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Inducible mutagenesis and biofilm formation in *Streptococcus uberis*

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ACADEMIC DISSERTATION

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Light microscopy image of *Streptococcus uberis* strain PV164 growing in THY broth

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ABSTRACT

The evolutionary success of bacteria depends on genetic variability. This variability may be the result of beneficial mutations in the genome or from the uptake of genetic elements that increase viability under stress conditions. Bacteria are repeatedly exposed to agents such as antibiotics and host immune responses, which may induce in bacteria a variety of survival strategies, including mutagenic mechanisms. The development of antibiotic resistance is one of the serious consequences of these mechanisms. Bacteria increase genetic variability and survive DNA damage by using horizontal gene transfer, competence and DNA repair mechanisms such as the SOS response, homologous recombination and mismatch repair. Besides genetic rearrangements, bacteria have other mechanisms to persist in hostile environments. Biofilms are sessile, often polysaccharide-encased bacterial growth forms that are particularly tolerant to mechanical shearing, antibiotics and host immune responses.

Bovine udder infection, or mastitis, is an inflammation of the mammary gland caused by bacteria and it is usually recognized by clinical signs including abnormalities in the milk and the udder. Mastitis is the most common disease in the dairy industry and account for a significant proportion of the antibiotic treatments in dairy cows. *Streptococcus uberis* is a Gram-positive pathogenic bacterium and a member of the pyogenic group of the genus *Streptococcus*. *S. uberis* mainly infects the udder from environmental sources and is one of the most common causative agents of bovine mastitis. However, the molecular biology of *S. uberis* has been relatively unknown.

The members of the Streptococcaceae family have been considered to lack the classical SOS response. In the first part of this research, the stress tolerance and mutagenic mechanisms of *S. uberis* were characterized. In study I, a novel self-regulated SOS -response gene cassette was identified and the expression of this cassette was induced by UV -light as well as the antibiotic ciprofloxacin. *S. uberis* was shown to perform mutagenic DNA -repair after UV -exposure and the repair was mediated by error-prone polymerase UmuC coded by the SOS -response gene cassette. This mutagenesis was shown to promote the development of antibiotic resistance and, according to the database searches, the homologs of this SOS gene cassette are found in several other streptococcal species. In study II, exposure to the fluoroquinolone antibiotic ciprofloxacin was also shown to induce antibiotic resistance-promoting mutations in *S. uberis*, but the mutagenesis was not UmuC-mediated. Unlike the model organism of the SOS response, *Escherichia coli*, *S. uberis* has distinct mechanisms for UV- and ciprofloxacin-induced mutagenesis.

In study III, the effects of a sublethal concentration of ciprofloxacin on *S. uberis* were studied by proteomics. As a result of ciprofloxacin stress, *S. uberis* differentially expressed 20 proteins. The proteins were identified by mass spectrometry as enzymes involved in oxidative stress tolerance, NADH generation and nucleotide biosynthesis. The results suggest that ciprofloxacin exposure causes oxidative damage in *S. uberis*. The changes in enzymes involved in nucleotide balance suggest that nucleotide biosynthesis is a mechanism to stimulate mutagenesis leading to the development of antibiotic resistance.

In the final part of the work, the ability of *S. uberis* to form biofilms was characterized and the biofilm formation of clinical and subclinical *S. uberis* isolates was investigated. The strains differed in their ability to form biofilms, ranging from low-producing strains to strains producing thick, multi-layered biofilms. To determine whether biofilm production by *S. uberis* is an inducible event, the effect of proteins from the host was tested. Milk, the natural growth medium of mastitis bacteria, induced biofilm formation in most strains, even at low concentrations. Further analyses indicated that the milk components casein and especially α- and β-casein are the primary inducing agents of biofilm production. The proteolytic activity of *S. uberis* is involved in this induction process, possibly by releasing peptides from caseins.

Research on stress inducible systems provides us with information on how bacteria evolve, develop antibiotic resistance and escape the host immune system. The effects of antibiotic usage on bacteria are one of the most important questions in modern medicine, and knowledge of these effects helps in evaluating the necessity of antibiotic therapy. The biofilm formation of *S. uberis* provides a possible explanation for the ability of *S. uberis* to cause persistant mastitis, independent of antibiotic treatment.

LIST OF ABBREVIATIONS

2-DE	two-dimensional gel electrophoresis
ABC	ATP-binding cassette
AFM	atomic force microscopy
ATCC	American Type Culture Collection
bp	base pair
BSA	bovine serum albumin
CF	ciprofloxacin
Clp	caseinolytic protein
CSP	competence-stimulating peptide
DIC	dichloroisocoumarin
DIGE	difference gel electrophoresis
EMSA	electromobility shift assay
FM	fluorescence microscopy
FQ	fluoroquinolone
HdiR	heat and DNA damage inducible regulator
HEX	hexachloro-6-carboxyfluorescein
HGT	horizontal gene transfer
HTH	helix-turn-helix
ICE	integrating conjugative element
IEF	isoelectric focusing
IR	inverted repeat
IS	insertion sequence
kb	kilobase
LB	Luria-Bertani
MALDI-TOF	matrix-assisted laser desorption/ionization time-of-flight
MIC	minimum inhibitory concentration
MGE	mobile genetic element
NADH	nicotinamide adenine dinucleotide
ORF	open reading frame
PIA	polysaccharide intercellular adhesion
PCR	polymerase chain reaction
QRDR	quinolone resistance-determining region
RAD	RecA-deficient mutant strain
RT	room temperature
RIF	rifampin
SaPI	*S. aureus* pathogenicity island
THY	Todd Hewitt broth with yeast extract
TLS	translesion synthesis
TSYE	tryptic soy broth with yeast extract
UTC	urea-thiourea-CHAPS

LIST OF ORIGINAL PUBLICATIONS AND MANUSCRIPT

I Varhimo E, Savijoki K, Jalava J, Kuipers OP and Varmanen P. 2007. Identification of a novel streptococcal gene cassette mediating SOS mutagenesis in *Streptococcus uberis*.
Journal of Bacteriology. 189(14): 5210-5222.

II Varhimo E, Savijoki K, Jefremoff H, Jalava J, Sukura A and Varmanen P. 2008. Ciprofloxacin induces mutagenesis to antibiotic resistance independent of UmuC in *Streptococcus uberis*.
Environmental Microbiology. 10(8): 2179-2183.

III Poutanen M, Varhimo E, Kalkkinen N, Sukura A, Varmanen P and Savijoki K. 2009. Two-dimensional difference gel electrophoresis analysis of *Streptococcus uberis* in response to mutagenesis-inducing ciprofloxacin challenge.
Journal of Proteome Research. 8(1): 246-255.

IV Varhimo E, Varmanen P, Fallarero A, Skogman M, Pyörälä S, Iivanainen A, Sukura A, Vuorela P and Savijoki K. Alpha- and β-casein components of host milk induce biofilm formation in the mastitis bacterium *Streptococcus uberis.*
Submitted to *Veterinary Microbiology*.

1 INTRODUCTION

The ability to survive stress and protect the cells from the harmful effects of physical and chemical attacks of the environment is the basic prerequisite for all living organisms. In DNA-damaging conditions, error-free replication pathways can mostly repair the damage, but sometimes the death of the cell seems unavoidable, unless mutagenic replication mechanisms are exploited (Tippin *et al.*, 2004). In bacteria, the activation of such mechanisms can include the acquisition of beneficial mutations or uptake of genetic elements, which both ensure increased viability under life-threatening conditions (Cirz *et al.*, 2006a).

Stress inducible systems and DNA repair pathways are best studied in model bacteria such as *Escherichia coli* and *Bacillus subtilis* (Au *et al.*, 2005; Foster, 2007). However, the stress response and mutation mechanisms vary between bacterial species (Ohmori *et al.*, 2001; Tippin *et al.*, 2004; Ambur *et al.*, 2009), indicating that stress responses in less well-studied bacteria also should be elucidated. As a result of genomic plasticity, multi-drug antibiotic resistance has already been emerging in pathogenic streptococci such as *Streptococcus pneumoniae* and *Streptococcus agalactiae* (Reinert, 2004; Sorlozano *et al.*, 2009) and in staphylococci especially in *Staphylococcus aureus* (reviewed in Jensen & Lyon, 2009; Wilcox, 2009; Woodford & Livermore, 2009).

The severity of the current situation is enhanced by the ability of many bacteria to switch to a biofilm mode of growth under certain conditions. This phenomenon is of considerable interest in the context of food and clinical hygiene, as this type of growth clearly increases the ability of cells to persist once challenged, for instance, with antibiotics and/or host defences (Kumar & Anand, 1998; Costerton *et al.*, 1999; Stephens, 2002; Trautner & Darouiche, 2004). Biofilm growth can also induce genetic diversity even without drug challenge, as evidenced in pathogens such as *S. pneumoniae* and *S. aureus* (Allegrucci & Sauer, 2007; Yarwood *et al.*, 2007; Allegrucci & Sauer, 2008). This highlights the importance of biofilm growth as a strategy to speed up the evolution of resistance.

The Streptococcaceae family contains members displaying a wide pathogenicity range, from non-pathogenic, commensal species to severe pathogens of humans and animals. For example, *Lactococcus lactis*, a member of this family, is a non-pathogenic bacterium used by the dairy industry as a starter in cheese fermentation (Bolotin *et al.*, 2001). The family also includes pathogenic streptococci of humans such as *Streptococcus pyogenes*, the causative agent of scarlet fever, pharyngitis, toxic shock syndrome and impetigo (Holden *et al.*, 2007), *S. pneumoniae*, a common cause of pneumonia, otitis media and sepsis (Tettelin *et al.*, 2001) and *Streptococcus mutans*, which causes dental caries (Ajdic *et al.*, 2002). Examples of the commensal members of this family are the oral bacteria *Streptococcus oralis* (Do *et al.*, 2009) and *Streptococcus salivarius* (Wescombe *et al.*, 2009). Many species are opportunistic pathogens or include both virulent and commensal strains, such as *S. agalactiae*, which is both a member of the normal genital flora of adults and a causative agent of neonatal sepsis (Glaser *et al.*, 2002). The pathogenic streptococci of animals include *Streptococcus equi*,

which causes strangles in horses (Timoney *et al.*, 1997), and *Streptococcus suis*, responsible for meningitis and septicemia in pigs and occasional outbreaks in humans (Wertheim *et al.*, 2009).

Streptococcus uberis is a Gram-positive pathogenic bacterium and one of the most common causative agents of bovine mastitis. The molecular biology of *S. uberis* has been relatively unknown, despite its ability to cause persistant infection of the mammary gland and costly effects in the dairy industry. In this thesis, the stress-induced mutagenic mechanisms of *S. uberis* were studied. We also investigated the effects of induced mutagenesis on the development of antibiotic resistance, as well as the effects of antibiotic treatment on the protein expression of *S. uberis*. Finally, biofilm formation, another means by which bacteria resist environmental stress, was examined in several *S. uberis* strains.

2 REVIEW OF THE LITERATURE

2.1 Bovine mastitis

Bovine udder infection, or mastitis, is an inflammatory response of the mammary gland caused by bacteria and it is usually recognized by clinical signs such as abnormalities in the milk and the udder (Viguier *et al.*, 2009). It is the most common disease and most common cause of antibiotic treatment in dairy cows. Mastitis has impacts on animal production and welfare and the quality of milk. It has been estimated that up to 50% of all lactating cows are infected each year in at least one of the four secretory quarters of the mammary gland (Hillerton & Berry, 2005). In Finland, the prevalence of mastitis was 30.6% in 2001 (Pitkälä *et al.*, 2004). The approximated annual financial losses to the dairy industry due to mastitis are $2 billion in the US and £300 million in the UK (Donovan *et al.*, 2005; Hillerton & Berry, 2005). The financial costs mainly derive from medical costs and the loss of milk and meat production because of antibiotic treatments and premature culling of infected animals (Hillerton & Berry, 2005; Viguier *et al.*, 2009). Many mastitis cases (10-15%) occur as milder infections, or subclinical mastitis, which increases the leukocyte concentration and bacterial content in milk and reduces milk production (Hillerton & Berry, 2005). Subclinical mastitis reduces the value of milk both as a food and in monetary terms, and forms an infection risk to the other animals of the herd.

In the UK, the implementation of a five-point control plan resulted in a dramatic decline in mastitis cases in the early 1980s. This plan included the use of correctly maintained milking equipment, post-milking teat disinfection, therapeutic and prophylactic antibiotics and the culling of persistently infected animals (Bramley, 1984). The strategy proved to be efficient on the control of contagious mastitis bacteria, including *S. aureus, Streptococcus dysgalactiae* and *S. agalactiae,* but was inefficient in reducing mastitis cases caused by *E. coli* and *S. uberis,* which infect the cattle from environmental sources (Booth, 1988). These environmental pathogens are in several countries the most frequent cause of mastitis in both lactating and nonlacting cows, especially in well-managed farms (Bradley, 2002).

In order to infect the milk compartments, the mastitis bacteria have to cross the teat canal barrier, proliferate in the milk phase and then resist or avoid the immune defence of the host (Sandholm *et al.*, 1990). Acute mastitis often converts to chronic inflammatory disease, which causes tissue damage. To treat mastitis, antibiotic therapy is almost always required. However, the outcome of antibiotic treatment in mastitis is often disappointing due to several factors, including the lack of pharmacokinetic data on the compatibility of antibiotics with milk, their concentration at the site of infection and their interactions with endogenous inhibitors (Owens *et al.*, 1990; Milne *et al.*, 2005). In most mastitis cases where antibiotic therapy is necessary, the antibiotic used in the treatment of mastitis is penicillin G (Hillerton & Berry, 2005; Pyörälä, 2009). The antibiotics are given in the form of intramammary preparations or intramuscular injections. Mastitis-causing streptococci are generally penicillin-susceptible, but penicillin-resistance is quite common among *S. aureus* (Pitkälä *et al.*, 2004). In some

mastitis cases, antimicrobial drugs are not required and pain medication and frequent milkings are sufficient to treat the disease.

2.1.1 Streptococcus uberis

S. uberis is a mastitis causing bacterium that infects the udder from environmental reservoirs such as bedding materials and pastures (Leigh, 1999). *S. uberis* can also be found in the tonsils, genital tracts, rumen and coat of a cow. It has been estimated to be responsible for 13% and 33% of bacterial mastitis cases in Canada and the UK, respectively (Hillerton & Berry, 2005; Olde Riekerink *et al.*, 2008). In Finland, *S. uberis* has been recorded in 11.86% of mastitis milk samples and in 14.55% of samples from clinical mastitis, and is the third most common finding in mastitis samples after coagulase-negative staphylococci and *S. aureus* (Koivula *et al.*, 2007).

The mastitis cases caused by *S. uberis* and *E. coli* are more common during the pasture season in summer and autumn. In experimentally induced infection of the lactating mammary gland, *S. uberis* is predominantly found in the luminal areas of secretory alveoli and ductular tissues. This indicates that bacterial growth mainly takes place in residual and newly synthesized milk (Thomas *et al.*, 1994; Taylor *et al.*, 2003). *S. uberis* often causes chronic infections that can persist through lactation or even for multiple lactations (Oliver *et al.*, 1998). It has been proposed that persistant *S. uberis* infection might be due the internalization of the bacteria in the host's epithelial cells. In cell culture experiment *S. uberis* has been shown to survive in host cells for up to 120 h without causing damage to the cells (Tamilselvam *et al.*, 2006). Internalized *S. uberis* could possibly survive antibiotic treatment and serve as a reservoir for persistent infections. *S. uberis* mastitis requires antibiotic treatment and the strains are in general penicillin susceptible (Pitkälä *et al.*, 2004). In experimental *S. uberis* mastitis infection, a non-treated control group was completely unsuccessful in resolving the infection and intervention was required for animal welfare (Hillerton & Kliem, 2002).

Comparative analyses of 16S rDNA, *sodA* and *cpn60* (*groEL*) sequences indicate that *S. uberis* is a member of the pyogenic group of *Streptococcus* (Facklam, 2002; Alber *et al.*, 2004). Other members of this group include the human pathogen *S. pyogenes* and equine pathogen *S. equi.* The genomic sequence of the clinical *S. uberis* strain 0140J has been published (Ward *et al.*, 2009). This strain is one of the most thoroughly studied and pathogenic to both lactating and non-lacting bovine mammary glands. The sequencing project revealed that compared to other pyogenic streptococci, *S. uberis* possesses fewer mobile genetic elements. Comparative genome analysis indicated that *S. uberis* has most similarities with *S. agalactiae* and *S. equi* subsp. *zooepidemicus*. In comparison to *S. pyogenes*, *S. uberis* has a broader variety of metabolic and transport pathways. According to the genome analysis, *S. uberis* has, for instance, two distinct metabolic routes for energy production, fermentation and respiration, and this versatility might indicate adaptation to the different environmental niches it occupies (Ward *et al.*, 2009). However, compared to *S. pyogenes*, *S. uberis* lacked many of the classical virulence genes, including genes involved in the production and anchoring of the M protein (Holden *et al.*, 2007).

2.2 Molecular and cellular mechanisms of bacteria for defence against mutagenesis-inducing agents

Bacteria are repeatedly exposed to mutagenesis-inducing agents such as antibiotics, irradiation, chemicals and host immune responses, which are likely to induce a variety of survival strategies for enhancement of viability and virulence. Bacteria seem to be able to adjust their mutation rates depending on the environmental conditions, and have also been shown to speed up their adaptation when in conditions interfering with genome integrity (Blazquez *et al.*, 2002; Cirz *et al.*, 2006a; Goerke *et al.*, 2006). Bacteria possess several mechanisms to repair damage in DNA, which under certain conditions can promote chromosomal rearrangements and/or alteration in the genetic information, thereby enabling the acquisition of new traits through mutations. The strategies employed include competence, activation of the SOS response, DNA mismatch repair pathways as well as the biofilm mode of growth.

2.2.1 The SOS response

The SOS response is a classical stress response to DNA damage, which is induced when DNA replication forks are stalled because of a DNA lesion (Friedberg *et al.*, 2006). This mechanism was first characterized in *E. coli* (Radman, 1975) and subsequently in several bacterial species including *Salmonella enterica* serovar Typhimurium (Benson *et al.*, 2000), *Caulobacter crescentus* (Galhardo *et al.*, 2005) and *Mycobacterium tuberculosis* (Davis *et al.*, 2002), but has not been observed in eukaryotic cells.

In a model based on results obtained with *E. coli*, the SOS response can be induced by exposure to UV light or mitomycin C, which results in the expression of genes involved in DNA replication, repair and mutagenesis (Figure 1) (Friedberg *et al.*, 2006). The exposure of single-stranded DNA (ssDNA) after interruption of replication functions as a signal to the RecA protein, which coats the exposed ssDNA areas, forming a nucleoprotein filament (RecA*). The coprotease activity of RecA* stimulates the self-cleavage reaction of LexA, the negative regulator of the SOS response. After a self-cleavage reaction, the N-terminal fragment of LexA is degraded by the ClpXP protease complex (Flynn *et al.*, 2003). The release of repression by LexA leads to the transcription of over 30 SOS genes and the synthesis of enzymes promoting DNA repair, recombination and DNA synthesis. Under non-damaging conditions, LexA is bound to its consensus binding sites located in the promoter regions of the target that include *lexA* itself, the *umuDC* operon and *recA* (Fernandez De Henestrosa *et al.*, 2000; Friedberg *et al.*, 2006).

Since the first description of the *E. coli* SOS response, functional LexA homologs have been characterized in several species, including *B. subtilis* (Wojciechowski *et al.*, 1991), *M. tuberculosis* (Movahedzadeh *et al.*, 1997), *Rhodobacter sphaeroides* (Fernandez de Henestrosa *et al.*, 1998) and *Thermotoga naepolitana* (Zverlov & Schwarz, 1999). In the Gram-positive bacterium *B. subtilis*, 33 genes have been characterized that have LexA binding sites and are also RecA-dependently induced by the SOS inducers mitomycin C and UV (Au *et al.*, 2005). Although the number of SOS-induced genes in *B. subtilis* is very similar

to *E. coli*, only 8 of these genes have homologous counterparts in *E. coli*. The *B. subtilis* SOS system is also induced in competent cells in the absence of any DNA-damaging treatment (Yasbin, 1977; Love *et al.*, 1985; Yasbin *et al.*, 1992).

Some bacteria, such as the members of the Streptococcaceae family, lack the classical LexA-regulated SOS response (Gasc *et al.*, 1980). However, RecA from *S. pneumoniae*, for instance, has been shown to cleave LexA of *E. coli*, which demonstrates the universal features of the SOS systems between bacterial species (Steffen & Bryant, 2000). *L. lactis* has been shown to possess a gene encoding a negative transcriptional regulator, HdiR (the heat and DNA damage inducible regulator), which possesses several characteristics similar to LexA, such as induction by mitomycin C (Savijoki *et al.*, 2003). HdiR is also capable of a self-cleavage reaction, but self-cleavage does not inactivate the protein, as in LexA. The N-terminal domain of HdiR has a helix-turn-helix (HTH) motif, which is used for binding of the target DNA. The N-terminal domain preserves its DNA-binding activity after self-cleavage and the DNA-binding activity is only lost after the Clp protease-mediated degradation of the cleavage product. The self-cleavage of *L. lactis* HdiR occurs between amino acids Ala126 and Gly127, which corresponds to the self-cleavage site (Ala84 and Gly85) determined for the LexA of *E. coli* (Little, 1984).

The SOS response is induced by antibiotics in several bacterial species. In *E. coli*, ciprofloxacin, rifampin, β-lactams

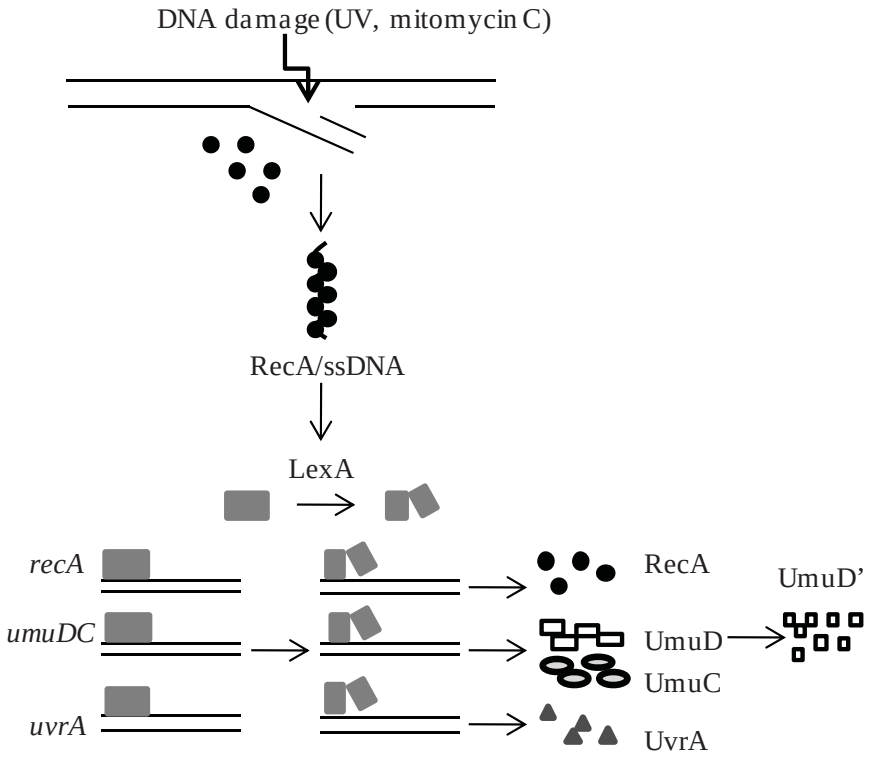

Figure 1. Induction of the SOS response in *E. coli* (modified from Sutton *et al.*, 2000)

and trimethoprim have been shown to induce the SOS response (Lewin & Amyes, 1991; Power & Phillips, 1992; Drlica & Zhao, 1997; Miller *et al.*, 2004; Cirz *et al.*, 2005). Antibiotics of the quinolone class, such as nalidixic acid and ciprofloxacin, especially promote the development of antibiotic resistance by SOS-dependent and other pathways. The quinolones trap in DNA the enzymes gyrase and topoisomerase IV, which coil and uncoil DNA during its replication. Induction of the SOS response and mutagenesis by quinolones mediates the development of antibiotic resistance to these antibiotics in *E. coli* (Cirz *et al.*, 2005). In *E. coli*, all three LexA-regulated error-prone polymerases Pol II, Pol IV and PolV are involved in this mutagenic response. Quinolones can also induce antibiotic resistance in other ways than error-prone replication. Exposure of *Vibrio cholerae* to ciprofloxacin leads to movement of conjugative elements (ICEs) that in some cases carry genes that confer antibiotic resistance (Beaber *et al.*, 2004).

