Is there a link between Streptococcus intermedius competence and antibiotic stress? Investigation of biofilm and susceptibility responses.

Introduction:

Bacteria have the ability of living both in a planktonic form, as free floating cells, and in biofilm communities. To survive against the many different factors which may affect their ability to grow in their habitat, bacteria may switch from planktonic to biofilm form and visa versa. A biofilm consists of bacteria with a three dimensional organization, enveloped in extra cellular polymeric substances, as well as networks of intervening water channels. The water channels deliver necessary nutrients and remove wastes so the bacteria deep in biofilm may survive (Marsh 1999). Bacteria living in a biofilm may endure longer due to their trait to communicate with one another and exchange genetic information. Biofilm formation essentially depends on stability and suitability of a surface like the hard tissue of teeth. Both dental caries and periodontal diseases are caused by bacteria growing in biofilms. A large number of other human bacterial infections are also related to biofilm formation, like for instance osteomyelitis, bacterial prostatitis, infective endocarditis and cystic fibrosis (Carol Potera 1999).

Bacteria are able to sense their population density by cell to cell communication (Aragao, Macedo et al. 2003). This property termed quorum sensing is particularly important in biofilms, in which high cell densities are often present. At low densities, small amounts of

signal molecules are detected, but as soon as the bacterial density reaches a specific threshold, changes in gene expression occur. Bacteria use quorum sensing to regulate a variety of phenotypes, including exopolysaccharide production, biofilm formation, virulence, competence, symbiosis sporulation and motility (Miller & bassler, 2001). Two quorum sensing systems have been identified in streptococci; one is mediated by secreted peptides (Kleerebezem, Quadri et al. 1997); (Scheie and Petersen 2004) and the other is influenced by a secreted furan derivative called auto inducer 2 (Schauder and Bassler 2001; Xavier and Bassler 2003).

Oral streptococci play an important role in biofilm formation due to being the primary colonizers of the microflora formed on teeth. Other bacteria such as actinomyces and porphyromonas adhere to the receptors provided by streptococci covering the tooth surfaces. The genus streptococcus consists of faculatively anaerobic, Gram-positive cocci which are either arranged in chains or pairs. There are many species within this genus which are pathogens to humans or major commensals. The oral streptococci include 5 main groups; anginosus, mitis, sanguinis, salivarius and mutans. The bacterium which we focused on in this study was *Streptococcus. intermedius* which is an oral streptococcus. *S. intermedius* is in the anginosus group. (Summanen, Rowlinson et al. 2009)

The anginosus group (previously called Milleri group) is found in the biofilms developed around the tooth margins, gastrointestinal and genitourinary tracts. *S. intermedius* has been cultured also from liver and brain abscesses (Whily et al. 1992). Earlier studies have shown the association between *S. intermedius* and periodontal diseases and implantitis (Tanner et al.1997).

Stress conditions induce a SOS response in several bacteria. SOS is a bacterial global response to severe DNA damage. This postreplicational DNA repair system is carried out by RecA which is involved in LexA repressor activation. Antibiotics such as ciprofloxacin (Power and Phillips 1993), trimethoprim ((Lewin and Amyes 1991), rifamycins (Cirz, Chin et al. 2005) and beta lactams (Miller, Thomsen et al. 2004) are able to trigger a SOS response in a variety of bacteria and induce antibiotic resistance.

Earlier studies indicate that streptococcal species do not have the classical SOS repair system as they lack LexA. So how streptococci protect themselves in stress conditions? Studies conducted by Varhimo introduced a SOS-like system in which HdiR is the DNA binding protein which acts as LexA in lactococci and probably also in S. uberis (Varhimo, Savijoki et al. 2007). Some streptococci including *S.pneumoniae* lack also Hdir. In *S. pneumoniae*, the competence system is hypothesized to protect the cells from DNA damage. Supporting this is the finding that intact competence system is required to induce RecA by aminoglycosides (Claverys, Prudhomme et al. 2006).

Competence is defined as a physiological state in which bacteria become competent to take up and incorporate extracellular DNA into the chromosome. This can be achieved both directly by specific inducing peptides, but in *S.pneumoniae* competence has also been induced by different stress factors like antibiotics.

