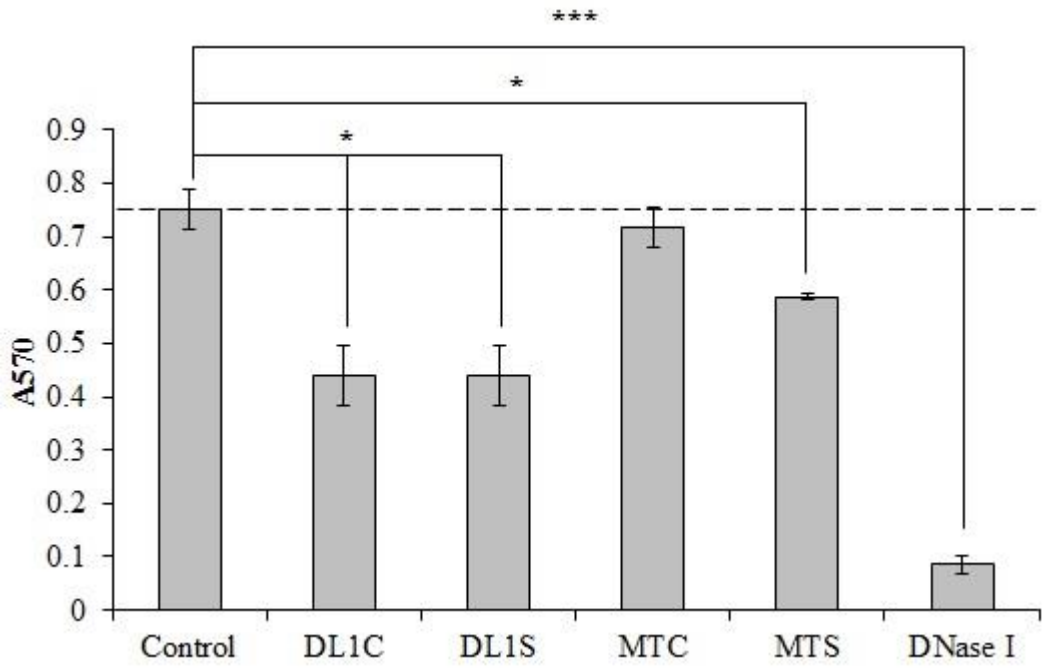

Figure 6.3 *Streptococcus mutans* GS-5 biofilm disruption by *Streptococcus gordonii*. *Streptococcus mutans* GS-5 biofilms were grown anaerobically, at 37°C, for 20 h. Pre-formed biofilms were then incubated with *S. gordonii* DL1 (DL1C) or *S. gordonii* Δ*ssnA* (MTC) cells, and *S. gordonii* DL1 (DL1S) or *S. gordonii* ΔssnA (MTS) supernatant for 1 hour at 37°C. Biofilm extent was quantified by the crystal violet assay. DNase I (5 µg/mL) was included as a positive control, to confirm the eDNA dependence of *S. mutans* GS-5. Control biofilms were treated with PBS only. Standard error from the mean (3 replicates) is indicated by the error bars. Statistical significance was calculated with the two sample *t*-test.

Catabolite Responsive Element

Potential Site: AAGAAAACGTTTGCGA
-35 box: TTGCGA
-10 box: CTATATAATAT

AAATACTATTTTGCAGTGAGGAAAGTTGATTTTTAGATATCGGTTTTCTTCACTGTTTTTATATATTG
ACTGATTTATCAAAACTTATAACAGAAATGGAAGTATGCATGAGTTTAAGAAAACGTTTGCGAAA
ATATTATTTTTTCTATATAATATAAACAGTATAGTAATTAAGTTTCATTAGGAAAAGGAGAACTTTAT
GAAGAAACAAGCTTATCAAAAGCGTCTTTTTTATTCAGCAGCAGTATTAAGTGCTTTTCTACCTTT
GCTTTCCTTGGCTGTTCCGGTCGGAGCTGAAGAGACGGAAAATTCTTCTAGTTCA

MalR Binding Site

Potential Site: TTAAGAAAACGTTTGCGAAA
-35 box: TTGCGA
-10 box: CTATATAATAT

AAATACTATTTTGCAGTGAGGAAAGTTGATTTTTAGATATCGGTTTTCTTCACTGTTTTTATATATTG
ACTGATTTATCAAAACTTATAACAGAAATGGAAGTATGCATGAGTTTAAGAAAACGTTTGCGAAA
ATATTATTTTTTCTATATAATATAAACAGTATAGTAATTAAGTTTCATTAGGAAAAGGAGAACTTTAT
GAAGAAACAAGCTTATCAAAAGCGTCTTTTTTATTCAGCAGCAGTATTAAGTGCTTTTCTACCTTT
GCTTTCCTTGGCTGTTCCGGTCGGAGCTGAAGAGACGGAAAATTCTTCTAGTTCA

Figure 6.4 Consensus binding sites in the *ssnA* promoter region. Contained within the promoter region of *ssnA* are areas that may bind to the CcpA and MalR regulatory proteins. Hypothetical binding sites are shown within the promoter sequence (black text) by underlining nucleobases. The start of the *ssnA* ORF is highlighted in colour (pink). Promoter sites were predicted using BPROM (SoftBerry).

were chosen as targets for gene deletions, to study the regulation of *ssnA* in response to carbon sources.

The *malR* and *ccpA* genes were deleted by an overlap extension PCR mutagenesis approach (see Materials and Methods 2.9), and gene deletions were confirmed by sequencing. *Streptococcus gordonii* Δ*ccpA* and *S. gordonii* Δ*malR* were grown on DNase test agar containing no sugar, glucose, maltose or galactose. As previously discussed, DNase test agar contains DNA and is a method that can confirm extracellular deoxyribonuclease activity. *Streptococcus gordonii* DL1 degraded DNA when no sugar, or the non-repressing sugar, galactose was present (Figure 6.5). However, it did not produce nuclease activity when glucose or maltose was added to DNase test agar, indicating the repression of *ssnA* by sugars. When *S. gordonii* Δ*ccpA* was cultured on the two repressing sugars, glucose and maltose, enzyme activity was still visible (Figure 6.5). This was in contrast to the *malR*-deficient mutant, which still did not produce extracellular deoxyribonuclease activity when grown with repressing sugars present, including maltose (Figure 6.5). *Streptococcus gordonii* Δ*malR*, along with the wild-type, also did not produce nuclease activity on DNase Test agar supplemented with a maltooligosaccharide mixture (2%) and maltodextrin (2%). These data indicate that CcpA down-regulates the extracellular nuclease of *S. gordonii* when optimal carbon sources such as glucose are present. It appears that MalR does not repress SsnA activity. The bpDNase I (1 mg/mL) positive control created a zone of DNA degradation on all media. Furthermore, the SsnA deficient mutant did not produce extracellular nuclease activity on any of the media tested.

6.4.2 Sugar fermentation and acid inhibition

Following the DNase test agar experiments with repressing sugars, nuclease activity was assessed quantitatively using the FRET nuclease assay (see Materials and Methods 2.8.7). This assay allows extracellular deoxyribonuclease activity to be quantified in cell or supernatant fractions. *Staphylococcus aureus* FH7 and *S. gordonii* DL1 were grown in broth culture for 20 h in BHY medium or BHY supplemented with glucose, maltose, fructose, galactose or inulin. Thereafter, cells and spent media were separated and nuclease activity was measured. *Staphylococcus aureus* FH7 was used as a positive control for extracellular deoxyribonuclease production, as it was shown previously to produce a DNase in Chapter 4. Lower extracellular DNase activity was measured in *S.*

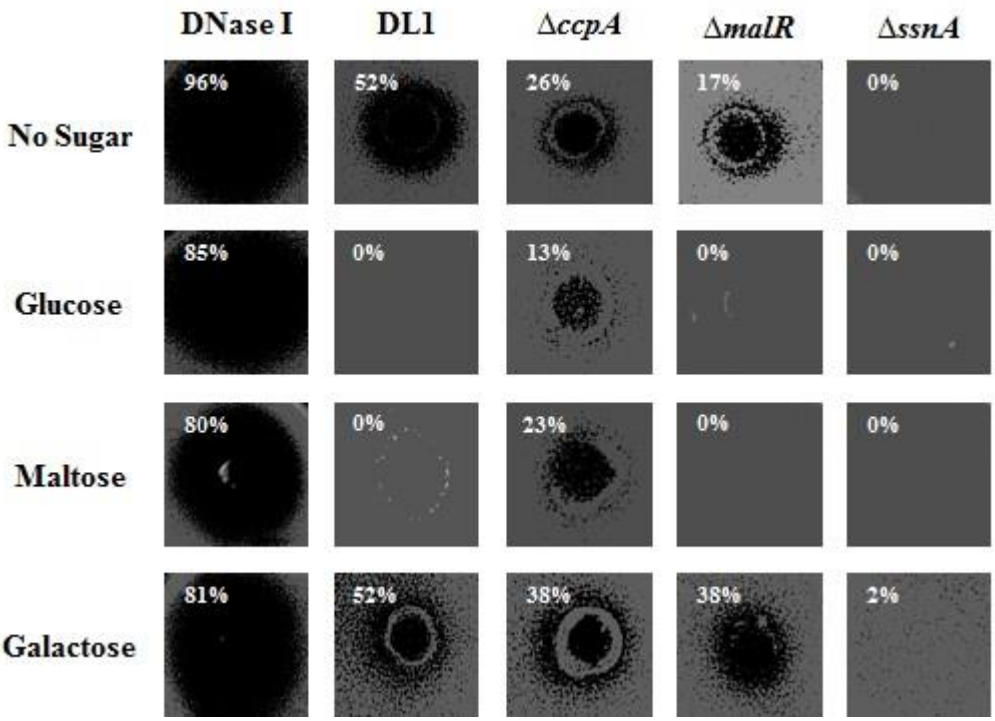

Figure 6.5 Nuclease expression by *S. gordonii*, and mutants, on DNase Test agars containing repressing and a non-repressing sugar. Nuclease activity was screened by culturing micro-organisms on DNase Test agar for 48 h, and then flooding the plates with 1N HCl to precipitate DNA. Sugars were added to a concentration of 2%, with glucose and maltose being repressing sugars, galactose is non-repressing. Black areas are zones of DNA degradation, whereas grey is an area where dsDNA is still present. The percentage area of DNA digestion was calculated per 5112 pixel (area) box (364.81 mm^2) by converting to binary and then measuring the percentage of black and white pixels. Image analysis was performed with ImageJ. *Streptococcus gordonii* Δ*ssnA* was included as a negative control; DNase I (1 mg/mL) was included as a positive control.

gordonii DL1 cell and supernatant fractions across all 5 sugars tested in comparison to BHY medium containing no sugar (0.2 % glucose) (Figure 6.6). This included the repressing sugars glucose, fructose and maltose, and non-repressing sugars, galactose and inulin. There was up to a 4-fold decrease in cell-bound nuclease activity, and a 40-fold decrease in non-cell-bound nuclease activity, although in absolute terms the biggest decrease was in cell-bound DNase activity. Similar decreases in extracellular deoxyribonuclease activity was shown in *S. aureus* FH7 (produces a nuclease which is not cell-bound) across all sugars except for inulin, where activity was only reduced by 2-fold (Figure 6.6). The decrease in extracellular nuclease activity in *S. gordonii* DL1 in the presence of either repressing or non-repressing sugars suggested that sugar fermentation, and subsequent acid production, may be responsible for the lack of SsnA activity. There was a 50 % decrease in *S. aureus* nuclease activity in response to inulin supplementation, which was less than with other carbon sources. It may be that *S. aureus* inefficiently ferments this sugar, producing less acid, although this requires further study. No significant nuclease activity was measured for the SsnA deficient mutant in any of the media.

To test the acidification of growth media in response to sugar fermentation the pH of each medium was measured after overnight culture (Figure 6.7A). The pH of BHY (no sugar) after *S. gordonii* growth was 5.6. BHY-based media containing sugars all had greatly reduced pH (ranging from pH 4 to 4.6) in comparison to standard BHY. To further assess the role of low pH in modulating SsnA activity, *Streptococcus gordonii* DL1 was cultured in BHY acidified to pH 5.5 (Figure 6.7B). As expected, extracellular DNase activity was greatly inhibited (8-fold reduction) in the presence of acid (Figure 6.7B). The pH of acidified BHY, after overnight culture, was 4.2, which was similar to BHY media that had sugars present.

The dynamics of SsnA inhibition in response to acidic conditions were further analysed by placing *S. gordonii* DL1 cells, grown in BHY (0.2 % glucose) overnight, in low pH buffers. Initial extracellular DNase activity was measured by placing cells in PBS (pH 7.1), from here, cells were split and placed into a pH 5.5 and a pH 4.5 buffer. Nuclease activity was quantified over the next 4 h. There was a gradual decline in extracellular DNase activity in the PBS buffer, after 4 h DNase activity was 76% of the starting value (Figure 6.7C). Cell-bound nuclease activity was moderately decreased (to 56% of the original) when cells were placed in the pH 5.5 buffer for 2 h. Extracellular DNase activity was only 2% of the starting activity after 2 h in the pH 4.5 buffer (Figure 6.7C). Clearly, between pH 5.5 and 4.5 there was a rapid inhibition of SsnA enzyme

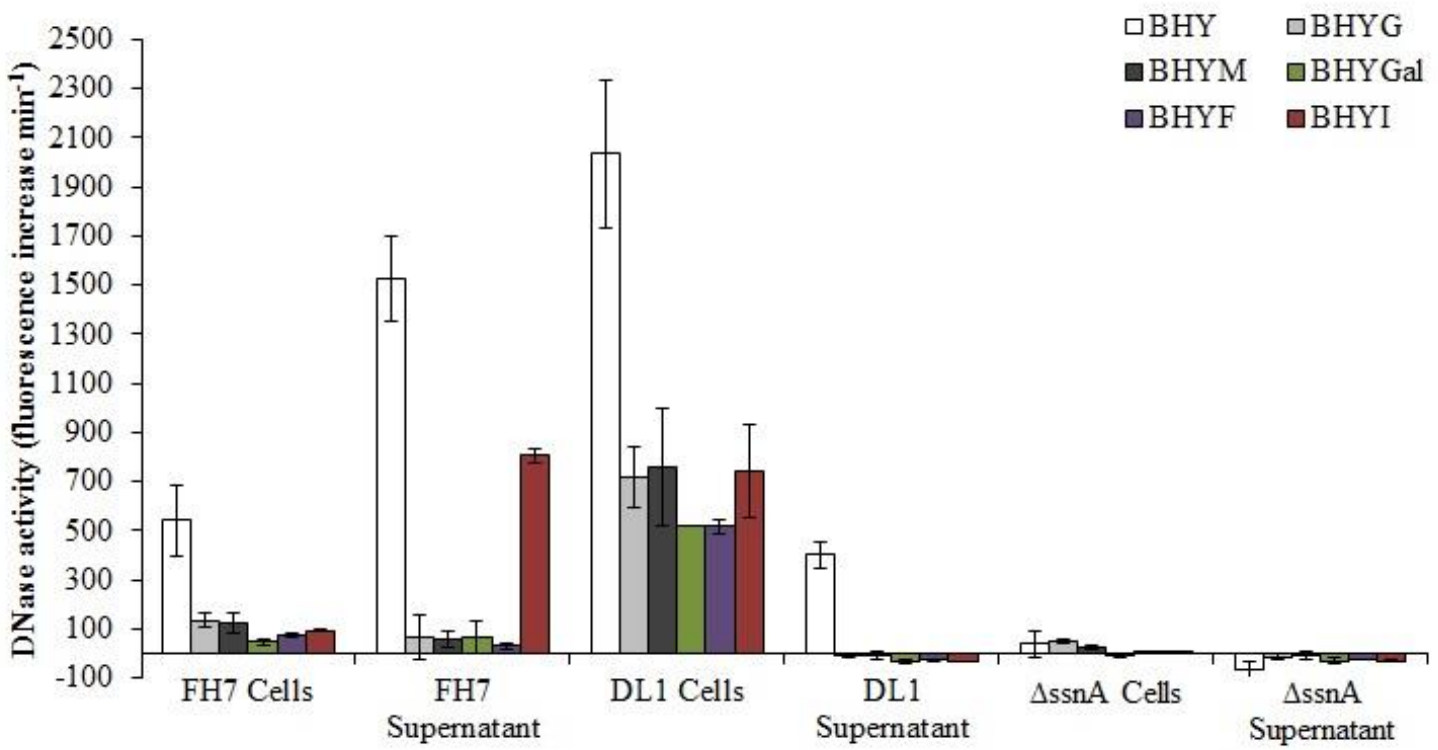

Figure 6.6 Förster resonance energy transfer assay of *S. gordonii* DNase activity in the presence of sugars. Bacterial strains were cultured overnight in BHY (+ 2% sugar), aerobically, at 37°C. Planktonic cultures were split into cell and supernatant fractions and the extracellular nuclease activity of both was quantified. *Staphylococcus aureus* FH7 nuclease activity was assayed as a positive control, and to understand if DNase activity in this species is regulated in a similar manner to *S. gordonii*. *Streptococcus gordonii* Δ*ssnA* was included as a negative control. BHYG (glucose), BHYM (maltose), BHYGal (galactose), BHYF (fructose), and BHYI (inulin). Standard error from the mean (3 replicates) is indicated by the error bars.

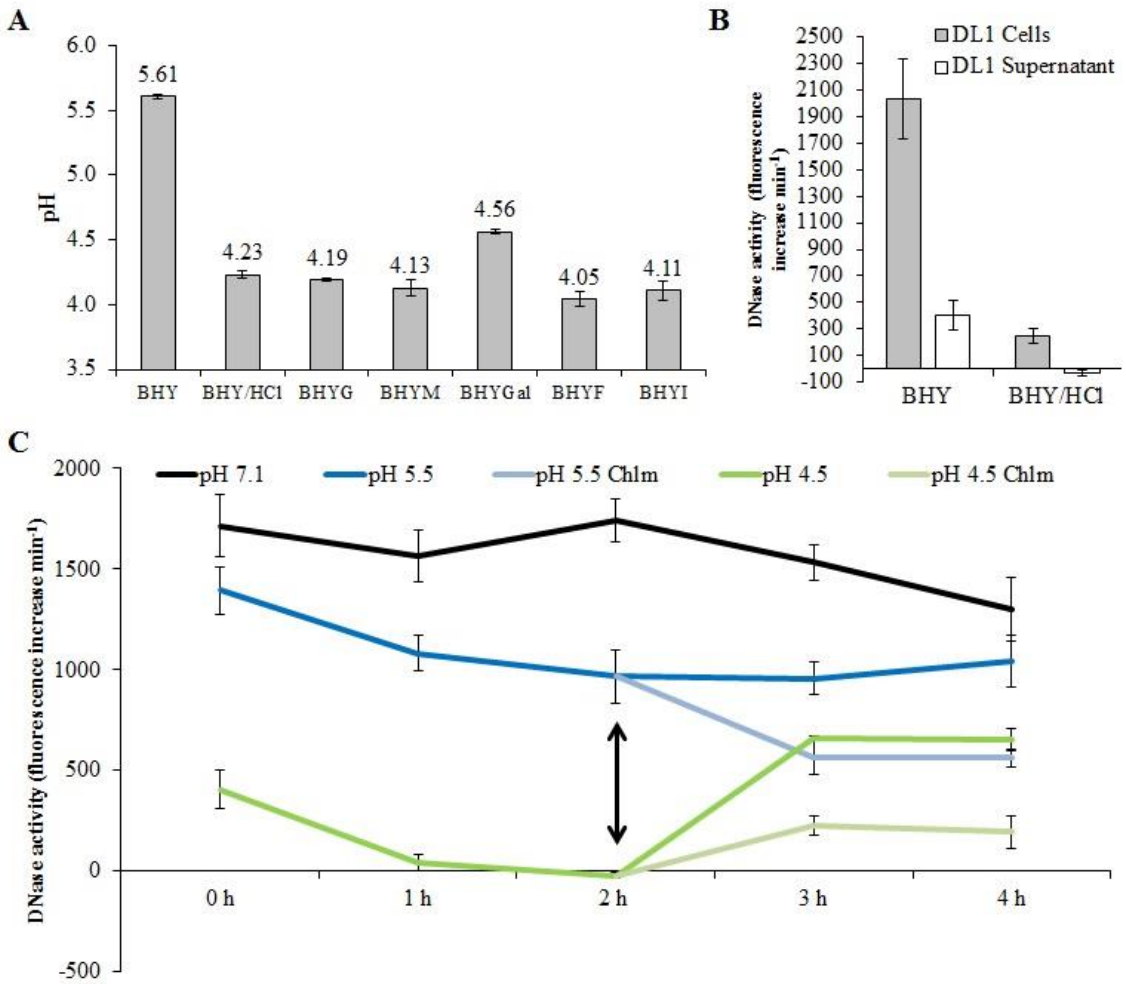

Figure 6.7 Inhibition of *S. gordonii* SsnA in response to acid. (A) The pH of each conditioned medium (20 h planktonic growth) was quantified. BHY/HCl is an acidified (pH 5.5) medium. (B) *Streptococcus gordonii* DL1 was cultured overnight in acidified BHY and SsnA activity was quantified using the FRET assay. (C) Cell fractions of overnight cultured *S. gordonii* DL1 were suspended in a neutral buffer (PBS) (pH 7.1), and two acidic buffers (pH 5.5 or pH 4.5). Cells were incubated for 2 h, and activity was measured at intervals. After 2 h the cells in acidic buffers were re-suspended in PBS for the remaining 2 h of the assay (double black arrow). Once returned to PBS, the cells were split in half and chloramphenicol (chlm) (12.5 μg/mL) was included to inhibit protein synthesis in one fraction. Nuclease activity was assayed for a further two h. Standard error from the mean (3 replicates) is indicated by the error bars.

activity. After 2 h, cells were moved from the pH 4.5 and 5.5 buffers to PBS, to analyse the ability of SsnA to regain activity. To account for new protein synthesis cells were also placed in PBS plus chloramphenicol. There was a 2-fold increase in SsnA activity, compared to cells with inhibited protein synthesis, when cells were moved from pH 5.5 to pH 7.1 (Figure 6.7C). Furthermore, SsnA activity was increased 3-fold when cells were placed in PBS from the pH 4.5 buffer (Figure 6.7C). These results indicate that SsnA activity is irreversibly inhibited by the presence of acid but can be restored by *de novo* protein synthesis.

6.5 GST-SsnA protein expression and purification

To further study the role of SsnA in *S. mutans* antagonism, and confirm the DNase activity of this protein, the *S. gordonii ssnA* gene was fused to a Glutathione S-transferase (GST) tag in an *Escherichia coli* protein expression system (Nick Jakubovics, unpublished data). Using this system, GST-tagged proteins are expressed in *E. coli* cytoplasm in high amounts. To optimise protein production, *Escherichia coli* was cultured in Luria Bertani broth, Terrific broth or YT-G and growth rates and GST-SsnA expression were monitored. Ultimately, *E. coli* grown in YT-G were found to produce the highest amounts of GST-SsnA, and therefore this media was used thereafter (see Materials and Methods 2.8.4). Figure 6.8A shows how protein expression changed over 4 h, compared to an un-induced control. GST-SsnA was subsequently purified using affinity chromatography (Figure 6.8A). GST-tagged proteins have a high binding affinity for glutathione (GSH coated) resins. Cell lysates were run through a GSH matrix after which free reduced GSH was added. Free reduced GSH competitively displaces immobilized GST-tagged SsnA, allowing capture of the protein. A diagram of this process is included in Appendix E. After this step up to 300 μg of >80% pure protein was available for further applications.

Purified GST-SsnA was exchanged from the elution buffer to TNC buffer using Amicon (Merck Millipore) centrifugal filters. TNC buffer is a reaction solution that allows optimal thrombin cleavage of the GST tag from the recombinant protein. Thrombin cleavage was optimised using a variety of incubation periods and temperatures. Cleavage of the GST tag was found to be optimal when incubated for 72 h, at room temperature (25°C), using 1 U of thrombin per 50 μg of GST-SsnA. The GST tag (26 kDa), SsnA (85 kDa), and GST-SsnA (111 kDa) were visualised on a 12% SDS-PAGE gel after thrombin cleavage (Figure 6.8B).

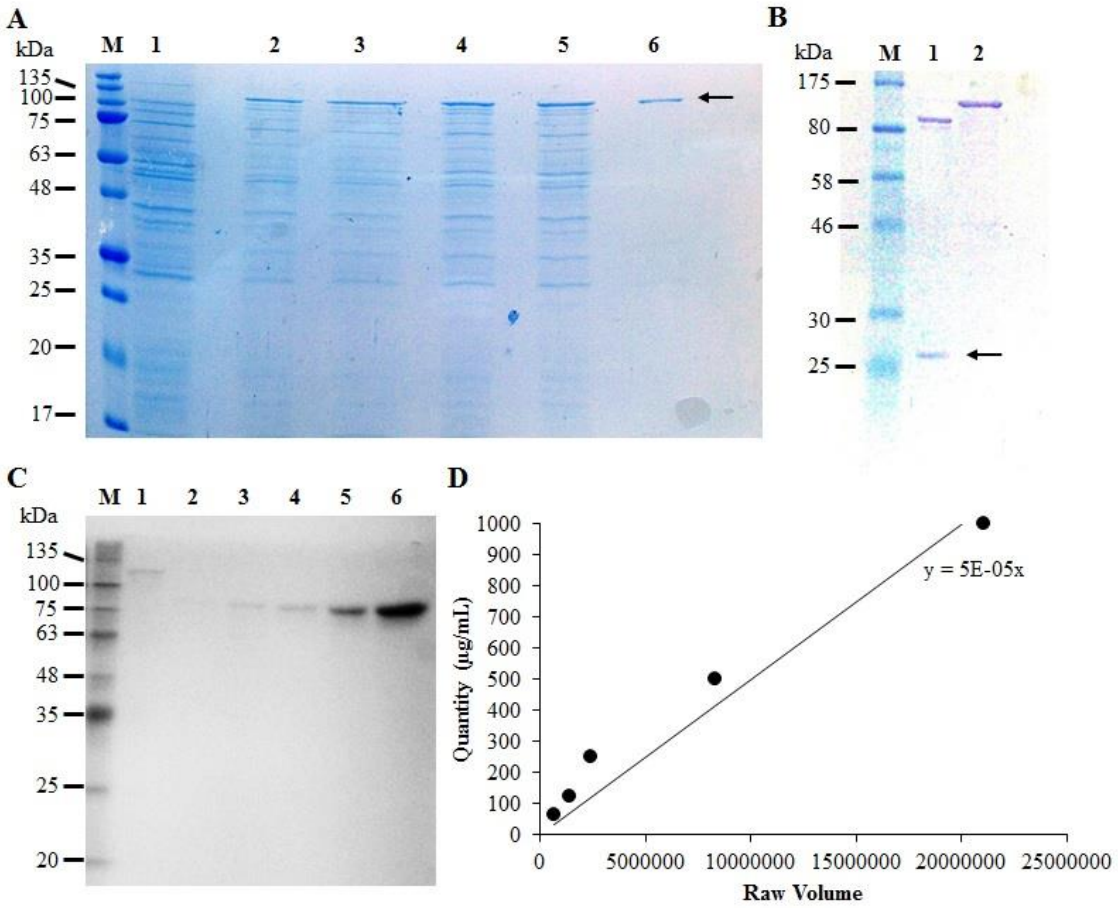

Figure 6.8 *Streptococcus gordonii* GST-SsnA protein expression and purification. (A) 12% SDS-PAGE detection of induced GST-SsnA expression in *E. coli*. Lanes: M) protein molecular weight marker; 1) cell culture without IPTG induction; 2) 1 h IPTG induction; 3) 2 h IPTG induction; 4) 3 h IPTG induction; 5) 4 h IPTG induction; 6) eluted fraction after glutathione affinity chromatography. GST-SsnA is indicated by the black arrow (111 kDa). (B) Thombin cleavage of the GST-tag from SsnA. Lanes: M) protein molecular weight marker; 1) SsnA (thrombin treated) (90 kDa) and GST (26 kDa) (black arrow); 2) GST-SsnA (111 kDa). (C) Protein concentration determination using BSA standards and densitometry after SDS-PAGE. Lanes: M) protein molecular weight marker; 1) GST-SsnA; 2) 62.5 µg/mL BSA; 3) 125 µg/mL BSA; 4) 250 µg/mL BSA; 5) 500 µg/mL BSA; 6) 1000 µg/mL BSA. (D) Graph of BSA standards after densitometry analysis using SynGene GeneTools. Line of best fit through the origin, with equation displayed in upper right corner.

GST-SsnA was excised from a 12% SDS-PAGE gel, digested by trypsin, and analysed by peptide mass fingerprinting (PMF). The mass fingerprinting of the digested peptides identified the protein as the putative DNase, SsnA (see Appendix F). Also identified by PMF was the GST tag.