2.2.1.1 Error-prone polymerases

In the case of DNA damage, high-fidelity DNA polymerases cannot use damaged DNA as templates for synthesis. When a replicative polymerase encounters a severe DNA lesion, the high-fidelity replicative subunit is replaced with low-fidelity subunit (Friedberg *et al.*, 2006). This translesion synthesis (TLS) is performed by specialized polymerases called error-prone polymerases that are part of the Y family (PolIV and PolV) or the C family (DnaE) and their transcription is mostly induced by RecA and the SOS response. The Y family of polymerases were originally characterized by their ability to bypass lesions in synthetic DNA templates *in vitro* and lack of an associated exonuclease. They also tend to copy undamaged DNA with remarkably poor fidelity (Goodman & Tippin, 2000), suggesting their importance in natural selection and evolution. The active PolIV consists of a single polypeptide encoded by the *dinB/dinP* gene, but to obtain functional PolV, the polymerase subunit UmuC must associate with two UmuD subunits that have undergone a RecA-stimulated self-cleavage reaction (UmuD') (Schlacher *et al.*, 2006). The UmuC-like polymerases are only present in prokaryotes and have separate branches in Gram-positive and -negative bacteria. The DinB-like polymerases are widely distributed among the archaea, bacteria and eukaryotes, but their essentiality varies between species (Ohmori *et al.*, 2001).

In Gram-positive bacteria, the Y-family polymerases are less well characterized. In *B. subtilis*, two putative error-prone polymerases, YqjH and YqjW, have been characterized and they have 36% identity to *E. coli* DinB and 26% identity to *E. coli* UmuC, respectively (Sung *et al.*, 2003). Deletion of YqjH and YqjW leads to decreased UV-induced mutagenesis and deletion of YqjH also reduces UV-tolerance. DinP/DinB homologs have also been characterized in Streptococcaceae. All of the three sequenced strains of *S. pneumoniae* code for Y-family polymerase DinP/DinB (Hoskins *et al.*, 2001; Tettelin *et al.*, 2001; Lanie *et al.* 2007). The natural *dinB* deletion mutant of *S. pneumoniae* showed a reduced mutation frequency compared to the *dinB* carrying strains when exposed to the antibiotic trimethoprim, indicating that DinB plays a role in trimethoprim-induced mutagenesis (Henderson-Begg *et al.*, 2006). The *S. pneumoniae* chromosome

lacks a UV-inducible error-prone system, but the conjugative transposon Tn*5252* carries genes with homology to *umuC/mucB* (ORF13) and *umuD/mucA* (ORF14), and these genes have an association with an SOS-like response (Munoz-Najar & Vijayakumar, 1999). ORF14 is also very similar to several transcriptional regulators of Gram-positive bacteria, and the similarity is highest in three domains involved in RecA-mediated cleavage of these proteins (Perry *et al.*, 1985; Munoz-Najar & Vijayakumar, 1999). The *umuC* and *umuD* homologs are part of an operon-like sequence of four open reading frames. Cloning of this operon into a plasmid and transformation of the plasmid to *E. coli* and UV-sensitive *S. pneumoniae* increased their survival rates and mutation frequencies after UV exposure. Deletion of a fragment of *umuC* abolished the effect, indicating that *umuC* is essential for this mutation mechanism. In pneumococci, this was the first demonstrated mutagenesis dependent on UV-induced SOS response. Munoz-Najar and others (1999) proposed that these SOS genes and the benefits they provide might also facilitate the persistence of the Tn*5252* element in the *S. pneumoniae* genome when antibiotic selection is not present.

In most Gram-positive organisms there are two essential polymerase subunits with high sequence similarity to the replicative α-subunit of *E. coli* polymerase (Dervyn *et al.*, 2001; Inoue *et al.*, 2001; Barnes *et al.*, 2002; Foster *et al.*, 2003). These polymerases are known as Pol C and DnaE. Pol C is an essential replicative polymerase but DnaE has a very different role as it has the capacity to by-pass certain DNA lesions. The absence of DnaE leads to the loss of UV-induced mutagenesis in *B. subtilis* (Le Chatelier *et al.*, 2004). In *S. pyogenes*, DnaE has been shown to be a highly error-prone C family polymerase, producing frame-shift and point mutations during TLS and on undamaged DNA *in vitro* (Bruck *et al.*, 2003). This polymerase is also essential in *S. pyogenes.*

In *C. crescentus,* an operon composed of *dnaE2, imuA* and *imuB* is damage-inducible in a *recA*-dependent manner after UV and mitomycin C exposure (Galhardo *et al.*, 2005). This operon is also responsible for most of the UV- and mitomycin C-induced mutations. The ImuB has similarities with Y-family proteases and possibly co-operates with DnaE2 in lesion bypass. The genes *imuA* and *imuB* are present in several bacterial species, such as the α-proteobacteria branch, that lack *umuDC* orthologs, and it has been proposed that *imuAB* orthologs replace *umuDC* in damage-inducible mutagenesis in these bacteria. However, the mutations arising as a result of the function of this operon are G:C to C:G type, which is rather unusual for mutations caused by UV. In *E. coli*, the hallmark of UV mutagenesis is the G:C to A:T transitions (Friedberg *et al.*, 2006).

The diversity of error-prone polymerases in bacterial species shows that bacteria have distinct mutagenic repair machineries that help them to survive DNA damage. However, the ability to repair DNA damages seems to be equally important in all kingdoms of life.

2.2.1.2 Excision repair and homologous recombination

Homologous recombination is a process that occurs during normal cellular processes such as chromosome replication, but also after DNA damage (Thoms &

Wackernagel, 1998; Gruss & Michel, 2001). In homologous recombination, double-stranded DNA breaks are repaired by replacing the damage with homologous DNA fragments from another source. In *S. pneumoniae*, this capacity is strongly linked to development of competence, a natural transformation capacity (Claverys *et al.*, 2006). The DNA damage can also be repaired by the removal of damaged nucleotides by endo- and exonucleases and the synthesis of new replacement DNA by excision repair (Kornberg & Baker, 2005). In Gram-positive bacteria, the exonuclease/helicase RexAB complex is essential for double-stranded DNA repair and is a functional homologue of the RecBCD enzyme of *E. coli* (Kowalczykowski *et al.*, 1994; Quiberoni *et al.*, 2001; Halpern *et al.*, 2004). RexAB of Streptococcaceae appears to consist of two nuclease activities that each degrades one of the two strands until a certain signal sequence (Chi sequence) is reached. UV irradiation induces DNA damage that is in many bacteria corrected by excision repair. If a replication fork encounters this excision repair site, a double-stranded break is formed. In *S. pneumoniae*, *rexAB* mutants are UV sensitive compared to the wild type, but not as sensitive as the *recA* mutant, which lacks the RecA regulator involved in many DNA repair pathways (Halpern *et al.*, 2004).

The glycosyl bond of deoxyribonucleotides is unstable at low pH, so the loss of purines and pyrimidines from DNA can occur in acid conditions, leading to the formation of an abasic site (Lindahl & Nyberg, 1972). The abasic site is commonly repaired by AP (apurinic/apyrimidinic) endonucleases, which remove the damaged nucleotide. In *S. mutans*, an AP endonuclease that shows higher activity in cells grown in an acidic pH has been identified (Hahn *et al.*, 1999). This endonuclease is coded by the *smx* gene and is functionally very similar to other DNA repair enzymes, especially *E. coli* exonuclease III and *S. pneumoniae* ExoA (Rogers & Weiss, 1980; Puyet *et al.*, 1989; Faustoferri *et al.*, 2005). Smx removes 5' abasic residues similarly as Exo III, but differs in its efficiency in removal of additional bases. The loss of *smx* resulted in a higher sensitivity to oxidative stress when *S. mutans* was grown at a low pH. In *S. mutans*, the expression of a gene with 67% identity to *B. subtilis* UV repair excinuclease *uvrA* was also shown to increase at a low pH (Hanna *et al.*, 2001). The gene *uvrA* is part of the UvrABC protein complex, which locates and excises bulky DNA lesions. Deletion of this gene leads to sensitivity to UV irradiation and lower survival rates in acidic conditions. These results suggest that *uvrA* and the excision repair pathway are essential for acid adaptation in *S. mutans.*

2.2.1.3 Horizontal gene transfer

Horizontal gene transfer (HGT) is a process where a bacterium acquires genetic elements from different bacterial species or from another member of the same species. HGT plays an important role in creating genetic diversity and includes the exchange of genomic material of prophages, plasmids, transposable elements and pathogenicity islands in bacteria.

Integrating conjugative elements (ICEs), also termed conjugative transposons, are genomic islands that excise, self-transfer by conjugation, and integrate in the genome of the recipient bacterium. They often carry useful traits such as genes providing resistance to

antibiotics or heavy metals. Several ICEs have been characterized in streptococcal species. For instance, ICESde3396 isolated from *S. dysgalactiae* is an element carrying functional genes and operons from several bacterial species and has the ability to transfer from *S. dysgalactiae* to *S. pyogenes* and *S. agalactiae* (Davies *et al.*, 2009). This element carries genes promoting heavy metal resistance, but no antibiotic resistance genes. However, ICESde3396 carries homologs of genes found in other mobile genetic elements (MGE) that carry antibiotic resistance genes, suggesting that gene transfer between MGEs can occur. In *S. pyogenes*, bacteriophages are the most common mobile genetic elements and each *S. pyogenes* strain sequenced so far has at least one prophage sequence, but in *S. agalactiae* bacteriophages are rare and ICEs more common (Ferretti *et al.*, 2004). Other forms of MGEs are pathogenicity islands. They are genomic regions with atypical nucleotide compositions that are acquired by gene transfer. *S. pneumoniae* pathogenicity island PPI1, for instance, carries an ABC transporter needed for iron uptake and full virulence in mouse models (Brown *et al.*, 2001; Brown *et al.*, 2002; Brown *et al.*, 2004).

Macrolide resistance in *S. pyogenes* is promoted by *mef(A)*, *erm(A)* and *erm(B)* genes, and these genes are frequently acquired by HGT of transposons, prophages and plasmids (Schalen *et al.*, 1995; Banks *et al.*, 2003; Santagati *et al.*, 2003; Giovanetti *et al.*, 2005). The conjugative transposon Tn1207.3 of *S. pyogenes* that carries the macrolide resistance gene *mef(A)* could be transferred by conjugation to *S. pneumoniae* and *S. gordonii*. Also the non-conjugative transposon Tn1207.1 of *S. pneumoniae* that carries macrolide-resistance genes is possibly a derivative of Tn1207.3 (Santagati *et al.*, 2000; Santagati *et al.*, 2003).

Environmental stress conditions may affect the frequency of horizontal gene transfer, and in many cases HGT happens via SOS-mediated pathways. Many pathogenicity islands, including *S. aureus* pathogenicity islands (SaPIs), are excised from the genome by SOS induction. For instance, fluoroquinolone antibiotics are fully effective in mobilizing *S. aureus* pathogenicity island SaPIbov1 (Ubeda *et al.*, 2005). Induction of the SOS response increases the rate of HGT after exposure to fluoroquinolones and β-lactams in *S. aureus* by inducing the excision and replication of prophages 80α, Φ11 and Φ147 that carry SaPIs, and these phages can spread the virulence genes of *S. aureus* (Ubeda *et al.*, 2005; Maiques *et al.*, 2006). In *V. cholerae*, the SOS response triggered by ciprofloxacin also mobilizes an ICE called SXT that carries genes promoting resistance to several antibiotics (Beaber *et al.*, 2004).

The genome sequencing of *S. uberis* 0140J revealed that this strain has a lower number of mobile genetic elements than other pyogenic streptococci. However, two bacteriophage-derived islands and a putative genomic island were identified, whilst insertion sequences (IS) were completely missing (Ward *et al.*, 2009).

2.2.2 Competence

Some bacteria, such as *S. pneumoniae*, have evolved alternative systems to the traditional SOS response in order to cope with DNA damaging stress conditions. Bacterial natural transformation, known as competence, allows bacteria to take up DNA from the environment and to integrate the DNA into the genome. In

general, competence is used to acquire three factors: 1) raw material for genetic exchange, 2) templates for DNA repair and 3) nutrients (Claverys *et al.*, 2006).

In response to antibiotic stress, *S. pneumoniae* exhibit competence to survive the stress. The *com* regulon needed for competence consists of 105 to 124 genes, including *recA* (Mortier-Barriere *et al.*, 1998; Dagkessamanskaia *et al.*, 2004; Peterson *et al.*, 2004). In *S. pneumoniae* competence, double-stranded donor DNA is converted to single-stranded DNA and is passed through the cytoplasmic membrane in a 3'-5' polarity (Mejean & Claverys, 1988). The development of competence is triggered by competence-stimulating peptide (CSP), which is encoded by the *comC* gene (Havarstein *et al.*, 1995). CSP stimulates ComD, a membrane-bound histidine kinase that phosphorylates the ComE regulator. Phosphorylated ComE leads to the expression of the *comX* gene, which encodes an alternative sigma factor ComX, the central regulator of competence genes. This leads to the activation of a cascade of competence genes (Claverys *et al.*, 2006). The production of CSP is increased in response to changes in environmental conditions, suggesting that competence is a general response, like the SOS response, in several other bacteria. When pneumococci are cultivated in a competence-promoting medium *in vitro*, competence develops spontaneously at OD_{550nm}= 0.15-0.2 and is rapidly lost after 30 minutes (Tomasz, 1966). External signals inducing development of competence in laboratory cultures include high phosphate concentration, bovine serum albumin, $CaCl_2$ and a slightly alkaline pH (Claverys & Havarstein, 2002). The DNA-damaging agent mitomycin C and antibiotics such as norfloxacin, levofloxacin, moxifloxacin, kanamycin and streptomycin also induce competence (Prudhomme *et al.*, 2006). It has been shown that competent cells have the ability to kill non-competent sister cells, acquiring suitable DNA for homologous recombination (Claverys & Havarstein, 2007).

Since the characterization of competence in pneumococci, *comC* has been identified in several other *Streptococcus* species of the mitis phylogenetic group, including *S. mitis*, *S. oralis* and *S. gordonii* (Havarstein *et al.*, 1996; Havarstein *et al.*, 1997; Whatmore *et al.*, 1999), and in a few species of the anginosus group, including *S. anginosus* (Havarstein *et al.*, 1997). However, the amino acid sequences of CSP peptides in these species show considerable diversity. The regulation network of competence is very complex, as the level of transcription of over > 180 genes is altered after induction of the competent state in *S. pneumoniae*. However, only 23 of these genes have been shown to directly affect natural transformation (Burghout *et al.*, 2007; Johnsborg & Havarstein, 2009).

It has been proposed that *S. pneumoniae* compensates for the lack of an SOS response with competence as a means to acquire genetic variability and overcome stress (Claverys *et al.*, 2006). Competence has proved to be very efficient in spreading β-lactam resistance in pneumococci, and has undermined the effect of pneumococcal vaccines. In *S. pneumoniae*, β-lactam resistance develops through mutations in genes of penicillin-binding protein, which leads to a lowered affinity to penicillin, and competence has efficiently spread these mutated genes (Hakenbeck *et al.*, 1980). Natural transformation might also be responsible for serotype switching, which has been identified in

serotypes used in pneumococcal vaccines (Brueggemann *et al.*, 2007; Johnsborg *et al.*, 2008). It has been proposed that multiple species biofilms in the human naso- and oropharynx, the natural habitat of pneumococci and related species, are an efficient source of gene transfer by competence (Johnsborg & Havarstein, 2009).

2.2.3 Other DNA repair pathways

The mismatch repair system corrects potentially mutagenic mismatches resulting from errors in DNA replication and homologous recombination, and the inactivity of the genes of the mismatch repair system mostly results in a hypermutator phenotype. In *Streptococcus* species, the mismatch repair is mediated by the Hex system which corrects point mutations, short insertions and deletions (Claverys & Lacks, 1986). The Hex system recognizes mismatches and induces the complete excision of the misinformative strand. This system corresponds to the *mutS-mutL* system described in *E. coli* (Yang, 2000). Inactivation of either of the *hex* genes, *hexA* or *hexB*, results in a mutator phenotype with an abolished mismatch repair system, indicating that a functional Hex system is important for avoiding mutations (Humbert *et al.*, 1995; Mortier-Barriere *et al.*, 1997). The mutation frequency of mismatch repair-deficient variants is rarely higher than 10-fold compared to the wild type, but this still provides an advantage in acquiring traits to overcome the environmental challenges (Negri *et al.*, 2002). Bacterial stress has been shown to promote and select for hypermutable variants (Oliver *et al.*, 2000; Miller *et al.*, 2002).

Another mismatch repair system was identified in *S. pneumoniae*, which converts A/G mismatches to CG pairs (Samrakandi & Pasta, 2000). This is mediated by the *mutY* homolog of *E. coli*. MutY of *E. coli* is an adenine glycosylase specific for removing the adenine from A/G mispairs mainly caused by 8-oxoG. The resulting apurinic site is removed by AP endonuclease and replaced by the correct GC pair. In a *mutY*-deficient strain of *S. pneumoniae* the emergence of rifampin-resistant mutants due to the CG-to-AT transversion was increased five-fold, indicating that in the wild-type strain the MutY system rescues the A/G mispairs to CG pairs. In *E. coli*, MutY cooperates with MutM and MutT to form an antimutator system specialized against the mutation potential of 8-oxoG and homologs for *mutM* and *mutT* genes have also been identified in *S. pneumoniae*, indicating a similar system (Mejean *et al.*, 1994; Tajiri *et al.*, 1995; Samrakandi & Pasta, 2000).

2.2.4 Biofilm growth

Sessile multispecies biofilms are probably the most common form of bacterial growth in nature (Costerton *et al.*, 1999). Biofilms are defined as structured, surface-adherent communities of bacterial cells enclosed in a self-produced polymeric matrix (Costerton *et al.*, 1999). Biofilms help bacteria to survive in hostile conditions, including rapid flow, cold, heat, desiccation and in the presence of antimicrobial chemicals. In natural environments, biofilms are often a mixture of several species of bacteria or fungi. The extra-cellular, polymeric matrix of a biofilm consists of various polysaccharides, proteins and even nucleic acids (Branda *et al.*, 2005). Recently, it has been acknowledged that biofilms form highly structured matrixes that are

suggestive of multicellular organisms in the communication between cells. The ability to form a biofilm is very important virulence factor for many species, including *S. mutans*, the causative agent of dental caries (Hamada & Slade, 1980). Microarray studies indicate that diverse model species including *Pseudomonas aeruginosa*, *V. cholerae*, *E. coli* and *S. aureus* differentially express as much as 10 % of their genome when in a biofilm as compared to planktonic growth conditions (Schoolnik *et al.*, 2001; Whiteley *et al.*, 2001; Schembri *et al.*, 2003; Beenken *et al.*, 2004).

The stages of biofilm formation include attachment to the surface and aggregation of the attached cells. The latter stages may include the production of a tight network of extracellular polysaccharides that facilitate the adherence of the cells to each other and to the surface, eventually forming differentiated three-dimensional biofilm architecture. The growth potential of any biofilm is limited at least by the deprivation of nutrients. This leads to the detachment of the outermost cell layer, planktonic growth of detached cells and colonization of other surfaces (Dunne, 2002; Stoodley *et al.*, 2002; Melchior *et al.*, 2006a) (Figure 2).

The biofilm mode of growth has been relatively extensively studied in oral streptococci *S. mutans* and *S. gordonii* (Kreth *et al.*, 2008), but biofilm formation in several other species, including *S. pyogenes*, *S. suis* and *S. pneumoniae*, has also been reported *in vitro* (Donlan *et al.*, 2004; Baldassarri *et al.*, 2006; Bonifait *et al.*, 2008). However, in many cases, very little is known about the importance of the biofilm mode of growth to streptococci. In *S. mutans* biofilms it has been shown that proteins required for the development of genetic competence were up-regulated and the cells growing in biofilms were able to incorporate foreign DNA 10-to 600-fold more efficiently via competence than planktonic cells (Li *et al.*, 2001; Rathsam *et al.*, 2005). In some bacteria a bacterial intercellular communication mechanism called quorum sensing also plays a role in biofilm formation. Quorum sensing is a bacterial system that regulates diverse physiological processes in

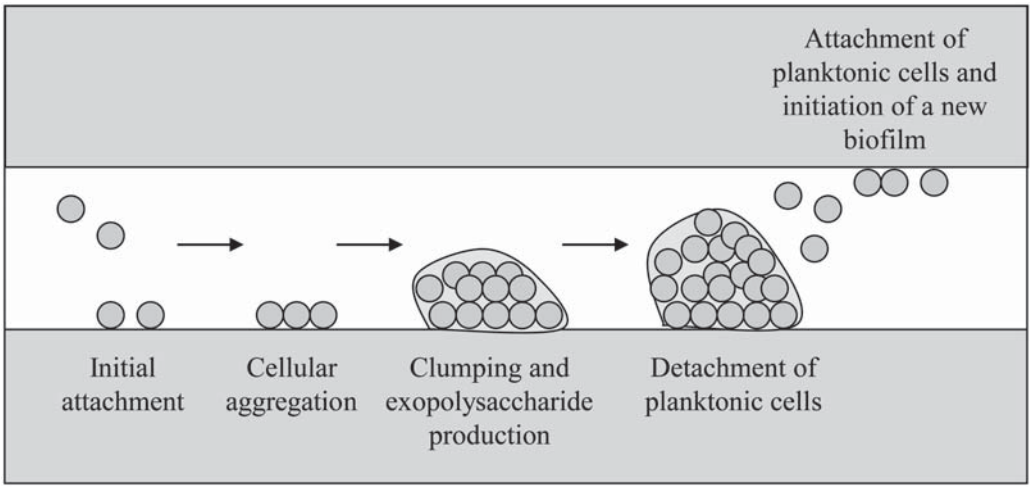

Figure 2. Biofilm formation and maturation (modified from Melchior *et al.*, 2006a)

response to increasing population density (Kleerebezem & Quadri, 2001). The inactivation of the *com* quorum-sensing system essential for competence leads to an abnormal biofilm with an altered architecture and reduced biomass in *S. mutans* (Li *et al.*, 2002).