In streptococci of the mitis and probably the anginosus group, competence stimulating peptide (CSP) is the signal triggering competence (Claverys et al, 2006). The product of *comc* is a CSP precursor which gene in most transformable streptococci is organized in an operon together with *comD* and *comE* (Håvarstein et al, 1997). Histidine kinase (ComD) is the sensor

necessary for recognizing the CSP. After ComD autophosphorylation and transferring of a phosphoryl group to ComE (Response Regulator), the expression activation of the *comCDE* operon as well as other early competence genes, encoding for instance the CSP exporter ComAB starts. ComAB cleaves ComC and separates the leader peptide from the CSP signal (Petersen et al). ComE is responsible for activation of ComX which has a role in activating transcription of genes involved in the binding and uptaking of extracellular DNA. Activation of late com genes is dependent on activation of *comX* (Morrison et al, 1999).

The CSP system is a global response which controls the expression of at least 130 genes in the streptococci, including the genes described above. Not all the proteins encoded by com genes are required, however, for DNA uptake and transformation. In fact, most of them are dispensable. Some of these dispensable genes are linked to the transformation process, by mediating fratricide, whereas the function of some of the upregulated genes has not yet been identified. Studies conducted by Claverys and Håvarstein represent the importance of these genes in fratricide and providing exogenous DNA for competent cells to utilize in transformation. (Claverys, Martin et al. 2007).

Competence leads therefore to DNA uptake, and killing of siblings (fratricide). This extracellular DNA may be used to acquire new genetic traits, but also to repair DNA damage. The mechanisms involved in increased biofilm formation by competent cells are not well understood, but it appears that the released extracellular DNA may be utilized as a matrix component in biofilm.

Biofilm formation leads to increased antibiotic resistance by mechanisms such as restricted penetration of antibiotics (Stewart 1996) , decreased growth by nutrient limitation

and differential gene expression under stress conditions (Brown and Lea 1988) (Xu, Stewart et al. 1998).

Although antibiotics first relieved human from infectious diseases which were the main reason for death throughout history, immediate bacterial response to these new weapons destroyed this hope. Today many people die due to antibiotic resistance. Inactivating the drugs, modifying the target of the drugs or decreasing the drug penetration are some of the ways practiced by bacteria to overcome the antibiotics. An important mechanism for resisrance is the ability of the bacteria to undergo changes in their DNA. The DNA modifications can be achieved by either de novo mutations, acquisition of exogenous DNA or natural transformation (mediated via competence).

In this study we investigated whether *comCDE* was involved in *S. intermedius* biofilm formation. Thereafter we tested the hypothesis that antibiotic induction of the competence system might be related to the known involvement of competence in biofilm formation.

Spectinomycin and streptomycin were the two antibiotics used in this study. These antibiotics belong to aminoglycoside family; spectinomycin inhibits protein synthesis by binding to 30S rRNA while streptomycin binds to 16S rRNA of the bacterial ribosome. (Moazed and Noller 1987), (Kirthi, Roy-Chaudhuri et al. 2006)

The aminoglycosides streptomycin and kanamycin have been shown to induce the competence response in *S. pneumoniae*.

Aims:

(1) To investigate the mechanism by which CSP influences *S. intermedius* biofilm formation.

(2) To examine whether subinhibitory antibiotic concentrations of streptomycin and spectinomycin affect *S. intermedius* biofilm formation.

(3) To investigate whether a possible effect on biofilm formation would be associated with the ability of the cells to communicate via CSP.

Materials and methods:

Bacterial strains and media:

The *S. intermedius* strains used in this study were NCTC 11324 (wild type) and three isogenic mutants; SI009 (*comC* deletion), SI003 (*comDE* deletion), and SI004 (*comE* deletion) (Figure1; a, b, c and d). The bacteria were stored at -70°C in 15% glycerol. At the beginning of each experiment, the bacteria were cultured on Todd-Hewitt broth agar plates (THB) and incubated at 37°C in a 5% CO_2 aerobic atmosphere. Trypticase soy broth (TSB) was the medium used for the biofilm and growth assays. The antibiotic range of concentrations used were: spectinomycin (0,2-200µg/mL) and streptomycin (0,1-256µg/mL) (Sigma).