To quantify the amount of GST-SsnA produced by this system the protein was run on a 12% SDS-PAGE gel with bovine serum albumin (BSA) standards. This method was chosen over the Bradford assay as it is more sensitive, and more appropriate for a partially purified protein. GST-SsnA was exchanged from the elution buffer to a DNase buffer by Slide-A-Lyzer® dialysis cassettes (Thermo Scientific). This method was chosen as the cellulose membrane has low binding characteristics. Initial attempts at buffer exchange with the Amicon system led to marked loss of protein, and therefore the Slide-A-Lyzer® system was used instead. Once placed in the DNase buffer, 10 μL were run alongside BSA standards (2-fold dilution from 1000 to 63 μg/mL) on a 12% SDS-PAGE gel (Figure 6.8C). The dilution series was not linear, which was likely due to the experimental technique used. Protein concentration was estimated using SynGene GeneTools densitometry software (Figure 6.8D). Although yields of GST-SsnA protein in cell lysates were circa 300 μg/mL, the exchange between buffers often led to a loss of protein. Final concentrations were approximately 100 μg/mL, with a final yield of 500 μL.

6.6 Zymography of SsnA

After purification of the enzyme, the activity against dsDNA was assessed using *in gel* zymography, an electrophoretic technique that provides a substrate for hydrolytic enzymes in a SDS-PAGE gel (see Materials and Methods 2.8.5). In this case salmon sperm DNA was included in the SDS-PAGE gel, as a substrate for the DNases. Samples of GST-SsnA, SsnA, and bpDNase I, were prepared in a non-reducing buffer without boiling, were run through a SDS-PAGE gel and were re-activated post-electrophoresis. DNA degradation was then visualised by staining the SDS-PAGE gel with ethidium bromide and pictured using ultraviolet light. Areas of DNA digestion appear as dark bands against the brightly stained background (ethidium bromide intercalating with dsDNA). DNA digestion was shown for both GST-SsnA and SsnA, and was comparable with bpDNase I activity when similar amounts were loaded (Figure 6.9A). Thrombin cleavage did not fully remove all GST tags from SsnA therefore leaving

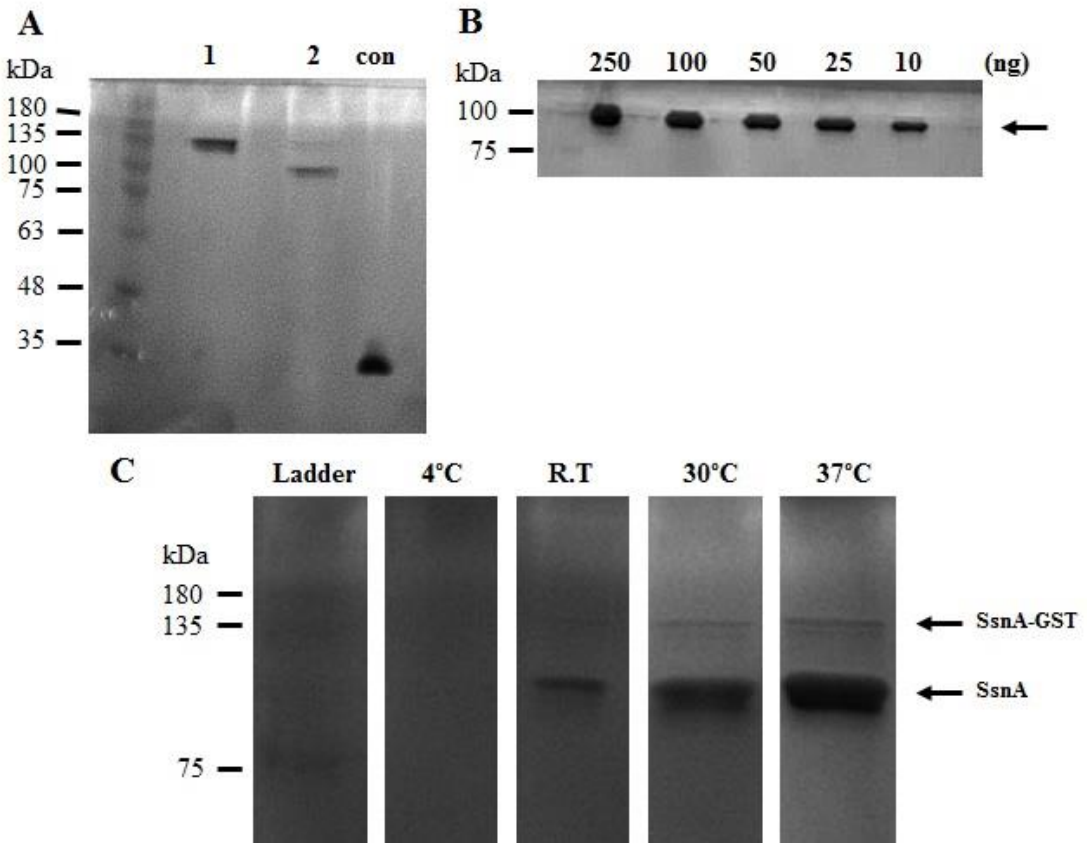

Figure 6.9 *in gel* zymography of GST-SsnA DNase activity. (A) GST-SsnA (1) (111 kDa), SsnA (2) (90 kDa), and DNase I (con) (29 kDa) were ran on a 12% SDS-PAGE gel containing dsDNA. Extracellular nucleases were reactivated and gels were stained with ethidium bromide. Images were visualised with an UV-light source (Syngene, G:Box) and dark zones indicate areas of DNA digestion. (B) SsnA (90 kDa) (black arrow) was loaded on a zymogram in amounts ranging from 250 to 10 ng. (C) During reactivation of SsnA activity SDS-PAGE gels were incubated at 4°C, room temperature (25°C), 30°C, and 37°C. Some residual GST-SsnA activity was present, likely due to incomplete thrombin cleavage of all GST-SsnA protein.

behind some DNase activity at a higher molecular weight (111 kDa- GST-SsnA) in the *in gel* zymograms. SsnA enzyme was visualised on a zymogram in amounts as low as 10 ng (Figure 6.9B). Lastly, SsnA was reactivated inbuffer incubated at 4°C, room temperature (25°C), 30°C, and 37°C to compare DNA degradation activity across these temperatures (Figure 6.9C). DNA degradation was greatest following incubation at 37°C, and the least at 4°C. As the temperature decreased, less DNase activity was present. This result suggests that the optimal temperature for SsnA activity is 37°C, of the temperatures tested. However, reactivation of the enzyme depends on the removal of SDS from the SDS-PAGE gel, and it is unknown how this changes in response to temperature. This may account for some of the decreased activity at lower temperatures.

6.7 Activity of GST-SsnA against *S. mutans*

It was shown in Section 6.3 that *S. mutans* GS-5 biofilms are dispersed by *S. gordonii* DL1 cells and not by an *ssnA* mutant. To test the effects of SsnA on *S. mutans* GS-5 biofilms directly, the partially purified GST-SsnA was added to pre-grown biofilms. GST-SsnA was applied as it has similar DNase activity to SsnA (Figure 6.9A) and can be produced in greater quantities. *Streptococcus mutans* GS-5 biofilms were grown anaerobically (37°C), for 20 h, and treated with 5 μg/mL GST-SsnA for 1 hour at 37°C. The biofilm biomass of *S. mutans* GS-5 was significantly decreased by >90% when GST-SsnA was present (Figure 6.10). This was compared to a control where only PBS was included with pre-formed biofilms. bpDNase I also removed up to 90% of *S. mutans* GS-5 biofilm, as shown in Chapter 5. The activity of GST-SsnA was also tested against pre-formed biofilms of *S. gordonii* DL1 and *S. gordonii* Δ*ssnA*. Neither of these strains was significantly dispersed. This corresponds with previous data that showed *S. gordonii* was not susceptible to bpDNase I treatment.

6.8 Discussion

Streptococcus gordonii is a constituent of dental plaque and is an initial colonizer of the tooth surface. Dental plaque is formed via a series of aggregations between several different species of bacteria, and *S. gordonii* is involved in many different co-aggregation interactions. Since *S. gordonii* co-localises with other bacteria in dental plaque, the enzymes produced by *S. gordonii* could profoundly shape the species

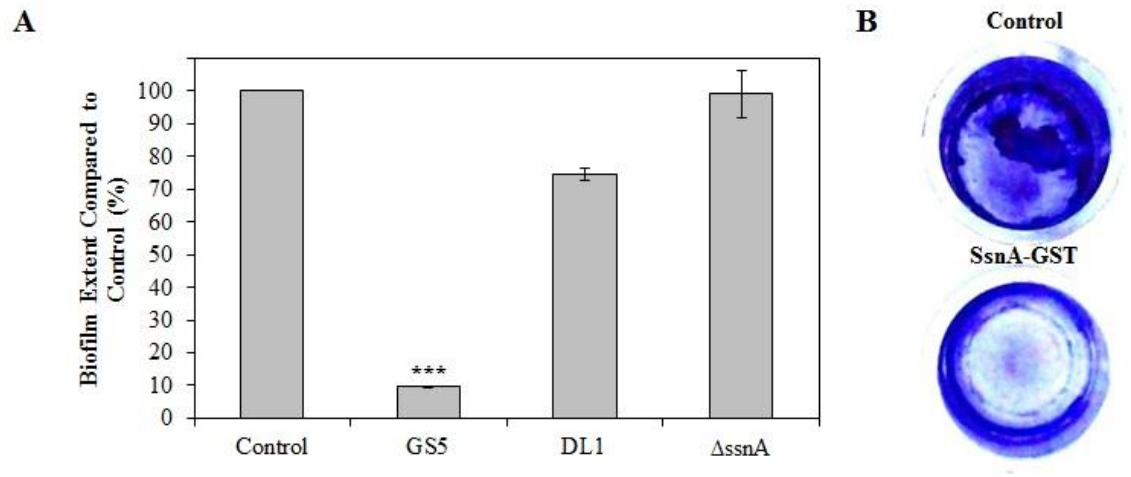

Figure 6.10 Anti-biofilm activity of GST-SsnA. Biofilms of *S. mutans* GS-5, *S. gordonii* DL1, and *S. gordonii* Δ*ssnA* were grown in 96-well microtiter plates overnight, at 37°C, in aerobic conditions. After 20 h biofilms were incubated in the presence of GST-SsnA (5 µg/mL) or PBS only. (A) Biofilm dispersal was calculated as the percentage biomass remaining after the addition of GST-SsnA, compared to the PBS treated control. Standard error from the mean (3 replicates) is indicated by the error bars. (B) *Streptococcus mutans* GS-5 biofilm dispersal was visualised by staining with crystal violet. Statistical significance was calculated with the two sample *t*-test.

composition of dental plaque. We identified an extracellular nuclease, SsnA, produced by *S. gordonii*. The aim of this study was to characterize SsnA in terms of its anti-biofilm activity against the cariogenic bacterium S. mutans and its regulation in response to carbon sources.

Several *Streptococcus* spp. produce excreted deoxyribonucleases (Brown, 1950; Smith and Bodily, 1967; Palmer *et al.*, 2012). DNases produced by streptococci that have been studied in detail include: *Streptococcus suis* SsnA (Fontaine *et al.*, 2004; de Buhr *et al.*, 2013), *Streptococcus pyogenes* SpnA (Hasegawa *et al.*, 2010), *Streptococcus pneumoniae* EndA (Beiter *et al.*, 2006), and *Streptococcus agalactiae* NucA (Derré-Bobillot *et al.*, 2013). Both *S. suis* SsnA and *S. pyogenes* SpnA are homologues of the extracellular nuclease of *S. gordonii*. These proteins, like *S. gordonii* SsnA, contain a Gram positive LPxTG cell wall anchor domain, and are predominantly cell-surface located. When analysed with the FRET assay extracellular nuclease activity was highest in the cell fraction of *S. gordonii* cells, suggesting that SsnA is also cell-bound. There was nuclease activity in the supernatant fraction, which is suggestive of some cell-bound SsnA becoming unattached, possibly during cell wall synthesis. This was in contrast to the SsnA-deficient mutant that produced no nuclease activity in the supernatant fraction, therefore ruling out the presence of intracellular DNase enzymes in wild-type supernatant. *Staphylococcus aureus*, produced higher DNase activity in its conditioned media. This bacterium produces at least two extracellular DNases, one cell wall located (Nuc2), and the other unbound (Nuc). Interestingly, both SpnA and *S. suis* SsnA contain three OB-fold domains. These small structural motifs may be important for nucleic acid binding (Theobald *et al.*, 2003). However, *S. gordonii* SsnA is predicted to have one OB-fold domain. Chang *et al*., (2011) created truncated versions of SpnA that contained all, two or no OB-fold domains. At least two OB-fold domains were required for DNase activity as shown by *in gel* zymography. It is unknown whether *S. gordonii* SsnA requires the OB-fold to bind DNA, or why these three homologues differ in this regard.

The biological role proposed for the extracellular deoxyribonucleases produced by streptococci is that they are linked to virulence, although other functions are possible. The virulence hypothesis exists because of the finding that neutrophil extracellular traps (NETs) can engulf, and kill bacteria, through use of eDNA (Brinkmann *et al.*, 2004). Therefore, bacteria produce extracellular nucleases to escape NETs by degrading NET eDNA. Group A *Streptococcus* (Sumby *et al.*, 2005; Buchanan *et al.*, 2006), *S. suis* (de Buhr *et al.*, 2013), *S. agalactiae* (Derré-Bobillot *et al.*, 2013), and *S. pneumoniae*

(Beiter *et al.*, 2006) produce one or several DNases that have been shown to have anti-NET activity. Extracellular DNases may also facilitate the spreading of Group A *Streptococcus* through lung mucus that contains high levels of eDNA (Bisno *et al.*, 2003). However, *S. gordonii* is not viewed as pathogenic in the oral cavity, so the role of the extracellular DNase in this organism is unclear. Extracellular DNase enzymes can also regulate DNA uptake in species such as *Vibrio cholerae*, by degrading DNA and therefore reducing uptake of DNA (Blokesch and Schoolnik, 2008). However, there was no difference in transformation efficiency between wild-type *S. gordonii* and the *ssnA*-deficient strain (David Taylor, unpublished work). It is also plausible that SsnA degrades eDNA that may have a deleterious effect on *S. gordonii*, for instance inhibiting biofilm accumulation (Berne *et al.*, 2010), or causing cell lysis via increased concentrations of cations (Mulcahy *et al.*, 2008). However, we propose that *S. gordonii* SsnA has a biofilm reducing effect on oral bacteria such as *S. mutans*, that require eDNA for biofilm stabilisation.

Gene deletion of *ssnA* demonstrated that this was the gene responsible for extracellular nuclease activity in *S. gordonii* DL1. Nuclease-deficient cells of *S. gordonii* were incapable of reducing *S. mutans* biofilm extent, whereas the parental strain of *S. gordonii* reduced colonisation. Although this reduction was less than bpDNase I, it demonstrates that *S. gordonii* SsnA may have a role in influencing biofilm species composition. Interestingly, both strains of *S. gordonii* produced supernatants that had an anti-biofilm effect against *S. mutans*. *Streptococcus gordonii*, cultured in the presence of oxygen, has been shown to have a growth limiting effect on *S. mutans* through the production of H_2O_2 (Kreth *et al.*, 2008). Although H_2O_2 wasn't measured it may account for the biofilm reduction caused by both strain supernatants, as they were grown aerobically. Cell fractions were washed in PBS and so it is unlikely that there were significant levels of residual H_2O_2.

Purified, recombinant SsnA has deoxyribonuclease activity and disperses biofilms formed by *S. mutans*. The amount of biofilm dispersion is similar to that seen with bpDNase I and NucB (Chapter 5). Wild-type *S. gordonii* cells and partially purified SsnA was shown to disperse *S. mutans* biofilms. However, the extent of dispersal was lower with *S. gordonii* cells than with GST-SsnA. Naturally SsnA is a cell-membrane-associated enzyme, which may explain the lower biofilm dispersal. *Streptococcus gordonii* conditioned media also contains nuclease activity, so both may contribute to *S. mutans* dispersal *in vivo*. Interestingly, SsnA does not have a significant effect against *S. gordonii* biofilms. This suggests that *S. gordonii* does not produce this enzyme to

actively degrade eDNA and disseminate biofilm cells in the experimental conditions used. It is possible that a small percentage of *S. gordonii* cells are susceptible to host-produced SsnA and that this is missed by the crystal violet assay. *Staphylococcus aureus* produces an extracellular nuclease that has been shown to reduce host biofilm extent *in vitro* (Kiedrowski *et al.*, 2011) as has *Neisseria gonorrhoeae* Nuc (Steichen *et al.*, 2011). However, *S. gordonii* biofilm matrices do not contain eDNA in great quantities in the models used during these studies (Chapter 5). It is unknown if eDNA contributes to stability in other growth conditions and models. Therefore this hypothesis requires further research.

Inactivation of the *ccpA* gene validated its role in regulating *ssnA* expression in response to carbon sources like glucose. CcpA has been shown to be essential for carbohydrate catabolite repression in *S. gordonii* (Dong *et al.*, 2004). Interestingly, loss of CcpA function reduces interspecies signalling interactions, via amylase, between *S. gordonii* and *Veillonella atypica* (Johnson *et al.*, 2009). CcpA has also been linked to penicillin tolerance in *S. gordonii* (Bizzini *et al.*, 2007). Clearly, the global regulator CcpA has a wide control on gene expression in low GC Gram positive bacteria. For example, up to 300 genes are regulated by CcpA in *Bacillus subtilis* (Moreno *et al.*, 2004). The primary function of CcpA is related to sequential utilization of carbon sources. The extracellular DNases of *Shewanella* spp. and *Helicobacter pylori* utilize eDNA as a source of nutrients, such as carbon, phosphorus, or purines (Pinchuk *et al.*, 2008; Heun *et al.*, 2012; Liechti and Goldberg, 2013). Therefore, the finding that CcpA regulates SsnA suggests that this DNase may be required for eDNA digestion, and when preferred carbon sources, like glucose, are present, *ssnA* expression is reduced. Recently, the cell bound nuclease of *S. suis*, SsnA, which shares homology (44%) to *S. gordonii* SsnA, was found to be down- regulated by CcpA during the stationary phase (Willenborg *et al.*, 2014). The majority of genes influenced by CcpA in *S. suis* are related to carbon metabolism, however, it is unknown if *S. gordonii* can survive on eDNA as a sole carbon source. This is an important aspect of SsnA production that needs further exploration, with the use of defined culture media and the *ssnA*-deficient mutant. If glucose is present in the oral cavity, and *ssnA* is down-regulated, this may have an indirect effect on *S. mutans* colonisation. Furthermore, acid production during sugar fermentation has been shown to reduce SsnA activity, therefore providing another reason for *S. gordonii* to not produce this enzyme when carbon sources are present. It is unknown if the *ssnA* gene is down-regulated in these conditions and this requires further study.

Although gene regulation in response to preferred carbon sources is an important consideration in SsnA anti-biofilm dynamics, it appears that acid-mediated inhibition of SsnA might have a larger impact. Lactic acid bacteria, like *S. gordonii*, are lactic-acid producing during carbohydrate fermentation (Platt and Foster, 1958; Axelsson, 2004). Planktonic culture in five sugars (glucose, maltose, galactose, fructose and inulin) led to media that were 1 pH unit lower than un-supplemented BHY media. Further analysis demonstrated that SsnA was rapidly inhibited in low pH environments. Interestingly, *S. aureus* nuclease activity was also lost in response to carbohydrate fermentation. Lower extracellular nuclease activity was demonstrated in the presence of glucose previously for *S. aureus*, although this was not linked to acid production (Kiedrowski *et al.*, 2011; Beenken *et al.*, 2012). The catalytic properties of SsnA are unknown but DNase enzymes work optimally over a wide-range of pH, from 4.2 with *Aspergillus* nuclease S1 (Vogt, 1973), to 9-10 with *S. aureus* nuclease (Cuatrecasas *et al.*, 1967). SsnA has a hypothetical binding site for Mg^{2+} and it may be that lactic acid is acting as a metal chelator, thereby reducing DNase activity. *Streptococcus pyogenes* SpnA, an SsnA homologue, requires Mg^{2+} and Ca^{2+} for optimal activity (Chang *et al.*, 2011). Organic acids are metal chelators, and can be used in heavy metal detoxification of soils (Wasay *et al.*, 2001). However, it should be noted that DNase activity was irreversibly lost in acid, and was only restored by protein synthesis.

Sugar fermentation and acid production is of relevance to the potential interaction between *S. gordonii* SsnA and *S. mutans* biofilm formation. *Streptococcus mutans* is recognised as the primary microbiological cause of dental caries (tooth decay) (van Houte, 1994). Although the shift to a cariogenic (acid-producing/acid-tolerating) oral biofilm is complex there is an increase in basal levels of cariogenic micro-organisms such as *S. mutans* and *Streptococcus sobrinus* in response to dietary sucrose. Therefore, carbon sources have a direct impact on the species composition of dental plaque. *Streptococcus mutans* biofilm formation can be dependent on eDNA (Chapter 5; Perry *et al.*, 2009). Furthermore, SsnA has a dispersal effect on *S. mutans* GS-5 biofilms. However, the inhibition of SsnA by organic acids produced during sugar metabolism may add an extra dimension to the shift to a cariogenic biofilm. *Streptococcus mutans* is strongly acidogenic and therefore may inhibit SsnA activity, resulting in less anti-biofilm effect against *S. mutans*. Interestingly, glucose reduces *S. gordonii* H_2O_2-mediated inhibition of *S. mutans* (Kreth *et al.*, 2008) and sucrose increases *S. mutans* bacteriocin production (Wang and Kuramitsu, 2005). Carbon sources may have a major role in the interaction between *S. gordonii* and *S. mutans*.

It appears that extracellular DNases are a conserved trait of many, but not all, streptococci and therefore these enzymes likely have an important role in the biology of this genus of micro-organisms. The studies presented here demonstrate that *S. gordonii* SsnA is an active DNase responsible for essentially all the extracellular DNase activity under the conditions tested. Further, SsnA is regulated by carbon source availability, CcpA, and inhibited by acidic pH. The most significant finding is the SsnA-mediated dispersal of *S. mutans*. *Streptococcus gordonii* SsnA may have an impact on *S. mutans* colonisation. However, acid production by *S. mutans* may be a defence response since acid inhibits SsnA. Future research should focus on the interaction between these two species, although it will be challenging to reproduce this interspecies antagonism *in vitro*.

Chapter 7: Final Perspectives

Staphylococcus aureus was found to produce extracellular DNA over 50 years ago (Catlin and Cunningham, 1958). However, this early description was not extensively followed up until research by Whitchurch *et al*., (2002) demonstrated the key role eDNA plays in facilitating initial biofilm formation in *Pseudomonas aeruginosa*. The last decade of eDNA research has created a paradigm, whereby eDNA is important for biofilm formation and holding mature biofilms together. Furthermore, exogenous and host-produced deoxyribonucleases have been found to have a key effect on some biofilm-forming micro-organisms. Therefore, the start of this PhD thesis, in November 2010 was placed in a key moment during the expansion of eDNA knowledge. The research contained within this thesis provides novel studies into biofilm matrix eDNA in three important biofilm systems. Lastly, applying exogenous DNase enzymes to biofilms has been investigated as a potential therapeutic approach across the biofilm systems studied.

7.1 Summary of chapters

The thesis began with a preliminary investigation into the role of eDNA in maintaining biofilm stability in TESV biofilms (Chapter 3). TESV biofouling is an issue that costs the Freeman Hospital, Newcastle (NHS) £44,000 a year in new valves (Shakir *et al.*, 2012). Valves have an average lifespan of as little as 108 days, due in part to biofilm colonisation (Kress *et al.*, 2013). Microscopic observations were consistent with previous research characterising TESV biofilms as large aggregations of fungal and bacterial micro-organisms. By adding a further fluorescent stain, DAPI, to the CLSM technique used by Kania *et al*., (2010), it was possible to visualise eDNA in the biofilm matrix of TESV biofilms. These types of microscopic observations do not always provide conclusive evidence for eDNA. However, it was also possible to quantify eDNA and visualise it by agarose gel electrophoresis. This was the first example of eDNA within the biofilm matrix of TESV biofilms, and one of few investigations describing eDNA in mixed-species biofilms. Lastly, exogenous NucB was used as an anti-biofilm enzyme. This approach showed promise at removing TESV biofilm-forming micro-organisms from the surface of TESVs (Shakir *et al.*, 2012). Several micro-organisms associated with TESV biofouling have been shown to be susceptible to DNase treatment, including: *Candida albicans* (Martins *et al.*, 2010), *Lactobacillus*

plantarum (Muscariello *et al.*, 2013), and Staphylococcus spp. (Qin *et al.*, 2007; Kaplan *et al.*, 2012). This investigation showed the clear role of eDNA in maintaining TESV biofilm matrix stability.

Again, working in collaboration with the ENT department of the Freeman Hospital, Newcastle, the second results chapter of this thesis (Chapter 4) looked in great detail at the role of eDNA in maintaining the biofilm stability of CRS isolates. Chronic rhinosinusitis is a complex disease that affects up to 10% of the adult European population (Hastan *et al.*, 2011). The pathogenesis of the disease is unclear but there is evidence that suggests biofilms are a major factor in the development of the disease (Foreman *et al.*, 2012). Therefore, this provided a strong rationale for examining the disease further, with the major objective of observing the potential of NucB as an anti-biofilm enzyme in CRS. There was a large clinical microbiology element to this investigation, with 75 bacterial strains being isolated from 22 patients on four different agar types. Bacterial strains were similar to those previously observed, using culture-dependent and culture-independent techniques (Brook, 2006; Araujo *et al.*, 2007; Feazel *et al.*, 2012). The microbial population was dominated by *S. aureus*, coagulase-negative staphylococci and α-haemolytic streptococci. Bacteria were isolated from 'obstructive mucin', a thick mucus-like material that is found in some CRS patient sinuses. This mucin was visualised in greater detail using TEM, whilst sinus mucosa was visualised with CLSM. These experiments suggested that 'obstructive mucin' was not colonised by microbial biofilms but that sinus mucosa was. The sinus mucosa biofilms were similar to those observed by Sanderson *et al*., (2006). Twenty-four CRS isolates were shown to produce microbial biofilms, with over half (14) being significantly dispersed by the NucB enzyme. Furthermore, 15 of the isolates contained eDNA in their biofilm matrices that was detectable by agarose gel electrophoresis. It was therefore shown, that NucB has potential in controlling biofilms formed by CRS isolates (Shields *et al.*, 2013).

Chapter 5 of this thesis examined the biofilm adhesin role of eDNA in the oral biofilm, with a clear aim of expanding on the previous two chapters results. Four important oral bacteria, *Streptococcus gordonii*, *Actinomyces oris*, *Streptococcus mutans* and *Fusobacterium nucleatum* were examined. Of these four micro-organisms only *S. gordonii* was found to not be susceptible to DNase I treatment. This micro-organism was also a poor producer of eDNA, although other authors have measured eDNA produced in significant quantifies by this bacteria (Kreth *et al.*, 2009; Itzek *et al.*, 2011; Liu and Burne, 2011). Previously, *F. nucleatum* had been shown to not be

susceptible to DNase I (Ali Mohammed *et al.*, 2013). Interestingly, four *S. mutans* strains all differed in their susceptibility to NucB. This research provided further evidence for strain differences in eDNA importance, a feature that was noted in CRS isolates. A major part of Chapter 5 was the 6 week research visit to the University of Michigan, MI, USA in 2013. Whilst working in Dr. Alexander Rickard's laboratory a microfluidic biofilm modelling technique was learnt. This technique provided 3D images of *S. gordonii*, *S. mutans*, and mixed-species saliva biofilms. Furthermore, the expertise gained during the collaboration may mean a similar system is employed in Dr. Jakubovics's laboratory soon.

The last results chapter characterised the cell-wall-bound extracellular nuclease of *S. gordonii*. Extracellular DNase activity appears to be a conserved trait of streptococci. This research had a molecular biological focus that added many techniques to the clinical and applied skills learned earlier in the thesis. Bioinformatic analysis showed that *S. gordonii* SsnA shared most homology with a 733-aa endonuclease produced by *Streptococcus anginosus*. Deletion of the *ssnA* gene removed the extracellular nuclease activity of *S. gordonii*, both on DNase test agar and in a FRET DNase assay. The SsnA enzyme was found to be regulated both by carbon sources and lactic acid production during sugar fermentation. Interestingly, *S. gordonii* SsnA may have an impact on *S. mutans* colonisation in the oral cavity as both *S. gordonii* cells and purified GST-SsnA significantly dispersed *S. mutans* GS-5 biofilms. However, *S. mutans* is strongly acidogenic and therefore this may disrupt SsnA activity. DNases produced by streptococci that have been studied in detail include: *Streptococcus suis* SsnA (Fontaine *et al.*, 2004), *Streptococcus pyogenes* SpnA (Hasegawa *et al.*, 2010), *Streptococcus pneumoniae* EndA (Beiter *et al.*, 2006), and *Streptococcus agalacitae* NucA (Derré-Bobillot *et al.*, 2013). The novel aspect of this investigation was the finding that a streptococcal extracellular deoxyribonuclease can disperse biofilms.