Infections that involve a biofilm mode of growth are often persistent and difficult to treat, because biofilms show innate resistance to clearance by host defence mechanisms, disinfectants and antibiotics (Costerton *et al.*, 1999; Mah & O'Toole, 2001). Compared to the minimum inhibitory concentrations (MIC) of planktonic cells, cells in biofilm can tolerate significantly higher antibiotic concentrations. Some strains of *S. aureus* and *Staphylococcus epidermidis*, are susceptible to antibiotics in susceptibility tests, but resist antibiotic treatment when grown as a biofilm *in vivo* (del Pozo & Patel, 2007). In these cases, the treatment of infection requires surgical removal of infected tissues (Costerton *et al.*, 2003). The growth in biofilms leads to up to 500 higher tolerance of penicillin G, cloxacillin, streptomycin, ceftiofur, tetracycline and ampicillin in the mastitis bacteria *S. agalactiae* and *S. aureus* (Olson *et al.*, 2002; Melchior *et al.*, 2006b). The slow growth rate of the bacterial cells and insufficient penetration of antibiotics are partly responsible for the decreased susceptibility of mature biofilms to antimicrobial agents (Brown *et al.*, 1988; Stewart & Costerton, 2001). In *P. aeruginosa*, specific proteins have also been charaterized that partially mediate the increased antibiotic resistance of biofilms. The inactivation of *ndvB* required for the synthesis of periplasmic glucans and PA1874-1877 operon encoding of a novel putative efflux pump reduce the antibiotic resistance of biofilms (Mah *et al.*, 2003; Zhang & Mah, 2008). The efflux pump PA1874-1877 is more highly expressed in biofilm cells than in planktonic cells, and expression of these genes in planktonic cells increases their resistance to antibiotics. These results indicate that biofilms could have an active mechanism for antibiotic resistance and thus be more than simply a diffuse barrier.

Antibiotics can also induce biofilm formation. Ciprofloxacin, tetracycline and tobramycin enhance biofilm formation at concentrations below the minimum inhibitory concentration in *P. aeruginosa* (Linares *et al.*, 2006). In *E. coli*, β-lactams induce the synthesis of colanic acid, one of the molecules involved in attachment to surfaces, and this possibly enhances biofilm formation (Sailer *et al.*, 2003).

In some cases it has been shown that components from the host induce biofilm formation. In *P. aeruginosa*, biofilm formation in the tympanostomy tube is enhanced by exposure to dry and fresh blood (Malaty & Antonelli, 2008). *P. aeruginosa* is also highly dependent on iron from the host organism in biofilm formation, and iron-chelating agents have been proposed as a tool to inhibit biofilms (O'May *et al.*, 2009). In *Enterococcus faecalis*, the addition of 0.5-1% glucose to the growth medium enhanced biofilm formation (Baldassarri *et al.*, 2001; Kristich *et al.*, 2004; Pillai *et al.*, 2004).

The biofilm form of growth can also be a way to provide virulence and avoid the host's immune responses. Bacteria growing in a biofilm are well protected and antibodies may only eliminate the planktonic cells shed from the biofilm and possibly damage the surrounded tissue instead (Costerton *et al.*, 1999; Clutterbuck *et al.*, 2007). *P. aeruginosa* biofilms induce a weak phagocyte response, and phagocytic cells that

form the first line of immunological defence have difficulties to envelop and destroy bacteria in biofilms (Kharazmi, 1991). The polysaccharide matrix of *S. epidermidis* biofilms can also interfere with macrophage phagocytic activity (Shiau & Wu, 1998). Biofilm formation is a major virulence determinant in *S. aureus* causing chronic infections (Cucarella *et al.*, 2001; Cucarella *et al.*, 2002; Melchior *et al.*, 2006a). With *S. aureus* biofilms it has been shown that human leukocytes are able to penetrate the biofilms, but for an unknown reason they are unable to engulf the bacteria within (Leid *et al.*, 2002). In *S. epidermidis* the inactivation of the *ica* operon, which codes for the production of the extracellular polysaccharide known as polysaccharide intercellular adhesion (PIA), has been shown to reduce virulence (Rupp *et al.*, 1999a; Rupp *et al.*, 1999b). The absence of PIA also increases the phagocytic killing of *S. epidermidis* by human leukocytes and the susceptibility of the bacteria to major antibacterial peptides such as defensin and dermcidin of the host (Vuong *et al.*, 2004). Biofilm-negative strains of *S. epidermidis* carrying the *ica* locus, that usually promotes biofilm formation, were induced to produce biofilms by hyperosmolarity and the addition of tetracycline, proposing that this biofilm formation system can be inducible (Cho *et al.*, 2002). Bacteria growing in biofilms also utilize host proteins in escaping the immune defense. Biofilms on heart valves are embedded in a fibrin capsule, which protects the bacteria from the leukocytes and leads to infective endocarditis (Durack, 1975).

The differentiation of biofilm cells can also lead to new phenotypes by mutagenic mechanisms. In *P. aeruginosa* it has been shown that bacteria in biofilms displayed an up to 105-fold increase in their mutation rate compared to planktonic bacteria (Driffield *et al.*, 2008). Recent results have described the formation of microcolonies in subpopulation of *P. aeruginosa* within a biofilm, and the mutation frequency in these microcolonies is up to 100-fold higher than in planktonic cultures (Conibear *et al.*, 2009). The description of microcolonies provides an explanation for the structural and genetic heterogeneity of bacterial biofilms. These microcolonies are possible hotspots of cell division, mutagenesis and shedding of cells in biofilms. A link between the stress response and biofilm formation pathways is also acknowledged in *S. mutans* (Inagaki *et al.*, 2009). The stress response regulator RecA has been shown to have significance in biofilm production, and a RecA-deficient mutant strain (RAD) produced a lower density biofilm than the wild type. The cells in the biofilm formed by the mutant strain also had a lower viability and reduced acid tolerance (Inagaki *et al.*, 2009).

2.3 Mechanisms of drug resistance

Drug resistance can occur by drug inactivation or modification, alteration of the drug target site, alteration of metabolic pathways, and reduced drug accumulation by a decrease in drug permeability and/or increase in active efflux (pumping out) of the drugs across the cell wall (Wright, 2003).

The evolution of antibiotic resistance is one outcome of the genetic variation in bacterial populations. Antibiotic resistance can be achieved by mutagenesis or by acquiring exogenous DNA. The latter may occur by natural transformation or in the form of plasmids or other mobile genetic elements from members of the

same bacterial species or from other species (reviewed in Cirz *et al.*, 2006a). Interspecies horizontal gene transfer is particularly common between closely related species that inhabit the same environment. The acquirement of these pre-tested resistance mechanisms is an efficient way to achieve antibiotic resistance but in environments with a low supply of genetic material, mutagenesis or genomic rearrangements are the most likely option (Blazquez *et al.*, 2002).

Fluoroquinolone antibiotics such as norfloxacin and ciprofloxacin interact with two essential DNA topoisomerases, gyrase and topoisomerase IV, which are involved in the regulation of chromosome supercoiling during DNA replication (Drlica & Zhao, 1997; Drlica, 1999). Fluoroquinolone (FQ) resistance mostly develops by mutations in the *parC* gene encoding the topoisomerase IV subunit and the *gyrA* gene encoding the gyrase subunit. Mutations in the quinolone resistance-determining region (QRDR) of the *parC* gene generally results in low-level FQ resistance, while high-level resistance requires mutations in both of the target genes (Janoir *et al.*, 1996; Munoz & De La Campa, 1996; Kawamura *et al.*, 2003). In *S. pyogenes*, fluoroquinolone resistance has been shown to develop by spontaneous mutations in the *parC* gene and horizontal dissemination from *S. dysgalactiae* (Pletz *et al.*, 2006). In *S. pneumoniae*, the donors of fluoroquinolone resistance are the viridans group of streptococci, *S. mitis* and *S. oralis* (Balsalobre *et al.*, 2003) and in *S. agalactiae* the donor is *S. difficile* (Kawamura *et al.*, 2003).

Several antibiotics induce the SOS response and mechanisms of mutagenesis and may in fact actively promote the development of antibiotic resistance. Fluoroquinolone antibiotics induce the SOS response in many bacterial species and induce prophage genome replication and expression of shiga toxin in *E. coli* (Zhang *et al.*, 2000). The ICE SXT is also mobilized by SOS induction caused by fluoroquinolones in *V. cholerae*, and this ICE carries several antibiotic resistance genes (Beaber *et al.*, 2004). Ciprofloxacin induces phage mobilization and enhances the expression of phage-encoded virulence factors in *S. aureus*, and this induction was most likely mediated by a *rec*A-mediated pathway (Goerke *et al.*, 2006). In fluoroquinolone-susceptible methicillin-resistant *S. aureus*, growth in the presence of a subinhibitory concentration of FQ enhances methicillin resistance and also increases fluoroquinolone resistance (Venezia *et al.*, 2001; Tattevin *et al.*, 2009).

UV irradiation is one of the factors causing antibiotic resistance by activating error-prone DNA repair mechanisms. The mutation types typically synthesized by UV irradiation are point mutations, especially A:T transitions, but in *C. crescentus*, G:C to C:G type mutations have been characterized (Galhardo *et al.*, 2005; Friedberg *et al.*, 2006). However, it has been proposed that SOS induction by antibiotics might play a more important role *in vivo* than SOS induction by radiation and other chemicals (Ubeda *et al.*, 2005). The SOS response is induced in *E. coli* by β-lactam antibiotics when penicillin-binding protein 3 is inactivated and cell wall synthesis is halted (Miller *et al.*, 2004). This induction occurs via a DpiAB two-component signalling system and delays cell division in β-lactam stress, which provides the bacteria with temporary protection from β-lactam lethality.

Results from several studies where antibiotic treatment has been shown to accelerate evolution, the spread of antibiotic resistance and dissemination of virulence factors indicate that the antibiotic treatment of pathogens can cause "collateral damage" to commensal microbes such as *S. pneumoniae* (Cirz *et al.*, 2006a).

2.4 Virulence and mutation mechanisms *in vivo*

Virulence factors are components that enable bacteria to infect the host and cause disease. Virulence factors can vary widely from classical factors such as toxins to metabolic factors that help bacteria to obtain nutrition from the host. However, it is widely accepted that thorough knowledge of the cell-wall-exposed proteins/outer surfaces of pathogenic bacteria are of great importance in understanding their pathogenesis (Navarre & Schneewind, 1999; Maione *et al.*, 2005). The surface proteins are implicated in bacterial defense machineries and they are involved in several virulence-associated behaviours such as adhesion.

In *S. pyogenes* the key virulence factor is the M-protein, a coiled-coil peptidoglycan-attached polypeptide conferring for example anti-phagocytic properties. The N-termini of the M proteins from different *S. pyogenes* strains have areas with dissimilar sequences and based on these variable areas the strains can be divided into more than 125 different serotypes (Facklam *et al.*, 2002). This variability hinders the immune system from recognizing and clearing the pathogen. In *S. pneumoniae*, the virulence factor protein hyaluronate lyase or hyaluronidase is a secreted protein that degrades hyaluronic acid and increases the permeability of the host tissues and mucosa. A connection between hyaluronidase activity and the capacity to induce meningitis has been shown in several studies (Berry *et al.*, 1994; Kostyukova *et al.*, 1995; Berry & Paton, 2000; Zwijnenburg *et al.*, 2001). In *S. suis*, an important pathogen of pigs and a cause of zoonotic infections in humans, hyaluronate lyase has a highly variable area. The variation occurs by several mechanisms, including point mutations, deletions, and insertions, and there is evidence that genetic recombination is involved in the variability of this gene (King *et al.*, 2004). However, the significance of hyaluronate lyase to virulence is less clear in *S. suis* than in *S. pneumoniae*.

It has been known for a long time that capsular polysaccharides are an essential virulence factor in *S. pneumoniae*, and capsule production enhances colonization and prevents phagocytosis (reviewed in Preston & Dockrell, 2008). The expression of a capsule is also important for survival of *S. pneumoniae* in the blood and is associated with the ability of pneumococci to cause invasive disease (Bentley *et al.*, 2006). The pneumococcal seven-valent polysaccharide vaccine induces antibody formation against surface-exposed capsular polysaccharides and protects against pneumococcal disease caused by the seven selected serotypes (McEllistrem, 2009). Genetic variation in the capsular gene clusters leads to the antigen variation on which the serotype classification is based. It has been proposed that new capsular polysaccharide genes leading to new serotypes are introduced by horizontal gene transfer from other species (Bentley *et al.*, 2006). In *S. pneumoniae*, tandem duplication, deletion and single nucleotide polymorphism in the serotype-

specific capsular gene *cps3D* leads to the formation of an acapsular phenotype, which has a significantly higher capacity to form biofilm (Waite *et al.*, 2001; Allegrucci & Sauer, 2007; McEllistrem *et al.*, 2007). Mutations in the genome can also lead to the differentiation of serotypes by antigen variation, and this is a major problem for vaccination efficiency. Another well-known pneumococcal virulence factor is pneumolysin, which is a cytotoxin released during cell lysis and is involved in forming pores in host epithelial cells (Tilley *et al.*, 2005).

A link between DNA repair and virulence has also been shown. In *M. tuberculosis*, the SOS response-induced DnaE2 polymerase confers the development of antibiotic resistance in response to UV irradiation and the synthesis of C to T transitions (Boshoff *et al.*, 2003). Interestingly, deletion of *dnaE2* not only resulted in reduced survival after UV damage, but also reduced the development of antibiotic resistance *in vivo* and reduced virulence in mice. It has been proposed that translesion synthesis and the generation of mutations provides better survival and virulence in *M. tuberculosis* by protecting the cells from DNA-damaging reactive oxygen species and nitrogen intermediates generated by the host's immune response.

Virulence of *S. uberis* is mainly associated with growth in milk, and only a few virulence factors have been characterized. *S. uberis* has the ability to activate bovine plasminogen to the serine protease plasmin with a specific activator, PauA, and bind plasmin to its surface (Leigh, 1993; Leigh, 1994; Leigh & Lincoln, 1997; Rosey *et al.*, 1999). The proteolytic activity of plasmin derives peptides from milk casein and provides amino acids to *S. uberis* in the early stages of mammary gland colonization. In experimental bovine mastitis challenge, vaccination with PauA reduced the rate of colonization and decreased the incidence of challenge (Leigh *et al.*, 1999). However, the virulence of a *pauA* deletion mutant was similar to the wild type, indicating that the significance of PauA in virulence is still unclear (Ward *et al.*, 2003). A mutant strain of *S. uberis* lacking an *mtuA* gene is unable to grow in milk and failed to colonize the mammary gland, even when applied in 100- to 1000-fold higher doses than those sufficient for the wild type strain to cause mastitis (Smith *et al.*, 2003). The *mtuA* gene codes for a lipoprotein receptor antigen that is required for the assimilation of Mn^{2+} during growth in milk (Smith *et al.*, 2003). It has homology with PsaA and MtsA of *S. pneumoniae* and *S. pyogenes*, which have been shown to be responsible for the high uptake of metal ions from the surrounding media (Kolenbrander *et al.*, 1998; Janulczyk *et al.*, 1999). An oligopeptide binding protein (OppA2) has also been shown to be greatly up-regulated in *S. uberis* during growth in milk (Taylor *et al.*, 2003). OppA2 of *S. uberis* binds small peptides and possibly has a role in virulence and cell-cell signalling.

3 AIMS OF THE STUDY

The aim of this study was to gain an understanding of the mechanisms exploited by the mastitis bacterium *S. uberis* to induce mutagenesis and to survive drug exposure.

The specific goals of the present study were:

1) To study the potential of *S. uberis* 0140J for SOS-induced mutagenesis and to identify some SOS genes in mutagenesis and its regulation.
2) To reveal the presence of a new mechanism in *S. uberis* mediating mutagenesis under ciprofloxacin challenge.
3) To investigate the effects of mutagenesis-inducing ciprofloxacin on the proteome of *S. uberis* 0140J using two-dimensional difference gel electrophoresis coupled with mass spectrometric identifications.
4) To investigate the biofilm forming ability of several isolates of *S. uberis* from clinical and subclinical mastitis and to search for mechanisms triggering enhanced biofilm growth in milk.

4 MATERIALS AND METHODS

All materials and methods are described in detail in the original publications I-IV.

4.1 Bacterial strains and plasmids

Table 1. Bacterials strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Article	Source or reference
Strains			
S.uberis			
ATCC BAA-854 (0140J)	Genome sequencing strain (the EMBL/GenBank database accession number AM946015)	I-IV	ATCC (Ward *et al.*, 2009)
EH 78	ATCC BAA-854 derivative containing pEH55, Tet^r	I	This work
EH 58	ATCC BAA-854 derivative Δ*umuC* (1.4 kb deletion) Tet^S	I, II	This work
EH 79	EH58 containing pEH79, Tet^r	I	This work
EH 80	EH58 derivative, Tet^S, with *umuC* reverse allelic exchange	I	This work
PV161	Isolate from clinical mastitis	IV	This work
PV162	Isolate from clinical mastitis	IV	This work
PV164	Isolate from subclinical mastitis	IV	This work
PV165	Isolate from clinical mastitis	IV	This work
PV166	Isolate from subclinical mastitis	IV	This work
PV167	Isolate from subclinical mastitis	IV	This work
PV168	Isolate from clinical mastitis	IV	This work
PV169	Isolate from subclinical mastitis	IV	This work
PV170	Isolate from subclinical mastitis	IV	This work
E.coli			
JM109	*endA1*, *recA1*, *gyrA96*, *thi*, *hsdR17* (r_k^-, m_k^+), *relA1*, *supE44*, Δ(*lac-proAB*), [F´ *traD36*, *proAB*, *laq*I^qZΔM15].	I	Promega
EH55	JM109 containing pEH55	I	This work
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacl*^{q}*Z* Δ *M15 Tn10*(Tet^r)]^c	I	Stratagene
S. parauberis			
PV163	Isolate from subclinical mastitis		This work
S. dysgalactiae			
PV171	Isolate from clinical mastitis		This work
S. epidermidis			
RP62A (ATCC35984)	Biofilm-positive strain (Accession number NC_002976)	IV	ATCC (Gill *et al*., 2005)
ATCC12228	Biofilm-negative strain (Accession number NC_004461)	IV	ATCC (Zhang *et al*., 2003)

Strain or plasmid	Relevant characteristics	Article	Source or reference
Plasmids			
pGhost8	Tet^r, a thermosensitive derivative of pGK12	I	(Maguin *et al.*, 1996)
pEH55	Tet^r, pGhost with 1.0 kb *Xba*I-*Pst*I-*Sal*I fragment containing 1.4 kb non-polar deletion of *umuC*	I	This work
pEH79	Tet^r, pGhost with 3.0 kb *Xba*I-*EcoRI*I fragment containing *hdiR-umuC*-ORF3-ORF4 operon without the upstream region	I	This work
pBluescript-II SK+	Am^r , cloning vector	I	Stratagene
pQE-30	Am^r, vector for overexpression of proteins with N-terminal His(6)-tag	I	Qiagen
pQE-*hdiR*	Am^r, pQE derivative for overexpression of His-tagged HdiR	I	This work
pBluescript-IR	Am^r, *E. coli* vector carrying 26 bp fragment containing the IR preceding the *hdiR*	I	This work
pBluescript-ctrl	Am^r, *E. coli* vector carrying 26 bp fragment from the region preceding the *hdiR* (not containing the IR)	I	This work

4.2 Bacterial culture conditions

All *S. uberis* strains were routinely grown at 37 °C using TSYE agar [3% (w/v) tryptic soy broth, 0.3% (w/v) yeast extract and 1.5% (w/v) Bacto agar] or THY broth [Todd-Hewitt broth with 1% (w/v) yeast extract] without shaking. *E. coli* strains were grown at 37 °C in Luria-Bertani (LB) agar or LB broth with shaking (220 rpm). *S. epidermidis* strains were grown at 37°C in Tryptic Soy Broth (TS) and in THY broth with shaking.

4.3 Strain constructions

Molecular cloning techniques were performed essentially as described by Sambrook & Russell (2001). In study I, to create an in-frame deletion of the *umuC* gene of ATCC BAA-854, a replacement recombination technique was used. The *Xba*I/*Pst*I and *Pst*I/*Sal*I digested PCR products from the flanking areas of the *umuC* gene were cloned to an *Xba*I/*Sal*I digested thermosensitive pGhost8 plasmid (Maguin *et al.*, 1996). The resulting plasmid pEH55 was transformed to *E. coli* JM109 (Promega) by $CaCl_2$-transformation (Dagert & Ehrlich, 1979) according to manufacturer's instructions. *S. uberis* cells were transformed with pEH55 by electroporation.

For electroporation, cells were grown overnight first on TSYE agar plates and then in 5 ml THY. The overnight cultures were diluted 1:50 in fresh THY including 1% glycine (to weaken the cell wall) and cultured until OD_{600}=0.2-0.3, at which point the cells were harvested by centrifugation at 6500 x g, +4°C, 15 min. The cells were washed four times with OPL II washing buffer (10% glycerol, 6% sucrose) by centrifugation (9000 x g, +4 °C, 15 min). The cells were suspended in 500 µl OPL II and divided into 45 µl aliquots and stored at -80 °C. The electroporation was performed with a Biorad Genepulser using pulse of 25 kV/cm and 600Ω. The cells were recovered in THY supplemented with 6% sucrose for 2 hours at 28 °C and plated on TSYE agar with 1 µg/ml tetracycline. Plasmid

integration and excision were carried out as described elsewhere (Biswas *et al.*, 1993) using a temperature of 37.5 °C for integration of the plasmid into the genome and 28 °C for the excision and replication.

4.4 Overexpression of HdiR protein and electromobility shift assay

In study I, the *S. uberis* HdiR protein was overexpressed as a His-tagged protein in *E. coli* XL1-Blue cells from the pQE30-His-*hdiR* plasmid construct.

The overnight cells were diluted 1:50 in 100 ml fresh LB with 100 µg/ml ampicillin. They were then grown at 37 °C to OD_{600}= 0.6 and 1 mM IPTG was added. The cells were next grown for 2 hours and harvested with centrifugation. After this, the cells were disrupted with sonication and the protein was purified using a HisTrap HP 1-ml column and eluted with elution buffer (20 mM sodium phosphate, 0.5 M NaCl, 0.3 M imidazole, pH 7.4).