Figure 1: Model of the *S. intermedius* ComCDE function in competence signaling and the deletion mutants used in this study (a) wild type; (b) *comC* deletion (encoding the CSP); (c) *comD* deletion (encoding the histidine kinase); (d) *comE* deletion (encoding the response regulator).
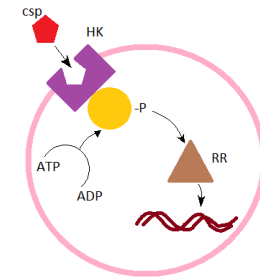
(a)
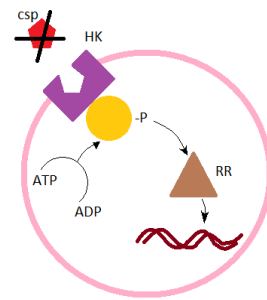
(b)
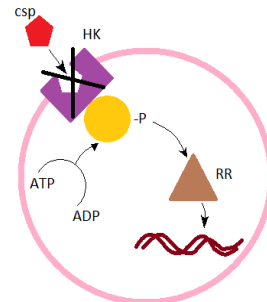
(c)
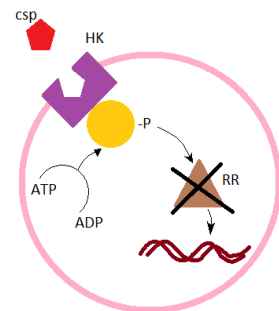
(d)

Synthetic CSP:

The synthetic CSP was ordered from MedProbe (CSP, amino acid sequence: NH2-DSRIRMGFDFSKLFGK-COOH) (>95% purity grade). The lyophilized CSP was reconstituted in distilled water and stored in small aliquots of 20µg/mL at -20˚C.

Biofilm assay:

The following strains were used in the biofilm assay: NCTC 11324, SI009, SI003 and SI004. The cells were taken from -70˚C and cultured on THB agar plates. Later 3 colonies of each strain were transferred into a glass tube containing 5mL TSB. These tubes were incubated at 37˚C for 20-24 h in a 5% CO_2 aerobic atmosphere.

The cultures were then diluted 1:100 and grown in the wells of microtiter plates (24 and 96 wells) in the presence or absence of exogenous CSP or antibiotics. The plates were incubated at 37˚C and 5% CO_2 for another 20-24 h. Figure 2 illustrates one of the plates used in the biofilm experiments.

First, optical densities at 600 nm (OD 600) were measured to estimate bacteria density, and then (250µL in 24-well plate or 150µL in 96-well plate) of safranin was added to each well. After 15 minutes, the plates were washed twice in distilled water. When the plates were totally dry, acetic acid was added (250µL in 24-well plate or 150µL in 96-well plate). The samples were then read at 530 nm.

Figure 2: Example of a 24-well microtiter plate with safranin stained biofilms.
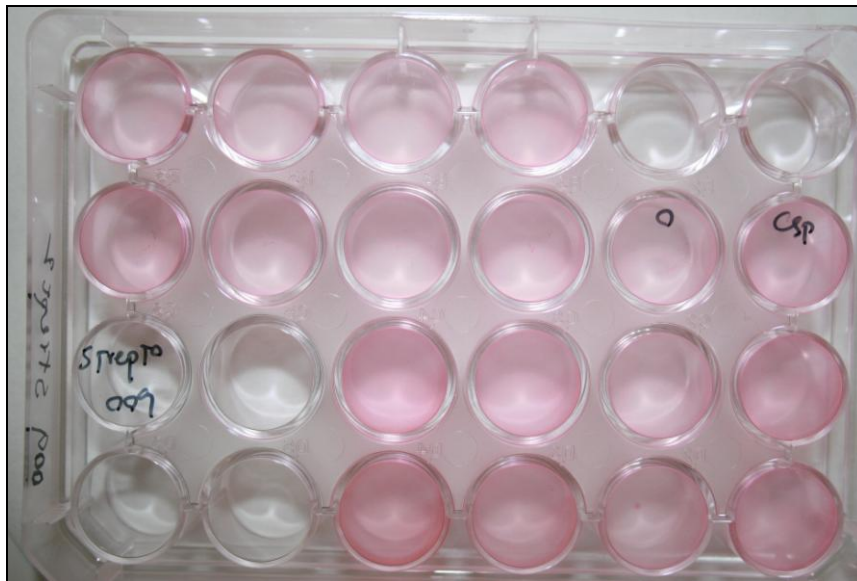
Growth curve assay:

In this assay NCTC 11324 and SI009 were tested in the presence of spectinomycin and streptomycin in two 96-well plates. This assay was repeated four times to adjust the antibiotic concentrations to subinhibitory levels and examine the effect of CSP on growth. The first overnight cultures were prepared and diluted as described above for the biofilm assay.