7.2 Thesis summary and impact

Each chapter of the thesis contributes to a greater understanding of the role of eDNA in maintaining biofilm stability. There have been many previous studies investigating eDNA and DNase dispersal but few have looked at chronic diseases and particularly mixed-species biofilms. The majority of research has examined eDNA production in model micro-organisms like *Staphylococcus aureus* (Mann *et al.*, 2009) and *Pseudomonas aeruginosa* (Whitchurch *et al.*, 2002). Furthermore, research based on

microbial extracellular DNases is often focussed on neutrophil extracellular trap (NET) degradation, not biofilm competition, although attention is moving towards this area. Therefore, this thesis has included novel investigations grounded on a paradigm that has existed for at least a decade. Interesting findings include the strain differences in eDNA importance, the overwhelming number of micro-organisms tested that are dispersed or inhibited by DNase enzymes and the high concentration of eDNA in most biofilm matrices tested. The research adds further weight to the theory that eDNA is an important component of microbial biofilm matrices.

Biofilms from three different areas were studied, the oral biofilm, biofilms in sinuses and biofilms that form on TESVs. These microbial systems all form on different substratum, from silicone with TESVs, to the hard tissue surface of a tooth (supragingival plaque), and lastly mucosal soft tissue in the sinuses. Biofilm-forming species were found to require eDNA for attachment in all these systems during investigations, implying that bacteria require eDNA during initial attachment across many surfaces. Bacterial carriage is also moderately different across these three environments, although there are commensal micro-organisms that are found in all. Chronic rhinosinusitis is dominated by staphylococci, the early oral biofilm by *Streptococcus* spp., and TESV biofouling is often caused by *Candida* spp. Again, although the microflora differed, micro-organisms from all three systems were susceptible to DNase treatment.

Streptococci can be isolated from sinuses, the oral environment and TESVs. There was variability in the efficacy of DNase enzymes versus *Streptococcus* spp. and strains. Other species that showed strain variability of DNase sensitivity included *S. aureus* and *S. mutans*. Recently, Grande *et al.*, (2014) also linked DNase efficacy to strain differences. In this case *S. aureus* UAMS-1 was not dispersed or inhibited by Pulmozyme, although biofilm morphology was altered. This strain of *S. aureus* produces two extracellular nucleases that reduce biofilm formation *in vitro* (Beenken *et al.*, 2012). Deoxyribonuclease activity may be altered due to many factors, including growth conditions, production of inhibitors, variance in eDNA structure, altered production of eDNA, variable efficacy of DNase enzymes against biofilm eDNA, and substratum used to culture biofilms. Clearly further investigations are required but this thesis has added further evidence to the variable nature of eDNA production and DNase efficacy amongst strains of the same species of micro-organism.

Importantly, mixed-species biofilms were studied during this thesis. These included TESV biofilms, oral saliva microcosms, and microfluidic modelling of oral biofilms.

Several biofilms of TESV biofouling and saliva microcosms were susceptible to DNase treatment. In particular, it is noteworthy that TESV biofilms that have existed for up to 2-3 months contain eDNA and are dispersed by NucB treatment. This suggests that eDNA is preserved in some biofilm systems for long periods of time, and is still exerting an influence on biofilm stability. Although eDNA has been shown to have importance in activated sludge biofilms (Dominiak *et al.*, 2011) it is poorly understood how eDNA use changes over time during biofilm formation. For the most part it has been suggested that eDNA exerts its largest role on biofilm formation during initial attachment of cells but our data clearly indicate a role for eDNA in mature biofilms.

Mixed-species biofilm research can be harder to interpret and have increased variability over single-species biofilms but it is important to demonstrate DNase potential in biofilms that more closely resemble those found in disease. With that in mind, oral biofilms were modelled using flowing human saliva in a microfluidics model. Contrasting static biofilms, with the BioFlux device it is clear that by using flowing nutrients biofilms can grow thicker, have a higher number of live cells (due to the removal of deleterious metabolites), and exhibit different colony morphology. It would be interesting to study these biofilms in more detail, in particular by treating with DNase enzymes.

The research contained within this thesis has had an impact on a wider audience through public engagement events and media attention (see Appendix C). In particular the use of an enzyme from a ‘seaweed bacterium’ has proved an accessible idea to those without a strong scientific background. The three biofilms studied all have a great impact on many people. Chronic rhinosinusitis affects 10% of the adult European population (Hastan *et al.*, 2011), TESV use, although rare, vastly increases the standard of living for users, and dental plaque affects anyone with teeth. Therefore this thesis contains research that everyone could have an interest in, and hopefully will positively impact society as a whole. I have enjoyed discussing it with friends, colleagues and complete strangers!

7.3 Research techniques

Many experimental techniques were used and designed during the course of the thesis. For example, to determine the anti-biofilm effect against TESV biofouling an experiment was designed to adequately measure biofilm dispersal. This has since been used by a further two students in Dr. Jakubovics’s laboratory (Edward Mason and

Syatirah Abdullah). The *in gel* zymography technique to visualise deoxyribonuclease DNA degradation has been shared with two laboratory groups (Prof. Grant Burgess and Dr. Paul Cooper). Microscopic analysis of TESV biofilms using a triple fluorescent stain CLSM approach (adapting Kania *et al*., (2010) technique) has been applied with great success by Sufian Al-Sammarraie to study mixed-species biofilm formation on denture acrylics. The adaptation of the FRET nuclease activity assay (Kiedrowski *et al.*, 2011; Beenken *et al.*, 2012) to work in Dr. Jakubovics's laboratory has also proved a success, with students' now testing nuclease activity in a variety of conditions (Richard Holliday and Syatirah Abdullah). Lastly, a microfluidics biofilm culture system (Cellix Ltd.), similar to the BioFlux (Fluxion) system used in Dr. Rickard's laboratory was recently tested in Dr. Jakubovics's laboratory by Jill Robinson using the knowledge gained whilst in the United States. It took well over a year to visualise bacteria on the surface of sinus mucosa, and the development of a PNA-FISH CLSM technique to do so was another challenge during the thesis research. Having started the PhD as a marine biologist all microbiological, clinical, and molecular biological research techniques were acquired 'on the job'.

7.4 Future research

The microflora of CRS patient sinuses, and briefly that of TESVs were studied during this thesis through the use of culture-dependent methods. It is potentially of value to further investigate the microbial populations of these two environments by applying culture-independent techniques. During the CRS investigation a sample of obstructive mucin from each patient was collected and frozen at -80°C so that it may be possible to study the microflora of these samples using a culture-independent technique, such as pyrosequencing of the 16S bacterial rRNA gene. However, there was not enough time to complete this study in full. Culture-independent analyses have been conducted previously in both CRS patients (Stressmann *et al.*, 2011; Feazel *et al.*, 2012; Boase *et al.*, 2013) and for TESVs (Buijssen *et al.*, 2012).

Whilst the research gathered during this thesis has undoubtedly furthered eDNA knowledge, it is important to consider future work, and the limitations of the study. In keeping with the majority of eDNA biofilm research, this work depended heavily on the high-throughput 96-well microtiter assay. However, it is unlikely that these types of biofilms closely resemble the biofilms observed in CRS patients or in the oral cavity. Therefore, biofilm modelling has to be improved before any *in vivo* testing can be

performed. It would be interesting to ascertain the efficacy of DNase enzymes in a more realistic system. For CRS biofilms, it would be important to incorporate host epithelial cells or a 3-dimensional tissue model. Oral biofilms may be more realistically modelled in a flowing system such as the microfluidic biofilm model (Nance *et al.*, 2013). Recently, eDNA has been found to be an important component of *Staphylococcus epidermidis* and *Candida albicans* biofilms grown in a subcutaneous catheter mouse model (Pammi *et al.*, 2013). Furthermore, DNase I has dispersed *Bordetella* spp. nasal biofilms in a mouse model (Conover *et al.*, 2011). These examples show the potential of deoxyribonucleases in treating biofilm diseases but investigations of this type should be expanded upon.

A key clinical issue is whether DNase enzymes can increase the efficacy of antibiotics against biofilms cells. There is some evidence that this might be the case for particular biofilm systems (Tetz *et al.*, 2009; Kaplan *et al.*, 2012). This would be an interesting area to expand the CRS research, by including antibiotics during NucB anti-biofilm assays. Theses assays could be performed using the Calgary Biofilm Device, which is designed to test antimicrobial efficacy against microbial biofilms (Ceri *et al.*, 1999). If deoxyribonuclease enzymes, like NucB, are to be used in a clinical setting it is important to prove their efficacy in treatment solutions. For TESV cleaning there are no major concerns as treatment solutions usually contain salts at low concentrations. However, DNase efficacy in oral hygiene products, that can contain chemicals like SDS, fluoride, and EDTA, is unknown. Work in the lab is currently underway to assess the compatibility of DNase enzymes with common constituents of oral hygiene products. It will be important to find a formulation that permits maximum activity of NucB if it is to prove useful at diminishing oral biofilms.

A potentially interesting topic for further investigation is the question of why different strains of the same species exhibit differences in eDNA production and in the reliance for eDNA to stabilise biofilms. Analysing the genomes of different strains, and comparing them, may allow the identification of important genes in regulating eDNA production. *Streptococcus mutans* would be an obvious target for this approach, since significant differences in DNase sensitivity were identified between several different *S. mutans* strains. The accumulation of eDNA also appears to be tightly regulated, and depends on factors such as growth phase, and nutrient availability. Further identification of the pathways that lead to the release of eDNA will help to understand how this molecule enters the biofilm matrix. This could be achieved by screening for eDNA defective mutants of a particular species by culturing in a high-throughput 96-well

model, and assaying for eDNA content with the PicoGreen stain. Staining eDNA in the microplate is a high-throughput technique that has been used by Rikke Meyer's laboratory (unpublished work). Possible eDNA defective mutants would be created through transposon mutagenesis. After discovering gene mutations that are responsible for lower eDNA content it would then be possible to target gene deletions in wild type species and analyse biofilm formation again. Ultimately, it may be possible to establish the regulators responsible for producing eDNA, and potential relationships between a number of regulators. Species that could be studied in this way could be *S. mutans*, *C. albicans* or *S. aureus*, as these micro-organisms all have a major impact on healthcare. Working with one species would be the aim, as this greatly simplifies research. This work could be expanded by using relevant animal models to analyse biofilm formation *in vivo*. Understanding the basic mechanism of eDNA release will make it easier to target this component of biofilm matrices with DNase enzymes or with novel compounds that target the DNA release step.

It is also interesting that *S. gordonii* produces a deoxyribonuclease that disperses *S. mutans* biofilms. Competition between the two species could change the oral biofilm significantly. However, this theory needs further investigation as there are many factors that could influence any interaction. This hypothesis could be analysed by culturing the two species together in a dual-species biofilm and determining the effect of acid production on *S. mutans* colonisation (inhibitory effect on SsnA activity). Biofilm extent by each species in different growth media could be measured both by quantitative PCR, and colony forming units on culture media. The dual-species biofilms would be cultured in sugar sources (plus minimal sugar), and SsnA activity measured by FRET, with the pH of the system also quantified. However, it would be important to consider the strain variability of DNase sensitivity in *S. mutans*.

Purely from an evolutionary prospective, it seems futile for an organism to devote extensive resources to create the molecule of hereditary, DNA, only to expel it into the environment. Clearly eDNA is an important aspect of microbial development, or its expulsion would not have evolved. It appears that we are only at the start of understanding the full dynamics of eDNA and DNase production.

7.5 Final conclusion

To conclude, the research in this thesis has made novel contributions to the characterisation of eDNA as a biofilm maintaining compound. Furthermore,

extracellular deoxyribonuclease enzymes have been shown to have powerful anti-biofilm activity across a wide range of micro-organisms in the head and neck. Microbial biofilms are an extremely important factor in the pathogenesis of many diseases and a greater understanding of them will allow us to eradicate them more easily. Additionally, DNase enzymes could potentially be developed for the treatment of many different chronic diseases with a biofilm pathogenesis.

Appendix A: Relevant Publications

Three papers were published during the thesis and are inserted below.

AMERICAN ACADEMY OF OTOLARYNGOLOGY–HEAD AND NECK SURGERY FOUNDATION

Otolaryngology–Head and Neck Surgery XX(X) 1–6

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http://otojournal.org
SAGE

Removal of Biofilms from Tracheoesophageal Speech Valves Using a Novel Marine Microbial Deoxyribonuclease

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Sponsorships or competing interests that may be relevant to content are disclosed at the end of this article.

Abstract

Objective. The growth of biofilms on tracheoesophageal speech valves shortens their life span and produces a reservoir of pathogens that may infect the respiratory tract. The authors have discovered a novel nontoxic deoxyribonuclease, NucB, from a marine isolate of Bacillus licheniformis that is effective at dispersing a variety of mono and mixed-species bacterial biofilms. The aim of this preliminary study was to determine whether NucB could also disrupt and remove mixed-species biofilms from tracheoesophageal speech valves.

Study Design. Laboratory-based treatment and analysis of discarded tracheoesophageal speech valves.

Setting. University human biology laboratory and the Department of Speech and Language Therapy at a tertiary referral hospital.

Subjects and Methods. Seventeen ex vivo tracheoesophageal speech valves fouled with natural human biofilms were collected and divided into 2 equal parts. One half was treated with NucB and the other half with a control buffer solution. Biofilm removal was measured by microscopy and by culture of dispersed biofilm organisms on agar plates.

Results. Significantly more organisms were released from biofilms using NucB than with buffer solution alone. On nonselective medium, more organisms were cultured in 11 samples (65%, n = 17, P > .05). Using growth media favoring fungi, more organisms were cultured in 14 samples (82%, n = 17, P >.05).

Conclusion. The nontoxic deoxyribonuclease NucB was effective in releasing more microorganisms from biofilms on tracheoesophageal speech valves. This reflects its potential ability to break up and disperse these biofilms. Future studies will aim to develop NucB as a novel agent to prolong the life span of tracheoesophageal speech valves, thus reducing health care costs.

Keywords

prosthesis-related infections, microbiology, larynx artificial, laryngectomy, larynx microbiology, biofilms, prosthesis fitting, prosthesis adverse effects, laryngectomy rehabilitation, valve

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A tracheoesophageal speech valve (TESV) contains a 1-way valve that diverts the air in the trachea into the throat and mouth to enable phonation with the tongue and lips, without leakage of the saliva or food from the neopharynx into the airway. These valves are placed in a tracheoesophageal fistula that is surgically created to allow the passage of air into the upper esophagus and, subsequently, the oropharynx and oral cavity. In situ, silicone TESVs rapidly become colonized with bacteria and fungi, some of which are opportunistic pathogens.[1] Soiling of the airway and respiratory tree leads to increased episodes of

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tracheal aspiration and tracheal irritation, leading to coughing.[2] Another symptom that heralds the end of a TESV life is increased patient effort required to generate phonation, and subsequently speech becomes very disturbed.[3] Furthermore, TESV failure impairs communication between the patient and his or her environment.[4]

The degradation of TESVs also leads to increased health care costs as valves have to be changed more frequently depending on the rate of colonization in a particular individual. In the Department of Otolaryngology, Freeman Hospital, Newcastle upon Tyne, UK, there were 64 patients on our records in 2010 who required TESVs. In the period from January 2010 to December 2010, 413 TESVs were used at a cost of $68,500 (excluding taxes and staff costs).

Cleaning of TESVs is particularly difficult because the micro-organisms that colonize them form biofilms.[1] Standard current advice given to patients with regard to cleaning their TESV is to immerse it in warm water or to use baby bottle cleaning agents and gently mechanically clean the valve with a cotton bud or cleaning brush often supplied with the TESV. Some TESVs can be removed and cleaned intermittently. However, even frequent cleaning does not prolong the life of the device beyond approximately 3 to 5 months.[5] At present, there are no mechanisms to clean permanently indwelling TESVs, and these devices are generally replaced every 2 to 3 months.

We have recently identified a novel biofilm-degrading enzyme, NucB, from the marine bacterium Bacillus licheniformis EI-34-6.[6] In vitro, NucB could disperse biofilms produced by a different strain of Bacillus (B licheniformis DSM13) and by a range of medically relevant gram-positive and gram-negative organisms. NucB is a deoxyribonuclease specifically produced by B licheniformis when growing in biofilms. NucB targets extracellular DNA (eDNA), an important component of the biofilm matrix.

Treatment of young Pseudomonas aeruginosa biofilms with a commercially available nuclease, DNase I, resulted in dispersal of cells from the surfaces.[7] It was also noticed that DNase I was more effective at releasing early P aeruginosa biofilms than at clearing established biofilms from surfaces.[7] Therefore, the assessment of dispersed microbes using colony forming units (CFUs) could be an indirect method of assessing the effect of nuclease in dispersing at least the newly formed biofilms. Similarly, addition of DNase I to planktonic P aeruginosa cultures retarded the de novo formation of new biofilm.[7]

Bacillus licheniformis NucB is smaller than DNase I and potentially may migrate through biofilms more efficiently. Concordantly, our preliminary in vitro studies indicate that NucB is more efficient than DNase I at removing biofilm.[6]

The aim of this preliminary study was to investigate the potential of the NucB enzyme to disperse natural mixed-species biofilms from TESVs. In the long term, the use of a nuclease in cleaning TESVs may make cleaning easier and prolong the lifetime of these devices.

Materials and Methods

Study Design

This study was an anonymized primary research into the efficacy of NucB nuclease in a laboratory setting, with a case controlled design. We obtained the approval of the local National Health Service (NHS) Trust Research and Development (R&D) Department to conduct this research, and it was confirmed that ethical approval was not required. The study was anonymous and did not involve any sampling of human tissue. No specific patient subgroup among TESV users was selected; valves were obtained on a ''next-available'' basis over a 4-month period.

Specimens

Ex vivo TESVs (Blom-Singer, Forth Medical Ltd, Newbury, Berkshire, United Kingdom) that were due to be discarded were obtained from the hospital's Speech and Language Therapy Department (Freeman Hospital, Newcastle Upon Tyne) and immediately placed in phosphate buffer solution (PBS) and stored at 4 C for up to 3 days before analysis.

NucB Treatment of TESVs

Valves were divided into 2 equal parts along their longitudinal axes using a sterile scalpel (Swann-Morton, Sheffield, UK). There were 2 treatment arms. The active treatment used our agent, NucB DNase (3 mg/mL), in PBS at pH 7.3. The control treatment used PBS alone. Sterile polystyrene 12-well microtiter plates (Greiner Bio-One, Monroe, North Carolina) were used for the treatment of TESV halves. The half valves were each washed 3 times with sterile PBS and subsequently placed for 1 hour at 20 C either in a treatment well containing 500 mL NucB solution (3 mg/mL)[6] and 2.5 mL sterile PBS solution or in a control well containing 3.0 mL sterile PBS.

Triplicate samples of the supernatant were diluted, and 50-mL aliquots were spread onto nutrient agar (Oxoid, Basingstoke, UK) or Sabouraud-dextrose agar (Oxoid). Plates were incubated for 16 to 20 hours at 37 C under aerobic conditions. Nutrient agar was chosen as a nonselective medium. Sabouraud-dextrose selects for fungi, including Candida spp. Following incubation of plates for 24 hours, colonies 2 mm were counted, and total colony-forming units (CFUs) released were calculated. Only plates with 10 to 400 colonies were counted. Representative colonies were picked, and strains were isolated by subculturing 3 times of solidified media. Isolates were analyzed by gram staining and microscopy. This method of detecting biofilm change was chosen over direct microscopy of valves before and after treatment, as the fixative process for scanning electron microscopy (SEM) destroys bacteria and alters the biofilm appearance by dehydration and the biofilm flora within.[8] In addition, SEM can only demonstrate qualitatively the surface of the TESV or the biofilm upon it,[9] making it hard to standardize the data. It is very difficult to locate 2 extremely similar biofilms, with reference to their

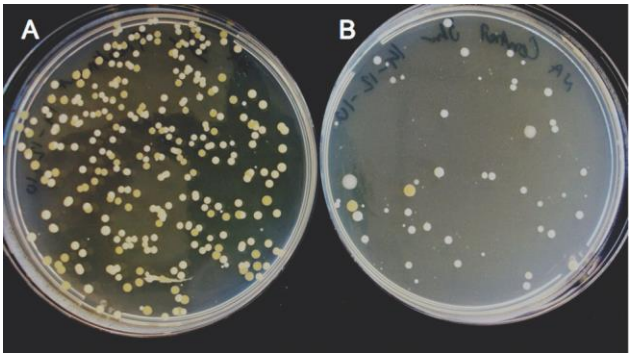

Figure 1. Post 16-hour, 37 C incubation of nutrient agar plates. (A) Culture of the tracheoesophageal speech valve (TESV) bath containing NucB. (B) Control only.

thickness, amount of DNA present, and species composition, on the same valve.

Statistical Analysis

Statistical analysis was performed on each TESV, comparing the triplicates from each half of the valve. Means derived from treatment and control CFU triplicate plate counts for each TESV half were used for the ratio of treatment and control values, standard error calculation, and x^2 comparison testing. From this, P values and statistical significance ($P > .05$) were derived.

Results

As this was a laboratory study, no patients were affected. Twenty TESVs were obtained. The results of 3 TESVs were excluded from our study; 1 TESV culture solution did not produce any CFUs, and 2 TESV treatment and control solutions produced too many colonies to be counted accurately and were excluded. The results of the remaining 17 TESV culture plates were used for analysis.

Figure 1 represents the colonies grown from the supernatant from a valve. Half of the valve was treated with NucB nuclease and the other half with PBS alone. The NucB treatment clearly released more viable micro-organisms than PBS alone. These plates demonstrate that our novel NucB nuclease liberated more micro-organisms from TESV biofilms compared with the control treatment. This suggests that NucB nuclease has the potential to disrupt TESV biofilms. We hypothesize that the mechanism of action is by the degradation of eDNA, which holds the biofilm together, in the biofilm matrix on the TESV surface.

From the CFU data, it was possible to determine which treatment generated higher CFU counts per valve. Table 1 shows which treatment produced significantly higher CFU counts in all included valves. Higher CFU counts result from more micro-organisms being liberated from TESV surfaces. The active treatment with NucB generated higher CFU counts on both nutrient and Sabouraud agar, indicating that more micro-organisms and fungi, in particular, were released by treatment with NucB. Table 2 shows the data from the 17 valves included in the study, confirming that more organisms were cultured in 11 samples (65%) on non-selective nutrient agar plates. The P values for each valve are included. Table 3 shows the use of Sabouraud agar plates, favoring fungi, from all the included valves. It demonstrates that more organisms were cultured in 14 samples (82%). Treatment of TESVs with NucB released 5.7-fold more CFUs vs control (standard error [SE], 3.7-7.7) on selective Sabouraud agar and 5.0-fold more CFUs vs control on nonselective nutrient agar (SE, 3.1-6.9), as shown in Figure 2.

Each valve yielded a variety of bacteria and fungi, and preliminary analysis using gram staining indicated the presence of gram-positive cocci and gram-negative rods. Tests identifying particular strains were not performed. Colony and organism morphology suggested a high prevalence of Candida spp. This is consistent with current literature.[10,11]

Discussion

The aim of this study was to determine the ability of an eDNA degrading nuclease NucB to disrupt and remove mixed-organism biofilms from TESVs. The application of NucB to fouled TESVs resulted in significantly higher numbers of micro-organisms being released from the TESV surfaces compared with the control. NucB was effective at releasing organisms from the majority of the 17 TESVs analyzed ($P > .05$ in 11 of 17 nutrient agar plates). The benefits of NucB were similar when Sabouraud-dextrose agar (selective for fungi, $P > .05$ in 14 of 17 plates) was used, illustrating a general applicability of this method on mixed-species biofilms.

It is well known that micro-organisms in biofilms are protected against antimicrobials and other external stresses. For example, the control of Candida albicans biofilm requires approximately 4 times higher concentrations of fluconazole than are needed to eliminate planktonic (free-floating) cells.[12] Long-term treatment with antifungals is undesirable as it encourages selection of resistant strains.[13]

Table 1. Summary of Which Treatment Generated Higher CFU Counts Cultured from Each TESV (n = 17)

	Active (NucB)	Control (Phosphate Buffer Solution)	Insignificant Difference
Nutrient agar	11[a]	1[a]	5
Sabouraud-dextrose agar	14[a]	2[a]	1

Significance level was calculated by comparing the 2 halves of each valve. Abbreviations: CFU, colony-forming unit; TESV, tracheoesophageal speech valve.
[a]$P > .05$.

Table 2. Mean of Triplicate Plate CFU Count Using NucB Treatment and Control on Nutrient Agar

Valve No.	Treatment CFU	Control CFU	P Value
1	10,230	2760	.0001
2	55,740	78,120	.0001
3	2520	600	.0001
4	21,960	21,840	.941
5	2910	90	.0001
6	21,000	12,360	.0001
7	1890	300	.0001
8	1710	1110	.145
10	27,960	24,300	.0387
11	10,080	3930	.0001
12	6420	1470	.0001
14	9690	8850	.426
15	5922	582	.0001
16	28,020	20,760	.0001
17	3820	2640	.058
19	2400	200	.0001
20	15,400	15,040	.79

Note that valves 9, 13, and 18 were excluded. The bold P values show that NucB released more colony-forming units (CFUs), whereas the italic ones reflect valve solutions when the control produced more CFUs.

Table 3. Mean of Triplicate Plate CFU Count Using NucB Treatment and Control on Sabouraud Agar

Valve No.	Treatment CFU	Control CFU	P Value
1	4410	12,120	.0001
2	61,440	93,120	.0001
3	2370	540	.0005
4	39,120	31,800	.0038
5	7470	600	.0001
6	12,360	8880	.0021
7	1380	150	.0001
8	2520	1710	.108
10	14,880	5910	.0001
11	5790	3510	.0023
12	2670	90	.0001
14	7530	4050	.0001
15	6900	480	.0001
16	30,480	21,600	.0001
17	5838	4122	.0264
19	2360	140	.0001
20	15,720	8960	.0001

Note that valves 9, 13, and 18 were excluded. The bold P values show that NucB released more colony-forming units (CFUs), whereas the italic ones reflect valve solutions when the control produced more CFUs.

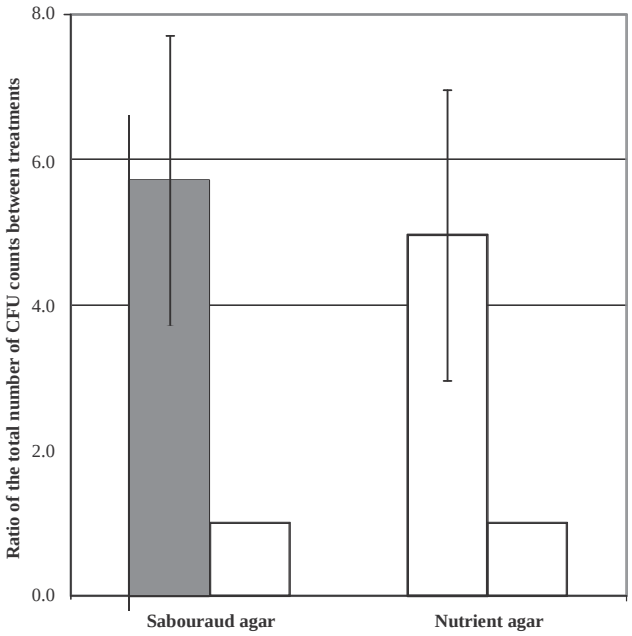

Figure 2. The ratio of micro-organisms released by NucB compared with control. Standard error bars are shown. NucB is in gray and control in white. CFU, colony-forming unit.

Biofilms protect bacteria and fungi from antimicrobial agents by several mechanisms, including reduced penetration of antimicrobial agents into the cell colonies, slow growth of micro-organisms in biofilms,[14] the presence of highly resistant ''persister'' cell types in biofilms,[15,16] and the upregulation of specific antimicrobial genes, such as those encoding efflux pumps[17] in biofilms. A key component of biofilms is the extracellular matrix, which glues micro-organisms to the surface and provides a barrier to mass transfer of antimicrobial agents. There is now evidence that some bacteria in biofilms produce biofilm-degrading enzymes to enable the liberation of bacterial cells to new sites in a host in vivo. These same enzymes have been employed to disperse bacterial biofilms to good effect.[18]

The antibiofilm properties of the B licheniformis NucB enzyme were reported for biofilms formed by a variety of aerobic bacteria in model monospecies cultures.[6] The current work demonstrates that NucB is also effective against natural mixed-species biofilms on TESVs. Of particular note is the observation that Candida spp. were released from biofilms by NucB. Candida spp. are common colonizers of TESV surfaces. In one study, the total Candida spp. cultures constituted more than 50% of organisms grown from TESVs at both the tracheal and esophageal surfaces of valves.[19] It is noteworthy that Candida spp. have previously been shown to employ eDNA to stabilize their biofilms, and treatment with DNase I promotes biofilm removal.[20] The eDNA therefore is an important component of mixed microbial biofilms and is produced in different ways: by cell lysis,[21] autolysis-dependent cell lysis,[22] or active excretion of eDNA from cells, for example, in vesicles.[23] The eDNA is an important factor in the life span of the biofilm, notably as a microbial aggregator[24] or as an important molecule in biofilm stability.[25]

An additional factor influencing the stability of biofilms is the composition of micro-organisms within them. The

presence of Candida spp. has been demonstrated to encourage the attachment of Streptococcus gordonii and the development of mixed-species biofilm communities in the oral cavity.[26] This study has shown a link between the behavior of Candida spp. and bacteria, and similar interactions may also occur on prosthetic TESV surfaces.[10]

The use of biosurfactants to treat TESV biofilms has been investigated; TESV biofilms were treated with a solution extracted from cultured Streptococcus thermophilus, but the active compound was not described.[27] Another study used an active antibiofilm enzyme that was tested by measuring the altered surface tension of water droplets, although not against prosthetic surfaces.[28] The study presented here is the first example of a bacterial nuclease being used to remove multispecies biofilms from TESVs.