The binding of HdiR to DNA was tested with the potential binding site, a DNA fragment (167 bp) covering -157 to +10 relative to the *hdiR* start codon. An internal 154-bp fragment of *hdiR* (from +4 to +157) was used as control DNA. The DNA fragments were created by using hexachloro-6-carboxyfluorescein (HEX) fluorescent-labelled primers. To test the binding of HdiR to the inverted repeat (IR) structure, 26-bp oligonucleotide pairs containing the IR sequence or control sequence with overhangs creating *Eco*RI and *Xba*I compatible ends were annealed, treated with T4 polynucleotide kinase (MBI Fermentas), and ligated with T4 ligase (Roche) to *Xba*I/*Eco*RI cut pBluescript-II SK+ to obtain pBluescript-IR and pBluescript-ctrl, respectively. The M13 rev and M13 uni primers were used to amplify 230-bp fragments from pBluescript-IR and pBluescript-ctrl for the gel mobility assay.

The purified HdiR protein was used for electromobility shift assays (EMSA) according to Savijoki *et al.* (2003) with modifications. EMSA reactions (15 µl) were assembled by mixing the PCR-amplified fragment (35 ng) and the His_6-HdiR protein (0 to 80 ng) in gel shift buffer [20 mM Tris-HCl, pH 8.0, 60 mM KCl, 1 mM dithiothreitol, 10% glycerol, 0.1 mg/ml bovine serum albumin, and 2 µg poly(dI-dC)]. Gel shift reactions were incubated at 25 °C for 15 min followed by electrophoresis on a 5% polyacrylamide gel with 0.5 X TBE at room temperature. Following electrophoresis, the gels with pBluescript-cloned DNA samples were stained with ethidiumbromide. The gels were scanned with a Fuji FLA-5100 scanner (Fuji Photo Film Co., Ltd., Japan) using an excitation laser at 532 nm, an output voltage of 400 V, and an LPG emission filter. Images were analyzed using the Aida Image Analyzer software v. 4.03 (Raytest GmbH, Straubenhardt, Germany).

4.5 RNA extraction and Northern blotting

In studies I & III, gene expression was identified by Northern blotting. For total RNA isolation, *S. uberis* ATCCBAA854 overnight cultures were diluted 1:500 in THY broth and grown at 37 °C to an optical density OD_{600}= 0.2 for a UV stress assay and OD_{600}= 0.2 (study III) and OD_{600}= 0.4 (study I) for ciprofloxacin (CF) stress assays. Samples were taken 0, 30 and 60 minutes after UV exposure with 50 J/m^2 or 0, 30, 60 and 180 min after the addition of 0, 0.5 or 1.0 µg/ml CF. Cells were disrupted with 250 mg glass beads (≤106 µm, Sigma) and a Fastprep

FP120 –homogenizer (ThermoSavant). RNA extraction was performed using RNeasy Mini kit (QIAgen) according to the instructions of the supplier.

The DNA probes for putative *umuC*, *dnaE*, *recA*, *hdiR*, *dinP*, *recG*, *recF*, *uvrA* and *ruvA* in study I and *nrdA*, *ahpF*, *prsA* and *deoC* in study III from ATCC BAA-854 were amplified by PCR. For Northern analysis, the separation and transfer of RNA were carried out using a Latitude® precast gel (1.25% Seakem® Gold gel, Cambrex, CA., USA) and a Hybond-XL (GE Healthcare, CA., USA) uncharged nylon membrane following the instructions provided by the manufacturer. The probe DNAs were labelled with [α-^{33}P]ATP (>92.5 TBq mmol^{-1}) using the DNA Megaprime labelling kit (GE Healthcare) following the manufacturer's instructions. Northern hybridization was carried out as described elsewhere (Varmanen *et al.*, 2000). The membrane was scanned and quantified using a FLA-5100 scanner (Fujifilm Europe GmbH, Germany) and AIDA software version 4.03.031 (Raytest Isotopenmessgeraete Gmbh, Germany). The differences in RNA amounts were corrected by quantitating the 16S rRNA amounts in each lane.

4.6 UV- and ciprofloxacin-induced mutagenesis assays

In studies I & II, the mutation response after UV light and CF exposure, respectively, were determined by the development of rifampin (RIF) resistance. The overnight cultures of *S. uberis* ATCC BAA-854 (0140J) and EH58 strains were diluted 1:500 in THY broth and allowed to grow to OD_{600}= 0.2. At this point, the cultures for CF induction were diluted 7.5-fold in fresh THY supplemented with 0.3 or 0.5 μg/ml CF and grown overnight. In the UV-induction assay, 2 ml of the culture was centrifuged at 4500 x g, 10 min, RT and cells were resuspended in 0.9% NaCl. The cells were subjected to a UV dose of 0, 25 and 50 J/m^2 using a Spectrolinker XL-1000 UV cross-linker and grown overnight in 15 ml THY. The UV- and CF-induced overnight cultures were plated on TSYE agar supplemented with 2 μg/ml RIF and the UV-induced cells also onto TSYE supplemented with 2 μg/ml CF. In all of the mutagenesis assays, appropriate dilutions were plated on TSYE agar to determine the viable counts.

4.7 Pyrosequencing

In study I, the ciprofloxacin resistance-determining mutations of *S. uberis* were analyzed by pyrosequencing. *S. uberis* cells were grown and a UV-induced mutagenesis assay was performed as described above, but to ensure independent mutational events, the cultures were divided into aliquots, plated and a single ciprofloxacin-resistant colony from each plate was picked for sequencing analysis. DNA regions covering bases 228 to 255 and 293 to 314 from the *parC* gene were analyzed by pyrosequencing. The area was amplified by PCR using a biotin-labelled primer and pyrosequencing was performed with streptavidin-coated Sepharose beads (GE Healthcare), a PSQ 96 MA instrument, a vacuum prep workstation, and Pyro Gold SQA reagents (Biotage AB, Uppsala, Sweden) according to the instructions of the manufacturer.

4.8 Protein extractions and CyDye labelling

In study III, protein extraction for two-dimensional gel electrophoresis (2-DE) was performed as follows. Four

independent cultures of *S. uberis* 0140 J (ATCC BAA-854) cells were diluted 1:100 in THY broth and allowed to grow until OD_{600}= 0.2. Cultures were divided into 15 ml aliquots and 0, 0.5 and 1.0 µg/ml CF was added to the cultures. The cultures were allowed to grow until OD_{600}= 0.5 and the cells were harvested by centrifugation and washed with cold Tris-HCl (pH 8.0) + 30% EtOH. The proteins were extracted by disrupting the cells with glass beads in 30 mM Trizma base using FastPrep FP120 homogenizer (ThermoScientific, USA). Proteins were solubilised in 400 µl of UTC buffer (7 M urea, 2 M thiourea, 4% CHAPS and 30 mM Trizma base) and incubated at RT for 1 h. The disrupted cells and glass beads were removed by centrifugation and proteins were extracted using a 2-D Clean-up Kit (GE Healthcare, Sweden) according to manufacturer's instructions. The proteins were solubilised in 20 µl of UTC buffer and stored at -20 °C before 2-DE.

Samples were labelled with CyDye Fluor minimal cyanine dyes (GE Healthcare) according to the manufacturer's instructions. The pH of samples was adjusted to pH 8.5 with 2 M Trizma base and two 25 µg protein samples were labelled with 200 pmol of Cy3 and Cy5 dyes, respectively. An internal standard was created by combining 12.5 µg of each sample and labelling it with Cy2 dye. Labelling reactions were performed for 30 min on ice in the dark and stopped with 1 µl of 50 mM lysine.

4.9 Two-dimensional gel electrophoresis (2-DE)

In study III, proteins were identified with 2-DE. The isoelectric focusing (IEF) of the CyDye-labelled samples from four independent cultures were performed using ReadyStrip™ IPG Strips (BioRad) and Protean IEF Cell (BioRad). The strips were rehydrated overnight in UTC buffer supplemented with 1% BioLyte 3-10 buffer, 50 mM DTT and 4 mM tributylphosphine. Protein samples of 75 µg were loaded onto rehydrated IPG Strips by anodic cup loading. After IEF the strips were equilibrated first with 50 mM Tris-HCl pH 6.8, 6 M urea, 2% SDS, 20% glycerol and 2% DTT and then with 2.5% IAA. The second dimension was run on an Ettan DALTsix Large Vertical System (GE Healthcare) using self-made 12% gels. The gels were scanned immediately after the second dimension with a FLA 5100 scanner and analyzed using DeCyder 5.02 software (GE Healthcare). The gels were fixed and stained with silver for visualization and spot cutting. The proteins were degraded with trypsin and peptides were extracted and identified with MALDI-TOF (matrix-assisted laser desorption/ionization time-of flight) mass spectrometry.

4.10 Biofilm assays

In study IV, *S. uberis* biofilms were grown on clear polystyrene flat-bottomed tissue-culture-treated 96-well (Becton&Dickinson Falcon™ product number 353072) or 24-well (BD Falcon™ product number BDAA353047) microtiter plates. Colonies from overnight cultured agar plates were suspended in 15 ml THY broth to achieve an OD_{600}= 0.025. The cultures were grown at 37 °C until the OD_{600}= 0.2. At this point, $2x10^2$ cells in 200 µl THY broth were added to the microtiter wells, and the cells were grown at 37 °C for 18 h without shaking in aerobic conditions. Biofilm formation of *Staphylococcus epidermidis* strains

RP62A (ATCC35984) and ATCC12228 was tested as previously described (Polonio *et al*., 2001).

The effect of different factors on biofilm formation was tested as follows. The growth medium was supplied with skim milk (0.1% and 0.5% w/v), bovine serum albumin (5 mg/ml), fibrinogen from bovine plasma (5 mg/ml), lactoferrin from bovine milk (1 mg/ml), α-casein (3 mg/ml), β-casein (3 mg/ml), κ-casein (3 mg/ml) or casein hydrolyzate (3 mg/ml and 5 mg/ml) from bovine milk (Sigma, St Louis, MO, USA) before applying the medium to the microtiter plate.

In experiments employing protease inhibitors, the strains were grown in THY supplemented with or without skim milk (0.5% w/v), casein hydrolyzate (3 mg/ml), α-casein (2 mg/ml) or β-casein (2 mg/ml) and the protease inhibitors E-64, dichloroisocoumarin (DIC) and 1-10-phenanthroline monohydrate (Sigma, St. Louis, MO, USA) at a final concentration of 1 mM (E-64 and DIC) or 100 μM (1-10-phenanthroline).

The stability of *S. uberis* biofilms against protease treatment was tested as follows. Mature biofilms cultivated in 96-well microtiter plates were washed two times with PBS, treated with 1 mg/mL Proteinase K (Sigma) in 100 mM Tris-HCl (pH 8.0) for 24 h at 37°C and stained with crystal violet. *S. epidermidis* RP62A was used as control because it produces a protease-resistant biofilm.

4.10.1 Biofilm staining assays

For crystal violet staining, the planktonic suspension was removed from the wells following incubation and the wells were washed twice with 200 μL of PBS followed by the addition of 100 μL of crystal violet solution (0.5% w/v in MilliQ) (Sigma–Aldrich, Munich, Germany), and incubated at room temperature for 15 min. The excess stain was washed off with tap water. The stained biofilms were then dissolved in 200 μL of 33% acetic acid by incubation at room temperature for 30 min. The absorbance at 545 nm was measured with the PerkinElmer Victor^3 multilabel microtiter plate reader.

For resazurin staining, the 96-well plates were washed with PBS as above and 200 μL of fresh THY was added. To each well, 10 μl of 50 μg/ml resazurin solution was added (=0.5 μg/well) and the plates were incubated at 37 °C for 30 min or 1h. Fluorescence was measured with the Victor^3 multilabel microtiter plate reader using an excitation wavelength of 550 nm and emission wavelength of 590 nm.

4.10.2 Visualization of S. uberis biofilms with atomic force microscopy (AFM) and fluorescence microscopy (FM)

Bacterial biofilms were visualized with AFM and FM. For AFM, the *S. uberis* strains 0140J, PV162 and PV168 were grown on sterile pieces of polystyrene. 2 ml of the bacterial suspension (10^8 CFU/mL) in THY was added to the 24-well polystyrene plate and the biofilm cultures were grown on polystyrene pieces (1 cm in diameter) placed on the bottom of a microtiter well for 18 h. Following incubation, the planktonic suspension was removed. In 0 h samples, the bacterial suspension (10^8 CFU/mL) was added to the polystyrene pieces and then immediately removed. A Nanoscope IIIa scanning probe microscope equipped with a J-scanner (Digital Instruments, Inc., Santa Barbara, CA) was used for imaging the sample surfaces. The AFM imaging is described in detail in study IV.

For FM, the biofilms of 0140J, PV162 and PV168 were grown in 96-well microtiter plates as described above. Determination of viable and non-viable cells was performed using the LIVE/DEAD® BacLight™ Bacterial Viability Kit (L7007, Molecular Probes Invitrogen Detection technologies) according to the manufacturer's instructions. The biofilm cells were stained and kept in the dark for 15 min and then examined with a Zeiss AxioVert 200M fluorescence microscope using a FITC filter for the SYTO-9 label and a TRITC filter for propidium iodide, after which photographs were taken with a digital camera.

4.10.3 Statistical analysis

Resazurin fluorescence and crystal violet absorption data were checked for normality using Shapiro-Wilk's test and for homogeneity of variances using Bartlett's test. The data were then subjected to analysis of variance. If a normal distribution of data or homogeneity of variances could not be confirmed, nonparametric variant of ANOVA was applied. In estimating the effect of DIC on casein-induced biofilm formation, the fluoresence in the absence of casein was substracted from the casein-induced values. The magnitude of induction was then compared in the absence vs. presence of DIC. The statistical analysis was performed with R software (R version 2–6.0 [2007-11-03]: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0; http://www.R-project.org).

4.10.4 Mutation rate determination

In order to determine the spontaneous mutation rate of planktonic and biofilm cells, colonies from overnight culture agar plates were suspended in 15 ml THY broth to achieve OD_{600}= 0.025. The cultures were grown at 37°C until $0D_{600}$= 0.2. The cultures were diluted in THY broth and 20 µl aliquots of the dilution containing $1x10^4$ cells were added to 24-well microtiter plates with 2 ml fresh THY including 3 mg/ml casein hydrolysate. The cultures were grown for 24 h and the medium was changed to fresh THY with 3 mg/ml casein hydrolysate, after which the plates were grown for an additional 16 h. The planktonic cells from 3 wells of technical replicas were combined and harvested by centrifugation (4500 g, 15 min, RT). The biofilms from the same wells were suspended in a total volume of 6 ml of PBS and the cells were harvested with centrifugation. The planktonic cells and the cells from biofilms were plated on TSYE agar containing 2 µg/ml RIF. Appropriate dilutions of the samples were also plated on TSYE agar for viability determination. The plates were grown 2 days at 37 °C and the numbers of colonies were counted. The proportion of mutations was the ratio of resistant cells/viable cells.

5 RESULTS AND DISCUSSION

5.1 Characterization of a new inducible stress response operon in *S. uberis*

Previously, it has been stated that the lack of a LexA homolog in streptococcal species indicates that streptococci do not have an SOS response system (Gasc *et al.*, 1980). However, sequence analysis of the *S. uberis* 0140J genome indicated that *S. uberis* has the gene SUB0899 with 33% identity to *Lactococcus lactis* LexA-like HdiR regulator, which is upregulated in heat shock and in DNA damaging conditions (Savijoki *et al.*, 2003). In *S. uberis*, this regulator is also followed by the SUB0898 gene homologous to *umuC*, the replicatory subunit of error-prone polymerase PolV, and two open reading frames (SUB0897 and SUB0896) with unknown function (study I). In study I, the role of this new operon was examined in order to identify a possible SOS-regulated DNA repair mechanism.

To study possible stress-induced DNA repair mechanims, *S. uberis* cells were subjected to UV light. This stress condition induced mutagenic responses detected by the formation of RIF- or CF-resistant colonies on agar plates. After UV-induction with 50 J/m^2, the number of RIF- and CF-resistant colonies increased 209- and 48-fold compared to uninduced cultures, respectively. The types of mutations synthesized after UV stress were characterized by sequencing of the *parC* region of CF-resistant mutants, where the mutations promoting CF resistance most commonly occur (Janoir *et al.*, 1996; Pan & Fisher, 1996; Orscheln *et al.*, 2005). The sequencing performed with pyrosequencing techniques revealed mainly G:C → A:T transition type mutations, which are considered to be the hallmark of UV-induced mutations in *E. coli* (Friedberg *et al.*, 2006). This suggested that *S. uberis* produces mutations in DNA-damaging conditions. To test which genes might play a role in this process, we searched the genome sequence of *S. uberis* ATCC BAA-854 for SOS response candidates, especially error-prone polymerases. The expression of putative error-prone polymerases (DinP, DnaE, UmuC) was tested by Northern blot analysis after UV exposure and after the addition of a sublethal concentration of CF. Both UV light and CF are known to induce the SOS response in other bacterial species (Cirz *et al.*, 2005; Ubeda *et al.*, 2005; Cirz *et al.*, 2007). Northern blot analysis revealed that only expression of the *umuC* gene increased after UV and CF stress. Changes in the expression of the other error prone polymerases were not detected in these stress conditions. To further study the importance of *umuC*

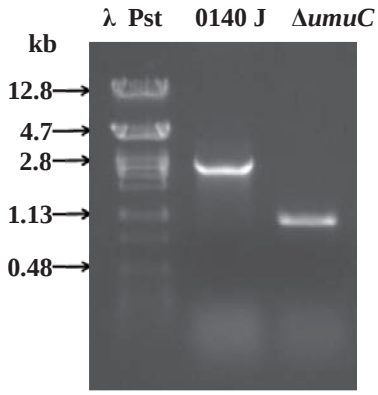

Figure 3. PCR fragments amplified from the *hdiR-umuC*-ORF3-ORF4 area using as templates the genomic DNA from the wild type (0140J) strain and from the Δ*umuC* strain with a 1.4 kb *umuC* deletion. The DNA fragments were separated in a 0.8 % agarose gel.

in stress conditions, we deleted in-frame the 1.4 kb *umuC* gene by using a replacement recombination technique and thermosensitive plasmid (Figure 3). The deletion of *umuC* led to lower tolerance of UV and mitomycin C and dramatically reduced the emergence of CF- and RIF-resistant mutants after UV induction. The results thus indicate that in *S. uberis* *umuC*-dependent stress response is used for survival in CF- and UV-stress.

The mRNA transcript of *umuC* (3.0 kb) is significantly longer than the 1,425 bp coding region predicted for *umuC* according to the sequence analysis (study I). The transcript recognized by the *umuC*-specific probe in Northern blotting was also recognized by the *hdiR*-specific probe, indicating that *umuC* is organized in an operon structure with *hdiR*. The operon structure would also explain the length (3.0 kb) of the mRNA transcript.

We characterized the properties of HdiR in order to determine whether HdiR could be the LexA-like regulator of the SOS response in *S. uberis* and the regulator of *umuC* expression (study I). We found that the HdiR protein of *S. uberis* has similar properties as *L. lactis* HdiR. *S. uberis* HdiR binds to the 26-bp IR structure preceding the *hdiR-umuC*-ORF3-ORF4 operon and undergoes a self-cleavage reaction at a high pH *in vitro*. Although the overall amino acid sequence identity of *S. uberis* HdiR with *L. lactis* HdiR is only 33%, the self-cleavage site (Ala126-Gly127 in *L. lactis* and Ala117-Gly118 in *S. uberis*) and two other amino acids involved in RecA-mediated self-cleavage (Ser160 as well as Lys200 in *L. lactis* and Ser150 as well as Lys187 in *S. uberis*) are conserved in both sequences (Savijoki *et al.*, 2003, study I). In *E. coli*, the model organism of SOS response, self-cleavage of LexA inactivates the protein (Friedberg *et al.*, 2006). We tested the DNA-binding ability of *S. uberis* HdiR after self-cleavage in an EMSA assay and noticed that the HdiR of *S. uberis* has the ability to bind DNA after self-cleavage in a similar manner as the *L. lactis* HdiR (Savijoki *et al.*, 2003).

Sequence data analysis of other streptococcal species revealed that gene sequences homologous to *hdiR, umuC,* ORF3 (SUB0897) and ORF4 (SUB0896) of *S. uberis* are present in several of them. A similar operon structure of these genes was present in at least one strain of *S. agalactiae, S. pyogenes, S. sanguinis* and *S. mitis*. The putative HdiRs were also preceded by an IR structure with similarities to IR structure preceding *S. uberis* HdiR. Interestingly, putative homologs of *hdiR, umuC,* ORF3 and ORF4 were also found in streptococcal plasmids, transposons and other mobile genetic elements. The *S. pyogenes* transposon Tn1207.3 also carries the macrolide resistance promoting *mef(A)* gene (Pozzi *et al.*, 2004). The results suggest that the *hdiR-umuC*-ORF3-ORF4 gene cassette might mediate survival, virulence or other benefits in streptococci and is therefore transferred from one bacterium to another in mobile genetic elements. This hypothesis is supported by studies on the *S. pneumoniae* Tn5252 transposon, in which the *umuDC*-like genes were previously characterized and shown to restore UV-inducible mutagenic DNA repair in bacteria defective in error-prone repair (Munoz-Najar & Vijayakumar, 1999).

Several attempts were made to delete the *hdiR* gene, the DNA-binding helix-turn-helix(HTH)-motif of HdiR or the self-cleavage site (Ala117-Gly118) of HdiR, but none of these attempts were successful. The deletion of *hdiR* probably

leads to the constitutive expression of the mutagenic gene cassette and production of lethal mutations. This reflects the importance of the HdiR regulator and suggests that HdiR might also have other regulation targets besides the *hdiR-umuC*-ORF3-ORF4 operon.

The possible roles of ORF3 and ORF4 in the SOS-inducible gene cassette of *S. uberis* remain to be elucidated. The UmuC protein forms the functional PolV when associated with two molecules of activated UmuD (UmuD') in *E. coli* (Friedberg *et al.*, 2006). These *umuC* and *umuD* genes are expressed in an operon. However, in Gram-positive bacteria the UmuD proteins have not been found. It is possible that ORF3 and ORF4 are proteins involved in assembling a functional error-prone polymerase PolV together with UmuC.

The significance of UmuC-like Y-family polymerase in UV-induced mutagenesis varies between bacterial species. The *umuC/D* genes are absent in several bacterial species, especially among Gram-positive bacteria. However, these bacteria have putative error-prone polymerases that might replace the function of PolV (UmuDC). In *B. subtilis*, the UmuC-like polymerase YqjW and the DinB-like polymerase YqjH have been characterized (Sung *et al.*, 2003). Deletion of the *yqjW* gene leads to reduced mutagenesis after UV induction, but the deletion of *yqjH* has even a greater effect on UV-induced mutagenesis. In *C. crescentus*, which lacks UmuDC homologs, the products of DNA-damage-inducible operon *dnaE2-imuA-imuB* replace the function of UmuDC in UV- and mitomycin C-induced DNA damage. ImuB has been characterized as a Y-family polymerase that is present in several bacterial species lacking *umuC* homologs. The mutation types created by the function of the *dnaE2-imuA-imuB* operon are G:C to C:G transversions, which differ from the G:C to A:T transitions commonly produced by *E. coli* PolV and *S. uberis* UmuC (Friedberg *et al.*, 2006; study I). In our study, it was shown that UmuC is the only putative error-prone polymerase induced by UV and mitomycin C stress, and was to a large extent responsible for the UV-induced mutagenic repair. This is in contrast to results from *E. coli*, where SOS-induced mutagenesis after CF exposure is abolished only if all three inducible, SOS-regulated polymerases PolII (PolB), PolIV (DinB) and PolV (UmuC) are deleted (Cirz *et al.*, 2005).