To investigate the role of *comCDE* on biofilm formation, 24-well plates were used. The plates were divided into half, the first half included overnight cultures of NCTC 11324 and the second half included SI009. The first row in the plates was filled with 200μL of TSB, which were used as background. In the second row, 100μL of diluted overnight culture and 0,5μL CSP were added to 100μL TSB. In the remaining wells 100μL diluted overnight culture plus 100μL of increasing antibiotic concentrations were added. The initial OD 600 was measured,

followed by incubation of the plates at 37˚C in 5% CO_2 for 4-5 h. Thereafter OD 600 was measured. at 30-60 min intervals, until a stable raise in the curve was observed. The last reading was done after approximately 24 h. Figure 3 illustrates one of the plates used in this assay.

Figure 3: An example of a 96 well-plate used for the growth curve assay.
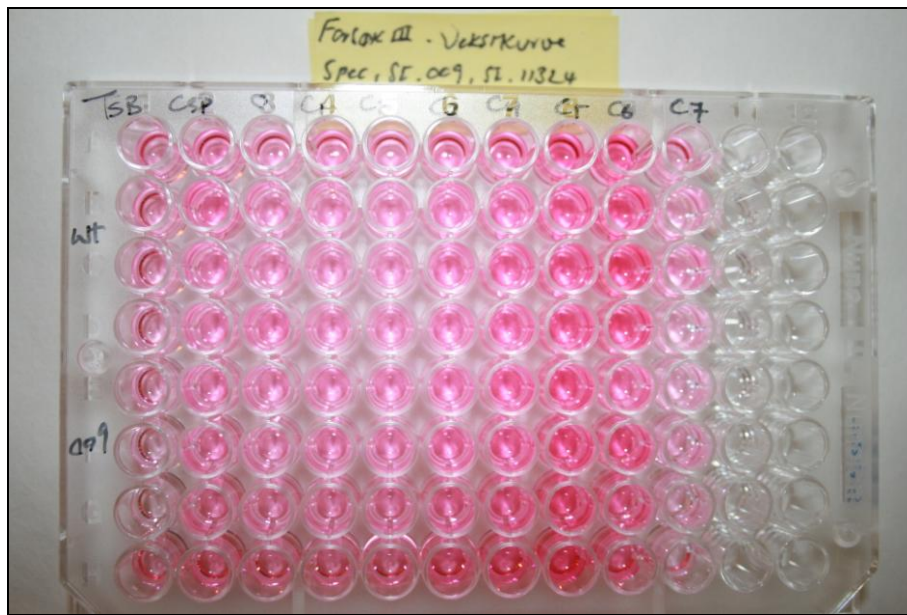
Scanning Electron Microscopy (SEM) images of the biofilms

The aim of this assay was to visualize the biofilms formed in the presence or absence of spectinomycin, streptomycin and CSP. Biofilms were allowed to form on discs placed on 24-well plates.

The plates included diluted overnight cultures of NCTC 11324, SI009 and SI004 and were incubated at 37˚C in 5% CO_2 for 20-24 h and then the discs were gently removed from the plates, placed in new empty plates and fixed with 1 mL fixation buffer (2.5% glutamate aldehyde in 0.1 M Sørensen's phosphate buffer. PH: 7.4). The plates were wrapped carefully

in plastic and placed in the refrigerator. These discs were later used to develop the SEM images.

Results:

CSP had an enhancing effect on biofilm formation that was dependent on the presence of a CSP receptor/response regulator. CSP induced biofilm formation in the wild type and in the *comC* deletion mutant SI009. In the absence of exogenous CSP, biofilm formation by the *comC* mutant SI009, as well as the *comDE* mutant SI003 and *comE mutant* SI004 was not affected by the presence of CSP (Figures 4 and 5). It was interesting to observe that in the presence of CSP, the wild type and SI009 mutant formed longer chains and some exhibited altered morfology as large and round spheres.