This study used the method of micro-organism culture and the counting of CFUs to measure degradation of TESV biofilms as it was a rapid and sensitive method for detecting biofilm breakdown. The results indicated that NucB is an extremely promising agent for the control of biofilms on TESVs. This is an indirect measure of the ability of NucB to disrupt the biofilms. However, a great deal of further work is required to develop this enzyme as a useful adjunct to current cleaning protocols for TESVs. Therefore, further studies will aim to develop treatment protocols that can be used by patients for maintaining biofilm-free TESVs. The future studies will aim at the direct examination of the TESVs to evaluate the effectiveness of NucB in preventing and disrupting biofilms. It is hoped that such treatments will prolong the life span of TESVs and reduce the risks of TESV-related infections.

Microbial degradation of TESVs has been clearly demonstrated by scanning electron microscopy.[9] Scanning electron microscopy has also been employed to study the microbial composition of TESV biofilms and has revealed that biofilms contain mixed populations of yeasts and bacteria.[9,11] Using confocal laser scanning microscopy (CLSM), we detected both bacteria and fungi in mixed-species biofilms on TESVs (data not shown), which is fully consistent with both the SEM studies and the CLSM analyses published by other groups.[10,29] Previous studies have documented reproducible spatial variations within TESV biofilms—for example, Candida spp. may be found deeper and adjacent to the silicone surface.[10]

In summary, this preliminary study has demonstrated that the use of the nuclease enzyme, NucB, from a marine bacterium, can release more microorganisms from biofilms found on TESVs in a statistically significant manner. This suggests its ability to disrupt biofilms on TESVs. This biofilm-degrading enzyme therefore has potential in the treatment and maintenance of TESVs, either as a sole treatment agent or in combination with other substances, to prolong the life span of TESVs.

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Author Contributions

Adam Shakir, literature search, collection of samples, helping in the laboratory work, analysis of data, writing and revising the article; Mohamed Reda ElBadawey, conception and design, literature search, collection of samples, analysis of data, writing and revising the article; Robert Colquhoun Shields, literature search, collection of samples, performing the laboratory work, analysis of data, collection of samples, writing and revising the article; Nicholas Stephen Jakubovics, conception and design, literature search, collection of samples, analysis of data, writing and revising the article; James Grant Burgess, conception and design, literature search, collection of samples, analysis of data, writing and revising the article.

Disclosures

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Efficacy of a Marine Bacterial Nuclease against Biofilm Forming Microorganisms Isolated from Chronic Rhinosinusitis

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Abstract

Background: The persistent colonization of paranasal sinus mucosa by microbial biofilms is a major factor in the pathogenesis of chronic rhinosinusitis (CRS). Control of microorganisms within biofilms is hampered by the presence of viscous extracellular polymers of host or microbial origin, including nucleic acids. The aim of this study was to investigate the role of extracellular DNA in biofilm formation by bacteria associated with CRS.

Methods/Principal Findings: Obstructive mucin was collected from patients during functional endoscopic sinus surgery. Examination of the mucous by transmission electron microscopy revealed an acellular matrix punctuated occasionally with host cells in varying states of degradation. Bacteria were observed in biofilms on mucosal biopsies, and between two and six different species were isolated from each of 20 different patient samples. In total, 16 different bacterial genera were isolated, of which the most commonly identified organisms were coagulase-negative staphylococci, Staphylococcus aureus and a-haemolytic streptococci. Twenty-four fresh clinical isolates were selected for investigation of biofilm formation in vitro using a microplate model system. Biofilms formed by 14 strains, including all 9 extracellular nuclease-producing bacteria, were significantly disrupted by treatment with a novel bacterial deoxyribonuclease, NucB, isolated from a marine strain of Bacillus licheniformis. Extracellular biofilm matrix was observed in untreated samples but not in those treated with NucB and extracellular DNA was purified from in vitro biofilms.

Conclusion/Significance: Our data demonstrate that bacteria associated with CRS form robust biofilms which can be reduced by treatment with matrix-degrading enzymes such as NucB. The dispersal of bacterial biofilms with NucB may offer an additional therapeutic target for CRS sufferers.

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Introduction

Chronic rhinosinusitis (CRS) is one of the most common upper respiratory tract diseases, affecting approximately 10% of the adult European population [1]. Rhinosinusitis is an inflammation of the paranasal sinuses that is almost always accompanied by inflammation of the nasal airway and is classified as 'chronic' if it lasts at least 12 consecutive weeks [2]. The symptoms of CRS include blockage or congestion of the nasal passages, nasal discharge, facial pain or pressure and/or a reduction or loss of sense of smell. The majority of cases of CRS are treated with medical therapy consisting of topical steroids and nasal douching [3]. Antibiotics such as clarithromycin or amoxicillin may be used at the outset of therapy or for acute exacerbations of disease. In cases of CRS that are recalcitrant to medical treatment, surgicaltechniques may be warranted to improve the drainage pathway of the sinuses and to remove polyps and obstructive mucin. Non- invasive surgical interventions, known collectively as functional endoscopic sinus surgery (FESS), are now widely used, although there is limited evidence to support their efficacy [4]. The successful treatment of CRS is hampered by the heterogeneity of the disease. Chronic rhinosinusitis is a spectrum of diseases with a variety of causes or contributing factors including smoking, allergies, underlying systemic diseases, invasive or non-invasive fungal infections, viruses and bacteria [2,5,6]. Increasingly, it is becoming clear that microbial biofilms are associated with many cases of CRS. The growth of microorganisms within biofilms presents unique problems for the management of CRS. Within biofilms, microorganisms are up to 1,000-fold more resistant to

antibiotics than free-living cells of the same species [7]. Increased resistance is due to many factors, including the presence of a viscous polymeric matrix that restricts the penetration of antimicrobials, slow growth of bacteria, resistant phenotypes, and altered chemical microenvironments [8–11]. In keeping with these observations, there is limited evidence that either topical or systemic antibiotics improve the outcome of CRS infections [12,13] underlining the need for new therapeutic approaches.

The role of biofilms in the initiation of CRS and their recalcitrance to treatment has received a great deal of attention over the last few years. A recent review of the literature identified 11 studies reporting the analysis of biofilms on sinus mucosa in CRS patients [14]. Several different techniques were employed to visualise biofilms. Arguably the most convincing method was fluorescence in situ hybridization, since this provides contrast between bacterial DNA and host cells. Biofilms on mucosal surfaces appear as punctate staining (bacterial cells), occasionally with some diffuse coloration, suggestive of extracellular nucleic acids [9]. All studies detected biofilms in a proportion of CRS patients, with prevalence varying from 25% to 100%. In contrast, only three of the eight studies that also analysed non-CRS controls identified biofilms on the non-CRS sinus mucosa, and these involved small numbers of patients. Collectively, these data clearly point to an association between biofilms on sinus mucosa and CRS. In addition, biofilms or biofilm-forming bacteria have been associated with unfavourable outcomes following FESS. For example, the presence of biofilms on paranasal sinus mucosa was correlated with persistent mucosal inflammation and requirements for lengthy post-surgical follow-up periods [15]. In a large cross-sectional study involving 518 CRS patients, the presence of sinus biofilms was significantly correlated with prior experience of sinus surgery, indicating either that biofilms contribute to CRS recurrence, or that FESS may promote the formation of biofilms [16]. Further, the capacity of paranasal sinus isolates of *Staphylococcus aureus* or *Pseudomonas aeruginosa* to form biofilms in vitro has been associated with the recurrence of CRS symptoms in the 12 month period following FESS [17].

There is strong evidence that the microflora of the maxillary sinus changes during acute sinusitis, towards a predominance of *Streptococcus pneumoniae*, *Haemophilus influenzae* and, less frequently, *Moraxella catarrhalis* [18,19]. However, the microbial population present in CRS more closely resembles that in non-inflamed paranasal sinuses. The most common organisms both in CRS and in healthy patients include *Staphylococcus aureus*, coagulase-negative staphylococci, a-haemolytic streptococci, *Corynebacterium* spp. and strict anaerobes such as *Prevotella* spp., *Peptostreptococcus* spp. and *Propionibacterium* spp. [19–22]. Therefore, the development of biofilms within the paranasal sinuses, and the subsequent host responses to biofilms, may be more important for the pathogenesis of CRS than the *de novo* colonization of the upper respiratory tract by specific pathogens.

In light of the resistance of biofilm bacteria to conventional antibiotics, a number of novel approaches for treating biofilms have been proposed, including interfering with chemical communication between micro-organisms or degrading the biofilm matrix with enzymes [23,24]. Biofilms associated with CRS are extremely heterogeneous, with many different organisms playing a role, and therefore any strategy to clear biofilms would need to target a component that is widely utilised by different microorganisms in the biofilm matrix. One possible target that has received significant interest in recent years is extracellular DNA (eDNA). The matrices of a many different bacterial and fungal biofilms contain eDNA, and this molecule has been shown to serve several critical functions including stabilising the biofilm structure [25–28], enhancing initial adhesion to surfaces [29,30], promoting the exchange of genetic information [31], and acting as a nutrient store that can be utilised during nutrient depletion [32]. Recently, we have identified an extracellular bacterial deoxyribonuclease, NucB, from a marine isolate of *Bacillus licheniformis* strain EI-34-6 that can disperse biofilms by degrading eDNA [33]. The exogenous addition of NucB to biofilms formed by *Escherichia coli*, *Bacillus subtilis* or *Micrococcus luteus* resulted in almost complete removal of bacterial cells from surfaces. Hence, NucB has the potential to remove biofilms formed by Gram-positive or Gram-negative bacteria. It is anticipated that the development of new methods for disrupting viscous biofilm matrices will improve the post-surgical outcomes of FESS. In addition, such approaches may facilitate the surgery itself if the obstructive mucin is also targeted. This study therefore aimed to characterise the potential of NucB to disperse biofilms formed by microorganisms associated with CRS.

Materials and Methods

Ethics Statement

Ethical approval for the study was granted by the National Research Ethics Service Committee (North East – Sunderland) and each patient gave informed consent before enrolment.

Collection of Specimens

A total of 20 patients undergoing FESS for the treatment of CRS at the Freeman Hospital, Newcastle upon Tyne, were recruited to this study. All patients met the CRS diagnosis criteria published by the Chronic Rhinosinusitis Task Force [2]. Patients were recruited to the study only if obstructive mucin was observed during the surgical procedure. During FESS, obstructive mucin that was dislodged surgically was collected with mucous traps (Sigma Aldrich) and immediately placed into sterile reduced transport fluid (RTF) [34]. Specimens were transferred to the laboratory and stored at 4 C. All samples were processed within 24 h.

Transmission Electron Microscope Analysis of Obstructive Mucin

Samples of obstructive material removed from patients during FESS were cut into 1 mm^3 pieces and placed into 2% glutaraldehyde immediately after surgery. These samples were dehydrated through a series of ethanol washes, embedded and sectioned at Electron Microscopy Research Services, Newcastle University. Sections were analysed in a transmission electron microscope (Philips, CM100).

Visualisation of Bacteria on the Surface of Sinus Mucosa

Fluorescence in situ hybridisation (FISH) was performed using a peptide nucleic acid (PNA) probe corresponding to the well-characterised EUB338 probe [35]. The probe was synthesized as a fluorescein amidite (FAM) conjugate by Panagene. Mucosal biopsy specimens were fixed in 10% formalin directly after surgery, and stored at 4 C for up to one month. For PNA-FISH analysis, specimens were transferred to 50% ethanol and incubated for 16 h at - 2 0 C. Biopsy material was transferred to 1 ml permeabilization buffer (10 mg/ml lysozyme in PBS) and incubated at 37 C for 30 min. Samples were immersed in 1 ml pre-warmed wash buffer (10 mM Tris-HCl pH 9.0, 1 mM EDTA) for 30 min at 55 C. Pre-warmed hybridization buffer (25 mM Tris-HCl pH 9.0, 100 mM NaCl, 0.5% SDS, 30% formamide) containing 150 pmol per ml of the PNA probe was added to samples and incubated in darkness for 90 min at 55 C. Unbound PNA probe was removed by incubating in pre-warmed

wash buffer for 30 min at 55 C. Eukaryotic cells were counter-stained by immersing the specimens in 1 ml PBS containing 2 mg ml^{21} 49,6-diamidino-2-phenylindole (DAPI) in darkness at 20 C for 15 min. Samples were glue-mounted onto a plastic surface and immersed in 2 ml PBS. Visualisation of surface bacteria and eukaryotic cells was performed using a Leica TCS SP2 microscope with an argon/neon laser for imaging FAM conjugates (excitation 495 nm, emission 520 nm), and DAPI (excitation 358 nm, emission 461 nm). Images were converted into z-stacks using Image J software [36].

Isolation and Culture of Micro-organisms

A variety of growth media were employed for the isolation and routine culture of micro-organisms. Blood agar contained (per litre) 37 g Brain Heart Infusion (Oxoid), 5 g Yeast Extract (Merck), and 15 g Bacteriological Agar. After sterilization, 5% (v/v) defibrinated horse blood (TCS Biosciences) was added. Chocolate agar was prepared using the same recipe except that, after the addition of horse blood, the medium was heated to 70uC for 10 min. Fastidious Anaerobe Agar (FAA) was purchased from LabM and Sabouraud Dextrose Agar was from Oxoid.

For isolation of micro-organisms, a portion of sinus aspirate from each patient was homogenized in sterile phosphate buffered saline (PBS) and inoculated onto blood agar, chocolate agar, FAA and two plates of Sabouraud Dextrose agar. Blood and chocolate agar plates were incubated in 5% CO_2 at 37uC. Pre-reduced FAA plates were incubated at 37 C anaerobically (Ruskinn, Bugbox Plus) in a gas mix consisting of 10% CO_2, 10% H_2 and 80% N_2. The Sabouraud Dextrose agar plates were incubated aerobically, one at 37 C and the other at 30 C. Plates were examined every 24–48 hours for at least seven days. Individual colonies were picked and sub-cultured three times to obtain pure isolates. Strains were stored at –80 C in BHY medium [Brain Heart Infusion 37 g/L (Oxoid) and Yeast Extract 5 g/L (Merck)] diluted to 50% strength by the addition of glycerol.

Identification of Isolates

All isolates were initially characterized by Gram staining, inspection of colony morphology and testing for catalase production, haemolysis and ability to grow aerobically or anaerobically. A single thick streak of each isolate was plated onto DNase agar (Oxoid) to test for extracellular nuclease activity. Plates were incubated aerobically or anaerobically at 37 C for between 24–96 h. Once colonies had grown, plates were flooded with 4 ml of 0.1% (w/v) toluidine blue (Sigma) to highlight nuclease production. The majority of clinical isolates were further identified to species level using a Matrix Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF) Mass Spectrometer (Bruker, Microflex) [37]. Isolates were streaked onto blood agar, incubated under 5% CO_2 or in the absence of oxygen, at 37 C for 24 h and transferred to the Pathology Department, Freeman Hospital, Newcastle upon Tyne, for identification.

In cases where MALDI-TOF analysis yielded ambiguous results, for example the majority of a-haemolytic streptococci, bacterial identification was confirmed by analysis of the 16S rRNA gene. Bacteria were cultured in BHY broth, and harvested by centrifugation at 4,000 g. Cells were resuspended in 150 ml spheroplasting buffer [26% (w/v) raffinose, 10 mM $MgCl_2$, 20 mM Tris-HCl, pH 6.8] supplemented with 37.5 mg lysozyme (Sigma) and 50 U mutanolysin (Sigma), and incubated at 37 C for 30 min. Following incubation, DNA was extracted using the MasterPure™ Gram Positive DNA Purification Kit (EpicentreH Biotechnologies) in accordance with manufacturer's instructions. The DNA was suspended in 25 ml elution buffer (10 mM Tris pH 8.5).

The gene encoding 16S rRNA was amplified from 2 ml of extracted DNA in a reaction that also contained 5 ml (25 pmol) each of oligonucleotide primers 0063F and 1387R [38], 25 ml ReddyMix Extensor PCR Master Mix (Thermo Scientific) and 13 ml dH_20. PCR reactions were run using a GeneAmp PCR System 9700 (Applied Biosystems) with steps as follows: denaturation at 94 C, 2 min, followed by 35 cycles of (i) 94 C, 10 sec, (ii) 55 C, 30 sec, (iii) 68 C, 1 min and a final elongation at 68 C, 7 min. PCR products were checked on a 1% agarose gel, and fragments of the expected size were sequenced by MWG Eurofins. Forward and reverse sequences were aligned and sequence matched using the Ribosomal Database Project website (http://rdp.cme.msu.edu/).

Purification of B. licheniformis NucB

NucB was produced using a previously described method [33]. Bacterial strain Bacillus subtilis NZ8900 containing plasmid pNZ8901 was inoculated into 5 ml sterile Luria Bertani broth (Sigma) containing 5 mg l^{21} chloramphenicol and cultured aerobically at 37 C for 18 h. The culture was adjusted to an optical density (OD_{600}) of 1.0, and 100 mL were transferred to 10 ml of sterile LB containing chloramphenicol and incubated aerobically at 37 C for 3 h until OD_{600} ~ 1.0 was reached. At this point, 5% v/v cell free supernatant of an overnight B. subtilis ATCC6633 culture was added to provide the subtilin required to induce NucB production, and this was incubated for a further 2.5 h. Cells were removed by centrifugation at 6000 g for 20 min and the supernatant was sterilized by passing through a 0.2 mm syringe filter. The concentration of NucB was estimated by comparison with bovine serum albumin standards on a SDS-PAGE gel. NucB was stored at 4 C for up to 3 months. NucB was purified from the supernatant using trichloroacetic acid (TCA) precipitation followed by Superose™ 12 gel filtration. Proteins in the active fractions were further concentrated by TCA precipitation again and analysed by SDS-PAGE. The concentration of protein was estimated by densitometry analysis, in comparison with standards of known concentration [33].

Growth and Biofilm Formation by CRS Isolates

Planktonic growth kinetics in batch culture were measured in BHY broth. Stock cultures of CRS isolates (see above) were diluted in BHY to a starting OD_{600} of ~ 0.1. Cultures were incubated at 37uC and at hourly intervals, 1 ml samples were removed and OD_{600} was determined. For biofilm assays, 8 ml of CRS isolate stock cultures (see above) were added to triplicate wells of a sterile polystyrene 96-well plate (Corning 3595) containing 200 ml BHY broth. The lid was replaced and plates were wrapped in parafilm and incubated without shaking aerobically at 37 C for 18 h. Following growth, 150 ml of the non-adherent planktonic cells were removed and transferred to a clean 96-well plate and the OD_{600} was read in a microplate reader (BioTek Synergy HT) to quantify growth in the planktonic phase. To quantify biofilm extent, 100 ml of 0.5% (w/v) crystal violet were added to each well and incubated at 20 C for 15 min. Wells were rinsed 3 times with 200 ml PBS. Residual crystal violet was dissolved in 100 ml 7% acetic acid and the A_{570} was read in a microplate reader (BioTek Synergy HT). Each assay was performed three times independently. To assess the efficacy of NucB for dispersing biofilms, biofilms were cultured as above, and washed three times with PBS. Purified NucB (3 mg ml^{21}) or PBS alone was added to wells and incubated for 1 h at 37 C. Remaining biofilms were quantified by staining with crystal violet.

Growth and Visualization of Biofilms on Glass Surfaces

The effect of NucB on biofilm architecture was visualized by confocal laser scanning microscopy (CSLM) or by scanning electron microscopy (SEM), using biofilms cultured on glass surfaces. Sterile 13 mm diameter glass coverslips were placed in wells of a six-well tissue culture plate containing 3 ml BHY. Wells were inoculated with 50 ml of stock bacterial cultures and incubated statically in air at 37 C for 18 h. Coverslips were removed and rinsed three times with PBS, and 1 ml NucB (3 mg ml^{21}) or 1 ml PBS (control) was added and incubated for 1 h at 37 C. For CSLM, coverslips were inverted onto a rubber O-ring that had been placed on a microscope slide and filled with Live/DeadH BacLight^{TM} stain (Molecular Probes). Biofilms were examined using a Leica TCS SP2 confocal microscope with an argon/neon laser for visualisation of SYTOH 9 (excitation 485 nm, emission 519 nm), and propidium iodide (excitation 536 nm, emission 617 nm). For SEM, coverslips were fixed in 2% (v/v) glutaraldehyde at 4uC for 16 h. Specimens were rinsed twice in PBS and dehydrated through a series of ethanol washes as follows: 25% ethanol 30 min, 50% ethanol 30 min, 75% ethanol 30 min, and two washes for 1 h in 100% ethanol. Samples were dried in a critical point dryer (Bal-tec), mounted on aluminium stubs and sputter coated with gold at Electron Microscopy Research Services, Newcastle University. Biofilms were visualised using a scanning electron microscope (Cambridge Stereoscan 240).

Extraction and Analysis of eDNA in Biofilms Formed In vitro

Bacterial isolates were cultured in 6-well tissue culture dishes (Greiner) containing 3 ml of BHY broth for 72 h aerobically at 37 C. During this time, broth was carefully removed every 24 h and replaced with fresh medium. At the end of the 72 h incubation, medium was removed and PBS (1.5 ml) was added. Biofilms were gently removed from the surface of the wells using a plastic tissue culture cell scraper. Cells from four wells were combined together, and eDNA was purified by a modification of the method of Kreth et al. [39]. Briefly, cells were mixed by vortexing for 20 s, and incubated at 37 C for 1 h in the presence of 5 mg ml^{21} of Proteinase K (Sigma Aldrich). Cells were separated from the supernatant, containing eDNA, by centrifugation at 16,000 g for 2 min. Extracellular DNA in the supernatant was extracted with phenol:chloroform:isoamyl alcohol (25:24:1). Samples were centrifuged at 16,000 g for 5 min to separate the phases, and the aqueous phase was collected. DNA was pre- cipitated by the addition of isopropanol. The DNA was pelleted by centrifuging at 16,000 g for 10 min, air dried, and re-suspended in 50 ml of 10 mM Tris pH 8.5. For intracellular DNA, pelleted cells
were resuspended in 150 ml spheroplasting buffer, and DNA was purified using the method described above.

The concentration and purity of DNA in each fraction was determined using a NanoDrop spectrophotometer. In addition, double stranded DNA (dsDNA) was quantified using the Pico-Green dsDNA reagent (Molecular Probes) and comparing with standards of known concentration according to the manufacturer's instructions. Intracellular and extracellular DNA was visualized by agarose gel electrophoresis.

Results

Microscopic Analysis of Obstructive Mucin and Mucosal Biopsies from CRS Patients

Obstructive mucin is sometimes observed in the paranasal sinuses of CRS patients with or without polyps in the absence of other symptoms that would indicate fungal rhinosinusitis. The removal of this mucin from the paranasal sinuses is the cornerstone of surgical treatment of CRS [40]. This material is extremely tenacious and removing it significantly extends the length of time required in the operating theatre. To investigate the structure of the obstructive mucin, a portion of the material from two different CRS patients was cut into small pieces ($\sim$1 mm^3) immediately after surgery and fixed in glutaraldehyde. The mucin was embedded and sectioned for TEM analysis (see Materials and Methods). The material from the two patients appeared similar in structure, and consisted predominantly of an acellular matrix either with a striated appearance punctuated by occasional degraded host cells and cell debris (Figure 1A), or with little structure and many pockets (Figure 1B). Erythrocytes were sometimes observed (not shown). However, areas containing large numbers of eosinophils were not observed in any field of view. Additionally, bacterial cells and fungi were not seen within the matrix.

The possibility that microbial cells were present but not directly observed could not be discounted. Therefore, mucosal biopsies were also collected from CRS patients, and analysed by confocal scanning laser microscopy (CSLM). Initially, propidium iodide was employed to stain bacteria. However, it was difficult to identify micro-organisms with confidence due to the lack of contrast between bacteria and host cells. Therefore, in subsequent samples bacteria were selectively stained by PNA-FISH with the EUB338 probe, and host cells were counterstained with DAPI. Using this approach, microbial cells were clearly identified on the mucosal surface (Figure 2). In yz and xz projections, it appeared that most of the micro-organisms were present in regions above the tissue surface or in a layer within the top 10 mm of the tissue. A three-dimensional representation of this image is shown in Figure S1. The EUB338 PNA probe targets bacterial 16S ribosomal RNA (rRNA) within metabolically active cells [41]. The detection of punctate staining with the EUB338 probe is indicative of microbial cells that were live prior to fixation. In addition to the sharp staining of cells, there were also patches of fluorescence from the PNA-FISH probe that were more diffuse (Figure 2B, arrows). Since PNA probes can hybridize with complementary DNA in addition to RNA [42], it is likely that this fluorescence represents extracellular microbial nucleic acids such as RNA or single stranded DNA.

Isolation and Identification of Micro-organisms Associated with CRS

Overall, 75 strains of bacteria were isolated from obstructive mucin, comprising a total of 16 different genera and 32 separate species (Table 1). The most prevalent organism associated with CRS aspirates was Staphylococcus epidermidis, which was isolated from 15 of 20 specimens (75%). Staphylococcus aureus and Streptococcus spp. were each isolated from 7 patients (35% of samples), Corynebacterium spp. were isolated from 6 patients (30%), and Propionibacterium spp. from 5 patients (25%). Other organisms that were less frequently isolated included Haemophilus influenzae, Moraxella catarrhalis, Neisseria spp., Finegoldia magna, and Enterobacter aerogenes. The majority of isolated bacteria were facultative anaerobes. However, both obligate aerobes (for example, Neisseria spp. and M. catarrhalis) and obligate anaerobes (F. magna, Propionibacterium spp.) were also commonly isolated. No fungi were isolated from any of the patient specimens.

The production of extracellular DNase enzymes by clinical isolates was assessed using DNase test agar and staining with toluidine blue. In total, 13 of the 75 isolates (17%) produced extracellular DNase (Table 1). All S. aureus isolates produced extracellular DNase, and other producers were Streptococcus

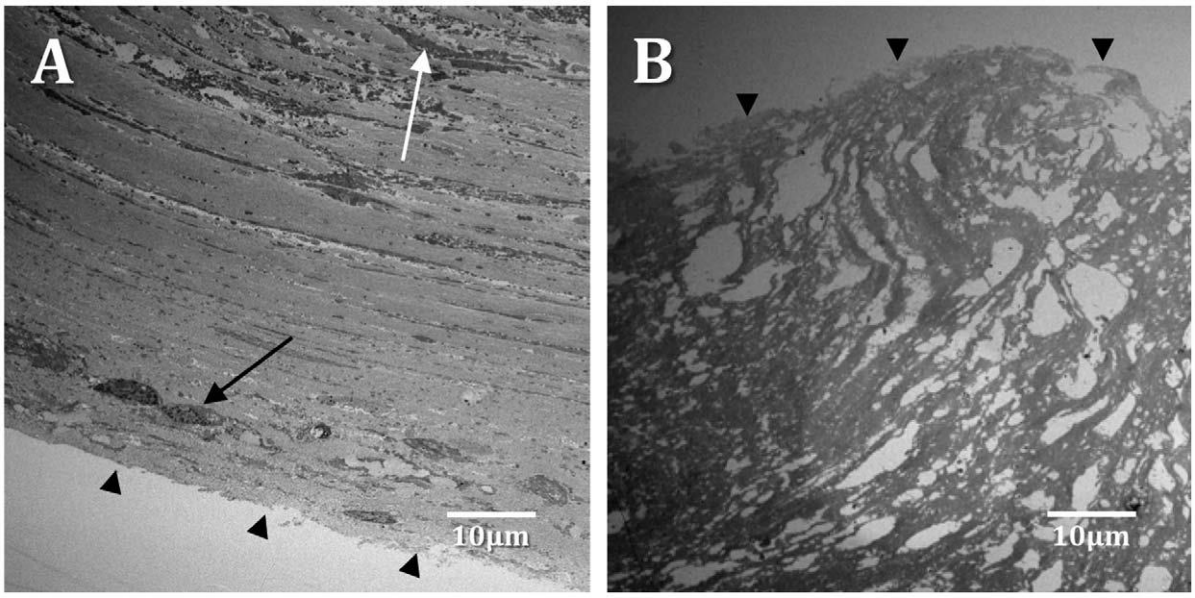

Figure 1. Transmission electron microscopy of obstructive mucin from CRS aspirates. In some cases (A), the mucin either formed a layered structure, with relatively intact cells towards the outer layers (black arrow) and more degraded cellular material further in (white arrow). (B) Alternatively, samples had little clear structure and the mucin was punctuated by pockets. The outermost layer of each sample is indicated by dark arrowheads.
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anginosus group (S. anginosus/S. constellatus/S. intermedius) strains (80% of strains), Staphylococcus lugdunensis (33% of strains) and Streptococcus salivarius (33% of strains). Extracellular nuclease producers were isolated from 11 out of 20 (55%) patients. In only two cases, more than one nuclease producing organism was isolated from the same patient sample.