To determine whether *hdiR* and the *hdiR-umuC*-ORF3-ORF4 are present in other *S. uberis* strains besides the culture collection strain 0140J (ATCC BAA-854), we performed a PCR reaction with *hdiR* and *hdiR-umuC*-ORF3-ORF4 specific primers using as a template DNA from two culture collection strains (0140J and DSMZ20569) and 11 *S. uberis* strains (PV161-PV171) from clinical and subclinical cases of mastitis (Table 1). The strains from clinical and subclinical cases of mastitis were identified as *S. uberis* based on phenotypes with conventional microbiological methods. With DNA from the culture collection strains and 9 of the clinical and subclinical strains as a template, PCR reactions with *hdiR* and *hdiR-umuC*-ORF3-ORF4 specific primers produced PCR products of 0.73 kb for *hdiR* and 3.0 kb for *hdiR-umuC*-ORF3-ORF4 (Figure 4). The sizes of the PCR products correspond to the predicted sizes based on the genome sequence. No PCR product was acquired from PV163 and PV171 strains. 16 S rDNA sequencing of the strains revealed that the strains PV163 and PV171 were *S. parauberis* and *S.*

dysgalactiae, respectively. All the other strains were confirmed by sequencing to be *S. uberis*. The PCR results from all of the *S. uberis* strains were positive, indicating that the *hdiR* gene and the *hdir-umuC*-ORF3-ORF4 operon are conserved in the different strains.

Inhibition of the LexA-regulated SOS response in *E. coli* prevents the development of antibiotic resistance by mutagenesis (Cirz *et al.*, 2005). This has led to the suggestion that the future development of antibiotic resistance could be prevented by the discovery of drugs, which inhibit the mechanisms that create resistance-promoting mutations. Especially interesting targets for this type of drug research are the genes specific to certain pathogens, such as the *hdiR-umuC* operon of *Streptococcus* or the *imuAB* of *C. crescentus* and α-proteobacteria (Galhardo *et al.*, 2005).

5.2 Ciprofloxacin induces mutagenesis leading to antibiotic resistance in *S. uberis*

The fluoroquinolone antibiotic CF was shown to induce the expression of the *hdiR* regulator and *umuC* coding for error-prone polymerase in *S. uberis* (study I). The effects of CF on the genome of *S. uberis* were further investigated in study II. The formation of RIF-resistant colonies

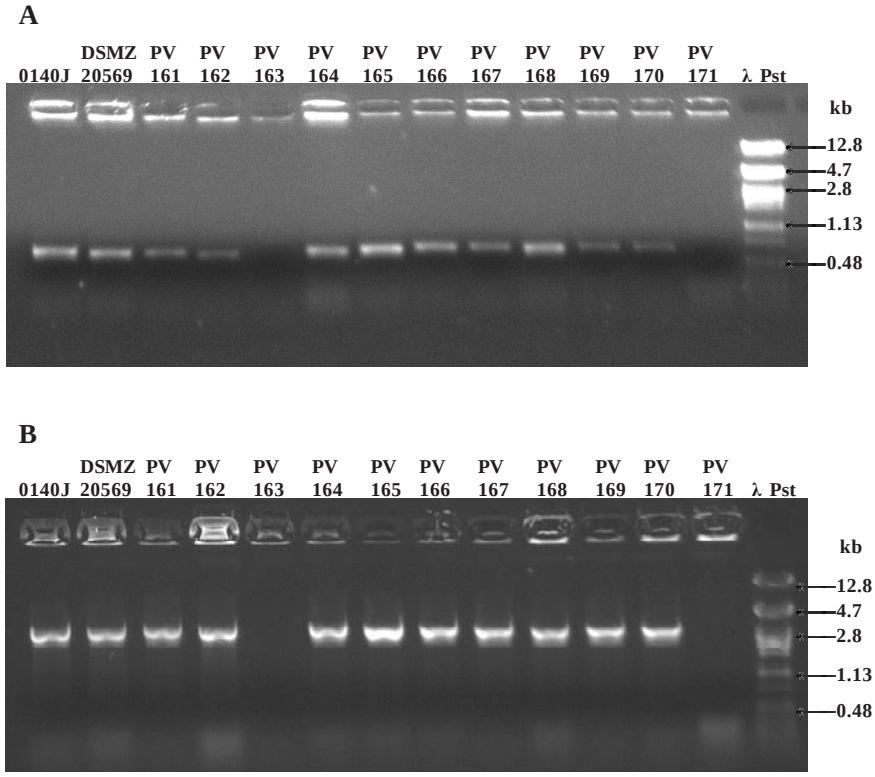

Figure 4. The DNA fragments from the PCR amplification of *hdiR* gene (A) and *hdiR-umuC*-ORF3-ORF4 operon (B). The template DNA was from *S. uberis* clinical and subclinical strains and the DNA fragments were separated in a 0.8% agarose gel. The numbers above the gels refer to the bacterial strains.

was used as a marker to observe the mutagenesis after overnight exposure to a sublethal (0.3-0.5 x MIC) concentration of CF. To examine the possible influence of *umuC* on the CF-induced response, the mutagenesis assay was performed on a wild type 0140J (ATCC BAA-854) *S. uberis* strain and the Δ*umuC* derivative (EH58) of this strain created in study I.

A sublethal concentration of CF resulted in a more than 100-fold increase in the formation of RIF resistance in both the wt and Δ*umuC* strain. This indicates that CF induces mutagenic mechanisms in *S. uberis*. Unlike the UV-induced mutagenesis in study I, CF also induces mutation mechanisms in the Δ*umuC* strain, suggesting an alternative, *umuC*-independent mutation mechanism repairing the DNA damage caused by CF. This is in contrast with the results from *E. coli*, where UV light and CF induce the SOS response in a similar manner and the products of the *umuDC* operon are required for mutagenesis (Cirz *et al.*, 2005; Friedberg *et al.*, 2006).

It has previously been shown that mutations in the *rpoB* area cause alterations in RIF target protein, the RNA polymerase β subunit, promoting RIF resistance. In order to determine the mutation types created after CF stress, a 702 bp fragment of the *rpoB* gene was sequenced. Forty-eight RIF-resistant mutants of both the wt and Δ*umuC* strain were sequenced, and 41 of the wt strains as well as 40 of the Δ*umuC* strains carried a mutation in this area. In clones derived from both strains, 50 % of the mutations were G:C to A:T transitions, which are the same type of mutations as those characterized after UV exposure in study I. The G:C to C:G transversions were slightly more common in the wt strain (4/41 mutants) than in the Δ*umuC* strain (1/40 mutants). Unlike after UV-exposure, insertions and deletions were also detected after CF exposure. The differences in CF-induced mutation types compared to UV-induced mutations support the hypothesis of *umuC*-independent mechanism in CF-induced mutagenesis. However, it is also possible that in *parC* and *rpoB* genes sequenced in study I and II, respectively, different kind of mutations promote antibiotic resistance.

The significance of error-prone polymerases in CF-induced mutagenesis remains to be elucidated. In study II it was shown that UmuC (PolV) is not required for this mutagenesis, and it has previously been demonstrated that in *S. pneumoniae*, CF also induces mutagenesis in a strain lacking PolIV (DinB) (Henderson-Begg *et al.*, 2006). Moreover, in study I, CF exposure did not increase the expression level of *dnaE* or *dinP*, the genes coding for other putative error-prone polymerases of *S. uberis*. In *P. aeruginosa*, CF has been shown to up-regulate the expression of the genes *dinB*, *dnaE2* and *imuB* coding for error-prone polymerases, but the significance of this finding in mutagenesis remains to be elucidated. Two of these polymerases (*dnaE2* and *imuB*) and several other recombination and replication genes are LexA-regulated (Cirz *et al.*, 2006b).

CF as an inducing agent of mutation mechanisms is significant when antibiotic treatments are considered in veterinary medicine. Although *S. uberis* infections are most commonly treated with penicillin, other bovine infections are treated with fluoroquinolones, such as enrofloxacin, which are metabolized to CF (McKellar *et al.*, 1999; Grobbel *et al.*, 2007). Residues of CF can also be found in environmental samples (Batt *et al.*, 2006). Thus, *S. uberis* strains are exposed to fluoroquinolones

posing a possible threat of development of mutations promoting antibiotic resistance. In *S. aureus*, exposure to CF has also been shown to enhance methicillin resistance (Tattevin *et al.*, 2009).

5.3 Ciprofloxacin induces expression of proteins involved in oxidative stress, nucleotide biosynthesis and NADH generation

The CF-induced mutagenesis in *S. uberis* characterized in study II was further elucidated in study III by investigating the effect of CF on *S. uberis* cultures using proteomics. With 2-DE the proteomes of untreated *S. uberis* cultures were compared with cultures treated with 0.5 µg/ml (0.5 x MIC) and 1.0 µg/ml (1 x MIC) CF. Altogether, 24 spots showed differential expression when 1.0 µg/ml CF-treated and untreated cultures were compared. Identification of the spots with MALDI-TOF mass spectrometry revealed that the spots represented 20 different proteins. With the lower CF concentration (0.5 µg/ml) only seven spots with altered expression were detected. These alterations were in parallel with the proteins detected from gels with 1.0 µg/ml CF-treated cultures, but an increase in the antibiotic concentration increased the difference in protein expression compared to the untreated cultures. In our study, 15 proteins were expressed at a higher level and 9 proteins at a lower level after exposure to 1 µg/ml CF. Previously, it has been stated that exposure to CF mostly triggers down-regulation of gene expression rather than up-regulation (Cirz *et al.*, 2006b; Marrer *et al.*, 2006). This has been proposed to be a survival strategy of the bacteria, because CF is more lethal to actively dividing cells than resting cells (Drlica & Zhao, 1997). However, the upregulation of gene expression in *S. uberis* proposes an active mechanism to survive the CF exposure.

The proteins found to have altered expression can be classified into three categories: i) oxidative stress proteins, ii) proteins related to NADH generation and iii) proteins involved in nucleotide biosynthesis and regulation of the dNTP pool balance.

CF has been shown to cause oxidative stress in *S. aureus* (Becerra & Albesa, 2002). In our study, the protein with the most efficient upregulation in expression level due CF stress was AhpF, alkyl hydroperoxide reductase, which is organized in an operon structure with *ahpC* in *S. pyogenes* (Pulliainen *et al.*, 2008) and also in *S. uberis* (study III). The products of this operon construct the functional alkyl hydroperoxide reductase (Jönsson *et al.*, 2007). The reduction of reactive oxygen species protects the bacterial cell from the damaging effects of oxygen and is mediated by the function of superoxide dismutases, catalases and peroxidises. As a member of the lactic acid bacteria, *S. uberis* does not produce oxidoreductase catalase, which is often considered to be essential in tolerance of the damaging effects of hydroperoxidase and in growth in an aerobic atmosphere. The ability of *S. uberis* to grow in an aerobic atmosphere indicates that it relies on other oxidoreductases in overcoming the damaging effects of oxygen and its radicals. In *S. pyogenes*, it has been shown that the product of the *ahpCF* operon protects the cell from H_2O_2 damage (Pulliainen *et al.*, 2008).

The downregulation of proteins involved in the generation of nicotinamide adenine dinucleotide (NADH) are possibly also due to the oxidative stress response. NADH is an electron acceptor and

carrier in many oxidation reactions. The proteins down-regulated in our study and involved in the generation of NADH were sorbitol-6-phosphate 2-dehydrogenase, fructose-6-phosphate amidotransferase, glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase. In *E. coli*, reduction of the amount of NADH by blocking the NADH-producing tricarboxylic acid cycle resulted in the decreased formation of reactive oxygen species and better survival under CF stress (Kohanski *et al.*, 2007). In *P. aeruginosa*, CF exposure down-regulates the expression of NADH dehydrogenase complex I, which is a similar response to that observed in acute H_2O_2 damage (Palma *et al.*, 2004; Cirz *et al.*, 2006b).

The increase in expression of proteins shown to regulate the dNTP pool possibly elucidates the mutation mechanism activated by CF. These proteins include ribonucleoside diphosphate reductase (NrdA), deoxyribose aldolase (DeoC), adenylosuccinate synthetase (AdsS), inositol-5-monophosphate dehydrogenase (ImpDH) and ribose-phosphate pyrophosphokinase (PrsA), and all of them were up-regulated in response to CF exposure by 2.2-, 1.6-, 1.5-, 2.0- and 1.7-fold, respectively. NrdA is the ribonucleotide reductase subunit, and ribonucleotide reductases catalyse the formation of dNTPs from ribonucleotides. This changes the composition of the cell's dNTP pool, and imbalance in the dNTP pool has been shown to cause insertions and deletions formed by replicative polymerases *in vitro* (Bebenek *et al.*, 1992). Intriguingly, also in study II the CF-induced mutational spectrum revealed a high frequency of insertions and deletions. The influence of CF on nucleotide metabolism has also been acknowledged in *S. aureus* transcriptional analysis, where the expression of ribonucleotide reductase encoding genes *nrdI*, *nrdE* and *nrdF* was upregulated 3.4-, 2.7- and 2.3-fold, respectively, when cells were subjected to 0.8 μg/ml CF for 120 minutes (Cirz *et al.*, 2007). A DNA microarray study on methicillin-resistant *S. aureus* exposed to a sub-inhibitory concentration of the fluoroquinolone levofloxacin revealed a 2- to 4-fold increase in ribonucleotide reductase *nrdE* expression and a 1.4- to 2.0-fold increase in *norR* expression, which has been shown to promote increased CF resistance mediated by efflux pumps without mutations in *gyrA* or *parC* genes (Truong-Bolduc *et al.*, 2003; Truong-Bolduc & Hooper, 2007; Tattevin *et al.*, 2009). Taken together the results indicate that like in other bacteria, also in *S. uberis* CF exposure significantly increased the abundance of some proteins involved in the dNTP pool. The changes in dNTP pool possibly mediate the CF-induced mutagenesis discovered in study II.

If *S. uberis* copes with oxidative stress caused by CF by decreasing the amount of available NADH, this might force the bacterium to use dNTPs as an energy source. This is supported by the higher expression of DeoC under CF stress. DeoC has been shown to play a role in the catabolism of dNTPs from dead cells (Sgarrella *et al.*, 1997). The enzymes involved in purine biosynthesis, AdsS and ImpDH, were also upregulated in our study. In *L. lactis*, it has been shown that genes involved in guanine metabolism are related to tolerance of acid, heat shock and glucose starvation, and the deletion mutants of *guaA*, GMP synthetase and (p)ppGpp synthetase *relA* were constitutively stress tolerant (Rallu *et al.*, 2000). This emphasizes the importance of the phosphate and purine nucleotide pools in

the multistress tolerance of *L. lactis*, as changes in purine nucleotide pools and an elevated (p)ppGpp concentration seem to be a signal for the stress response.

In study III, it was shown that *S. uberis* can alter its energy metabolism and nucleotide composition as a response to CF stress. The variety of metabolic options and the ability to alter its metabolism has been proposed as a survival mechanism in *S. uberis* (Ward *et al.*, 2009). Results from a genome sequencing project on *S. uberis* suggest that it is an opportunistic pathogen that has a wider variety of metabolic options than other related streptococci, and this helps in the adaptation of the bacterium to its specialized ecological niche and to challenging and changing environments.

5.4 Biofilm formation is induced by casein components and milk in *S. uberis*

Biofilms are sessile, surface-attached forms of growth characterized in several bacterial species and they in general tolerate high amounts of chemical and mechanical stress. In the mastitis bacteria *S. aureus* and *S. epidermidis*, biofilms are associated with recurrent and persistant mastitis (Melchior *et al.*, 2006a; Oliveira *et al.*, 2006), but the biofilm form of growth has not been characterized before in *S. uberis*. However, *S. uberis* mastitis often persists for unknown reasons and develops into chronic mastitis even though this bacterium is susceptible to antibiotics (Milne *et al.*, 2005). In study IV, biofilms as potential survival and persistence mechanisms in *S. uberis* were investigated.

Nine *S. uberis* strains isolated from clinical and subclinical mastitis confirmed as *S. uberis* by 16S rDNA sequencing were used in this study, together with the culture collection strain 0140J. As control strains we used the *S. epidermidis* strains RP62A (ATCC35984) and ATCC12228. RP62A is a reference biofilm-positive strain, which forms a thick and multilayered biofilm on polymer surfaces (Gill *et al.*, 2005), whereas ATCC12228 is a non-biofilm forming strain (Zhang *et al.*, 2003). First, the ability of these strains to form biofilm was assessed by cultivating the strains in 96-well polystyrene plates. The amount of biofilm was measured with crystal violet and resazurin staining. *S. uberis* had the ability to grow in biofilms, but the strains produced biofilm at varying efficiency, indicating that the biofilm producing ability is a strain-specific trait. Two of the *S. uberis* strains, PV162 and PV168, formed biofilm in amounts comparable to that formed by the *S. epidermidis* RP62A under the conditions used.

To study the differences in biofilm thickness and structure, the biofilms from strains 0140J (non-biofilm producer), PV162 and PV168 (strong biofilm producers) were examined with atomic force microscopy (AFM) and fluorescence microscopy (FM). PV162 and PV168 formed true biofilms with a multilayered structure, but 0140J formed only scattered, net-like monolayer structure.

The ability to form a biofilm varies between streptococcal species. *S. mutans* is the best studied biofilm producer, and the biofilm form of growth is essential for its virulence (Senadheera & Cvitkovitch, 2008). The biofilm form of growth has also been characterized in at least some strains of *S. suis, S. pneumoniae, S. pyogenes, S. gordonii, S. agalactiae* and *S. dysgalactiae* (Olson *et al.*, 2002; Gilmore *et al.*, 2003; Donlan *et al.*, 2004; Baldassarri *et al.*, 2006; Bonifait *et al.*, 2008), but in *S. pyogenes*, for instance, this ability varies strongly between strains (Baldassarri *et*

al., 2006). Inducible biofilm formation has been shown in several bacteria such as *S. epidermidis*, where biofilm-negative strains were induced to produce a biofilm by hyperosmolarity and the addition of tetracycline (Cho *et al.*, 2002), and in *S. suis*, where host component fibrinogen induces biofilm formation (Bonifait *et al.*, 2008).

To test whether proteins from the host could induce biofilm production in *S. uberis* strains producing low amounts of biofilm, we cultivated the strains with bovine serum albumin (BSA), fibrinogen and lactoferrin (unpublished results). Lactoferrin reduced the amount of biofilm produced in all strains. Lactoferrin is part of the host's innate immune system, and it possibly prevents infection by inhibiting the biofilm production of pathogens (Wakabayashi *et al.*, 2009). In *S. suis* fibrinogen induced biofilm production and possibly functions as a cross-bridging molecule in biofilms (Bonifait *et al.*, 2008). However, the effect of fibrinogen as well as BSA on *S. uberis* biofilm production varied between strains.

Milk is the natural growth medium of *S. uberis*, and it has previously been shown that milk or milk proteins enhance the adherence and internalization of *S. uberis* into mammary epithelial cells (Almeida *et al.*, 2003). In the presence of small concentrations (0.1% and 0.5%) of skim milk, biofilm formation in *S. uberis* strains PV161, PV164-PV167, PV169 and PV170 was markedly induced. In PV165, 0.5% milk increased biofilm formation by 800%. In most cases, even 0.1% milk was sufficient for the induction. When we further examined the possible inducing component in milk, casein hydrolysate and α- and β-casein were shown to increase biofilm formation. Biofilm formation was induced in all of the weak and moderate biofilm producers by casein hydrolysate and α- and/or β-casein, but κ-casein had no effect on any of these strains. The concentration of α-, β- and κ-casein in milk varies between cows due to genotypic variation (Ikonen *et al.*, 1999; Hallen *et al.*, 2008). Because of the different efficiencies of caseins in inducing biofilm formation, it is tempting to speculate that the milk protein composition of the host might affect the growth mode of *S. uberis* and the development of infection.

During *S. agalactiae* mastitis infection, the proportion of α- and β-casein decreases as a result of proteolytic activity, indicating that these proteins are degraded in mastitis (Saeman *et al.*, 1988). In *S. uberis*, both casein hydrolysate and intact α- and β-casein induced biofilm formation, suggesting that proteolytic activity of the bacterium might play a role in biofilm formation. The biofilm formation of two *S. uberis* strains (PV165 and PV166) was tested in the presence of milk, caseins and protease inhibitors E-64 (cysteine protease inhibitor), 1-10-phenanthroline (metalloprotease inhibitor) and DIC (serine protease inhibitor). Only DIC was shown to reduce biofilm production in the presence of milk, casein hydrolysate, α- and β-casein. The reducing effect of DIC on biofilm growth was particularly significant with α-casein (~50% reduction in biofilm growth in both PV165 and PV166) and least significant with casein hydrolysate (19% reduction in PV165 and 16% in PV166). The results suggest that bacterial proteolysis of intact casein fractions by serine proteases releases peptides that might function as cross-bridging peptides that promote intercellular adhesion or as a signal to switch from planktonic growth to the sessile form. Previously, it has been shown that *S. uberis* acquires amino acids

from milk by stimulating host protein plasmin with the plasminogen activator PauA, resulting in casein hydrolysis *in vivo* (Leigh, 1994; Kitt & Leigh, 1997). Our results indicate that the proteolytic activity of *S. uberis* might be important in the degradation of the caseins, but this remains to be elucidated.

In *S. epidermidis* strain RP62A the biofilm production is based on the expression of Polysaccharide intercellular adhesin (PIA), while in some strains proteinaceous structures seem to mediate biofilm formation in polysaccharide-independent manner (Heilmann *et al.*, 1996; Rohde *et al.*, 2005). In *S. uberis* the extracellular proteins play a crucial role in formation of biofilm (study IV). The overnight treatment of biofilms formed by the PV162 and PV168 strains with Proteinase K resulted in complete detachment of biofilms. The control biofilms formed by *S. epidermidis* strain RP62A were not affected by Proteinase K.

To our knowledge, biofilm production by *S. uberis* has not been previously described. In study IV, we proposed that the biofilm form of growth is a possible explanation for persistent *S. uberis* mastitis and the failure of antibiotic therapy. This has been shown in biofilm-positive *S. aureus* strains, which are more often associated with chronic infections than the biofilm-negative strains (Cucarella *et al.*, 2002; Cucarella *et al.*, 2004; Kwon *et al.*, 2008).

The inducible effects of milk and caseins have also not been characterized in other mastitis bacteria. Previously, internalization in the host cells has been evidenced as the main mechanism of persistant *S. uberis* infections (Tamilselvam *et al.*, 2006), but based on our results, we propose that biofilm formation and the induction of biofilm growth by components from the host might help *S. uberis* to reside and persist in the mammary gland, which is a harsh environment with high shearing forces and flow. The inducibility of the biofilm mode of growth might also be important for the virulence of *S. uberis*, which infects the udder from environmental sources and under suitable conditions in the mammary gland switches to become a causative agent of acute clinical infection.