Efficacy of NucB against Biofilm Forming Isolates

Twenty-four bacteria, isolated from patient specimens, were grown in 96-well microtiter plates to assay for biofilm formation. Representative strains of all species that produced extracellular DNase were selected for these studies, along with a similar number of non-producing organisms. Following incubation for 20 h in microtitre wells, all isolates had grown in the planktonic phase to

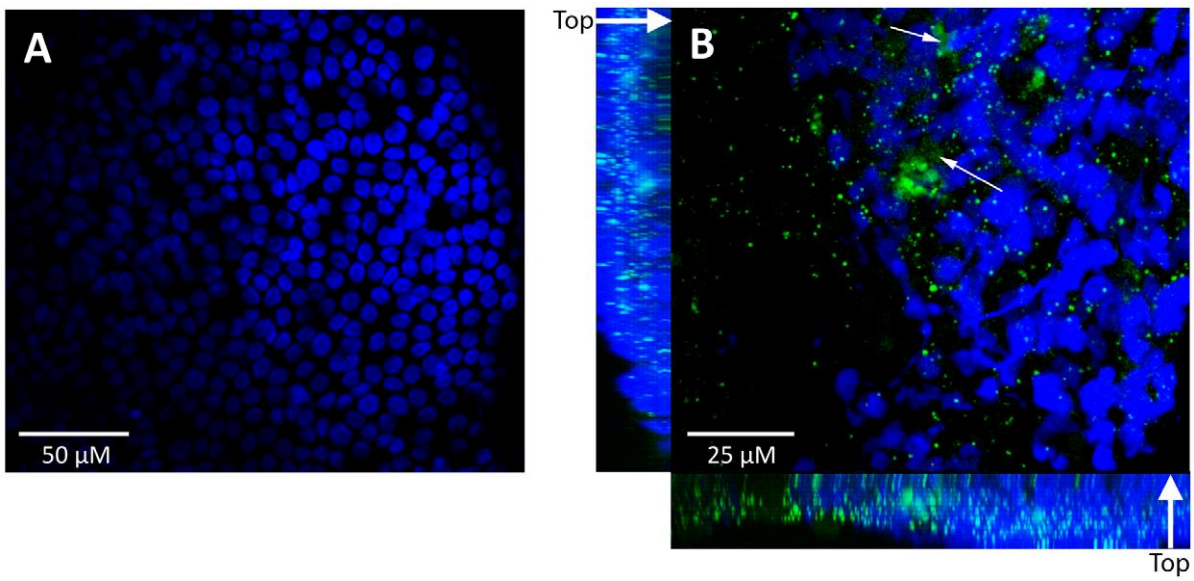

Figure 2. Confocal laser scanning microscopy of surface associated bacteria on mucosa removed from patients diagnosed with CRS. Bacterial DNA (green) was visualized using an EUB338 PNA-FISH probe, and epithelial cell nuclei (blue) were counterstained with DAPI. Maximum projection images are shown. In some fields, epithelial cells were observed in the absence of bacteria (A), and in other fields bacterial biofilm was evident (B). B includes z-stacks oriented from the outside of the mucosal biopsy specimen (labelled 'top') to the deeper layers (indicated by a thick white arrow). Small white arrows indicate patches of diffuse staining, consistent with the presence of extracellular nucleic acids.
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Table 1. Bacteria isolated from CRS aspirates.

Patient	Microbial Species Present[a]	Total number of isolates
1	Enterobacter aerogenes, Staphylococcus epidermidis, Streptococcus sp., Streptococcus pneumoniae, Streptococcus salivarius	5
2	Haemophilus influenzae, Micrococcus luteus, Staphylococcus epidermis, Staphylococcus hominis, Streptococcus pneumoniae	5
3	Moraxella catarrhalis, Staphylococcus aureus, Streptococcus anginosus	3
4	Klebsiella rhizophila, Staphylococcus epidermidis, Streptococcus constellatus, Streptococcus intermedius, Streptococcus salivarius	5
5	Esherichia coli, Staphylococcus aureus, Staphylococcus epidermidis	3
6	Corynebacterium pseudodiphtheriticum, Finegoldia magna, Klebsiella pneumoniae, Moraxella catarrhalis, Staphylococcus epidermidis, Staphylococcus lugdunensis	6
7	Staphylococcus aureus, Staphylococcus epidermidis, Staphylococcus warneri, Streptococcus constellatus	4
8	Corynebacterium propinquum, Staphylococcus epidermidis, Staphylococcus lugdunensis	3
9	E. aerogenes, Finegoldia magna, Propionibacterium sp., Streptococcus pneumoniae	4
10	Haemophilus influenzae, Moraxella catarrhalis, Staphylococcus epidermidis, Streptococcus pneumoniae	4
11	Staphylococcus epidermidis, Staphylococcus warneri	2
12	Citrobacter koseri, Staphylococcus epidermidis, Propionibacterium sp., Pseudomonas aeruginosa	4
13	Corynebacterium pseudodiphtheriticum, Propionibacterium granulosum, Staphylococcus aureus	3
14	Staphylococcus epidermidis, Staphylococcus pasteuri, Staphylococcus warneri	3
15	Staphylococcus epidermidis, Staphylococcus lugdunensis, Propionibacterium acnes, Propionibacterium granulosum	4
16	Corynebacterium propinquum, Neisseria meningitidis, Staphylococcus aureus	3
17	Neisseria sp., Staphylococcus epidermidis, Streptococcus anginosus, Streptococcus parasanguinis, Streptococcus salivarius	5
18	Staphylococcus aureus, Staphylococcus epidermidis	2
19	Corynebacterium pseudodiphtheriticum, Propionibacterium avidium, Staphylococcus aureus, Staphylococcus epidermidis	4
20	Corynebacterium accolens, Corynebacterium pseudodiphtheriticum, Lactobacillus sp.	3

[a]Strains highlighted in bold text produced extracellular deoxyribonuclease.
doi:10.1371/journal.pone.0055339.t001

OD_{600} ~ 0.1 with the exception of three strains: S. anginosus FH19, S. constellatus FH21 and S. pneumoniae FH26 (Table 2). Nevertheless, all of these strains produced biofilms that were detectable by crystal violet staining. In fact, S. pneumoniae FH26 produced a relatively strong biofilm (A_{570} = 1.87). Growth rates of each strain in BHY medium were determined in planktonic cultures (Table 2). No correlation was seen between the maximum growth rate of strains and the capacity to form biofilms. Generally, there was extensive variation in the extent of biofilm formation between different species and between different strains of the same species. For example, M. catarrhalis FH3 produced a very weak biofilm (A_{570} = 0.77), whereas M. catarrhalis FH4 formed extensive biofilms (A_{570} = 2.78). Of the strains tested, Streptococcus anginosus FH19 produced the least abundant biofilms (A_{570} = 0.22). The mean extent of biofilm formation by non-nuclease producers (A_{570} = 1.51, S.E. 0.19, n = 15) was not significantly different from that of nuclease producers (A_{570} = 1.48, S.E. 0.32, n = 9).

To assess the importance of eDNA in maintaining the structural integrity of biofilms, pre-formed biofilms were incubated for 1 h in the presence of the microbial DNase NucB (Table 2). Biofilms formed by 9 out of 9 (100%) nuclease producing strains were significantly reduced by NucB (T test comparing NucB treatment with buffer control, $p > 0.05$, n = 3). By contrast, only 5 out of 15 (33%) of the biofilms produced by non-nuclease producing bacteria were dispersed by NucB. In addition, 2 out of 15 (13%) non-nuclease producers had slightly increased levels of biofilm following incubation with NucB than without the enzyme. To assess whether NucB had detrimental effects on the cells themselves, four different isolates, S. aureus FH7, S. constellatus FH20, S. salivarius FH29 and M. catarrhalis FH4, were cultured to mid-exponential phase in THYE broth and challenged with 5 mg ml^{-1} NucB. These isolates were selected as representative Gram-positive and Gram-negative organisms to assess the general toxicity of NucB for bacterial cells. Since the production of extracellular nucleases by bacteria is widespread, it seemed unlikely that DNase activity itself would be toxic to bacteria. Nevertheless, it was important to assess whether the NucB protein could inhibit the growth of bacteria. No effects were observed on the growth rate of cells following the challenge (data not shown). The number of viable cells in each culture continued to increase following NucB addition, and 1 h after adding NucB there was no difference in the number of viable cells in cultures containing NucB compared with control cultures without the enzyme. Overall, these data suggest that eDNA is an important component of the EPS for over 50% of the CRS isolates, including strains that produce extracellular DNase enzymes, and that addition of NucB dislodges cells without killing or inhibiting bacteria.

Microscopic Analysis of in vitro Grown Biofilms

To obtain more detailed information about the effects of NucB, biofilms of selected organisms were cultured on glass coverslips and analysed by CLSM and SEM. This work focussed on staphylococci and streptococci, since these were the genera most commonly isolated from CRS patients. In the absence of NucB treatment, biofilms formed by S. constellatus FH20 were relatively thin and consisted primarily of a single cell layer that covered most of the surface (Figure 3). In places, clusters of cells projected from the surface to a depth of > 12 mm. Using BacLight Live/Dead stain, both live cells (green) and dead cells (red) were observed in biofilms. Biofilms that had been treated with NucB were clearly

Table 2. Biofilm formation and NucB sensitivity of selected isolates from CRS aspirates.

Strain	Planktonic Growth yield OD_{600} Mean (S.E.)	Doubling Time (min) Mean (S.E.)	Biofilm Growth A_{570} Mean (S.E.)	Nuclease Production[a]	Remaining Biofilm after NucB Addition (%)	P-value
Corynebacterium propinquum FH1	0.39 (0.06)	235 (11)	1.79 (0.78)	2	105	0.558
Corynebacterium pseudodiphtheriticum FH2	0.86 (0.24)	129 (2)	2.61 (0.43)	2	92	0.577
Moraxella catarrhalis FH3	0.39 (0.07)	232 (6)	0.77 (0.21)	2	127	0.349
Moraxella catarrhalis FH4	0.42 (0.14)	155 (5)	2.78 (0.21)	2	124	0.032
Staphylococcus aureus FH5	0.40 (0.11)	62 (3)	1.84 (0.34)	+	77	0.003
Staphylococcus aureus FH6	0.50 (0.08)	74 (0.02)	0.71 (0.12)	+	59	0.000
Staphylococcus aureus FH7	0.79 (0.26)	61 (3)	1.23 (0.22)	+	40	0.000
Staphylococcus epidermidis FH8	0.27 (0.09)	81 (6)	2.29 (0.41)	2	114	0.077
Staphylococcus epidermidis FH10	0.48 (0.06)	90 (1)	1.59 (0.22)	2	67	0.001
Staphylococcus epidermidis FH11	0.54 (0.13)	104 (4)	1.52 (0.24)	2	74	0.010
Staphylococcus lugdunensis FH12	0.74 (0.03)	73 (4)	1.16 (0.23)	2	49	0.001
Staphylococcus lugdunensis FH13	0.78 (0.05)	70 (0.2)	0.57 (0.10)	2	66	0.001
Staphylococcus lugdunensis FH14	0.78 (0.13)	73 (16)	0.53 (0.05)	+	69	0.001
Staphylococcus warneri FH15	0.59 (0.18)	72 (4)	0.89 (0.25)	2	126	0.005
Staphylococcus warneri FH17	0.88 (0.16)	65 (3)	2.40 (0.55)	2	90	0.319
Streptococcus anginosus FH18	0.16 (0.05)	54 (3)	1.16 (0.07)	+	34	0.000
Streptococcus anginosus FH19[b]	0.07 (0.00)	90 (15)	0.22 (0.02)	+	59	0.015
Streptococcus constellatus FH20	0.22 (0.04)	103 (28)	1.90 (0.39)	+	44	0.001
Streptococcus constellatus FH21[b]	0.04 (0.03)	ND[c]	0.31 (0.05)	2	39	0.001
Streptococcus intermedius FH22	0.19 (0.02)	67 (2)	3.07 (0.80)	+	46	0.000
Streptococcus pneumoniae FH26	0.07 (0.03)	56 (1)	1.87 (0.31)	2	123	0.585
Streptococcus salivarius FH27	0.20 (0.06)	37 (3)	0.99 (0.04)	2	92	0.240
Streptococcus salivarius FH28	0.32 (0.04)	111 (29)	2.67 (0.96)	+	66	0.002
Streptococcus salivarius FH29	0.23 (0.03)	39 (0.7)	1.08 (0.04)	2	96	0.692

[a]Production of nuclease was measured on DNase agar, and is indicated by a '+' sign.
[b]Isolates grew poorly in both the planktonic and biofilm phase.
[c]ND, not determined.
doi:10.1371/journal.pone.0055339.t002

less extensive than the untreated controls, and consisted of sparsely distributed single cells or very small aggregates of ,10 cells (Figure 3B). To obtain higher resolution images, similar biofilms were analysed by SEM (Figure 4). Again, in the absence of NucB, cell aggregates were evident and a relatively large proportion of the surface was covered by micro-organisms (Figure 4A). By contrast, NucB-treated biofilms almost exclusively contained isolated cells or small clusters of cells (Figure 4B). In addition, extracellular material was apparent in untreated biofilms under high resolution SEM (Figure 4C), that was not seen in biofilms incubated with NucB (Figure 4D). Biofilms formed on glass surfaces by S. aureus FH7 or S. intermedius FH22 were also visualised by SEM (not shown). As with S. constellatus FH20, biofilms that were treated with NucB contained far less biomass than those incubated in buffer alone. However, extracellular polymers were not observed in these organisms.

Quantification of eDNA in Model Biofilms

To quantify levels of eDNA in model biofilms, eDNA and intracellular DNA (iDNA) was extracted from biofilm cultures of S. aureus FH7, S. constellatus FH20 and S. salivarius FH29. The eDNA was analysed by agarose gel electrophoresis (Figure 5). Sharp bands migrating at an apparent size of 30 kbp were observed in eDNA fractions of S. aureus FH7 and S. constellatus FH20. However, no high molecular eDNA bands were seen in S. salivarius FH29. Intracellular DNA from all three organisms appeared as a smear of high molecular weight fragments, probably due to binding of chromosomal DNA to cell wall fragments. In addition to the high molecular weight fragments, small fragments of DNA or RNA were seen at the bottom of the gel. Nucleic acids in each fraction were quantified using the Nanodrop spectrophotometer (Figure 5B). For each strain, eDNA represented approximately 5–10% of the total DNA present in the biofilm. To account for the possibility that samples may have contained RNA in addition to DNA, nucleic acids were also quantified using PicoGreen dye, which is strongly selective for double stranded DNA. No significant differences were observed between the total amount of eDNA in S. salivarius FH29 biofilms and eDNA in biofilms formed by the other two strains. Therefore, despite the lack of a clear band by agarose gel electrophoresis, it appears that eDNA was present in S. salivarius FH29 biofilms.

To assess whether eDNA was present in biofilms formed by other CRS isolates, biofilms of each strain were cultured in 6-well plastic dishes and eDNA was purified as described in the Methods section. Only two strains were omitted from this analysis: S. constellatus FH21 grew very poorly in biofilms and it was not possible to extract eDNA, and S. salivarius FH28 was prone to contamination and, after several attempts, it was decided not to

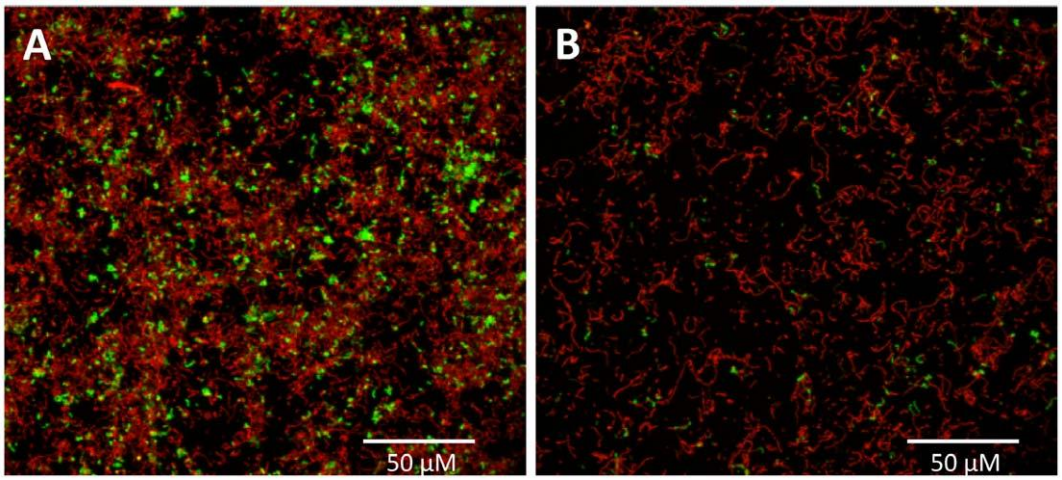

Figure 3. Confocal laser scanning microscopy of Streptococcus constellatus FH20 biofilms with or without NucB treatment. Biofilms were formed on glass surfaces and were visualised with CLSM using BacLight LIVE/DEAD stain, which stains compromised (dead) cells red and live cells green. (A) Biofilms treated with buffer alone, and (B) biofilms treated with NucB.
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pursue DNA purification from this strain. By agarose gel electrophoresis, sharp bands corresponding to high molecular weight eDNA products were observed in all Staphylococcus spp., S. constellatus FH20 and in S. intermedius FH22 (Figure 5C). By contrast, similar bands were not detected from Corynebacterium spp., S. anginosus or S. pneumoniae. Only M. catarrhalis and S. salivarius had inter-strain differences in the production of eDNA. Thus, eDNA was not visualised in M. catarrhalis FH3 biofilm extracts, whereas eDNA was clearly present in M. catarrhalis FH4 (Figure 5C). Similarly S. salivarius FH29 did not produce a band of eDNA on an agarose gel, whereas a sharp band was seen in S. salivarius FH27. The concentrations of eDNA in samples were determined using the Nanodrop spectrophotometer. Concentrations of eDNA in the extracts ranged from 206 ng ml^{21} to 917 ng ml^{21} (Figure 5C). Interestingly, there appeared to be little correlation between the concentration of eDNA and the presence or absence of a band on the agarose gel. For example, M. catarrhalis FH3 produced one of the highest concentrations of eDNA, but no band on the gel. Conversely, the eDNA concentration from S. intermedius FH22 was just 325 ng ml^{21} even though this strain clearly produced a band of eDNA on a gel. The production of an extracellular nuclease did not correlate with the presence of a band of eDNA on a gel. All strains of S. aureus (nuclease-positive) and S. epidermidis (nuclease-negative) produced clear bands of eDNA, for example. With the exception of S. anginosus FH18 and S. anginosus FH19, all strains that failed to produce a clear band of eDNA on an agarose gel were insensitive to NucB treatment.

Discussion

There is mounting evidence that microbial biofilms growing within paranasal sinuses are a major factor in the pathogenesis of CRS [14]. Bacterial biofilms have been most commonly detected on the sinus mucosa, whereas fungi tend to be more easily detected within the sinonasal mucous. Fungal growth is often accompanied by mucous secretions containing large numbers of intact or degraded eosinophils, known as 'eosinophilic mucin' or 'allergic mucin' [43–45]. The eosinophils appear to migrate intact from the tissues, and degrade or degranulate upon reaching the mucin, possibly in order to target fungi growing within the mucin. Allergic mucin may be present in the absence of fungi [46]. In our experience, a number of patients present with thick, tenacious mucin obstructing the paranasal sinsues, but without other evidence of fungal rhinosinusitis. We hypothesized that bacterial biofilms may contribute to the pathogenesis of CRS in these patients. The aim of this study was to characterize the microflora in paranasal sinuses of patients with obstructive mucin, and to assess the potential of a novel deoxyribonuclease enzyme for degrading biofilms formed by isolated micro-organisms.

Initially, the structure of obstructive mucin was investigated using TEM. This material contained relatively small numbers of degraded host cells. Therefore, this structure appears to be different from eosinophilic mucin [44]. In addition, fungi were not observed either by high-resolution TEM of obstructive mucin or by culture. The role of fungi in CRS is currently unclear. Culture-based studies have reported very low rates of isolation of fungi from CRS samples [20,22], whereas the direct microscopic analysis of eosinophilic mucin in some cases detects fungi in 100% of patient samples [45]. In the patient cohort analysed here, CRS appeared to be of a non-fungal aetiology. Bacterial cells were also not detected within the obstructive mucin. However, bacteria do not generally produce filamentous structures such as hyphae and can be difficult to detect in thin sections. By carefully examining the mucosal surfaces, bacterial biofilms could be observed. Bacteria appeared to be localised on top of the tissue or within the outer layer of epithelial cells. It is likely that the dehydration steps involved in sample processing for FISH would have compromised the outer barrier of the tissue, leading to an irregular surface and the appearance of bacterial nucleic acid staining in regions slightly below the surface of the tissue. Alternatively, a proportion of the bacterial cells may have been present within the host cytoplasm, as has previously been described for S. aureus [47]. Nevertheless, the structure of biofilms was consistent with those previously described in CRS patients [9]. In addition, more than one species of bacterium was isolated from each of the 20 samples tested.

In total, 75 strains of bacteria were isolated from 20 CRS patients. On the whole, the organisms identified in this study were very similar to those identified in previous culture-based investigations into the microflora of CRS patients. Thus, staphylococci (both S. aureus and coagulase-negative staphylococci) and a-haemolytic streptococci were the most commonly isolated

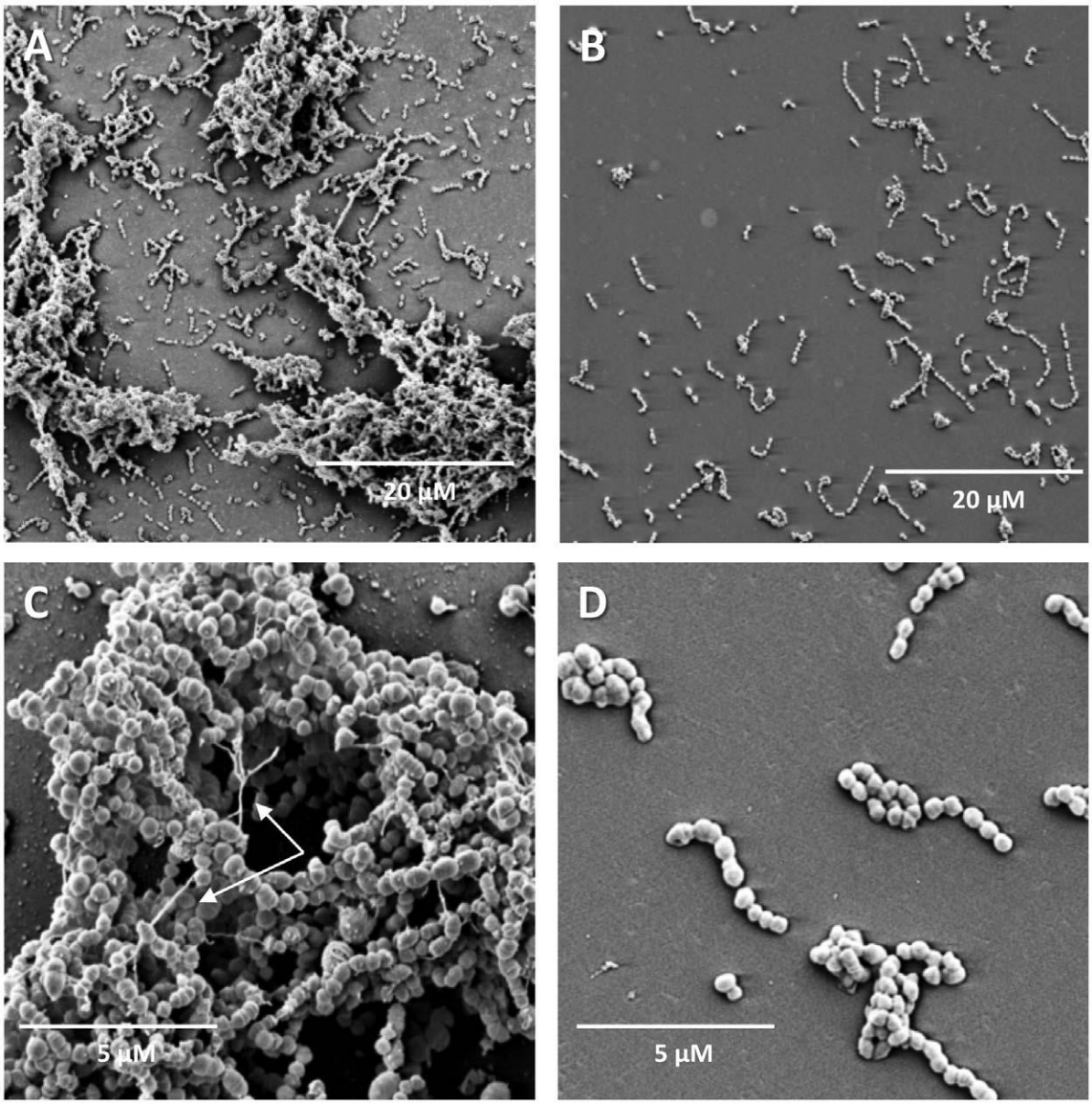

Figure 4. Scanning electron microscopy of Streptococcus constellatus FH20 biofilms treated with NucB or buffer control. Biofilms were visualised with SEM after treatment for 1 h with buffer (A) or with NucB (B). At higher magnification, extracellular material (white arrow) was observed in the absence of NucB treatment (C), but was not seen in NucB-treated biofilms (D).
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organisms, in agreement with published reports [18,20–22]. Corynebacteria were isolated from seven CRS patients, and several potential pathogens were identified, including M. catarrhalis, Neissseria spp. and H. influenzae. All of these species have been isolated from CRS cases. However, their contribution to disease pathogenesis is unclear [20,22]. Enterobacteriaciae have been frequently isolated from CRS patients [20–22], and this group of organisms was represented here by C. koseri, E. aerogenes, E. coli, and K. pneumoniae. There has been some debate about the presence of obligate anaerobes in paranasal sinuses of CRS patients. Thus, Doyle et al. [22] did not isolate anaerobes from chronic ethmoid sinusitis, whereas Brook [21] found that obligate anaerobes formed the majority of the bacteria isolated from chronic maxillary sinusitis. It is possible that the maxillary sinuses provide a more conducive environment for the growth and survival of anaerobes than the ethmoid sinuses. Here, samples were collected from a mixture of maxillary, sphenoid and ethmoid sinuses, and obligate anaerobes (Propionibacterium spp. or F. magna) were isolated from 6 of the 20 patients. The micro-organisms isolated and identified in this investigation are representative of the culturable microflora common in CRS patients. Recently, it has been shown that culture-independent, pyrosequencing analysis of the CRS microflora identifies a very similar microflora to that found by culture, although pyrosequencing has greater sensitivity for detecting difficult-to-culture or low-abundance micro-organisms [48].

The capacity of micro-organisms to form biofilms is likely to be important for the colonization of paranasal sinuses. Several studies have now provided direct evidence that bacteria are commonly present in biofilms on sinus mucosa in CRS patients [14].

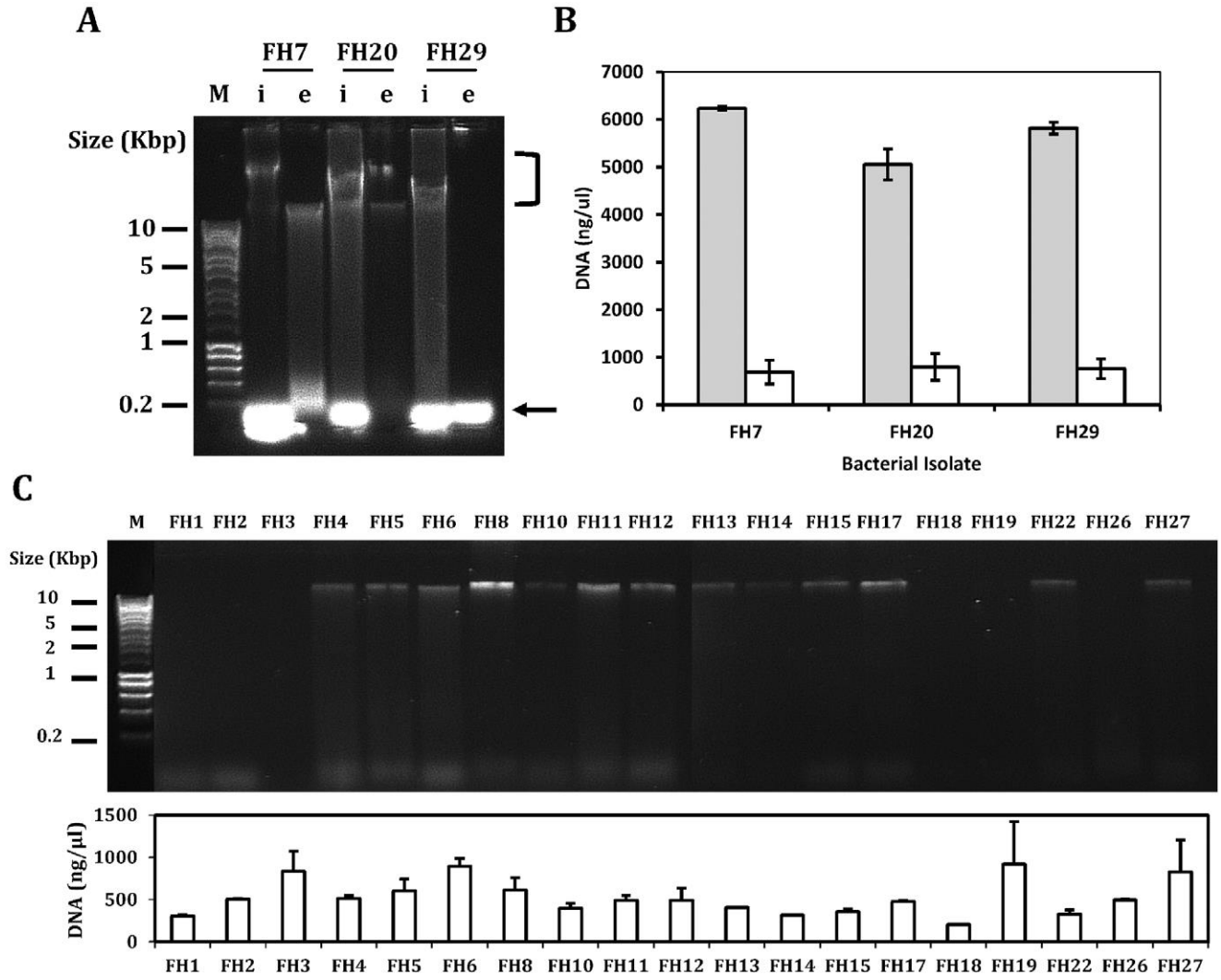

Figure 5. The visualization and quantification of eDNA from CRS isolates. (A) Intracellular DNA (i) or eDNA (e) was purified from bacterial biofilms of S. aureus FH7, S. constellatus FH20 or S. salivarius FH29, and analysed by agarose gel electrophoresis. High molecular weight chromosomal DNA is indicated by a black bracket; low molecular DNA or RNA is highlighted at the bottom of the gel by a black arrow. M; size marker. (B) The concentration of DNA in the intracellular (grey bars) and extracellular (white bars) fractions from bacterial biofilms was measured by NanoDrop spectrophotometry. Bars represent means of three independent extracts, and SEs are indicated. (C) Extracellular DNA concentration in biofilms was also visualised for another 19 isolates (see Table 1 for species names). In many cases, distinct bands were observed with an apparent migration at approximately 30 kbp. The total DNA concentration was measured by NanoDrop spectrophotometry, bars represent the average of three replicates and error bars are S.E.
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Nevertheless, little is known about whether these organisms are particularly well-suited to forming biofilms. The ability of biofilm bacteria on sinus mucosa to produce biofilms in vitro has been assessed by directly inoculating mucosal swabs into a Calgary biofilm device model [49]. P. aeruginosa biofilm-defective mutants (sad-31 and sad-36) were separately set up in the model to set the threshold value, below which samples were designated as non-biofilm formers. Using these criteria, 28.6% of 157 sinus aspirate samples produced biofilms. However, this is likely to be a significant underestimate of the total biofilm-forming capacity of mucosal bacteria since the biofilm experiments used only Luria-Bertani broth incubated aerobically, and therefore anaerobic or fastidious micro-organisms would not have grown. The capacity of clinical isolates of P. aeruginosa, S. aureus and coagulase-negative staphylococci, isolated ~1 year post-FESS treatment, to form biofilms has been assessed as a possible predictor of the long-term outcomes of treatment [17]. Over 50% of strains tested produced biofilms, and the ability of P. aeruginosa or S. aureus to form biofilms appeared to be correlated with a poor clinical evolution of disease. To the best of our knowledge, there have been no investigations into the biofilm-forming ability of bacteria freshly isolated from patients during CRS treatment. We aimed to establish whether isolated CRS bacteria form biofilms in vitro and, further, whether eDNA contributes to the integrity of the biofilm.

In total, 24 isolated strains were tested for biofilm formation in a microplate model system, and all strains produced biofilms to some extent. The ability to form biofilms was not closely related to the growth rate or yield in planktonic cultures. These data are in line with previous studies on Listeria monocytogenes or Salmonella enterica strains, which also found no correlation between the growth rate or yields of individual strains and their capacities to form biofilms in microplate model systems [50,51]. Representative

strains of many of the species found in this study have been shown to produce DNase I-sensitive biofilms including, for example, S. aureus [52,53], S. pneumoniae [54,55], Neisseria spp. [56,57], P. aeruginosa [29] and E. coli [53]. We have recently identified a novel DNase enzyme, NucB, from a marine strain of Bacillus licheniformis that has potent anti-biofilm activity against a number of bacteria including E. coli and M. luteus [33]. This enzyme is smaller in size (~12 kDa) than many other nucleases, including DNase I, and appears to be well adapted to breaking up bacterial biofilms even at low concentrations [33]. A key goal of this study was to establish whether freshly isolated CRS-associated bacteria produce biofilms that are sensitive to NucB. Overall, ~50% of the strains tested produced biofilms that were reduced upon treatment with NucB. In fact, the vast majority of staphylococci (8 of 10 strains tested) and streptococci (6 of 9 strains) produced NucB-sensitive biofilms. In contrast, two Corynebacterium spp. and two M. catarrhalis strains made biofilms that were not removed by NucB. In one case the M. catarrhalis biofilm was slightly, but significantly, increased by NucB treatment. Whilst eDNA commonly promotes adhesion and biofilm formation by bacteria, in rare cases eDNA has been shown to inhibit bacterial settlement [58]. It is possible that eDNA may be inhibitory to M. catarrhalis adhesion and that NucB-mediated eDNA degradation would therefore promote adhesion by this organism. This hypothesis requires further investigation.

The production of extracellular DNase enzymes by bacteria may influence the structure of biofilms. For example, isogenic nuclease-deficient mutants of S. aureus, Neisseria gonorrhoeae or Vibrio cholerae form thicker biofilms than their wild-type progenitor strains [57,59,60]. However, using in vitro or in vivo models of catheter biofilms, Beenken et al. [61] found that the total number of viable cells in biofilms of the clinical osteomyelitis isolate S. aureus UAMS-1 was not affected by mutation in either of two extracellular nuclease-encoding genes. Therefore, it is not clear whether microbial nucleases contribute to the gross biofilm structure in clinically relevant situations. Production of extracellular DNase enzymes has been reported for several of the genera isolated here. S. aureus is well-known to produce DNases, and DNase production is often used as a phenotypic test to differentiate S. aureus from coagulase-negative staphylococci. However, the test must be interpreted with caution, since some coagulase-negative staphylococci such as S. lugdunensis can produce nucleases [62]. In fact, one of the S. lugdunensis strains isolated here was found to produce DNase. Corynebacterium diphtheriae and N. gonorrhoeae have also been reported to be able to produce extracellular DNase enzymes [57,63], but DNases were not detected in any of the Corynebacterium spp. or Neisseria spp. identified in this study. Production of DNases is variable in a-haemolytic streptococci [64], and 5 of the 9 streptococci isolated here produced DNase activity. Representative strains of all species that produced nuclease were tested in biofilm assays. Interestingly, all 9 nuclease-producing strains made biofilms that were reduced by treatment with the exogenous addition of NucB. These data provide clear evidence that the ability of a strain to produce extracellular nucleases does not preclude the formation of biofilms that are stabilised by eDNA. The production of extracellular DNases is tightly regulated in bacteria. For example, in S. aureus, nuclease production is regulated by the stress response sigma factor B [59]. Within biofilms, nucleases may be produced at low levels or by only a small proportion of the cells.

Here, direct evidence for the presence of eDNA in biofilms formed by 22 CRS isolates was provided by extraction and quantification of eDNA. All strains produced significant amounts of eDNA that could easily be measured in the Nanodrop spectrophotometer. A more detailed analysis was conducted on three different CRS isolates, including two that produce nucleases (S. aureus FH7 and S. constellatus FH20). In S. constellatus FH20 biofilms, extracellular material was observed by SEM (Figure 4). Extracellular DNA purified from S. aureus FH7 and S. constellatus FH20 biofilms was visualised as sharp high molecular weight bands on agarose gels with an apparent migration similar to that of intracellular chromosomal DNA. However, eDNA from S. salivarius FH29 was not detected by this technique. Nevertheless, quantitative measures indicated that extracellular nucleic acids were present in S. salivarius FH29 biofilms. Interestingly, in contrast to S. aureus FH7 and S. constellatus FH20, S. salivarius FH29 biofilms were not sensitive to NucB. Therefore, it appears that S. salivarius FH29 does not rely on large fragments of eDNA to stabilise biofilms. A broader analysis of the CRS isolates identified six other strains that did not produce defined bands of eDNA when analysed on agarose gels. Of these, four strains were insensitive to NucB indicating that, like S. salivarius FH29, these strains do not utilise large eDNA fragments for biofilm stabilisation. The two strains of S. anginosus did not produce visible bands of eDNA on gels, even though both strains were sensitive to NucB. It is possible that eDNA from S. anginosus was partially degraded, to the point where it did not form a defined band on a gel, but was still present in sufficient quantities to be utilised for maintaining the biofilm structure.

Improving the surgical treatment of CRS requires new methods for controlling microbial biofilms in the paranasal sinuses. The data presented here demonstrate that many CRS-associated bacteria produce biofilms that can be reduced by treatment with a microbial nuclease NucB in vitro. Given the high prevalence of CRS, even a 50% reduction in the colonization of sinus mucosa by micro-organisms would be predicted to have significant clinical benefits on a population level. Of course, the current study has focussed on in vitro work and it is acknowledged that translating the findings to the clinic will require further investigations in animal models and ultimately in patients. Before this can be done, the safety of NucB for clinical use must be established. We are currently in the process of testing the safety of NucB with a view to conducting clinical trials in future. In addition, it would be interesting to determine whether matrix-degrading enzymes act synergistically with antibiotics to control biofilm growth since this would present additional therapeutic possibilities. Ultimately, the utility of DNase enzymes to aid the treatment of CRS will depend upon in vivo data. Nevertheless, we have shown that NucB has clear potential for the control of biofilms formed by clinically important strains of bacteria.

Supporting Information

Figure S1 Three-dimensional rotation showing micro-organisms associated with the outer layer of a mucosal biopsy. Bacterial DNA was hybridized with the EUB338 PNA-FISH probe, and appears green in the image. Host cell nuclei were counterstained blue. Bacterial cells (punctate green staining) are seen interacting with cells on the surface of the biopsy.
(AVI)

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Author Contributions

Conceived and designed the experiments: RCS MF MJH JGB MR NSJ. Performed the experiments: RCS NM. Analyzed the data: RCS JGB MR NSJ. Contributed reagents/materials/analysis tools: RCS JGB MR NSJ. Wrote the paper: RCS JGB MR NSJ.

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UNDER THE MICROSCOPE

Life after death: the critical role of extracellular DNA in microbial biofilms

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The death and lysis of microbial cells leads to the release of cytoplasmic contents, many of which are rapidly degraded by enzymes. However, some macromolecules survive intact and find new functions in the extracellular environment. There is now strong evidence that DNA released from cells during lysis, or sometimes by active secretion, becomes a key component of the macromolecular scaffold in many different biofilms. Enzymatic degradation of extracellular DNA can weaken the biofilm structure and release microbial cells from the surface. Many bacteria produce extracellular deoxyribonuclease (DNase) enzymes that are apparently tightly regulated to avoid excessive degradation of the biofilm matrix. Interfering with these control mechanisms, or adding exogenous DNases, could prove a potent strategy for controlling biofilm growth.

Introduction

DNA is the genetic coding material, the very essence of life. Yet when a cell dies, the DNA contained within it is not necessarily lost. In fact, DNA is a relatively stable molecule and, in a protected environment, can sometimes survive intact for thousands of years (Hebsgaard et al. 2005; Nicholls 2005). This extracellular DNA (eDNA) accumulates in the environment, where it can form a significant source of organic nutrients. For example, the top 10 cm of deep sea sediments contain an estimated 0 45 gigatons of eDNA, constituting approx. 50% of the total phosphorous pool for the resident micro-organisms (Dell'Anno and Danovaro 2005).

Extracellular DNA is known to cause problems for health. In cystic fibrosis, eDNA derived from neutrophils contributes to the sticky, viscous nature of mucous secretions (Manzenreiter et al. 2012). It is now also becoming clear that eDNA is an important factor in microbial biofilms. The diverse roles of eDNA include disseminating genes amongst different micro-organisms, acting as a nutrient store and strengthening the biofilm matrix. Intriguingly, whilst eDNA is a linchpin for biofilm formation by many micro-organisms, other species appear to have little or no dependence on eDNA. In rare cases, eDNA can even act as a barrier to microbial adhesion (Berne et al. 2010). Similarly, major differences exist in the way that different species release DNA into the environment, and in the capacities of different organisms to uptake free DNA and to incorporate it into their genetic code. Nevertheless, the widespread reliance of micro-organisms on eDNA makes this molecule an attractive target for new approaches to controlling microbial biofilms.

Early descriptions of eDNA

The pioneering experiments of Avery, MacLeod and McCarty in the 1940s demonstrated unequivocally that DNA was the transforming principle for Streptococcus pneumoniae and, in doing so, provided the first clear evidence that DNA is the genetic blueprint for life (Avery et al. 1979). At the same time, this work showed that DNA remains functional when outside bacterial cells. An enzymatic activity was observed in dense cultures of Strep. pneumoniae that was capable of degrading the transforming factor. Therefore, even at this early stage, a picture began to emerge of a dynamic interplay between

bacterial cells and free DNA in the environment. In the following decade, a number of descriptions appeared in the literature of eDNA as a viscous 'slime' surrounding bacterial cells and promoting the formation of a pellicle in broth cultures (Catlin 1956; Catlin and Cunningham 1958). Even Staphylococcus aureus, which produces extensive amounts of extracellular deoxyribonuclease (DNase), accumulated eDNA in broth cultures. In this case, the production of eDNA was enhanced under DNase inhibitory conditions, such as high salt, low pH or low calcium (Catlin and Cunningham 1958). The accumulation of eDNA by Pseudomonas fluorescens was dependent on the concomitant release of RNA, which inhibits extracellular DNase activity. Over the following decades, sporadic reports described physical effects of eDNA on cultured bacterial cells. For example, the slimy eDNA matrix was shown to promote cell clumping in Neisseria gonorrhoeae (Arko et al. 1979). In 2002, an important link was made between eDNA and the structure of biofilms. During investigations of the polysaccharide matrix of Pseudomonas aeruginosa biofilms, it was found that eDNA constituted a major component of the matrix material (Whitchurch et al. 2002). Importantly, treatment of nascent biofilms with DNase I resulted in almost complete removal of cells from the surface. These data demonstrated for the first time that eDNA plays a key structural role in biofilms, and suggested the possibility that DNase enzymes might be applicable for biofilm control.

Extracellular DNA provides structural support for biofilms

The physical properties of the DNA molecule make it ideally suited for providing adhesive support and protection to microbial cells. High molecular weight double-stranded DNA is viscous (Freund and Bernardi 1963) and, when bound to surface of bacterial cells, can promote adhesion to hydrophobic substrata (Das et al. 2011). There is now strong evidence that eDNA is also an important structural component of many different biofilms. For example, characterization of biofilm-inhibiting compounds from marine bacteria identified a potent anti-biofilm molecule produced by an isolate of Bacillus licheniformis recovered from the surface of seaweed (Nijland et al. 2010). After fractionation, the active agent was shown to be the DNase enzyme NucB. NucB dispersed biofilms formed by Micrococcus luteus, Escherichia coli, Bacillus subtilis or B. licheniformis, indicating that eDNA is critical for stabilizing biofilms of each of these organisms. This enzyme also promoted the dispersal of biofilms formed by over 50% of bacterial strains freshly isolated from the paranasal sinuses of patients with chronic rhinosinusitis (Fig. 1) (Shields et al. 2013). A number of studies have now demonstrated that different DNase enzymes can inhibit the formation of biofilms, or can disperse preformed biofilms, of many bacteria and fungi (Table 1). On the other hand, there are also several examples of biofilms that contain significant quantities of eDNA but are not dispersed by DNase enzymes (Lappann et al. 2010; Grande et al. 2011; Shields et al. 2013). Even in cases where DNase treatments do not remove biofilms, it is possible that they may weaken the biofilm structure. Thus, DNase treatment of biofilms has been shown to increase sensitivity to antimicrobials (Tetz et al. 2009; Tetz and Tetz 2010; Kaplan et al. 2012; Martins et al. 2012), sodium dodecyl sulfate (Allesen-Holm et al. 2006) or mechanical removal by sonication (Hu et al. 2012). Conversely, the addition of exogenous DNA to Ps. aeruginosa biofilms increases their tolerance to antimicrobial peptides and to aminoglycoside antibiotics such as tobramycin and gentimicin (Mulcahy et al. 2008; Chiang et al. 2013; Lewenza 2013).

Origins of eDNA in biofilms

Cell lysis provides a simple mechanism for releasing DNA into the environment. In some cases, active mechanisms

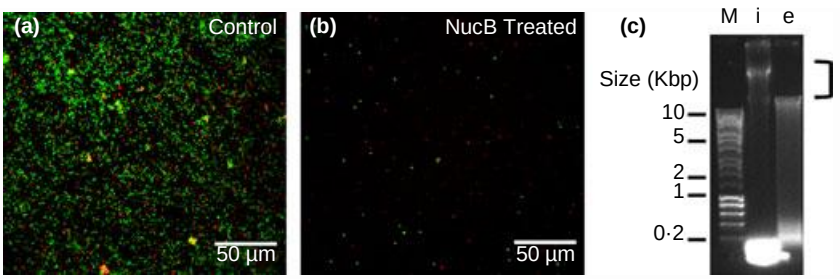

Figure 1 Extracellular DNA stabilizes biofilms of a clinical isolate of Staphylococcus aureus. Biofilms of Staph. aureus FH7 were cultured statically on glass coverslips and treated for 1 h at 37°C with PBS (a) or with PBS containing 3 **l**g ml ^{1} Bacillus licheniformis NucB (b). Cells were stained with BacLight LIVE/DEAD dye and living cells appear green, whereas dead cells stain red. (c) Extracellular DNA (e) extracted from the biofilm contains high molecular weight DNA (>10 kbp), as does intracellular chromosomal DNA (i), and indicated by a square bracket. A marker (M) is shown for comparison. Image was reproduced in part from Shields et al. (2013).

Table 1 Examples of inhibition or dispersal of biofilms by exogenous nucleases

Micro-organism	DNase enzyme	Inhibition (I) or Dispersal (D)*	Reference
Bacteria			
Acinetobacter baumanii, Escherichia coli, Haemophilus influenza, Klebsiella pneumoniae, Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus pyogenes	DNase I	D	Tetz et al. (2009)
Bordetella bronchiseptica, Bordetella pertussis	DNase I	D, I	Conover et al. (2011)
E. coli, Bacillus licheniformis, Bacillus subtilis, Micrococcus luteus	NucB (B. licheniformis)	D	Nijland et al. (2010) Tetz
E. coli, Staph. aureus	DNase I	I	and Tetz (2010) Thomas
Enterococcus faecalis	DNase I	I	et al. (2008) Harmsen et
Listeria monocytogenes	DNase I	D, I	al. (2010) Steichen et al.
Neisseria gonorrhoeae	Nuc (N. gonorrhoeae)	D	(2011) Whitchurch et al.
Ps. aeruginosa	DNase I	D, I	(2002) Nemoto et al.
Ps. aeruginosa	Varidase	D	(2003) Nemoto et al.
Staph. aureus	Varidase	D	(2000) Kaplan et al.
Staph. aureus, Staphylococcus epidermidis	hrDNase I	D, I	(2012) Shields et al.
Staph. aureus, Staph. epidermidis, Staphylococcus lugdunensis, Streptococcus anginosus, Streptococcus constellatus, Streptococcus intermedius, Strepcococcus salivarius	NucB (B. licheniformis)	D	(2013)
Streptococcus pneumoniae (clinical isolates)	hrDNase I	D	Hall-Stoodley et al. (2008)
Fungi			
Candida albicans	DNase I	D	Martins et al. (2010)
Aspergillus fumigatus	DNase I	D	Rajendran et al. (2013)

DNase I, bovine DNase I; hrDNase I, human recombinant DNase I; Varidase, a combination of streptokinase and streptodornase (streptococcal DNase).

*Inhibition of biofilm formation by inclusion of DNase during biofilm development or dispersal of preformed biofilms.

for initiating the lysis of a proportion of a cell population contribute to eDNA release. The existence of autolysis mechanisms has been known for many years, but their importance for releasing DNA and modulating biofilm formation is only beginning to be appreciated. In Enterococcus faecalis, for example, autolysis is mediated by fratricide. Microbial fratricide is a process by which a proportion of the cells in a population of micro-organisms, upon induction, release a factor that kills their siblings. In the case of Ent. faecalis, fratricide occurs in response to a quorum-sensing signal at high cell densities (Thomas et al. 2009). Fratricide is mediated by the protease GelE, which triggers a pathway that results in activation of the primary autolysin AtlA. Immunity to GelE is conferred by the AtlA-modifying protein SprE. A small proportion of cells within a population of Ent. faecalis lack SprE and undergo autolysis in response to GelE, releasing eDNA that stabilizes the biofilm (Thomas et al. 2009). However, the links between autolysis and biofilm formation in Ent. faecalis are still far from clear. Disruption of the alternative sigma factor $\mathbf{r}^{54}$ leads to reduced autolysis and DNA release from cells, but results in the development of relatively thick biofilms (Iyer and Hancock 2012). It is possible that compensatory systems enable biofilm formation in the $\mathbf{r}^{54}$ mutant. Nevertheless, there is accumulating evidence that autolytic and/or fratricidal mechanisms contribute to eDNA release and enhanced biofilm production in a wide range of micro-organisms including Gram-positive and Gram-negative bacteria, and fungi (Allesen-Holm et al. 2006; Yamada et al. 2009; Lappann et al. 2010; Hsu et al. 2011; Liu and Burne 2011; Bose et al. 2012; Christner et al. 2012; Ju et al. 2012; Rajendran et al. 2013). A variety of environmental signals and cues trigger DNA release. For example, in Ps. aeruginosa, the release of DNA is modulated in response to the pqs quorum-sensing system and to changes in the external iron concentration (Allesen-Holm et al. 2006; Yang et al. 2007).

The active release of DNA fragments via lysis-independent mechanisms has been described in bacteria. For example, many Gram-negative organisms produce membrane vesicles that contain DNA either in the lumen or on the outer surface (Dorward and Garon 1990; Schooling et al. 2009). Membrane vesicles originating from bacterial cell surfaces have been observed in Ps. aeruginosa monospecies biofilms and in natural mixed-species biofilms such as early dental plaque (Frank and Houver 1970; Schooling and Beveridge 2006). The production of membrane vesicles by Pseudomonas spp. promotes biofilm formation (Baumgarten et al. 2012). It is possible, although by no means confirmed, that vesicle-mediated delivery of DNA to the biofilm matrix contributes to

enhanced biofilm production. Cell lysis–independent DNA release has recently been described in an undomesticated strain of the Gram-positive species B. subtilis and appears to be linked to the early competence pathway (Zafra et al. 2012). In N. gonorrhoeae, an unusual type IV secretion system (T4SS) provides an alternative mechanism for the extrusion of DNA into the extracellular milieu (Hamilton et al. 2005). Unlike most T4SSs, the N. gonorrhoeae system does not require cell–cell contact to function. N. gonorrhoeae biofilms are stabilized by an extensive matrix of eDNA (Steichen et al. 2011). At present, the relative contributions of cell lysis, outer membrane vesicle production and the T4SS to the overall matrix composition are unknown.

Structural composition of eDNA in biofilms

The primary sequence of eDNA is no different from that of intracellular DNA (Zafra et al. 2012), but there is mounting evidence that there are other differences between eDNA and intracellular DNA, at least in some cases. DNA secreted by the N. gonorrhoeae T4SS, for example, requires an origin of transfer (oriT) and sequences of base pairs adjacent to oriT will therefore be enriched in eDNA compared with sites at distal portions of chromosome. The secreted DNA is single stranded and protected from 5′–3′ exonuclease digestion, possibly by interaction with a protein at the 5′ end (Salgado-Pabon et al. 2007). The binding of eDNA by proteins has also been observed in several other systems. In Staph. aureus biofilms, eDNA catalyses the covalent cross-linking of extracellular beta toxin, resulting in a stable nucleoprotein matrix (Huseby et al. 2010). The Strep. pneumoniae extracellular cell wall hydrolase LytC binds DNA in a nonsequence-specific manner and forms nucleoprotein complexes in biofilms (Domenech et al. 2013). Neisseria meningitidis also forms extracellular nucleoprotein structures through interactions of the heparin-binding antigen NhbA and the alpha-peptide of IgA protease with eDNA (Arenas et al. 2013). In the Gram-negative soil bacterium Myxococcus xanthus, eDNA forms complexes with exopolysaccharides resulting in biofilms with increased mechanical strength and adhesion capacity (Hu et al. 2012). Binding of exogenous DNA to membrane vesicles in the Ps. aeruginosa biofilm matrix has been observed, although it is not yet clear what role this plays in the structure or function of the biofilm (Schooling et al. 2009).

Extracellular DNA in biofilms can be visualized using fluorescent dyes, even in complex fungal/bacterial biofilms such as those present on fouled tracheoesophageal speech valves (Fig. 2). Imaging of eDNA can be enhanced using labelled anti-DNA immunoglobulins (Barnes et al. 2012). When extracted and purified from biofilms, eDNA often appears to be similar in structure to chromosomal DNA. In many cases, eDNA forms a sharp band on agarose gels with an apparent migration of >10 kbp (Fig. 1c) (Shields et al. 2013). Analysis of eDNA by multilocus PCR or by random amplification of polymorphic DNA (RAPD) PCR has shown that eDNA is very similar in sequence composition to chromosomal DNA in some organisms (Allesen-Holm et al. 2006; Qin et al. 2007; Rajendran et al. 2013),

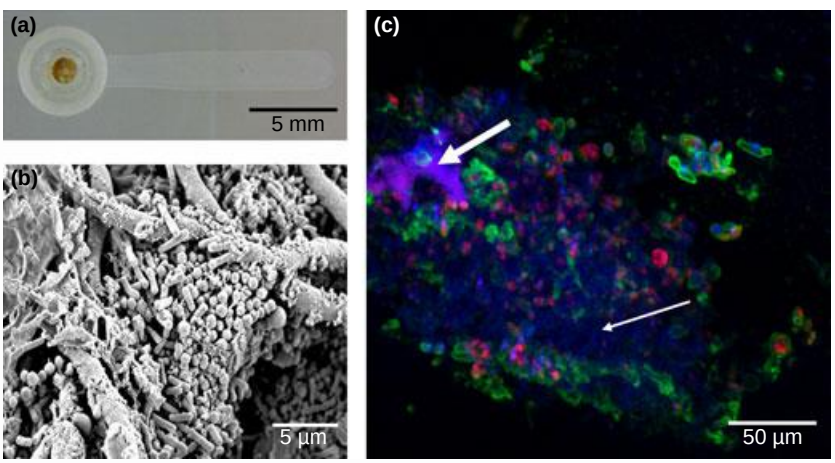

Figure 2 Observation of extracellular DNA (eDNA) in biofilms on fouled tracheoesophageal speech valves. (a) Voice prostheses become blocked during usage with a thick microbial biofilm which is visible to the naked eye (brown discolouration in the centre of the valve). (b) High-resolution imaging by scanning electron microscopy demonstrates that biofilms contain different types of cells including cocci and rod shaped bacteria, and fungal hyphae. (c) Biofilms were stained with propidium iodide (red) and 4′,6-diamidino-2-phenylindole (DAPI; blue) to highlight dead cells and eDNA, respectively, and Alex Fluor-conjugated concanavalin A (green), which labels fungal cell walls. eDNA is abundant within the biofilm (thin arrow). In some regions, propidium iodide co-localized with DAPI (thick arrow), probably indicating a localized region with a high concentration of eDNA.

but markedly different in others (Steinberger and Holden 2005; Böckelmann et al. 2006; Grande et al. 2011). In the latter case, differences may arise from selective DNA secretion, as exemplified by the N. gonorrhoeae T4SS described above, or from modification of eDNA within the biofilm matrix. However, it is not clear at present which sequences are enriched in eDNA, or whether the enrichment of sequences impacts on the rate of transfer of specific genes between cells. It is also possible that eDNA differs chemically from DNA inside cells, for example, by having different patterns of methylation. Clearly, there are many unanswered questions about the structure of eDNA within biofilms. Further investigations in this area will be important not only for the understanding of the function of eDNA in biofilms, but also for the accurate interpretation of data from studies that employ DNA amplification methodologies to analyse microbial biofilm communities.