5.5 Biofilm form of growth increases rifampicin resistance promoting mutation rate

Biofilm cultures display a higher mutation frequency compared to planktonic cultures in *P. aeruginosa* (Driffield *et al.*, 2008). To test whether the biofilms of *S. uberis* have a higher mutation frequency than planktonic cells and whether error-prone polymerase UmuC has significance in biofilms, the frequency of rifampicin resistance promoting mutations in the planktonic and the biofilm cells in strain 0140J and EH58 were determined. To induce biofilm formation, the strains were grown in the presence of 3 mg/ml casein hydrolyzate for 40 hours and the planktonic and biofilm cells were then plated on RIF agar plates. We discovered that the biofilms had a 4- and 5-fold higher mutation frequency compared to the planktonic cells in 0140J and EH58, respectively (Figure 5, unpublished results). The mutation frequencies of planktonic cultures in strains 0140J and EH58 were similar, being 15 RIF-resistant cells per 10^9 viable cells. The biofilm cells of 0140J and EH58 had mutation frequencies of 82 $RIF^R/10^9$ cells and 64 $RIF^R/10^9$ cells, respectively. The difference between the mutation frequency of the wild type strain and Δ*umuC* strain is

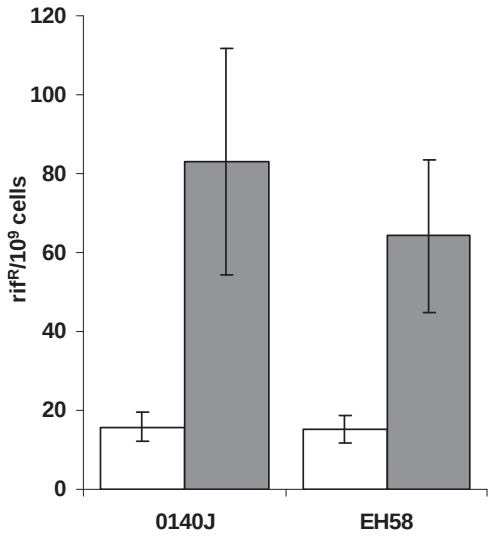

Figure 5. Mutation rate of wt *S. uberis* strain 0140J and the Δ*umuC* strain EH58 grown in planktonic culture and biofilm. The white bars represent the proportion of RIF resistant cells in planktonic cells and the grey bars in biofilms. The assay was performed with three biological replicas and the error bars represent the standard errors of the mean.

not statistically significant, indicating that other factors are involved in rifampicin resistance causing mutagenesis.

The cells in this assay were cultured for 40 hours, because the regular 16 hours of growth used in other assays is not sufficient for the biofilm to mature and the cells to differentiate. Because of the elongated incubation time, the growth medium had to be changed after 24 hours to obtain viable cells. This resulted in the original planktonic cells being discarded, but our hypothesis was that the planktonic cells harvested at the end of the experiment originated from the planktonic phase that remained at the bottom of the well. In fact, if the planktonic phase of the mutation frequency assay originates from cells detached from the biofilms, the observed mutation frequency is an underestimate of the true mutation frequency.

To our knowledge, increased mutation rates in biofilms compared to planktonic cells have previously been noted only in *P. aeruginosa* (Driffield *et al.*, 2008). In *P. aeruginosa* the mutation frequency in biofilms was 105-fold greater than in planktonic cells, but the experimental conditions with a longer growth period possibly enhanced the effect. Within *P. aeruginosa* biofilms, microcolonies with a higher mutation frequency have been characterized (Conibear *et al.*, 2009). These microcolonies also have a faster cell division rate and differentiation level than the surrounding biofilm. The description of microcolonies provides an explanation for the structural and genetic heterogeneity of *P. aeruginosa* biofilms.

6 CONCLUDING REMARKS AND FUTURE PROSPECTS

It has been proposed that the ability of *S. uberis* to survive in environmental reservoirs and switch from this life form to become a causative agent of udder infection requires mechanisms that respond efficiently to environmental changes and stimuli (Ward *et al.*, 2009). In this thesis, several mechanisms used by *S. uberis* to respond to environmental conditions and to survive stress have been characterized.

Research on stress-inducible mutation mechanisms provides information on how bacteria evolve, develop antibiotic resistance and escape the host immune system. In study I, a new inducible streptococcal mutagenesis gene cassette that promotes better survival and mutagenesis after UV light exposure was characterized. The presence of the *hdiR-umuC*-ORF3-ORF4 operon indicates that *S. uberis*, as well as some other streptococcal species, might have an SOS-response-like inducible mutagenesis system, which is regulated by the novel regulator HdiR instead of the SOS regulator LexA characterized in *E. coli* and *B. subtilis*. The *umuC* gene of this operon codes for a polymerase subunit of the error-prone polymerase PolV. The functional PolV of *E. coli* is composed of a UmuC subunit and two modified UmuD' subunits. According to our research, the UmuC subunit is encoded by the *umuC* gene of the *hdiR-umuC*-ORF3-ORF4 operon, but the gene coding for possible UmuD subunits remains to be elucidated. The potential candidates for UmuC cofactors are the proteins encoded by ORF3 and ORF4. Neither of these genes shares homology with previously found *umuD* genes and the significance of these genes is a potential topic for future research.

The fluoroquinolone antibiotic CF also induced the expression of the *hdiR-umuC*-ORF3-ORF4 operon. In study II, the potential induction of mutagenesis by CF in *S. uberis* was investigated. CF induced mutation mechanism in *S. uberis*, which resulted in the development of antibiotic resistance, but this mechanism was independent of UmuC, as the *umuC* deletion mutant had a similar mutation frequency to the wild type. To identify the mutation mechanisms and cellular responses induced by CF, changes in the protein composition of *S. uberis* in the presence of CF were identified by 2-D electrophoresis in study III. This revealed changes in the expression of proteins involved in oxidative stress, NADH generation and nucleotide biosynthesis. The oxidative stress caused by CF exposure is a possible explanation for most of the changes in protein expression, but changes in the expression of proteins regulating the dNTP pool and mediating nucleotide biosynthesis might elucidate the mechanism by which CF induces mutagenesis in *S. uberis*. For future studies we have constructed a transposon-based random mutagenesis library, which consists of 5500 clones of *S. uberis* with randomly deleted genes. Mutants with a reduced CF tolerance and with a reduced mutation frequency after CF exposure can be screened by growing these clones in the presence of CF. The identification of the genes deleted in CF sensitive mutants is likely to further reveal the mutagenic mechanisms of *S. uberis*.

Biofilms are a surface-attached growth form of bacteria that promotes better tolerance of stress factors such as desiccation, shearing forces, flow and chemicals. In study IV, the ability of *S. uberis* to grow as a biofilm was analysed and the switch from planktonic growth to biofilm

formation was induced by milk and caseins from the host. The characterization of biofilm growth in *S. uberis* further reinforces the idea that *S. uberis* has diverse and inducible mechanisms to adapt and survive in various environments. Serine proteases of *S. uberis* were shown to play a role in inducible biofilm formation by caseins and milk, but the molecular mechanism behind this remains to be elucidated. The proteolytic activity of *S. uberis* possibly releases peptides that trigger and assist biofilm formation. Biofilms often involve quorum sensing and thereby signalling between bacteria in the biofilm (Suntharalingam & Cvitkovitch, 2005), and these aspects in *S. uberis* biofilms are an interesting topic for future research. The bacterial cells in biofilms show genotypic and phenotypic variation proposing that this is a survival strategy in changing environmental conditions (Allegrucci & Sauer, 2007; Conibear *et al.*, 2009). The increased rifampicin resistance in *S. uberis* biofilms also indicate that compared to the planktonic growth from, biofilms have molecular mechanisms that are currently unknown.

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8 REFERENCES

Ajdic, D., McShan, W. M., McLaughlin, R. E., Savic, G., Chang, J., Carson, M. B. *et al.* (2002) Genome sequence of *Streptococcus mutans* UA159, a cariogenic dental pathogen. *Proc Natl Acad Sci U S A* 99: 14434-14439.

Alber, J., El-Sayed, A., Lammler, C., Hassan, A. A., & Zschock, M. (2004) Polymerase chain reaction mediated identification of *Streptococcus uberis* and *Streptococcus parauberis* using species-specific sequences of the genes encoding superoxide dismutase A and chaperonin 60*. *J Vet Med B Infect Dis Vet Public Health* 51: 180-184.

Allegrucci, M., & Sauer, K. (2008) Formation of *Streptococcus pneumoniae* non-phase-variable colony variants is due to increased mutation frequency present under biofilm growth conditions. *J Bacteriol* 190: 6330-6339.

Allegrucci, M., & Sauer, K. (2007) Characterization of colony morphology variants isolated from *Streptococcus pneumoniae* biofilms. *J Bacteriol* 189: 2030-2038.

Almeida, R. A., Luther, D. A., Nair, R., & Oliver, S. P. (2003) Binding of host glycosaminoglycans and milk proteins: possible role in the pathogenesis of *Streptococcus uberis* mastitis. *Vet Microbiol* 94: 131-141.

Ambur, O. H., Davidsen, T., Frye, S. A., Balasingham, S. V., Lagesen, K., Rognes, T., & Tonjum, T. (2009) Genome dynamics in major bacterial pathogens. *FEMS Microbiol Rev* 33: 453-470.

Au, N., Kuester-Schoeck, E., Mandava, V., Bothwell, L. E., Canny, S. P., Chachu, K. et al. (2005) Genetic composition of the *Bacillus subtilis* SOS system. *J Bacteriol* 187: 7655-7666.

Baldassarri, L., Creti, R., Recchia, S., Imperi, M., Facinelli, B., Giovanetti, E. *et al.* (2006) Therapeutic failures of antibiotics used to treat macrolide-susceptible *Streptococcus pyogenes* infections may be due to biofilm formation. *J Clin Microbiol* 44: 2721-2727.

Baldassarri, L., Cecchini, R., Bertuccini, L., Ammendolia, M. G., Iosi, F., Arciola, C. R. *et al.* (2001) *Enterococcus* spp. produces slime and survives in rat peritoneal macrophages. *Med Microbiol Immunol* 190: 113-120.

Balsalobre, L., Ferrandiz, M. J., Linares, J., Tubau, F., & de la Campa, A. G. (2003) Viridans group streptococci are donors in horizontal transfer of topoisomerase IV genes to *Streptococcus pneumoniae*. *Antimicrob Agents Chemother* 47: 2072-2081.

Banks, D. J., Porcella, S. F., Barbian, K. D., Martin, J. M., & Musser, J. M. (2003) Structure and distribution of an unusual chimeric genetic element encoding macrolide resistance in phylogenetically diverse clones of group A *Streptococcus*. *J Infect Dis* 188: 1898-1908.

Barnes, M. H., Miller, S. D., & Brown, N. C. (2002) DNA polymerases of low-GC gram-positive eubacteria: identification of the replication-specific enzyme encoded by *dnaE*. *J Bacteriol* 184: 3834-3838.

Batt, A. L., Bruce, I. B., & Aga, D. S. (2006) Evaluating the vulnerability of surface waters to antibiotic contamination from varying wastewater treatment plant discharges. *Environ Pollut* 142: 295-302.

Beaber, J. W., Hochhut, B., & Waldor, M. K. (2004) SOS response promotes horizontal dissemination of antibiotic resistance genes. *Nature* 427: 72-74.

Bebenek, K., Roberts, J. D., & Kunkel, T. A. (1992) The effects of dNTP pool imbalances on frameshift fidelity during DNA replication. *J Biol Chem* 267: 3589-3596.

Becerra, M. C., & Albesa, I. (2002) Oxidative stress induced by ciprofloxacin in *Staphylococcus aureus*. *Biochem Biophys Res Commun* 297: 1003-1007.

Beenken, K. E., Dunman, P. M., McAleese, F., Macapagal, D., Murphy, E., Projan, S. J. *et al.* (2004) Global gene expression in *Staphylococcus aureus* biofilms. *J Bacteriol* 186: 4665-4684.

Benson, N. R., Wong, R. M., & McClelland, M. (2000) Analysis of the SOS response in *Salmonella enterica* serovar *typhimurium* using RNA fingerprinting by arbitrarily primed PCR. *J Bacteriol* 182: 3490-3497.

Bentley, S. D., Aanensen, D. M., Mavroidi, A., Saunders, D., Rabbinowitsch, E., Collins, M. *et al*. (2006) Genetic analysis of the capsular biosynthetic locus from all 90 pneumococcal serotypes. *PLoS Genet* 2: e31.

Berry, A. M., & Paton, J. C. (2000) Additive attenuation of virulence of *Streptococcus pneumoniae* by mutation of the genes encoding pneumolysin and other putative pneumococcal virulence proteins. *Infect Immun* 68: 133-140.

Berry, A. M., Lock, R. A., Thomas, S. M., Rajan, D. P., Hansman, D., & Paton, J. C. (1994) Cloning and nucleotide sequence of the *Streptococcus pneumoniae* hyaluronidase gene and purification of the enzyme from recombinant *Escherichia coli*. *Infect Immun* 62: 1101-1108.

Biswas, I., Gruss, A., Ehrlich, S. D., & Maguin, E. (1993) High-efficiency gene inactivation and replacement system for gram-positive bacteria. *J Bacteriol* 175: 3628-3635.

Blazquez, J., Oliver, A., & Gomez-Gomez, J. M. (2002) Mutation and evolution of antibiotic resistance: antibiotics as promoters of antibiotic resistance? *Curr Drug Targets* 3: 345-349.

Bolotin, A., Wincker, P., Mauger, S., Jaillon, O., Malarme, K., Weissenbach, J. *et al*. (2001) The complete genome sequence of the lactic acid bacterium *Lactococcus lactis* ssp. *lactis* IL1403. *Genome Res* 11: 731-753.

Bonifait, L., Grignon, L., & Grenier, D. (2008) Fibrinogen induces biofilm formation by *Streptococcus suis* and enhances its antibiotic resistance. *Appl Environ Microbiol* 74: 4969-4972.

Booth, J. M. (1988) Update on mastitis. I. Control measures in England and Wales. How have they influenced incidence and aetiology? *Br Vet J* 144: 316-322.

Boshoff, H. I., Reed, M. B., Barry, C. E.,3rd, & Mizrahi, V. (2003) DnaE2 polymerase contributes to *in vivo* survival and the emergence of drug resistance in *Mycobacterium tuberculosis*. *Cell* 113: 183-193.

Bradley, A. (2002) Bovine mastitis: an evolving disease. *Vet J* 164: 116-128.

Bramley, A. J. (1984) *Streptococcus uberis* udder infection--a major barrier to reducing mastitis incidence. *Br Vet J* 140: 328-335.

Branda, S. S., Vik, S., Friedman, L., & Kolter, R. (2005) Biofilms: the matrix revisited. *Trends Microbiol* 13: 20-26.

Brown, J. S., Gilliland, S. M., & Holden, D. W. (2001) A *Streptococcus pneumoniae* pathogenicity island encoding an ABC transporter involved in iron uptake and virulence. *Mol Microbiol* 40: 572-585.

Brown, J. S., Gilliland, S. M., Spratt, B. G., & Holden, D. W. (2004) A locus contained within a variable region of pneumococcal pathogenicity island 1 contributes to virulence in mice. *Infect Immun* 72: 1587-1593.

Brown, J. S., Gilliland, S. M., Ruiz-Albert, J., & Holden, D. W. (2002) Characterization of Pit, a *Streptococcus pneumoniae* iron uptake ABC transporter. *Infect Immun* 70: 4389-4398.

Brown, M. R., Allison, D. G., & Gilbert, P. (1988) Resistance of bacterial biofilms to antibiotics: a growth-rate related effect? *J Antimicrob Chemother* 22: 777-780.

Bruck, I., Goodman, M. F., & O'Donnell, M. (2003) The essential C family DnaE polymerase is error-prone and efficient at lesion bypass. *J Biol Chem* 278: 44361-44368.

Brueggemann, A. B., Pai, R., Crook, D. W., & Beall, B. (2007) Vaccine escape recombinants emerge after pneumococcal vaccination in the United States. *PLoS Pathog* 3: e168.

Burghout, P., Bootsma, H. J., Kloosterman, T. G., Bijlsma, J. J., de Jongh, C. E., Kuipers, O. P., & Hermans, P. W. (2007) Search for genes essential for pneumococcal transformation: the RADA DNA repair protein plays a role in genomic recombination of donor DNA. *J Bacteriol* 189: 6540-6550.

Cho, S. H., Naber, K., Hacker, J., & Ziebuhr, W. (2002) Detection of the *icaADBC*

gene cluster and biofilm formation in *Staphylococcus epidermidis* isolates from catheter-related urinary tract infections. *Int J Antimicrob Agents* 19: 570-575.

Cirz, R. T., Gingles, N., & Romesberg, F. E. (2006a) Side effects may include evolution. *Nat Med* 12: 890-891.

Cirz, R. T., O'Neill, B. M., Hammond, J. A., Head, S. R., & Romesberg, F. E. (2006b) Defining the *Pseudomonas aeruginosa* SOS response and its role in the global response to the antibiotic ciprofloxacin. *J Bacteriol* 188: 7101-7110.

Cirz, R. T., Chin, J. K., Andes, D. R., de Crecy-Lagard, V., Craig, W. A., & Romesberg, F. E. (2005) Inhibition of mutation and combating the evolution of antibiotic resistance. *PLoS Biol* 3: e176.

Cirz, R. T., Jones, M. B., Gingles, N. A., Minogue, T. D., Jarrahi, B., Peterson, S. N., & Romesberg, F. E. (2007) Complete and SOS-mediated response of *Staphylococcus aureus* to the antibiotic ciprofloxacin. *J Bacteriol* 189: 531-539.

Claverys, J. P., & Havarstein, L. S. (2007) Cannibalism and fratricide: mechanisms and raisons d'etre. *Nat Rev Microbiol* 5: 219-229.

Claverys, J. P., & Havarstein, L. S. (2002) Extracellular-peptide control of competence for genetic transformation in *Streptococcus pneumoniae*. *Front Biosci* 7: d1798-814.

Claverys, J. P., & Lacks, S. A. (1986) Heteroduplex deoxyribonucleic acid base mismatch repair in bacteria. *Microbiol Rev* 50: 133-165.

Claverys, J. P., Prudhomme, M., & Martin, B. (2006) Induction of competence regulons as a general response to stress in gram-positive bacteria. *Annu Rev Microbiol* 60: 451-475.

Clutterbuck, A. L., Woods, E. J., Knottenbelt, D. C., Clegg, P. D., Cochrane, C. A., & Percival, S. L. (2007) Biofilms and their relevance to veterinary medicine. *Vet Microbiol* 121: 1-17.

Conibear, T. C., Collins, S. L., & Webb, J. S. (2009) Role of mutation in *Pseudomonas aeruginosa* biofilm development. *PLoS One* 4: e6289.

Costerton, J. W., Stewart, P. S., & Greenberg, E. P. (1999) Bacterial biofilms: a common cause of persistent infections. *Science* 284: 1318-1322.

Costerton, W., Veeh, R., Shirtliff, M., Pasmore, M., Post, C., & Ehrlich, G. (2003) The application of biofilm science to the study and control of chronic bacterial infections. *J Clin Invest* 112: 1466-1477.

Cucarella, C., Solano, C., Valle, J., Amorena, B., Lasa, I., & Penades, J. R. (2001) Bap, a *Staphylococcus aureus* surface protein involved in biofilm formation. *J Bacteriol* 183: 2888-2896.

Cucarella, C., Tormo, M. A., Knecht, E., Amorena, B., Lasa, I., Foster, T. J., & Penades, J. R. (2002) Expression of the biofilm-associated protein interferes with host protein receptors of *Staphylococcus aureus* and alters the infective process. *Infect Immun* 70: 3180-3186.

Cucarella, C., Tormo, M. A., Ubeda, C., Trotonda, M. P., Monzon, M., Peris, C. *et al.* (2004) Role of biofilm-associated protein Bap in the pathogenesis of bovine *Staphylococcus aureus*. *Infect Immun* 72: 2177-2185.

Dagert, M., & Ehrlich, S. D. (1979) Prolonged incubation in calcium chloride improves the competence of *Escherichia coli* cells. *Gene* 6: 23-28

Dagkessamanskaia, A., Moscoso, M., Henard, V., Guiral, S., Overweg, K., Reuter, M. *et al.* (2004) Interconnection of competence, stress and CiaR regulons in *Streptococcus pneumoniae*: competence triggers stationary phase autolysis of *ciaR* mutant cells. *Mol Microbiol* 51: 1071-1086.

Davies, M. R., Shera, J., Van Domselaar, G. H., Sriprakash, K. S., & McMillan, D. J. (2009) A novel integrative conjugative element mediates genetic transfer from group G *Streptococcus* to other {beta}-hemolytic streptococci. *J Bacteriol* 191: 2257-2265.

Davis, E. O., Dullaghan, E. M., & Rand, L. (2002) Definition of the mycobacterial SOS box and use to identify LexA-regulated genes in *Mycobacterium tuberculosis*. *J Bacteriol* 184: 3287-3295.

del Pozo, J. L., & Patel, R. (2007) The challenge of treating biofilm-associated bacterial infections. *Clin Pharmacol Ther* 82: 204-209.

Dervyn, E., Suski, C., Daniel, R., Bruand, C., Chapuis, J., Errington, J. *et al*. (2001) Two essential DNA polymerases at the bacterial replication fork. *Science* 294: 1716-1719.

Do, T., Jolley, K. A., Maiden, M. C., Gilbert, S. C., Clark, D., Wade, W. G., & Beighton, D. (2009) Population structure of *Streptococcus oralis*. *Microbiology* 155: 2593-2602.

Donlan, R. M., Piede, J. A., Heyes, C. D., Sanii, L., Murga, R., Edmonds, P. *et al*. (2004) Model system for growing and quantifying *Streptococcus pneumoniae* biofilms in situ and in real time. *Appl Environ Microbiol* 70: 4980-4988.

Donovan, D. M., Kerr, D. E., & Wall, R. J. (2005) Engineering disease resistant cattle. *Transgenic Res* 14: 563-567.

Driffield, K., Miller, K., Bostock, J. M., O'Neill, A. J., & Chopra, I. (2008) Increased mutability of *Pseudomonas aeruginosa* in biofilms. *J Antimicrob Chemother* 61: 1053-1056.

Drlica, K. (1999) Mechanism of fluoroquinolone action. *Curr Opin Microbiol* 2: 504-508.

Drlica, K., & Zhao, X. (1997) DNA gyrase, topoisomerase IV, and the 4-quinolones. *Microbiol Mol Biol Rev* 61: 377-392.

Dunne, W. M.,Jr. (2002) Bacterial adhesion: seen any good biofilms lately? *Clin Microbiol Rev* 15: 155-166.

Durack, D. T. (1975) Experimental bacterial endocarditis. IV. Structure and evolution of very early lesions. *J Pathol* 115: 81-89.

Facklam, R. (2002) What happened to the streptococci: overview of taxonomic and nomenclature changes. *Clin Microbiol Rev* 15: 613-630.

Facklam, R. F., Martin, D. R., Lovgren, M., Johnson, D. R., Efstratiou, A., Thompson, T. A. *et al*. (2002) Extension of the Lancefield classification for group A streptococci by addition of 22 new M protein gene sequence types from clinical isolates: emm103 to emm124. *Clin Infect Dis* 34: 28-38.

Faustoferri, R. C., Hahn, K., Weiss, K., & Quivey, R. G.,Jr. (2005) Smx nuclease is the major, low-pH-inducible apurinic/apyrimidinic endonuclease in *Streptococcus mutans*. *J Bacteriol* 187: 2705-2714.