Extracellular DNA turnover

Many bacteria produce extracellular DNase enzymes, which are anchored to the cell wall or secreted into the extracellular milieu. The ability of different strains to produce DNases does not appear to correlate with reduced dependence on eDNA for biofilm formation (Shields et al. 2013). However, studies with isogenic mutants have provided evidence that extracellular nucleases keep the growth of biofilms in check. In Shewanella oneidensis, the disruption of genes encoding two extracellular nucleases, ExeS and ExeM, resulted in altered biofilm formation and the accumulation of eDNA (Godeke et al. 2011). Disruption of a third nuclease-encoding gene, endA, had little effect on biofilm structure (Heun et al. 2012). Increased eDNA and biofilm formation has also been observed in DNase-defective mutants of Vibrio cholerae or N. gonorrhoeae (Seper et al. 2011; Steichen et al. 2011). In Staph. aureus, the disruption of nuc1 or nuc2 genes encoding two extracellular nucleases resulted in increased biofilm formation in vitro (Kiedrowski et al. 2011; Beenken et al. 2012). However, the introduction of nuclease mutants into a murine catheter biofilm model resulted in reduced biofilm, determined as an increase in daptomycin sensitivity compared with the isogenic wild type (Beenken et al. 2012). It is likely that these nucleases have additional roles in vivo that are not required in simple in vitro models. One key in vivo function of microbial nucleases is the degradation of neutrophil extracellular traps, which enables bacteria to escape from host immune systems (Beiter et al. 2006; Buchanan et al. 2006; Berends et al. 2010). It remains to be determined whether the degradation of host DNA is important for the development of Staph. aureus biofilms on artificial surfaces in vivo.

Extracellular microbial nucleases play a number of key roles in bacteria, and their functions are likely facilitated by the proximity between bacterial cells and eDNA in the biofilm matrix. For example, the degradation of eDNA by DNases mobilizes nucleotides that can be scavenged by micro-organisms as a source of carbon, nitrogen and phosphorous (Pinchuk et al. 2008; Mulcahy et al. 2010). Extracellular DNA chelates metal ions (Mulcahy et al. 2008), and it is possible that extracellular nucleases promote the scavenging of trace metals by biofilm bacteria. Extracellular DNases also contribute to genetic transformation. Thus, the DNase activity observed by Avery and colleagues (Avery et al. 1979), and later identified as the cell wall-bound enzyme EndA, nicks eDNA to provide single-stranded substrate for the DNA uptake machinery and is essential for transformation (Berge et al. 2002; Moscoso and Claverys 2004). It is not clear whether extracellular nucleases also have roles in the turnover of DNA within cells, or whether they remain inactive until they are outside the cell.

Potential of exogenous nucleases for biofilm control

The identification of eDNA as a critical molecule for diverse biofilms immediately suggests a potential approach for biofilm control by degrading the eDNA either with chemical agents or with enzymes. The latter approach is more likely to be viable because enzymes, unlike chemical agents, are highly selective for the eDNA component of the biofilm. Enzymatic degradation of eDNA could potentially be achieved either by up-regulating endogenous DNases within the biofilm or by adding nucleases exogenously. To induce the expression of endogenous DNases, the regulation of these systems must be understood. Many bacterial nucleases are controlled at the level of transcription. For example, in Staph. aureus, expression of Nuc is repressed by sigma factor B or glucose (Kiedrowski et al. 2011; Schulthess et al. 2011). Streptococcus pyogenes extracellular nucleases are regulated by the peroxide response regulator PerR and the nutrient stress-responsive transcriptional regulator CodY (Wen et al. 2011; McDowell et al. 2012). Nuclease production by V. cholerae is linked to quorum sensing and competence through the HapR regulator (Blokesch and Schoolnik 2008). HapR becomes active at high cell densities and represses nuclease expression, allowing the accumulation of eDNA for genetic transformation. In Clostridium perfringens, expression of the extracellular nuclease CadA is controlled by the virulence-related VirR/VirS two-component system (Okumura et al. 2005). Clearly, micro-organisms possess many different systems for regulating the expression of extracellular nucleases, and approaches

to control biofilm formation based on stimulating intrinsic nuclease expression are still some way off.

The exogenous addition of nucleases may be a simpler approach for biofilm control. Bacterial and mammalian DNases have been shown to disperse monospecies biofilms in vitro (Table 1). Bovine DNase I dramatically reduces the thickness of dual-species Ps. aeruginosa/Staph. aureus biofilms, and B. licheniformis NucB promotes the dispersal of mixed-species biofilms from tracheoesophageal speech valves (Yang et al. 2011; Shakir et al. 2012), indicating that DNases may be broadly applicable for control of natural mixed-species biofilms. Varidase and human recombinant DNase I (hrDNase I; marketed as Pulmozyme or Dornase Alfa) have been employed in patients. Varidase contains a DNase (streptodornase) and a plasminogen activator (streptokinase). This has previously been used to promote wound healing (Smith et al. 2011), but has been withdrawn in most countries. Human recombinant DNase is employed clinically as a mucolytic for the treatment of cystic fibrosis. There is some evidence that hrDNase I can disperse biofilms in vitro (Hall-Stoodley et al. 2008; Kaplan et al. 2012), although it is not clear whether this activity occurs in vivo. A drawback of mammalian DNases such as hrDNase I is that they require glycosylation for activity and cannot therefore be produced in bacterial expression systems (Fujihara et al. 2008), which dramatically increases the costs of production and limits their potential applications. The purification of bacterial DNases can also be problematic, because they are often poorly tolerated in E. coli. For example, expression of Strep. pneumoniae EndA in E. coli is difficult unless active site residues are mutated (Midon et al. 2012). Further medical and biotechnological application of nucleases will require systems for their improved production, such as the optimized B. licheniformis NucB expression system that has recently been described (Rajarajan et al. 2012).

Conclusions

Over the last decade or so, it has become clear that eDNA is a major structural component of many different microbial biofilms. Numerous reports have described the disruption of biofilms in vitro by digestion of the matrix with DNase enzymes. The next challenge is to move these studies into more natural biofilm systems, where conditions tend to be less than optimal for DNase enzyme activity. Ultimately, the ability to employ DNases as biofilm control enzymes may depend on the development of enzymes that are robust, safe and active over a wide range of temperatures and pH values. It is unlikely that a single enzyme will be a 'magic bullet', capable of destroying all problematic biofilms. However, even small successes in this field could provide major benefits for biofilm control.

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Conflicts of interest

No conflict of interest declared.

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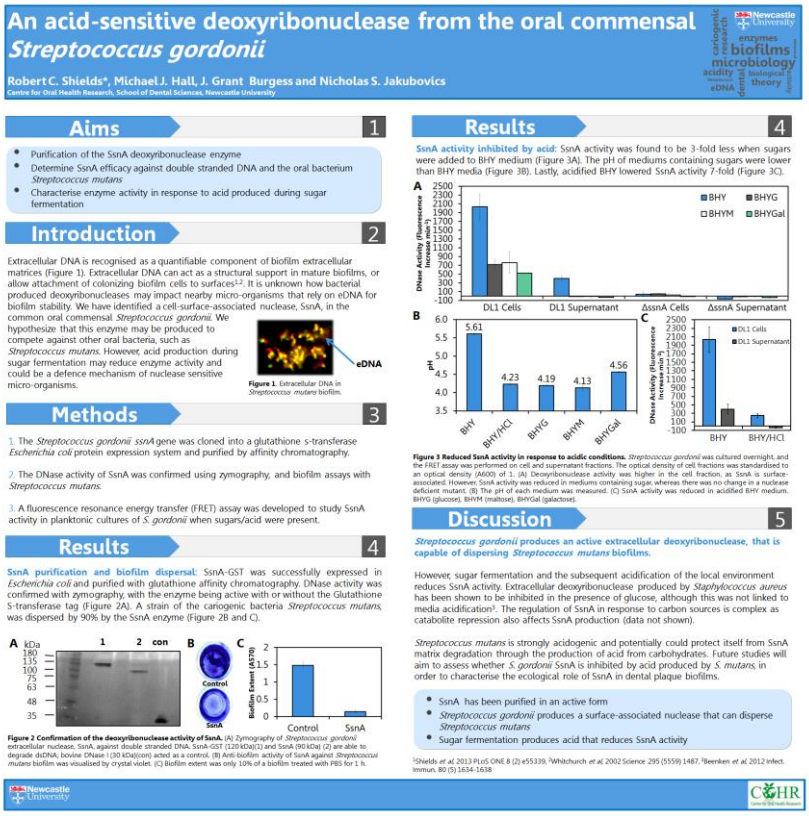
Appendix B: Conference and Meeting Attendance

31/10/2013: SfAM PECS Research Conference 2013, London (Poster Presentation)



Title: An acid-sensitive deoxyribonuclease from the oral commensal *Streptococcus gordonii*

The biofilm extracellular matrix can contain significant amounts of extracellular DNA which has a range of functions, including acting as a biofilm adhesin and increasing matrix stability. We have identified a surface-associated nuclease, SsnA, in the common oral commensal *Streptococcus gordonii*. The aim of this work was to assess the role of SsnA in disrupting biofilms produced by the cariogenic species *Streptococcus mutans* and the impact of acid on SsnA activity. The *S. gordonii ssnA* gene was fused to a Glutathione S-transferase (GST) tag in an *Escherichia coli* protein expression system and purified by affinity chromatography. The deoxyribonuclease activity of SsnA against double stranded DNA was confirmed using zymography. SsnA inhibited the attachment of *S. mutans* or dispersed *S. mutans* biofilms, grown in 96-well microtiter plates, by up to 90% compared with controls. Using fluorescence resonance energy

transfer assays, SsnA activity was rapidly inhibited by the presence of acid (pH 4.5). *S. mutans* is strongly acidogenic and potentially could protect itself from SsnA matrix degradation through the production of acid from carbohydrates. Future studies will aim to assess whether *S. gordonii* SsnA is inhibited by acid produced by *S. mutans* cultured with sucrose in order to characterise the ecological role of SsnA in dental plaque biofilms.

10/09/2013: Eurobiofilms 2013, Ghent University, Belgium (Oral Presentation)

Title: Removal of Oral Biofilms by a Marine Microbial Deoxyribonuclease

Objectives: The biofilm matrix can contain significant amounts of extracellular DNA (eDNA), which has a number of key functions including acting as an adhesin and a scaffold during biofilm formation. The aims of this study were to determine if eDNA is present in oral biofilms and to investigate whether biofilms are sensitive to removal by a novel marine bacterial nuclease, NucB.

Methods: Extracellular DNA in the biofilm matrices of *Streptococcus gordonii* DL1, *Actinomyces oris* MG1, *Streptococcus mutans* GS-5 and *Fusobacterium nucleatum* 25586 was extracted and quantified. Extracellular DNA was visualised in intact biofilms using confocal laser scanning microscopy (CLSM). Species were grown in 96-well plates and treated with bovine DNase I to assess its efficacy at removing forming, and pre-formed biofilms. A similar model was employed to evaluate anti-biofilm activity of NucB against mixed-species biofilms cultured from human saliva.

Results: *S. mutans*, *F. nucleatum* and *A. oris* produced eDNA that migrated as a sharp band during agarose gel electrophoresis. Microscopy showed eDNA in *A. oris* biofilms and less clearly in *S. mutans* and *F. nucleatum* biofilms. Biofilm biomass was reduced by up to 90% following treatment with DNase I. *S. gordonii* biofilms contained little eDNA and were not susceptible to DNase I. NucB caused a significant reduction in 10 of the 24 saliva biofilms tested.

Conclusions: The data provide clear evidence that biofilms formed by oral bacteria rely on eDNA for structural support. Further studies will determine whether NucB can be used clinically for oral biofilm control.

08/05/2013: COHR Research Afternoon, Newcastle (Oral Presentation) – 1st Place

Title: Oral Biofilms Contain Extracellular DNA and are Dispersed by DNase I

Objectives: Extracellular DNA (eDNA) is released by some oral bacteria but it is unknown if this contributes to oral biofilm matrix stability. The aims of this study were to determine if eDNA is present in oral biofilms and investigate if deoxyribonuclease I (DNase I) can disperse these biofilms.

Methods: The abundance of eDNA in the established biofilm matrices of 4 oral bacteria, *Streptococcus gordonii* DL1, *Actinomyces oris* MG1, *Streptococcus mutans* GS-5 and *Fusobacterium nucleatum* 25586 was quantified via DNA extraction. Extracellular DNA was visualised with confocal laser scanning microscopy using LIVE/DEAD® BacLight™ to stain DNA surrounding the bacterial cells. Each species was grown in a 96-well plate biofilm model and treated with bovine DNase I to assess its efficacy at removing forming, and pre-formed biofilms.

Results: *Streptococcus mutans*, *Fusobacterium nucleatum* and *Actinomyces oris* produced enough eDNA to visualise it as a sharp band during agarose gel electrophoresis. For *S. mutans* and *F. nucleatum*, eDNA accounted for between 20-50% of the total DNA present. Microscopy revealed eDNA in *A. oris* biofilms. For *S. mutans* and *F. nucleatum* eDNA was less visible although it appears to be cell bound. The addition of DNase I to *A. oris*, *S. mutans* and *F. nucleatum* biofilms caused dispersal and inhibition of up to 90%. By contrast, *Streptococcus gordonii* biofilms contained little eDNA and were not susceptible to DNase I.

Conclusions: The data provide clear evidence that some oral biofilms depend on eDNA for structural support. Further studies are needed on *in vivo* grown dental plaque to elucidate any therapeutic potential.

25/03/2013: ICaMB's Postgraduate Research Symposium, Dental Lecture Theatre D, Newcastle (Oral Presentation)

Title: From Seaweed to Sinuses – Extracellular DNA in Biofilms

A biofilm is a microbial community that is attached to a surface and encased in a hydrated polymeric matrix. In a biofilm, microbes exhibit greater resistance both to antimicrobial agents and the host immune system. Resistance is due, in part, to the robust polymeric matrix. Research has shown that the matrix contains significant amounts of extracellular DNA (eDNA) and that eDNA plays a number of key functions including acting as an adhesin and a scaffold for biofilm attachment. These observations pave the way for the development of novel anti-biofilm strategies employing DNA degrading enzymes (DNases). The aims of this project were to quantify the amount of eDNA and the potential of nucleases to disperse biofilms of relevance to the head and neck, including those associated with voice prostheses, chronic rhinosinusitis, and dental caries/periodontitis. Notably, this project began in marine science where a seaweed dwelling bacteria, *Bacillus licheniformis*, was found to produce an anti-biofilm compound that was later identified as a nuclease, NucB. Later, working in collaboration with the Ear, Nose and Throat department of the Freeman Hospital, Newcastle this enzyme was applied to testing its efficacy against biofilms that foul voice prostheses and those that cause chronic rhinosinusitis. Voice prostheses are given to patients who've received a total laryngectomy following cancer, as a way of rehabilitating speech. However, these silicon devices become damaged by the microbial biofilms that grow on the surface. With Chronic Rhinosinusitis, it is thought that bacterial biofilms contribute to the inflammation of the sinuses and/or block the sinuses. In both cases NucB was shown to be effective against these biofilms in the models employed. With chronic rhinosinusitis, a disease that affects 10% of the European population, the sinuses were found to contain many species of bacteria, of which several also produced eDNA, giving a reason for their susceptibility to NucB.

For dental plaque, a complex mixed-species biofilm, research began by quantifying the eDNA found in the biofilm matrices of *Actinomyces oris*, *Streptococcus mutans*, *Fusobacterium nucleatum* and *Streptococcus gordonii* – four important oral bacteria. These biofilms contained eDNA, and three of the species, *A. oris*, *S. mutans*, *F. nucleatum*, were dispersed by bovine DNase I. NucB was used to treat mixed-species

biofilms grown from human saliva inoculae and had a variable effect, leading to a reduction in 10 of the 24 biofilms tested. *Streptococcus gordonii*, an important early colonizer of dental plaque produces an extracellular nuclease, SsnA. To further understand the role of this enzyme in *S. gordonii* the *ssnA* gene was cloned into an *Escherichia coli* protein expression system and the protein was purified using affinity chromatography. The activity of the enzyme against dsDNA was verified using zymography. SsnA also dispersed the biofilm of *S. mutans* GS-5. Notably, this enzyme was down-regulated in response to the low pH environments created by sugar fermentation. This could have implications in the oral environment, which is characterized as having periods of acidity, thus stopping the use of this enzyme by *S. gordonii* at particular time points.

Taken together, the data indicate that eDNA is an important component in many clinically relevant biofilm forming bacteria. What has emerged is that total eDNA and the efficacy of nucleases against microbial biofilms is highly variable. This variation and the application of more complex mixed-species biofilm models is the next vital avenue of research. Fundamentally, why do bacteria expend energy creating the molecule of heredity, DNA, only to discard it into the matrix around them?

26/10/2012: North East Postgraduate Conference, The Hancock Museum, Newcastle (Poster Presentation)

Presented the same poster as that at the SGM Autumn Conference.

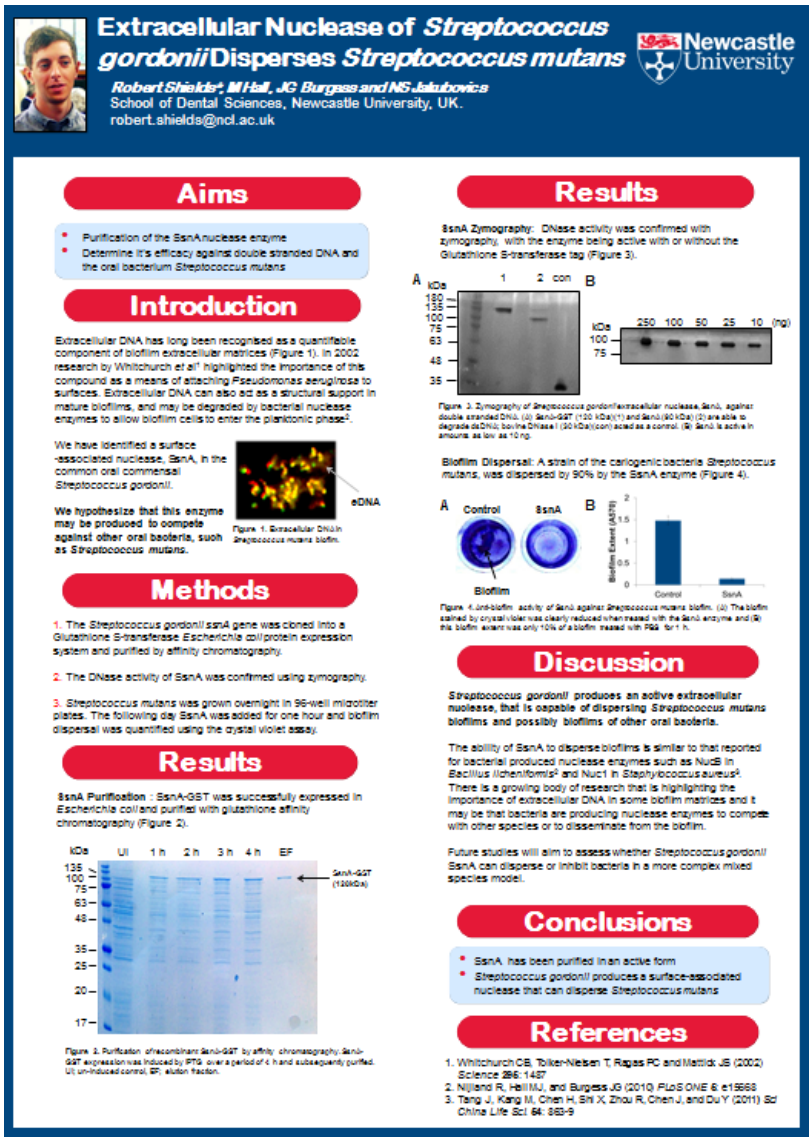
03/09/2012: SGM Autumn Conference 2012, Warwick University (Poster Presentation)

Title: Characterization of a Surface-associated Nuclease of *Streptococcus gordonii*

The biofilm extracellular matrix contains significant amounts of extracellular DNA (eDNA) which has a range of functions, including acting as a biofilm adhesin and a structural support. However, the eDNA may also be a target for matrix turnover and degradation. Thus, we have identified a surface-associated nuclease, SsnA, in the common oral commensal *Streptococcus gordonii*. The aim of this work was to assess the role of SsnA in disrupting biofilms produced by different oral bacteria. The

Streptococcus gordonii ssnA gene was cloned into a Glutathione S-transferase (GST) *Escherichia coli* protein expression system and purified by affinity chromatography. The activity of SsnA was confirmed using zymography, showing that the purified protein degraded double stranded DNA. SsnA was able to disperse or inhibit the attachment of oral bacteria, grown in 96-well microtiter plates. This included strains of the cariogenic bacteria *Streptococcus mutans*, which were dispersed by up to 90% by the enzyme. Future studies will aim to assess whether *S. gordonii* can disperse or inhibit bacteria in a mixed species model. It may be that this enzyme plays an important role in the plaque biofilm by reducing the abundance of oral bacteria that rely on extracellular DNA in their biofilm matrices.

Poster:



26/04/2012: Oral Microbiology and Immunology Group Postgraduate Meeting, Newcastle (Oral Presentation)

Title: Extracellular DNA in Biofilms Associated with Chronic Rhinosinusitis

Chronic Rhinosinusitis (CRS) affects approximately 10% of the adult European population. It is an inflammatory disease that is often triggered by bacterial biofilms in the paranasal sinuses. Healthy sinuses are non-sterile but there is an important microbiological flora change in CRS, with species such as *Streptococcus pneumoniae* and *Staphylococcus aureus* being more frequently isolated in disease. Oral bacteria, including *Fusobacterium* spp. and *Prevotella* spp. can also be found in the sinuses of CRS sufferers, indicating that there is a connection between the oral cavity and the paranasal sinuses. Increasingly, it appears that biofilm forming bacteria utilise extracellular DNA (eDNA) for initial adhesion to surfaces and for biofilm matrix stability. In addition, some bacteria produce extracellular DNases to remodel biofilm matrices. This project aimed to investigate the role of eDNA and microbial DNases in biofilms formed by CRS-associated bacteria.

Twenty patients diagnosed with CRS were recruited to the study. Microbiological analysis of aspirates removed during functional endoscopic sinus surgery revealed an average of 3.75 bacterial isolates per patient (75 isolates in total). Bacteria associated with CRS included coagulase-negative staphylococci, corynebacteria, streptococci, and *S. aureus*. Thirteen (17%) strains produced an extracellular DNase. All *S. aureus* isolates produced an extracellular DNase, and other producers were *Streptococcus milleri* group strains, *Staphylococcus lugdunensis* and *Streptococcus salivarius*. Selected isolates were cultured in 96-well microtiter plates to determine their ability to form biofilms. All tested isolates were capable of forming biofilms, although the extent of formation was species and strain specific. The role of eDNA was investigated by treating biofilms with a bacterial DNase. This enzyme removed between 0-60% of biofilm in different monoculture systems. Taken together, these results demonstrate that CRS-associated bacteria utilise eDNA in biofilms, and that some CRS bacteria have the potential to remodel this component of the matrix.

18/04/2012: Society for Applied Microbiology Spring Meeting, Stratford-upon-Avon (Attended Meeting)

17/11/2011: ICaMB Postgraduate Conference, Baddiley-Clark, Newcastle (Oral Presentation)

12/09/2011: British Society for Oral and Dental Research Annual Meeting, Sheffield (Oral Presentation)

Title: Reducing Dental Plaque Formation with a Novel Bacterial Nuclease

Objectives: Although extracellular DNA (eDNA) is known to be a significant component of many environmental and health-associated biofilm matrices, its role in oral biofilms is unclear. We have identified a novel extracellular nuclease (NucB) from *Bacillus licheniformis*, which can disperse biofilms by degrading constituent eDNA. The aim of this investigation was therefore to determine whether model dental plaque biofilms can be disrupted using this enzyme.

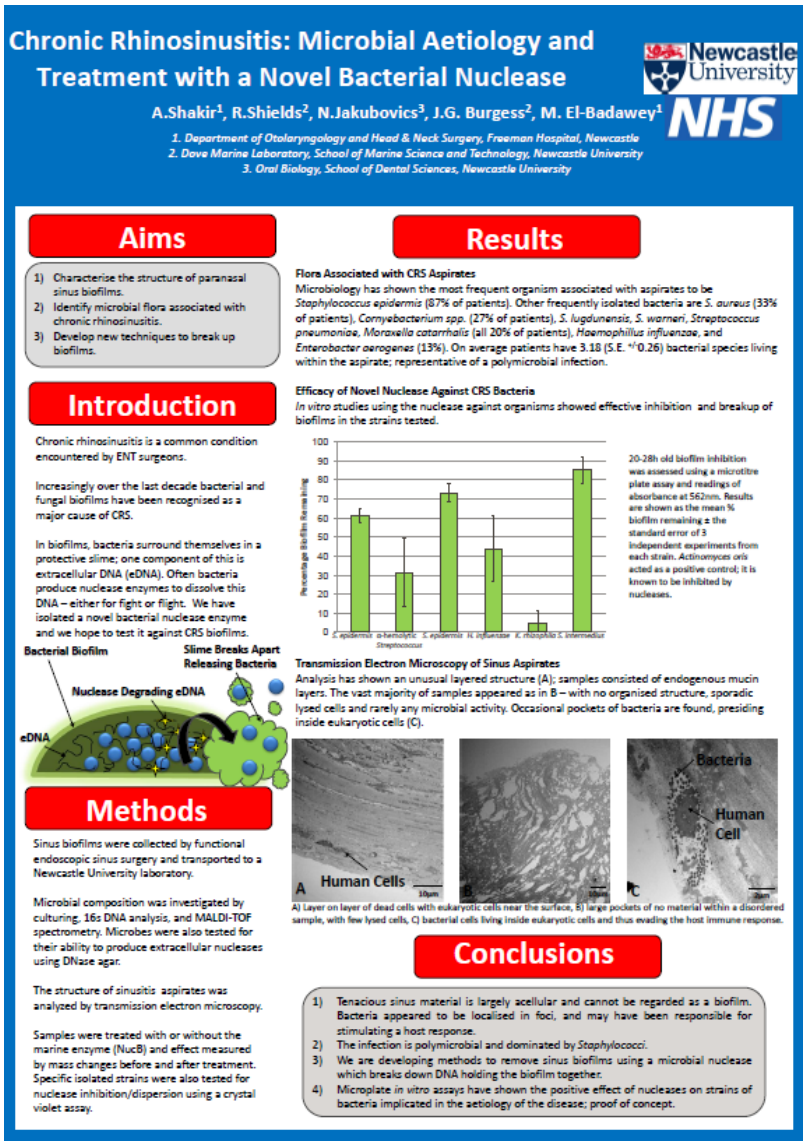
Methods: Biofilms were developed on the surface of polystyrene microtitre plates from strains of oral bacteria or from natural microbial populations in human saliva. Salivary inoculae from 6 volunteers were used for establishing mixed-species biofilm populations. Mixed species biofilms were incubated for up to 20 hours at 37^{o}C either aerobically in the presence of 2% sucrose or anaerobically. Single strains were incubated anaerobically. NucB (1 µg ml^{-1}) was applied to cultures either before or after biofilm development. Residual biofilm was quantified by staining with crystal violet.

Results: NucB inhibited and/or dispersed a variety of single strain and mixed-species oral biofilms. In mixed-species cultures, reductions in biofilm biomass of up to 66% were observed when NucB was added prior to biofilm formation compared with untreated controls. Addition of NucB to preformed biofilms resulted in up to 42% reduction of the attached biofilm. Certain individual bacterial strains were extremely sensitive to NucB; biofilm extent was reduced by up to 90% in the presence of the enzyme. Nuclease sensitivity of biofilms from natural salivary inoculae was dependent on incubation conditions (anaerobic versus aerobic with sucrose) and the time span of biofilm development.

Conclusions: We have shown that the addition of nuclease inhibits and disperses biofilms grown *in vitro*, suggesting that eDNA may play a role in oral biofilm formation *in vivo*. Therefore, microbial nucleases such as NucB may be highly effective at controlling the accumulation of dental plaque.