Fernandez de Henestrosa, A. R., Rivera, E., Tapias, A., & Barbe, J. (1998) Identification of the *Rhodobacter sphaeroides* SOS box. *Mol Microbiol* 28: 991-1003.

Fernandez De Henestrosa, A. R., Ogi, T., Aoyagi, S., Chafin, D., Hayes, J. J., Ohmori, H., & Woodgate, R. (2000) Identification of additional genes belonging to the LexA regulon in *Escherichia coli*. *Mol Microbiol* 35: 1560-1572.

Ferretti, J. J., Ajdic, D., & McShan, W. M. (2004) Comparative genomics of streptococcal species. *Indian J Med Res* 119 Suppl: 1-6.

Flynn, J. M., Neher, S. B., Kim, Y. I., Sauer, R. T., & Baker, T. A. (2003) Proteomic discovery of cellular substrates of the ClpXP protease reveals five classes of ClpX-recognition signals. *Mol Cell* 11: 671-683.

Foster, K. A., Barnes, M. H., Stephenson, R. O., Butler, M. M., Skow, D. J., LaMarr, W. A., & Brown, N. C. (2003) DNA polymerase III of *Enterococcus faecalis*: expression and characterization of recombinant enzymes encoded by the *polC* and *dnaE* genes. *Protein Expr Purif* 27: 90-97.

Foster, P. L. (2007) Stress-induced mutagenesis in bacteria. *Crit Rev Biochem Mol Biol* 42: 373-397.

Friedberg, E. C., Walker, G. C., Seide, W., Wood, R. D., Schultz, R. A., & Ellenberger, T. (2006) *DNA repair and mutagenesis*. 2nd edition edn. Washington, DC., American Society for Microbiology,

Galhardo, R. S., Rocha, R. P., Marques, M. V., & Menck, C. F. (2005) An SOS-regulated operon involved in damage-inducible mutagenesis in *Caulobacter crescentus*. *Nucleic Acids Res* 33: 2603-2614.

Gasc, A. M., Sicard, N., Claverys, J. P., & Sicard, A. M. (1980) Lack of SOS repair in *Streptococcus pneumoniae*. *Mutat Res* 70: 157-165.

Gill, S. R., Fouts, D. E., Archer, G. L., Mongodin, E. F., Deboy, R. T., Ravel, J., *et al*. (2005) Insights on evolution of virulence and resistance from the complete genome analysis of an early methicillin-resistant *Staphylococcus aureus* strain and a biofilm-producing methicillin-resistant *Staphylococcus epidermidis* strain. *J Bacteriol* 187: 2426-2438.

Gilmore, K. S., Srinivas, P., Akins, D. R., Hatter, K. L., & Gilmore, M. S. (2003) Growth, development, and gene expression in a persistent *Streptococcus gordonii* biofilm. *Infect Immun* 71: 4759-4766.

Giovanetti, E., Brenciani, A., Vecchi, M., Manzin, A., & Varaldo, P. E. (2005) Prophage association of mef(A) elements encoding efflux-mediated erythromycin resistance in *Streptococcus pyogenes*. *J Antimicrob Chemother* 55: 445-451.

Glaser, P., Rusniok, C., Buchrieser, C., Chevalier, F., Frangeul, L., Msadek, T. *et al*. (2002) Genome sequence of *Streptococcus agalactiae*, a pathogen causing invasive neonatal disease. *Mol Microbiol* 45: 1499-1513.

Goerke, C., Koller, J., & Wolz, C. (2006) Ciprofloxacin and trimethoprim cause phage induction and virulence modulation in *Staphylococcus aureus*. *Antimicrob Agents Chemother* 50: 171-177.

Goodman, M. F., & Tippin, B. (2000) The expanding polymerase universe. *Nat Rev Mol Cell Biol* 1: 101-109.

Grobbel, M., Lubke-Becker, A., Wieler, L. H., Froyman, R., Friederichs, S., & Filios, S. (2007) Comparative quantification of the *in vitro* activity of veterinary fluoroquinolones. *Vet Microbiol* 124: 73-81.

Gruss, A., & Michel, B. (2001) The replication-recombination connection: insights from genomics. *Curr Opin Microbiol* 4: 595-601.

Hahn, K., Faustoferri, R. C., & Quivey, R. G.,Jr. (1999) Induction of an AP endonuclease activity in *Streptococcus mutans* during growth at low pH. *Mol Microbiol* 31: 1489-1498.

Hakenbeck, R., Tarpay, M., & Tomasz, A. (1980) Multiple changes of penicillin-binding proteins in penicillin-resistant clinical isolates of *Streptococcus pneumoniae*. *Antimicrob Agents Chemother* 17: 364-371.

Hallen, E., Wedholm, A., Andren, A., & Lunden, A. (2008) Effect of beta-casein, kappa-casein and beta-lactoglobulin genotypes on concentration of milk protein variants. *J Anim Breed Genet* 125: 119-129.

Halpern, D., Gruss, A., Claverys, J. P., & El-Karoui, M. (2004) *rexAB* mutants in *Streptococcus pneumoniae*. *Microbiology* 150: 2409-2414.

Hamada, S., & Slade, H. D. (1980) Biology, immunology, and cariogenicity of *Streptococcus mutans*. *Microbiol Rev* 44: 331-384.

Hanna, M. N., Ferguson, R. J., Li, Y. H., & Cvitkovitch, D. G. (2001) *uvrA* is an acid-inducible gene involved in the adaptive response to low pH in *Streptococcus mutans*. *J Bacteriol* 183: 5964-5973.

Havarstein, L. S., Hakenbeck, R., & Gaustad, P. (1997) Natural competence in the genus *Streptococcus*: evidence that streptococci can change pherotype by interspecies recombinational exchanges. *J Bacteriol* 179: 6589-6594.

Havarstein, L. S., Coomaraswamy, G., & Morrison, D. A. (1995) An unmodified heptadecapeptide pheromone induces competence for genetic transformation in *Streptococcus pneumoniae*. *Proc Natl Acad Sci U S A* 92: 11140-11144.

Havarstein, L. S., Gaustad, P., Nes, I. F., & Morrison, D. A. (1996) Identification of the streptococcal competence-pheromone receptor. *Mol Microbiol* 21: 863-869.

Henderson-Begg, S. K., Livermore, D. M., & Hall, L. M. (2006) Effect of subinhibitory concentrations of antibiotics on mutation frequency in *Streptococcus pneumoniae*. *J Antimicrob Chemother* 57: 849-854.

Heilmann, C., Schweitzer, O., Gerke, C., Vanittanakom, N., Mack, D., & Götz, F. (1996) Molecular basis of intercellular adhesion in the biofilm-forming *Staphylococcus epidermidis*. *Mol Microbiol* 20: 1083-1091.

Hillerton, J. E., & Berry, E. A. (2005) Treating mastitis in the cow--a tradition or an archaism. *J Appl Microbiol* 98: 1250-1255.

Hillerton, J. E., & Kliem, K. E. (2002) Effective treatment of *Streptococcus uberis* clinical mastitis to minimize the use of antibiotics. *J Dairy Sci* 85: 1009-1014.

Holden, M. T., Scott, A., Cherevach, I., Chillingworth, T., Churcher, C., Cronin, A. *et al*. (2007) Complete genome of acute rheumatic fever-associated serotype M5 *Streptococcus pyogenes* strain Manfredo. *J Bacteriol* 189: 1473-1477.

Hoskins, J., Alborn, W.E. Jr., Arnold, J., Blaszczak, L.C., Burgett, S. *et al*. (2001) Genome of the bacterium *Streptococcus pneumoniae* strain R6. *J Bacteriol*. 183: 5709-5717.

Humbert, O., Prudhomme, M., Hakenbeck, R., Dowson, C. G., & Claverys, J. P. (1995) Homeologous recombination and mismatch repair during transformation in *Streptococcus pneumoniae*: saturation of the Hex mismatch repair system. *Proc Natl Acad Sci U S A* 92: 9052-9056.

Ikonen, T., Ojala, M., & Ruottinen, O. (1999) Associations between milk protein polymorphism and first lactation milk production traits in Finnish Ayrshire cows. *J Dairy Sci* 82: 1026-1033.

Inagaki, S., Matsumoto-Nakano, M., Fujita, K., Nagayama, K., Funao, J., & Ooshima, T. (2009) Effects of recombinase A deficiency on biofilm formation by *Streptococcus mutans*. *Oral Microbiol Immunol* 24: 104-108.

Inoue, R., Kaito, C., Tanabe, M., Kamura, K., Akimitsu, N., & Sekimizu, K. (2001) Genetic identification of two distinct DNA polymerases, DnaE and PolC, that are essential for chromosomal DNA replication in *Staphylococcus aureus*. *Mol Genet Genomics* 266: 564-571.

Janoir, C., Zeller, V., Kitzis, M. D., Moreau, N. J., & Gutmann, L. (1996) High-level fluoroquinolone resistance in *Streptococcus pneumoniae* requires mutations in *parC* and *gyrA*. *Antimicrob Agents Chemother* 40: 2760-2764.

Janulczyk, R., Pallon, J., & Bjorck, L. (1999) Identification and characterization of a *Streptococcus pyogenes* ABC transporter with multiple specificity for metal cations. *Mol Microbiol* 34: 596-606.

Jensen, S. O., & Lyon, B. R. (2009) Genetics of antimicrobial resistance in *Staphylococcus aureus*. *Future Microbiol* 4: 565-582.

Johnsborg, O., & Havarstein, L. S. (2009) Regulation of natural genetic transformation and acquisition of transforming DNA in *Streptococcus pneumoniae*. *FEMS Microbiol Rev* 33: 627-642.

Johnsborg, O., Eldholm, V., Bjornstad, M. L., & Havarstein, L. S. (2008) A predatory mechanism dramatically increases the efficiency of lateral gene transfer in *Streptococcus pneumoniae* and related commensal species. *Mol Microbiol* 69: 245-253.

Jönsson, T. J., Ellis, H. R., & Poole, L. B. (2007) Cysteine reactivity and thiol-disulfide interchange pathways in AhpF and AhpC of the bacterial alkyl hydroperoxide reductase system. *Biochemistry* 46: 5709-5721.

Kawamura, Y., Fujiwara, H., Mishima, N., Tanaka, Y., Tanimoto, A., Ikawa, S. *et al*. (2003) First *Streptococcus agalactiae* isolates highly resistant to quinolones, with point mutations in *gyrA* and *parC*. *Antimicrob Agents Chemother* 47: 3605-3609.

Kharazmi, A. (1991) Mechanisms involved in the evasion of the host defence by *Pseudomonas aeruginosa*. *Immunol Lett* 30: 201-205.

King, S. J., Allen, A. G., Maskell, D. J., Dowson, C. G., & Whatmore, A. M. (2004) Distribution, genetic diversity, and variable expression of the gene encoding hyaluronate lyase within the *Streptococcus suis* population. *J Bacteriol* 186: 4740-4747.

Kitt, A. J., & Leigh, J. A. (1997) The auxotrophic nature of *Streptococcus uberis*. The acquisition of essential acids from plasmin derived casein peptides. *Adv Exp Med Biol* 418: 647-650.

Kleerebezem, M., & Quadri, L. E. (2001) Peptide pheromone-dependent regulation of antimicrobial peptide production in Gram-positive bacteria: a case of

multicellular behavior. *Peptides* 22: 1579-1596.

Kohanski, M. A., Dwyer, D. J., Hayete, B., Lawrence, C. A., & Collins, J. J. (2007) A common mechanism of cellular death induced by bactericidal antibiotics. *Cell* 130: 797-810.

Koivula, M., Pitkälä, A., Pyörälä, S., & Mäntysaari, E. A. (2007) Distribution of bacteria and seasonal and regional effects in a new database for mastitis pathogens in Finland. *Acta Agric Scand Sect A-Anim Sci* 57: 89-96.

Kolenbrander, P. E., Andersen, R. N., Baker, R. A., & Jenkinson, H. F. (1998) The adhesion-associated *sca* operon in *Streptococcus gordonii* encodes an inducible high-affinity ABC transporter for Mn2+ uptake. *J Bacteriol* 180: 290-295.

Kornberg, A., & Baker, T. A. (2005) *DNA replication.* 2nd edition edn. Sausalito, California, University Science Books,

Kostyukova, N. N., Volkova, M. O., Ivanova, V. V., & Kvetnaya, A. S. (1995) A study of pathogenic factors of *Streptococcus pneumoniae* strains causing meningitis. *FEMS Immunol Med Microbiol* 10: 133-137.

Kowalczykowski, S. C., Dixon, D. A., Eggleston, A. K., Lauder, S. D., & Rehrauer, W. M. (1994) Biochemistry of homologous recombination in *Escherichia coli*. *Microbiol Rev* 58: 401-465.

Kreth, J., Zhang, Y., & Herzberg, M. C. (2008) Streptococcal antagonism in oral biofilms: *Streptococcus sanguinis* and *Streptococcus gordonii* interference with *Streptococcus mutans*. *J Bacteriol* 190: 4632-4640.

Kristich, C. J., Li, Y. H., Cvitkovitch, D. G., & Dunny, G. M. (2004) Esp-independent biofilm formation by *Enterococcus faecalis*. *J Bacteriol* 186: 154-163.

Kumar, C. G., & Anand, S. K. (1998) Significance of microbial biofilms in food industry: a review. *Int J Food Microbiol* 42: 9-27.

Kwon, A. S., Park, G. C., Ryu, S. Y., Lim, D. H., Lim, D. Y., Choi, C. H. *et al*. (2008) Higher biofilm formation in multidrug-resistant clinical isolates of *Staphylococcus aureus*. *Int J Antimicrob Agents* 32: 68-72.

Lanie, J.A., Ng, W.L., Kazmierczak, K.M., Andrzejewski, T.M., Davidsen, T.M., *et al*. (2007) Genome sequence of Avery's virulent serotype 2 strain D39 of *Streptococcus pneumoniae* and comparison with that of unencapsulated laboratory strain R6. *J Bacteriol*. 189: 38-51

Le Chatelier, E., Becherel, O. J., d'Alencon, E., Canceill, D., Ehrlich, S. D., Fuchs, R. P., & Janniere, L. (2004) Involvement of DnaE, the second replicative DNA polymerase from *Bacillus subtilis*, in DNA mutagenesis. *J Biol Chem* 279: 1757-1767.

Leid, J. G., Shirtliff, M. E., Costerton, J. W., & Stoodley, A. P. (2002) Human leukocytes adhere to, penetrate, and respond to *Staphylococcus aureus* biofilms. *Infect Immun* 70: 6339-6345.

Leigh, J. A. (1999) *Streptococcus uberis*: a permanent barrier to the control of bovine mastitis? *Vet J* 157: 225-238.

Leigh, J. A. (1994) Purification of a plasminogen activator from *Streptococcus uberis*. *FEMS Microbiol Lett* 118: 153-158.

Leigh, J. A. (1993) Activation of bovine plasminogen by *Streptococcus uberis*. *FEMS Microbiol Lett* 114: 67-71.

Leigh, J. A., & Lincoln, R. A. (1997) *Streptococcus uberis* acquires plasmin activity following growth in the presence of bovine plasminogen through the action of its specific plasminogen activator. *FEMS Microbiol Lett* 154: 123-129.

Leigh, J. A., Finch, J. M., Field, T. R., Real, N. C., Winter, A., Walton, A. W., & Hodgkinson, S. M. (1999) Vaccination with the plasminogen activator from *Streptococcus uberis* induces an inhibitory response and protects against experimental infection in the dairy cow. *Vaccine* 17: 851-857.

Lewin, C. S., & Amyes, S. G. (1991) The role of the SOS response in bacteria exposed to zidovudine or trimethoprim. *J Med Microbiol* 34: 329-332.

Li, Y. H., Lau, P. C., Lee, J. H., Ellen, R. P., & Cvitkovitch, D. G. (2001) Natural genetic transformation of *Streptococcus mutans* growing in biofilms. *J Bacteriol* 183: 897-908.

Li, Y. H., Tang, N., Aspiras, M. B., Lau, P. C., Lee, J. H., Ellen, R. P., & Cvitkovitch, D. G. (2002) A quorum-sensing signaling system essential for genetic competence in *Streptococcus mutans* is involved in biofilm formation. *J Bacteriol* 184: 2699-2708.

Linares, J. F., Gustafsson, I., Baquero, F., & Martinez, J. L. (2006) Antibiotics as intermicrobial signaling agents instead of weapons. *Proc Natl Acad Sci U S A* 103: 19484-19489.

Lindahl, T., & Nyberg, B. (1972) Rate of depurination of native deoxyribonucleic acid. *Biochemistry* 11: 3610-3618.

Little, J. W. (1984) Autodigestion of *lexA* and phage lambda repressors. *Proc Natl Acad Sci U S A* 81: 1375-1379.

Love, P. E., Lyle, M. J., & Yasbin, R. E. (1985) DNA-damage-inducible (*din*) loci are transcriptionally activated in competent *Bacillus subtilis*. *Proc Natl Acad Sci U S A* 82: 6201-6205.

Maguin, E., Prevost, H., Ehrlich, S. D., & Gruss, A. (1996) Efficient insertional mutagenesis in lactococci and other gram-positive bacteria. *J Bacteriol* 178: 931-935.

Mah, T. F., & O'Toole, G. A. (2001) Mechanisms of biofilm resistance to antimicrobial agents. *Trends Microbiol* 9: 34-39.

Mah, T. F., Pitts, B., Pellock, B., Walker, G. C., Stewart, P. S., & O'Toole, G. A. (2003) A genetic basis for *Pseudomonas aeruginosa* biofilm antibiotic resistance. *Nature* 426: 306-310.

Maione, D., Margarit, I., Rinaudo, C. D., Masignani, V., Mora, M., Scarselli, M. *et al.* (2005) Identification of a universal Group B streptococcus vaccine by multiple genome screen. *Science* 309: 148-150.

Maiques, E., Ubeda, C., Campoy, S., Salvador, N., Lasa, I., Novick, R. P. *et al.* (2006) beta-lactam antibiotics induce the SOS response and horizontal transfer of virulence factors in *Staphylococcus aureus*. *J Bacteriol* 188: 2726-2729.

Malaty, J., & Antonelli, P. J. (2008) Effect of blood and mucus on tympanostomy tube biofilm formation. *Laryngoscope* 118: 867-870.

Marrer, E., Satoh, A. T., Johnson, M. M., Piddock, L. J., & Page, M. G. (2006) Global transcriptome analysis of the responses of a fluoroquinolone-resistant *Streptococcus pneumoniae* mutant and its parent to ciprofloxacin. *Antimicrob Agents Chemother* 50: 269-278.

McEllistrem, M. C. (2009) Genetic diversity of the pneumococcal capsule: implications for molecular-based serotyping. *Future Microbiol* 4: 857-865.

McEllistrem, M. C., Ransford, J. V., & Khan, S. A. (2007) Characterization of *in vitro* biofilm-associated pneumococcal phase variants of a clinically relevant serotype 3 clone. *J Clin Microbiol* 45: 97-101.

McKellar, Q., Gibson, I., Monteiro, A., & Bregante, M. (1999) Pharmacokinetics of enrofloxacin and danofloxacin in plasma, inflammatory exudate, and bronchial secretions of calves following subcutaneous administration. *Antimicrob Agents Chemother* 43: 1988-1992.

Mejean, V., & Claverys, J. P. (1988) Polarity of DNA entry in transformation of *Streptococcus pneumoniae*. *Mol Gen Genet* 213: 444-448.

Mejean, V., Salles, C., Bullions, L. C., Bessman, M. J., & Claverys, J. P. (1994) Characterization of the *mutX* gene of *Streptococcus pneumoniae* as a homologue of *Escherichia coli mutT*, and tentative definition of a catalytic domain of the dGTP pyrophosphohydrolases. *Mol Microbiol* 11: 323-330.

Melchior, M. B., Vaarkamp, H., & Fink-Gremmels, J. (2006a) Biofilms: a role in recurrent mastitis infections? *Vet J* 171: 398-407.

Melchior, M. B., Fink-Gremmels, J., & Gaastra, W. (2006b) Comparative assessment of the antimicrobial susceptibility of *Staphylococcus aureus* isolates from bovine mastitis in biofilm versus planktonic culture. *J Vet Med B Infect Dis Vet Public Health* 53: 326-332.

Miller, C., Thomsen, L. E., Gaggero, C., Mosseri, R., Ingmer, H., & Cohen, S. N. (2004) SOS response induction by beta-lactams and bacterial defense against antibiotic lethality. *Science* 305: 1629-1631.

Miller, K., O'Neill, A. J., & Chopra, I. (2002) Response of *Escherichia coli* hypermutators to selection pressure with antimicrobial agents from different classes. *J Antimicrob Chemother* 49: 925-934.

Milne, M. H., Biggs, A. M., Barrett, D. C., Young, F. J., Doherty, S., Innocent, G. T., & Fitzpatrick, J. L. (2005) Treatment of persistent intramammary infections with *Streptococcus uberis* in dairy cows. *Vet Rec* 157: 245-250.

Mortier-Barriere, I., de Saizieu, A., Claverys, J. P., & Martin, B. (1998) Competence-specific induction of *recA* is required for full recombination proficiency during transformation in *Streptococcus pneumoniae*. *Mol Microbiol* 27: 159-170.

Mortier-Barriere, I., Humbert, O., Martin, B., Prudhomme, M., & Claverys, J. P. (1997) Control of recombination rate during transformation of *Streptococcus pneumoniae*: an overview. *Microb Drug Resist* 3: 233-242.

Movahedzadeh, F., Colston, M. J., & Davis, E. O. (1997) Characterization of *Mycobacterium tuberculosis* LexA: recognition of a Cheo (*Bacillus*-type SOS) box. *Microbiology* 143 (Pt 3): 929-936.

Munoz, R., & De La Campa, A. G. (1996) ParC subunit of DNA topoisomerase IV of *Streptococcus pneumoniae* is a primary target of fluoroquinolones and cooperates with DNA gyrase A subunit in forming resistance phenotype. *Antimicrob Agents Chemother* 40: 2252-2257.

Munoz-Najar, U., & Vijayakumar, M. N. (1999) An operon that confers UV resistance by evoking the SOS mutagenic response in streptococcal conjugative transposon Tn5252. *J Bacteriol* 181: 2782-2788.

Navarre, W. W., & Schneewind, O. (1999) Surface proteins of gram-positive bacteria and mechanisms of their targeting to the cell wall envelope. *Microbiol Mol Biol Rev* 63: 174-229.

Negri, M. C., Morosini, M. I., Baquero, M. R., del Campo, R., Blazquez, J., & Baquero, F. (2002) Very low cefotaxime concentrations select for hypermutable *Streptococcus pneumoniae* populations. *Antimicrob Agents Chemother* 46: 528-530.

Ohmori, H., Friedberg, E. C., Fuchs, R. P., Goodman, M. F., Hanaoka, F., Hinkle, D. *et al*. (2001) The Y-family of DNA polymerases. *Mol Cell* 8: 7-8.

Olde Riekerink, R. G., Barkema, H. W., Kelton, D. F., & Scholl, D. T. (2008) Incidence rate of clinical mastitis on Canadian dairy farms. *J Dairy Sci* 91: 1366-1377.

Oliveira, M., Bexiga, R., Nunes, S. F., Carneiro, C., Cavaco, L. M., Bernardo, F., & Vilela, C. L. (2006) Biofilm-forming ability profiling of *Staphylococcus aureus* and *Staphylococcus epidermidis* mastitis isolates. *Vet Microbiol* 118: 133-140.

Oliver, A., Canton, R., Campo, P., Baquero, F., & Blazquez, J. (2000) High frequency of hypermutable *Pseudomonas aeruginosa* in cystic fibrosis lung infection. *Science* 288: 1251-1254.

Oliver, S. P., Almeida, R. A., & Calvinho, L. F. (1998) Virulence factors of *Streptococcus uberis* isolated from cows with mastitis. *Zentralbl Veterinarmed B* 45: 461-471.

Olson, M. E., Ceri, H., Morck, D. W., Buret, A. G., & Read, R. R. (2002) Biofilm bacteria: formation and comparative susceptibility to antibiotics. *Can J Vet Res* 66: 86-92.

O'May, C. Y., Sanderson, K., Roddam, L. F., Kirov, S. M., & Reid, D. W. (2009) Iron-binding compounds impair *Pseudomonas aeruginosa* biofilm formation, especially under anaerobic conditions. *J Med Microbiol* 58: 765-773.

Orscheln, R. C., Johnson, D. R., Olson, S. M., Presti, R. M., Martin, J. M., Kaplan, E. L., & Storch, G. A. (2005) Intrinsic reduced susceptibility of serotype 6 *Streptococcus pyogenes* to fluoroquinolone antibiotics. *J Infect Dis* 191: 1272-1279.

Owens, W. E., Watts, J. L., Greene, B. B., & Ray, C. H. (1990) Minimum inhibitory concentrations and disk diffusion zone diameter for selected antibiotics against streptococci isolated from bovine intramammary infections. *J Dairy Sci* 73: 1225-1231.

Palma, M., DeLuca, D., Worgall, S., & Quadri, L. E. (2004) Transcriptome analysis of the response of *Pseudomonas aeruginosa* to hydrogen peroxide. *J Bacteriol* 186: 248-252.

Pan, X. S., & Fisher, L. M. (1996) Cloning and characterization of the *parC* and *parE* genes of *Streptococcus pneumoniae* encoding DNA topoisomerase IV: role in fluoroquinolone resistance. *J Bacteriol* 178: 4060-4069.

Perry, K. L., Elledge, S. J., Mitchell, B. B., Marsh, L., & Walker, G. C. (1985) *umuDC* and *mucAB* operons whose products are required for UV light- and chemical-induced mutagenesis: UmuD, MucA, and LexA proteins share homology. *Proc Natl Acad Sci U S A* 82: 4331-4335.

Peterson, S. N., Sung, C. K., Cline, R., Desai, B. V., Snesrud, E. C., Luo, P. *et al*. (2004) Identification of competence pheromone responsive genes in *Streptococcus pneumoniae* by use of DNA microarrays. *Mol Microbiol* 51: 1051-1070.

Pillai, S. K., Sakoulas, G., Eliopoulos, G. M., Moellering, R. C.,Jr, Murray, B. E., & Inouye, R. T. (2004) Effects of glucose on *fsr*-mediated biofilm formation in *Enterococcus faecalis*. *J Infect Dis* 190: 967-970.

Pitkälä, A., Haveri, M., Pyörälä, S., Myllys, V., & Honkanen-Buzalski, T. (2004) Bovine mastitis in Finland 2001 -prevalence, distribution of bacteria, and antimicrobial resistance. *J Dairy Sci* 87: 2433-2441.

Pletz, M. W., McGee, L., Van Beneden, C. A., Petit, S., Bardsley, M., Barlow, M., & Klugman, K. P. (2006) Fluoroquinolone resistance in invasive *Streptococcus pyogenes* isolates due to spontaneous mutation and horizontal gene transfer. *Antimicrob Agents Chemother* 50: 943-948.

Polonio, R. E., Mermel, L. A., Paquette, G. E., & Sperry, J. F. (2001) Eradication of biofilm-forming *Staphylococcus epidermidis* (RP62A) by a combination of sodium salicylate and vancomycin. *Antimicrob Agents Chemother* 45: 3262-3266.

Power, E. G., & Phillips, I. (1992) Induction of the SOS gene (*umuC*) by 4-quinolone antibacterial drugs. *J Med Microbiol* 36: 78-82.

Pozzi, G., Iannelli, F., Oggioni, M. R., Santagati, M., & Stefani, S. (2004) Genetic elements carrying macrolide efflux genes in streptococci. *Curr Drug Targets Infect Disord* 4: 203-206.

Preston, J. A., & Dockrell, D. H. (2008) Virulence factors in pneumococcal respiratory pathogenesis. *Future Microbiology* 3: 205-221.

Prudhomme, M., Attaiech, L., Sanchez, G., Martin, B., & Claverys, J. P. (2006) Antibiotic stress induces genetic transformability in the human pathogen *Streptococcus pneumoniae*. *Science* 313: 89-92.

Pulliainen, A. T., Hytönen, J., Haataja, S., & Finne, J. (2008) Deficiency of the Rgg regulator promotes H_2O_2 resistance, AhpCF-mediated H_2O_2 decomposition, and virulence in *Streptococcus pyogenes*. *J Bacteriol* 190: 3225-3235.

Puyet, A., Greenberg, B., & Lacks, S. A. (1989) The *exoA* gene of *Streptococcus pneumoniae* and its product, a DNA exonuclease with apurinic endonuclease activity. *J Bacteriol* 171: 2278-2286.

Pyörälä, S. (2009) *In vitro* studies as supporting evidence on the efficacy of antimicrobials for mastitis treatment. *J Appl Microbiol* 107: 2116-2117

Quiberoni, A., Rezaiki, L., El Karoui, M., Biswas, I., Tailliez, P., & Gruss, A. (2001) Distinctive features of homologous recombination in an 'old' microorganism, *Lactococcus lactis*. *Res Microbiol* 152: 131-139.

Radman, M. (1975) SOS repair hypothesis: phenomenology of an inducible DNA repair which is accompanied by mutagenesis. *Basic Life Sci* 5A: 355-367.

Rallu, F., Gruss, A., Ehrlich, S. D., & Maguin, E. (2000) Acid- and multistress-resistant mutants of *Lactococcus lactis* : identification of intracellular stress signals. *Mol Microbiol* 35: 517-528.

Rathsam, C., Eaton, R. E., Simpson, C. L., Browne, G. V., Berg, T., Harty, D. W., & Jacques, N. A. (2005) Up-regulation of competence- but not stress-responsive proteins accompanies an altered metabolic phenotype in *Streptococcus mutans* biofilms. *Microbiology* 151: 1823-1837.

Reinert, R. R. (2004) Resistance phenotypes and multi-drug resistance in *Streptococcus pneumoniae* (PROTEKT years 1-3 [1999-

20021). *J Chemother* 16 Suppl 6: 35-48.

Rogers, S. G., & Weiss, B. (1980) Cloning of the exonuclease III gene of *Escherichia coli*. *Gene* 11: 187-195.

Rohde, H., Burdelski, C., Bartscht, K., Hussain, M., Buck, F., Horstkotte, M. A., Knobloch, J. K. *et al*. (2005) Induction o*f Staphylococcus epidermidis* biofilm formation via proteolytic processing of the accumulation-associated protein by staphylococcal and host proteases. *Mol Microbiol* 55: 1883-1895.

Rosey, E. L., Lincoln, R. A., Ward, P. N., Yancey, R. J.,Jr, & Leigh, J. A. (1999) PauA: a novel plasminogen activator from *Streptococcus uberis*. *FEMS Microbiol Lett* 178: 27-33.

Rupp, M. E., Ulphani, J. S., Fey, P. D., & Mack, D. (1999a) Characterization of *Staphylococcus epidermidis* polysaccharide intercellular adhesin/hemagglutinin in the pathogenesis of intravascular catheter-associated infection in a rat model. *Infect Immun* 67: 2656-2659.

Rupp, M. E., Ulphani, J. S., Fey, P. D., Bartscht, K., & Mack, D. (1999b) Characterization of the importance of polysaccharide intercellular adhesin/hemagglutinin of *Staphylococcus epidermidis* in the pathogenesis of biomaterial-based infection in a mouse foreign body infection model. *Infect Immun* 67: 2627-2632.

Saeman, A. I., Verdi, R. J., Galton, D. M., & Barbano, D. M. (1988) Effect of mastitis on proteolytic activity in bovine milk. *J Dairy Sci* 71: 505-512.

Sailer, F. C., Meberg, B. M., & Young, K. D. (2003) beta-Lactam induction of colanic acid gene expression in *Escherichia coli*. *FEMS Microbiol Lett* 226: 245-249.

Sambrook, J., & Russell, D. W. (2001) *Molecular cloning. A laboratory manual*. 3rd edition edn. Cold Spring Harbor, NY, Cold Spring Harbor Laboratory Press,

Samrakandi, M. M., & Pasta, F. (2000) Hyperrecombination in *Streptococcus pneumoniae* depends on an atypical *mutY* homologue. *J Bacteriol* 182: 3353-3360.

Sandholm, M., Kaartinen, L., & Pyörälä, S. (1990) Bovine mastitis -why does antibiotic therapy not always work? An overview. *J Vet Pharmacol Ther* 13: 248-260.

Santagati, M., Iannelli, F., Oggioni, M. R., Stefani, S., & Pozzi, G. (2000) Characterization of a genetic element carrying the macrolide efflux gene *mef(A)* in *Streptococcus pneumoniae*. *Antimicrob Agents Chemother* 44: 2585-2587.

Santagati, M., Iannelli, F., Cascone, C., Campanile, F., Oggioni, M. R., Stefani, S., & Pozzi, G. (2003) The novel conjugative transposon tn1207.3 carries the macrolide efflux gene *mef(A)* in *Streptococcus pyogenes*. *Microb Drug Resist* 9: 243-247.

Savijoki, K., Ingmer, H., Frees, D., Vogensen, F. K., Palva, A., & Varmanen, P. (2003) Heat and DNA damage induction of the LexA-like regulator HdiR from *Lactococcus lactis* is mediated by RecA and ClpP. *Mol Microbiol* 50: 609-621.

Schalen, C., Gebreselassie, D., & Stahl, S. (1995) Characterization of an erythromycin resistance *(erm)* plasmid in *Streptococcus pyogenes*. *APMIS* 103: 59-68.

Schembri, M. A., Kjaergaard, K., & Klemm, P. (2003) Global gene expression in *Escherichia coli* biofilms. *Mol Microbiol* 48: 253-267.

Schlacher, K., Pham, P., Cox, M. M., & Goodman, M. F. (2006) Roles of DNA polymerase V and RecA protein in SOS damage-induced mutation. *Chem Rev* 106: 406-419.

Schoolnik, G. K., Voskuil, M. I., Schnappinger, D., Yildiz, F. H., Meibom, K., Dolganov, N. A. *et al*. (2001) Whole genome DNA microarray expression analysis of biofilm development by *Vibrio cholerae* O1 E1 Tor. *Methods Enzymol* 336: 3-18.

Senadheera, D., & Cvitkovitch, D. G. (2008) Quorum sensing and biofilm formation by *Streptococcus mutans*. *Adv Exp Med Biol* 631: 178-188.

Sgarrella, F., Poddie, F. P., Meloni, M. A., Sciola, L., Pippia, P., & Tozzi, M. G. (1997) Channelling of deoxyribose moiety of exogenous DNA into carbohydrate metabolism: role of deoxyriboaldolase. *Comp Biochem Physiol B Biochem Mol Biol* 117: 253-257.

Shiau, A. L., & Wu, C. L. (1998) The inhibitory effect of *Staphylococcus epidermidis* slime on the phagocytosis of murine peritoneal macrophages is interferon-independent. *Microbiol Immunol* 42: 33-40.

Smith, A. J., Ward, P. N., Field, T. R., Jones, C. L., Lincoln, R. A., & Leigh, J. A. (2003) MtuA, a lipoprotein receptor antigen from *Streptococcus uberis*, is responsible for acquisition of manganese during growth in milk and is essential for infection of the lactating bovine mammary gland. *Infect Immun* 71: 4842-4849.

Sorlozano, A., Gutierrez, J., Roman, J., Liebana, J., & Piedrola, G. (2009) Activity of daptomycin against multiresistant clinical isolates of *Staphylococcus aureus* and *Streptococcus agalactiae*. *Microb Drug Resist* 15: 125-127.

Steffen, S. E., & Bryant, F. R. (2000) Purification and characterization of the RecA protein from *Streptococcus pneumoniae*. *Arch Biochem Biophys* 382: 303-309.

Stephens, C. (2002) Microbiology: breaking down biofilms. *Curr Biol* 12: R132-4.

Stewart, P. S., & Costerton, J. W. (2001) Antibiotic resistance of bacteria in biofilms. *The Lancet* 358: 135-138.

Stoodley, P., Sauer, K., Davies, D. G., & Costerton, J. W. (2002) Biofilms as complex differentiated communities. *Annu Rev Microbiol* 56: 187-209.

Sung, H. M., Yeamans, G., Ross, C. A., & Yasbin, R. E. (2003) Roles of YqjH and YqjW, homologs of the *Escherichia coli* UmuC/DinB or Y superfamily of DNA polymerases, in stationary-phase mutagenesis and UV-induced mutagenesis of *Bacillus subtilis*. *J Bacteriol* 185: 2153-2160.

Suntharalingam, P., & Cvitkovitch, D. G. (2005) Quorum sensing in streptococcal biofilm formation. *Trends Microbiol* 13: 3-6.

Sutton, M. D., Smith, B. T., Godoy, V. G., & Walker, G. C. (2000) The SOS response: recent insights into *umuDC*-dependent mutagenesis and DNA damage tolerance. *Annu Rev Genet* 34: 479-497.

Tajiri, T., Maki, H., & Sekiguchi, M. (1995) Functional cooperation of MutT, MutM and MutY proteins in preventing mutations caused by spontaneous oxidation of guanine nucleotide in *Escherichia coli*. *Mutat Res* 336: 257-267.

Tamilselvam, B., Almeida, R. A., Dunlap, J. R., & Oliver, S. P. (2006) *Streptococcus uberis* internalizes and persists in bovine mammary epithelial cells. *Microb Pathog* 40: 279-285.

Tattevin, P., Basuino, L., & Chambers, H. F. (2009) Subinhibitory fluoroquinolone exposure selects for reduced beta-lactam susceptibility in methicillin-resistant *Staphylococcus aureus* and alterations in the SOS-mediated response. *Res Microbiol* 160: 187-192.

Taylor, D. L., Ward, P. N., Rapier, C. D., Leigh, J. A., & Bowler, L. D. (2003) Identification of a differentially expressed oligopeptide binding protein (OppA2) in *Streptococcus uberis* by representational difference analysis of cDNA. *J Bacteriol* 185: 5210-5219.

Tettelin, H., Nelson, K. E., Paulsen, I. T., Eisen, J. A., Read, T. D., Peterson, S. *et al*. (2001) Complete genome sequence of a virulent isolate of *Streptococcus pneumoniae*. *Science* 293: 498-506.

Thomas, L. H., Haider, W., Hill, A. W., & Cook, R. S. (1994) Pathologic findings of experimentally induced *Streptococcus uberis* infection in the mammary gland of cows. *Am J Vet Res.* 55: 1723-1728

Thoms, B., & Wackernagel, W. (1998) Interaction of RecBCD enzyme with DNA at double-strand breaks produced in UV-irradiated *Escherichia coli*: requirement for DNA end processing. *J Bacteriol* 180: 5639-5645.

Tilley, S. J., Orlova, E. V., Gilbert, R. J., Andrew, P. W., & Saibil, H. R. (2005) Structural basis of pore formation by the bacterial toxin pneumolysin. *Cell* 121: 247-256.

Timoney, J. F., Artiushin, S. C., & Boschwitz, J. S. (1997) Comparison of the sequences and functions of *Streptococcus equi* M-like proteins SeM and SzPSe. *Infect Immun* 65: 3600-3605.

Tippin, B., Pham, P., & Goodman, M. F. (2004) Error-prone replication for better or worse. *Trends Microbiol* 12: 288-295.

Tomasz, A. (1966) Model for the mechanism controlling the expression of competent state in *Pneumococcus* cultures. *J Bacteriol* 91: 1050-1061.

Trautner, B. W., & Darouiche, R. O. (2004) Role of biofilm in catheter-associated urinary tract infection. *Am J Infect Control* 32: 177-183.

Truong-Bolduc, Q. C., & Hooper, D. C. (2007) The transcriptional regulators NorG and MgrA modulate resistance to both quinolones and beta-lactams in *Staphylococcus aureus*. *J Bacteriol* 189: 2996-3005.

Truong-Bolduc, Q. C., Zhang, X., & Hooper, D. C. (2003) Characterization of NorR protein, a multifunctional regulator of *norA* expression in *Staphylococcus aureus*. *J Bacteriol* 185: 3127-3138.

Ubeda, C., Maiques, E., Knecht, E., Lasa, I., Novick, R. P., & Penades, J. R. (2005) Antibiotic-induced SOS response promotes horizontal dissemination of pathogenicity island-encoded virulence factors in staphylococci. *Mol Microbiol* 56: 836-844.

Varhimo, E., Savijoki, K., Jalava, J., Kuipers, O. P., & Varmanen, P. (2007) Identification of a novel streptococcal gene cassette mediating SOS mutagenesis in *Streptococcus uberis*. *J Bacteriol* 189: 5210-5222.

Varmanen, P., Ingmer, H., & Vogensen, F. K. (2000) *ctsR* of *Lactococcus lactis* encodes a negative regulator of *clp* gene expression. *Microbiology* 146 (Pt 6): 1447-1455.

Venezia, R. A., Domaracki, B. E., Evans, A. M., Preston, K. E., & Graffunder, E. M. (2001) Selection of high-level oxacillin resistance in heteroresistant *Staphylococcus aureus* by fluoroquinolone exposure. *J Antimicrob Chemother* 48: 375-381.

Viguier, C., Arora, S., Gilmartin, N., Welbeck, K., & O'Kennedy, R. (2009) Mastitis detection: current trends and future perspectives. *Trends Biotechnol* 27: 486-493.

Vuong, C., Voyich, J. M., Fischer, E. R., Braughton, K. R., Whitney, A. R., DeLeo, F. R., & Otto, M. (2004) Polysaccharide intercellular adhesin (PIA) protects *Staphylococcus epidermidis* against major components of the human innate immune system. *Cell Microbiol* 6: 269-275.

Waite, R. D., Struthers, J. K., & Dowson, C. G. (2001) Spontaneous sequence duplication within an open reading frame of the pneumococcal type 3 capsule locus causes high-frequency phase variation. *Mol Microbiol* 42: 1223-1232.

Wakabayashi, H., Yamauchi, K., Kobayashi, T., Yaeshima, T., Iwatsuki, K. & Yoshie, H. (2009) Inhibitory effects of lactoferrin on growth and biofilm formation of *Porphyromonas gingivalis* and *Prevotella intermedia*. *Antimicrob Agents Chemother*. 53: 3308-3316

Ward, P. N., Field, T. R., Rapier, C. D., & Leigh, J. A. (2003) The activation of bovine plasminogen by PauA is not required for virulence of *Streptococcus uberis*. *Infect Immun* 71: 7193-7196.

Ward, P. N., Holden, M. T., Leigh, J. A., Lennard, N., Bignell, A., Barron, A. *et al*. (2009) Evidence for niche adaptation in the genome of the bovine pathogen *Streptococcus uberis*. *BMC Genomics* 10: 54.

Wertheim, H. F., Nghia, H. D., Taylor, W., & Schultsz, C. (2009) *Streptococcus suis*: an emerging human pathogen. *Clin Infect Dis* 48: 617-625.

Wescombe, P. A., Heng, N. C., Burton, J. P., Chilcott, C. N., & Tagg, J. R. (2009) Streptococcal bacteriocins and the case for *Streptococcus salivarius* as model oral probiotics. *Future Microbiol* 4: 819-835.

Whatmore, A. M., Barcus, V. A., & Dowson, C. G. (1999) Genetic diversity of the streptococcal competence (*com*) gene locus. *J Bacteriol* 181: 3144-3154.

Whiteley, M., Bangera, M. G., Bumgarner, R. E., Parsek, M. R., Teitzel, G. M., Lory, S., & Greenberg, E. P. (2001) Gene expression in *Pseudomonas aeruginosa* biofilms. *Nature* 413: 860-864.

Wilcox, M. H. (2009) Future gazing in the management of multiply drug-resistant Gram-positive infection. *J Infect* 59 Suppl 1: S75-80.

Wojciechowski, M. F., Peterson, K. R., & Love, P. E. (1991) Regulation of the SOS response in *Bacillus subtilis*: evidence for a LexA repressor homolog. *J Bacteriol* 173: 6489-6498.

Woodford, N., & Livermore, D. M. (2009) Infections caused by Gram-positive bacteria: a review of the global challenge. *J Infect* 59 Suppl 1: S4-16.

Wright, G. D. (2003) Mechanisms of resistance to antibiotics. *Curr Opin Chem Biol* 7: 563-569.

Yang, W. (2000) Structure and function of mismatch repair proteins. *Mutat Res* 460: 245-256.

Yarwood, J. M., Paquette, K. M., Tikh, I. B., Volper, E. M., & Greenberg, E. P. (2007) Generation of virulence factor variants in *Staphylococcus aureus* biofilms. *J Bacteriol* 189: 7961-7967.

Yasbin, R. E. (1977) DNA repair in *Bacillus subtilis*. II. Activation of the inducible system in competent bacteria. *Mol Gen Genet* 153: 219-225.

Yasbin, R. E., Cheo, D. L., & Bayles, K. W. (1992) Inducible DNA repair and differentiation in *Bacillus subtilis*: interactions between global regulons. *Mol Microbiol* 6: 1263-1270.

Zhang, L., & Mah, T. F. (2008) Involvement of a novel efflux system in biofilm-specific resistance to antibiotics. *J Bacteriol* 190: 4447-4452.

Zhang, Y. Q., Ren, S. X., Li, H. L., Wang, Y. X., Fu, G., Yang, J., *et al.* (2003) Genome-based analysis of virulence genes in a non-biofilm-forming *Staphylococcus epidermidis* strain (ATCC 12228). *Mol Microbiol* 49: 1577-1193.

Zhang, X., McDaniel, A. D., Wolf, L. E., Keusch, G. T., Waldor, M. K., & Acheson, D. W. (2000) Quinolone antibiotics induce Shiga toxin-encoding bacteriophages, toxin production, and death in mice. *J Infect Dis* 181: 664-670.

Zverlov, V. V., & Schwarz, W. H. (1999) Organization of the chromosomal region containing the genes *lexA* and *topA* in *Thermotoga napolitana*. Primary structure of LexA reveals phylogenetic relevance. *Syst Appl Microbiol* 22: 174-178.

Zwijnenburg, P. J., van der Poll, T., Florquin, S., van Deventer, S. J., Roord, J. J., & van Furth, A. M. (2001) Experimental pneumococcal meningitis in mice: a model of intranasal infection. *J Infect Dis* 183: 1143-1146.