23/05/2011: Marine Science and Technology Postgraduate Symposium, Newcastle (Oral Presentation) – 1st Place

13/05/2011: British Rhinological Society Annual Meeting, Edinburgh (Poster Presentation) – 3rd Place

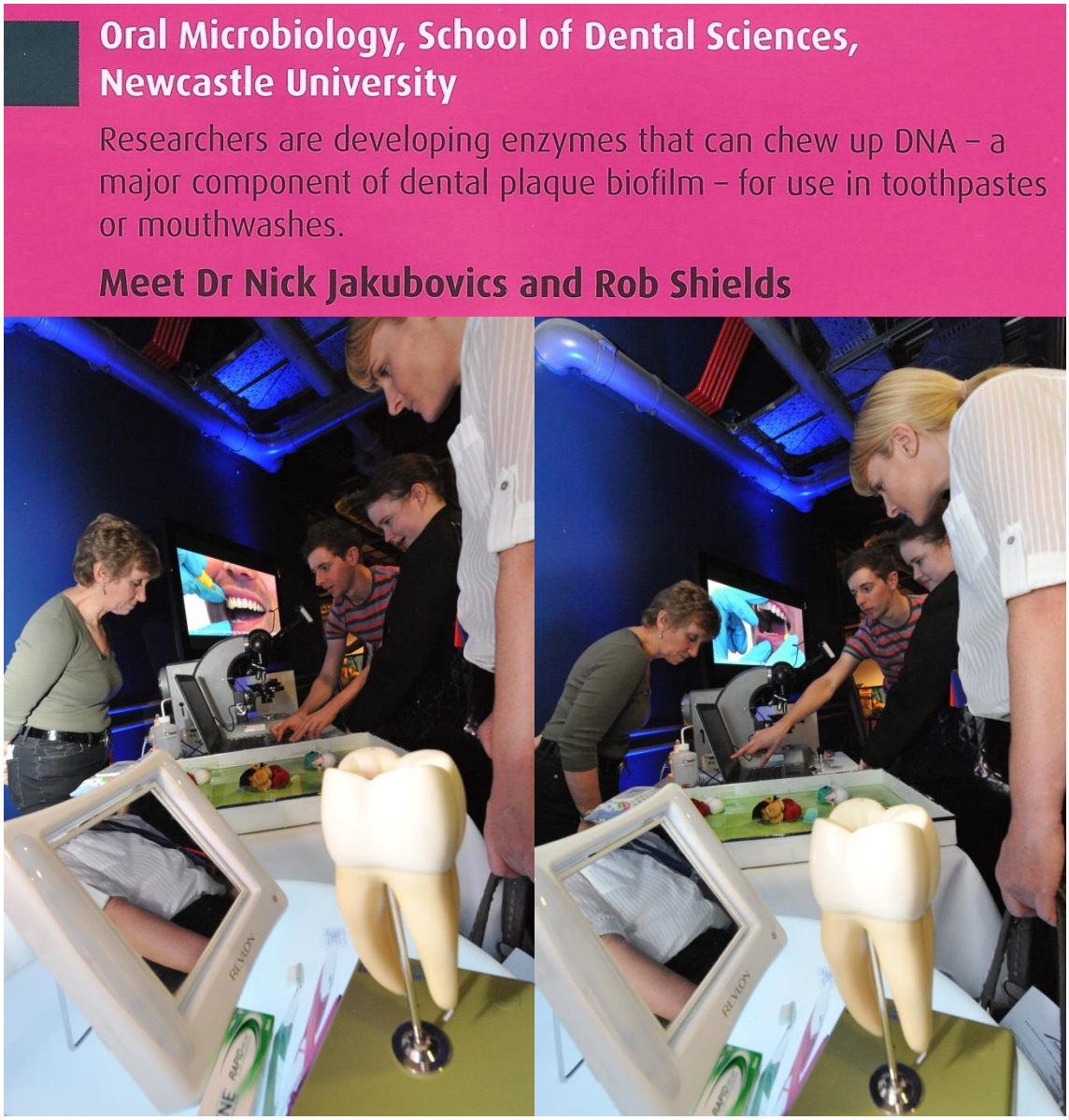


08/04/2011: Scottish and Northern Periodontal Research Group Conference 2011, Old Library Building (Attended Meeting)

Appendix C: Public Engagement

Newcastle Science City Showcase

Held at the Centre for Life in Newcastle city centre, this event was aimed at showcasing the rich diversity of scientific discovery in Newcastle. Our event allowed children and adults to explore the oral biofilm with active demonstrations of plaque bacteria with a microscope, plaque disclosing tablets and competitions for fluffy microbes!



Chronic Rhinosinusitis Press Release 2013

Coinciding with the publication of a paper in PLoS ONE there was a press release that featured on TV, radio and internationally in newspapers.

In Brief

Microbe found on seaweed could clear up sinusitis

A team of scientists and surgeons are developing a nasal spray from a marine microbe to help clear chronic sinusitis.

They are using an enzyme isolated from a marine bacterium, *Bacillus licheniformis*, found on the surface of seaweed, which scientists at Newcastle University were originally researching for cleaning the hulls of ships.

Publishing in *PLoS ONE*, they describe how in many cases of chronic sinusitis the bacteria form a slimy protective barrier, which can protect them from sprays or antibiotics. In vitro experiments showed that the enzyme dispersed the barrier.

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A solution to sinusitis from the sea

Share:

A team of scientists and surgeons from Newcastle are developing a new nasal spray from a marine microbe to help clear chronic sinusitis.

They are using an enzyme isolated from a marine bacterium *Bacillus licheniformis* found on the surface of seaweed which the scientists at Newcastle University were originally researching for the purpose of cleaning the hulls of ships.

Publishing today in PLOS ONE, they describe how in many cases of chronic sinusitis the bacteria form a biofilm, a slimy protective barrier which can protect them from sprays or antibiotics. In vitro experiments showed that the enzyme, called NucB dispersed 58% of biofilms.

Dr Nicholas Jakubovics of Newcastle University said: "In effect, the enzyme breaks down the extracellular DNA, which is acting like a glue to hold the cells to the surface of the sinuses. In the lab, NucB cleared over half of the organisms we tested."

Sinusitis with or without polyps is one of the most common reasons people go to their GP and affects more than 10% of adults in the UK and Europe. Mr Mohamed Reda Elbadawey, Consultant of Otolaryngology Head and Neck Surgery, Freeman Hospital – part of the Newcastle Hospitals NHS Foundation Trust – was prompted to contact the Newcastle University researchers after a student patient mentioned a lecture on the discovery of NucB and they are now working together to explore its medical potential.

Mr Elbadawey said: "Sinusitis is all too common and a huge burden on the NHS. For many people, symptoms include a blocked nose, nasal discharge or congestion, recurrent headaches, loss of the sense of smell and facial pain. While steroid nasal sprays and antibiotics can help some people, for the patients I see, they have not been effective and these patients have to undergo the stress of surgery. If we can develop an alternative we could benefit thousands of patients a year."

In the research, the team collected mucous and sinus biopsy samples from 20 different patients and isolated between two and six different species of bacteria from each individual. 24 different strains were investigated in the laboratory and all produced biofilms containing significant amounts of extracellular DNA. Biofilms formed by 14 strains were disrupted by treatment with the novel bacterial deoxyribonuclease, NucB.

www.chroniclelive.co.uk

Researchers discover marine microbe can help illness

Medical team makes waves with finding

HELEN RAE
Health Reporter

RESEARCH in Tyneside is helping to develop a nasal spray to clear sinusitis.

A team of scientists and surgeons have discovered that a marine microbe could be the answer to dealing with the chronic condition.

Experts are using an enzyme isolated from a marine bacterium Bacillus licheniformis found on the surface of seaweed, which scientists at Newcastle University were originally researching for the purpose of cleaning the hulls of ships.

Some forms of sinusitis is caused by a bacteria which forms a biofilm and acts as a glue to hold the cells to the lining of the sinuses, acting as a barrier to sprays or antibiotics.

Scientists have now found that a marine enzyme called NucB, dispersed 58% of biofilms and therefore could be used to help clear sinusitis.

Dr Nicholas Jakubovics of Newcastle University said: "In effect, the enzyme breaks down the extra-cellular DNA, which is acting like a glue to hold the cells to the surface of the sinuses. In the lab, NucB cleared over half of the organisms we tested.

"Sinusitis is a painful condition that affects 10-15% of the population.

"Symptoms are very uncomfortable and we can help those with the condition.

"Some patients have to undergo surgery to clear their sinuses and this takes approximately an hour in the operating theatre.

"If we can develop a treatment that can prevent the need for surgery then this will in turn save money for the NHS."

Scientists are now looking to test their groundbreaking nasal spray and are currently in talks with drug manufacturers to undergo toxicology tests to assess the safety of the treatment.

"We are just in the preliminary stages, but we believe the treatment is safe and effective," said Dr Jakubovics.

Seaweed to stop decay

SEAWEED from ships' hulls has also been identified as helping in the fight against tooth decay.

Newcastle University scientists discovered that seaweed could be used to destroy bacteria in plaque which can cause cavities.

It is believed this could lead to better dental products.

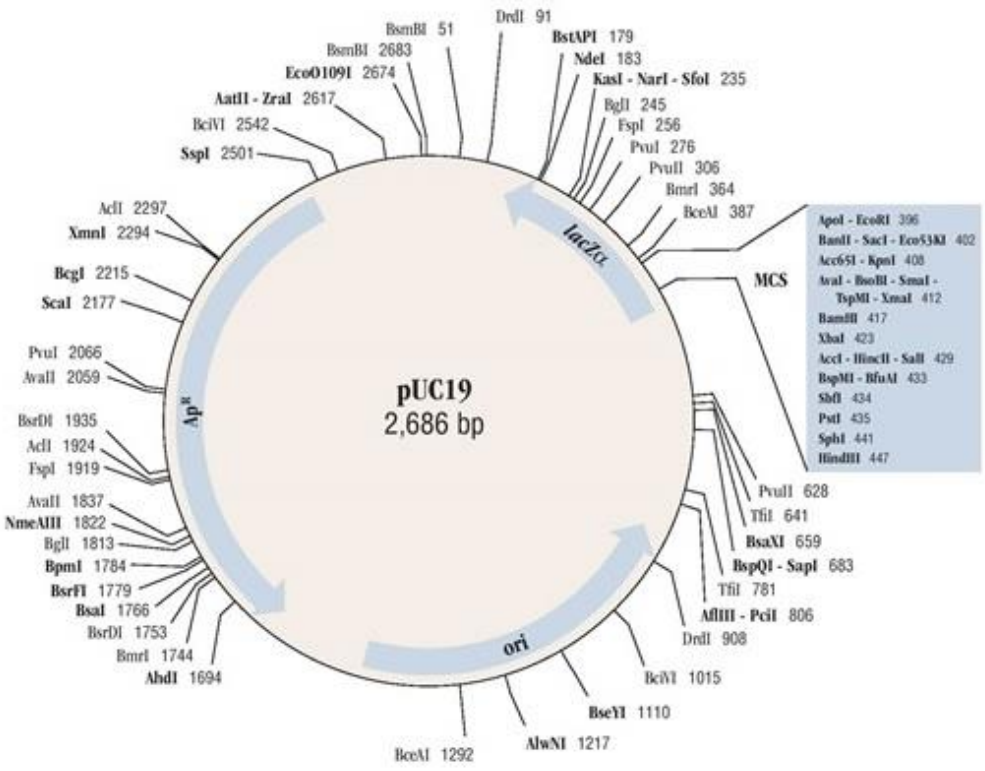
RESEARCH Prof Grant Burgess, Mohamed Reda Elbadawey and Dr Nicholas Jakubovics worked on the discovery of the marine microbe helping sinusitis

British Science Festival 2013 – What's hiding in Your Mouth?

I helped organise an event at the British Science Festival, held in Newcastle in 2013. This free event aimed to promote knowledge about oral hygiene and the microbiology of the mouth.

Appendix D: Vector Maps

pUC 19



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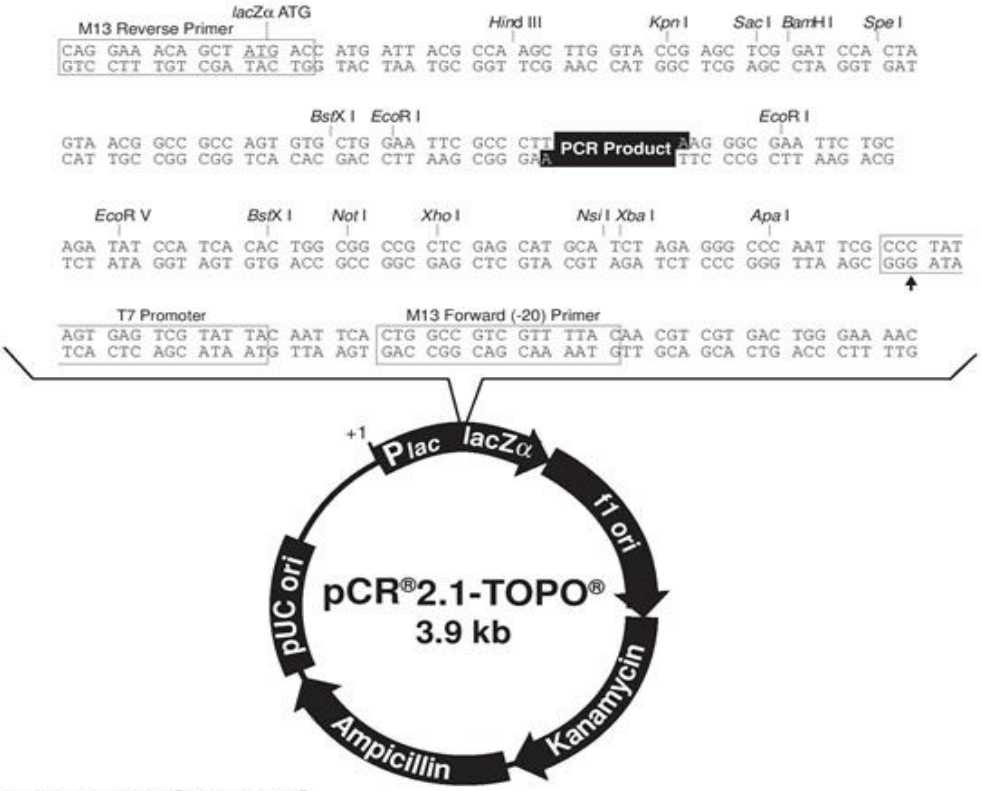

Comments for pCR®2.1-TOPO®
3931 nucleotides

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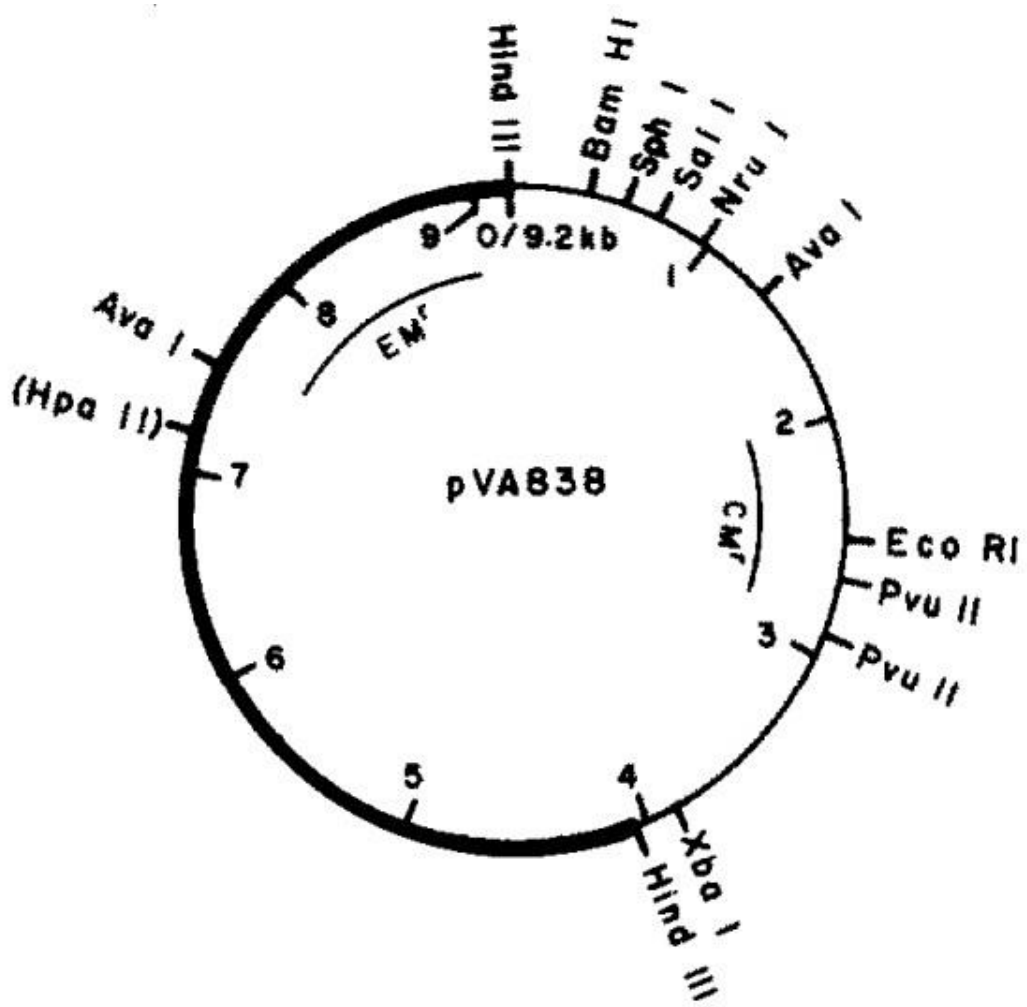
Multiple cloning site: bases 234-357
T7 promoter/priming site: bases 364-383
M13 Forward (-20) priming site: bases 391-406
f1 origin: bases 548-985
Kanamycin resistance ORF: bases 1319-2113
Ampicillin resistance ORF: bases 2131-2991
pUC origin: bases 3136-3809

Invitrogen™
life technologies

pVA838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Bam HI
Sph I
Sal I
Nru I
Ava I
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1
2
3
4
5
6
7
8
9
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Pvu II
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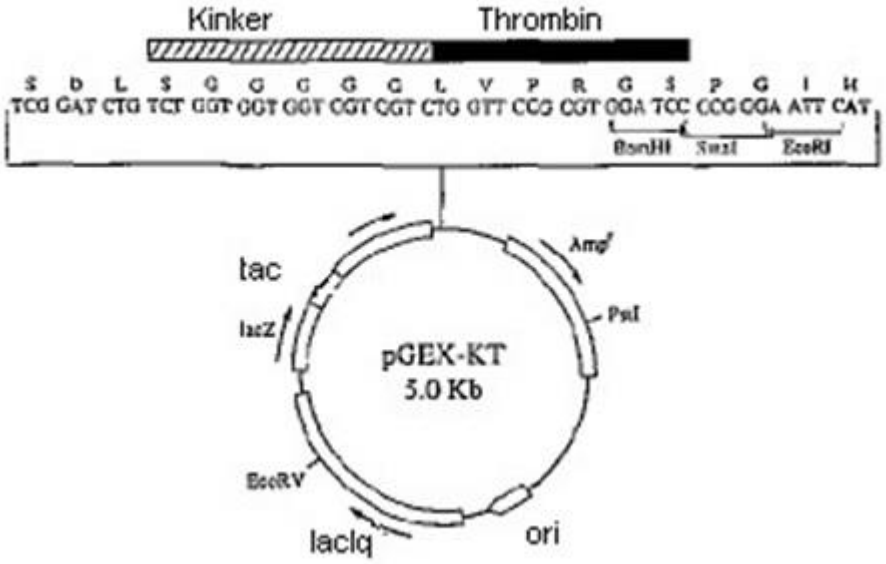
Xba I
Hind III

pGEX-KT


Kinker
Thrombin
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BamHI
SmaI
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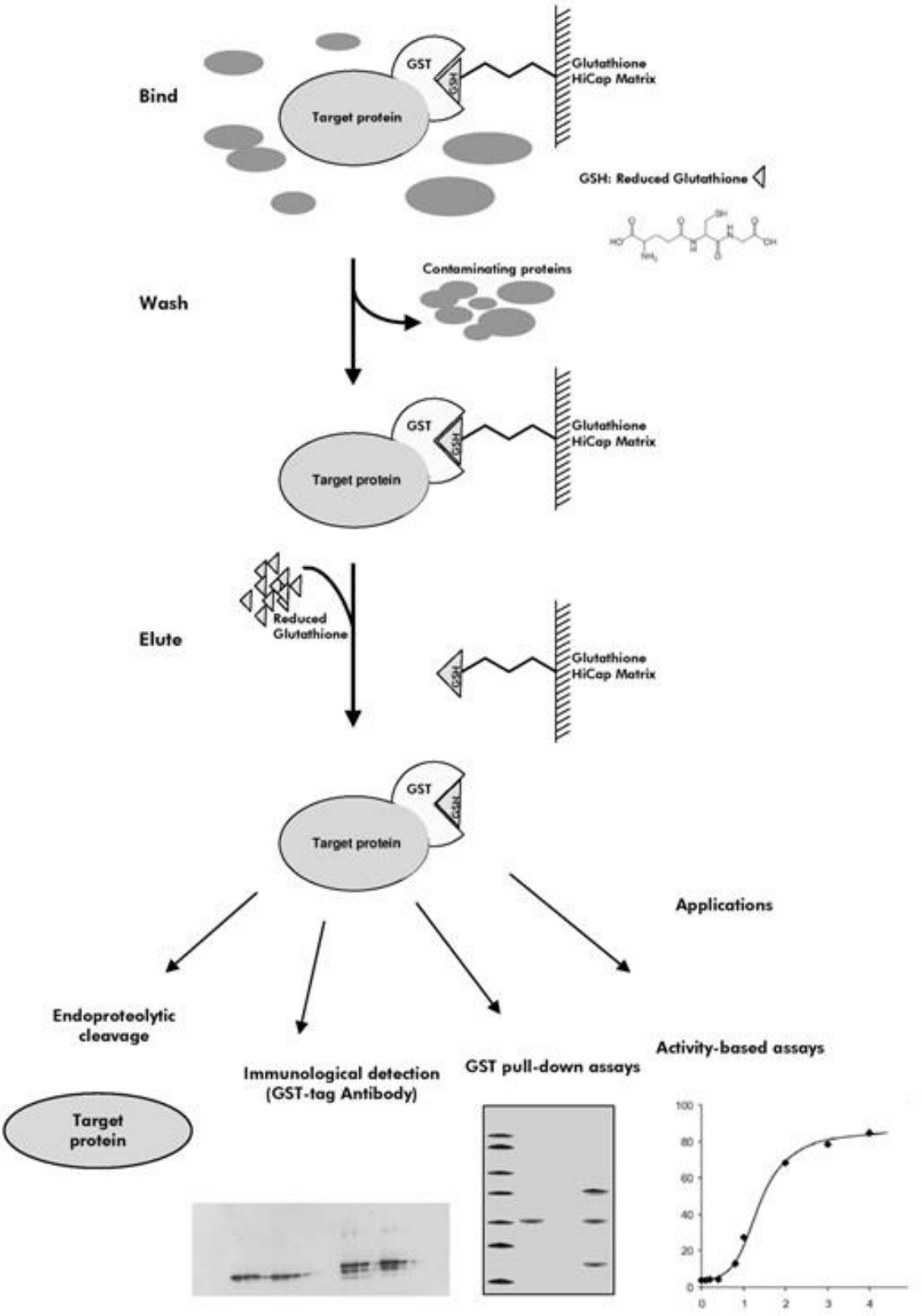
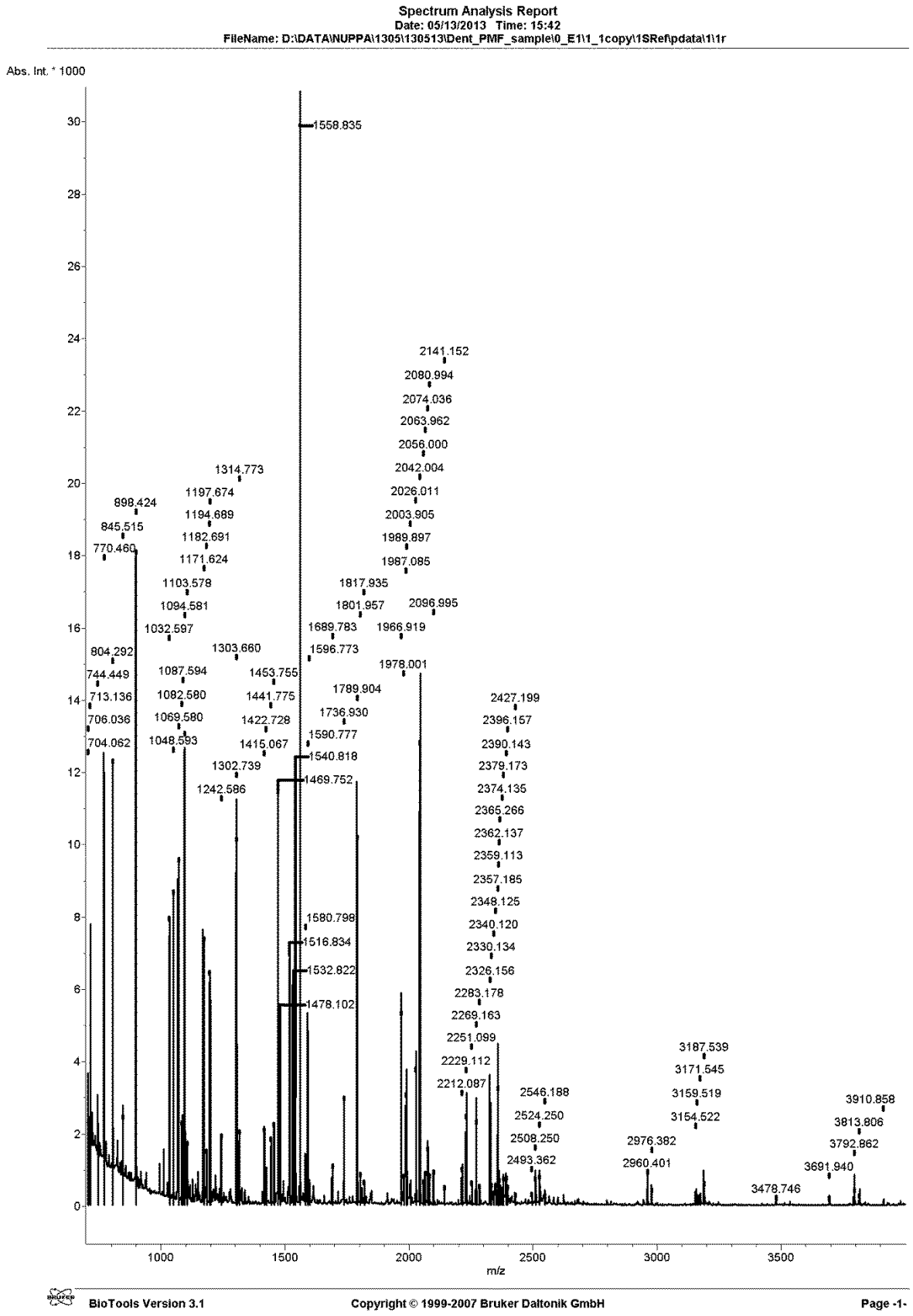
lacIq
ori

Appendix E: GST-tag purification diagram



Appendix F: GST-SsnA Peptide Mass Fingerprinting Results



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Sequence Coverage MS/MS:	0.0%	pI (isoelectric point):	0.0

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Processing Parameter:

Display Parameter:

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7	845.515	2303.258	8	898.424	17537.714	9	1032.597	7656.946
10	1048.593	8358.558	11	1069.580	9230.576	12	1082.580	2139.994
13	1087.594	2298.814	14	1094.581	12635.252	15	1103.578	1588.967
16	1171.624	7093.070	17	1182.691	1379.574	18	1194.689	6182.040
19	1197.674	5408.967	20	1242.586	1781.150	21	1302.739	9795.354
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25	1422.728	882.794	26	1441.775	1693.277	27	1453.755	2093.454
28	1469.752	11123.276	29	1478.102	1006.990	30	1516.834	6740.907
31	1532.822	5984.861	32	1540.818	2913.806	33	1558.835	31204.597
34	1580.798	1386.048	35	1590.777	4721.767	36	1596.773	620.771
37	1689.783	973.697	38	1736.930	2807.922	39	1789.904	9831.564
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peak 702390.143 - 645.100 - - -
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peak 732493.362 - 210.884 - - -
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peak 823187.539 - 507.900 - - -
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peak 853792.862 3792.852 317.954 0.010 2.513 82 - 118 1 EGTVAAQAVSSTAESVANLGTIQGESQVSPYQDKDVR

References

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