Figure 4: Effect of CSP on *S.intermedius* biofilm formation in wild type and mutants. The green column is without CSP and the blue with CSP addition to the culture.
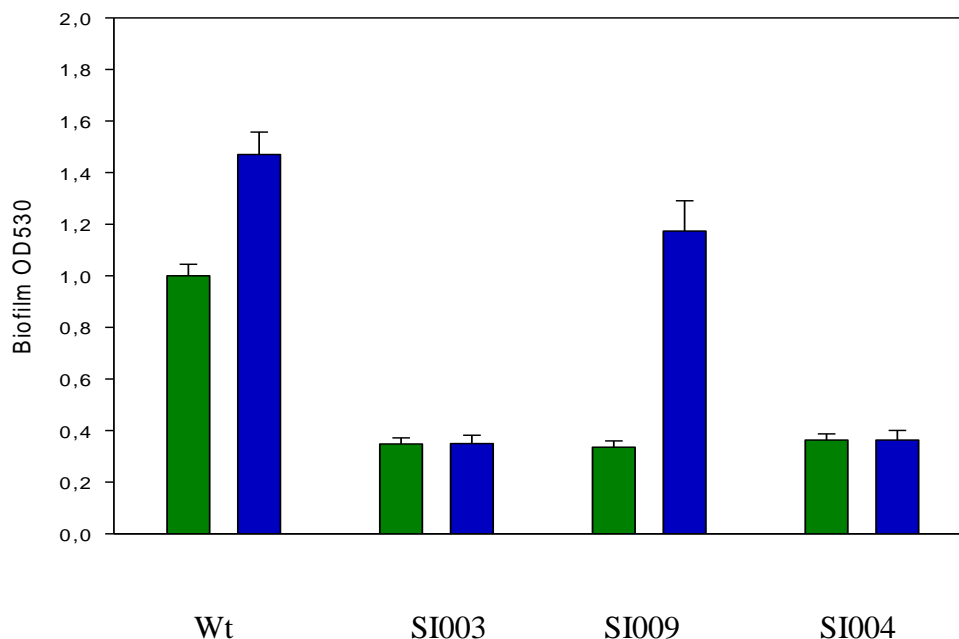
Figure 5: SEM images of the wild type and SI009.

NCTC 11324
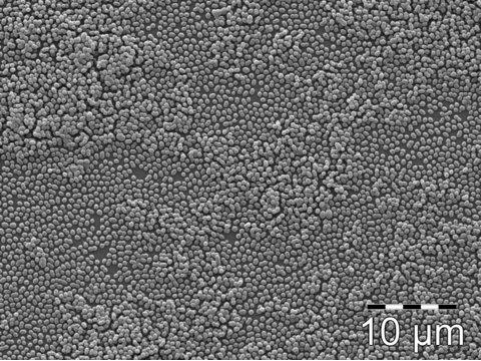
NCTC 11324 + CSP
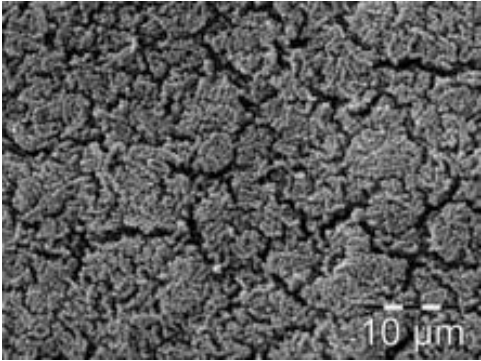
SI009
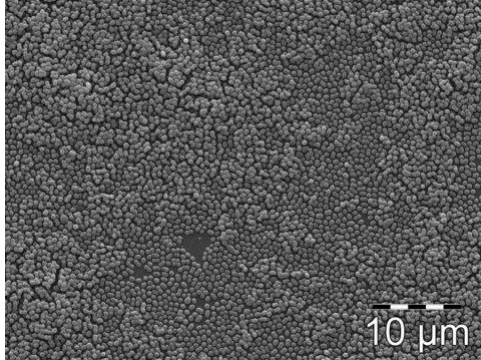
SI009 + CSP
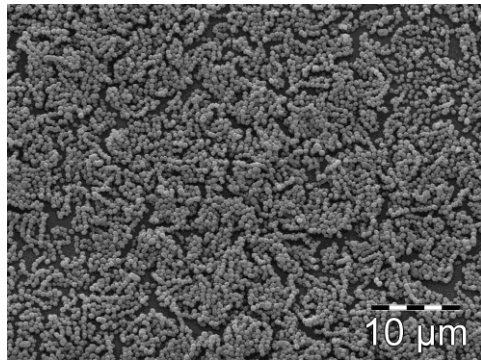
SI004
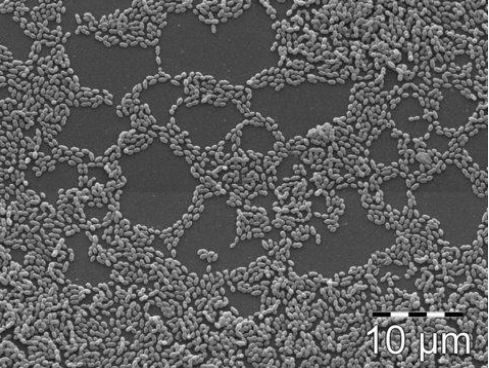
SI004 + CSP
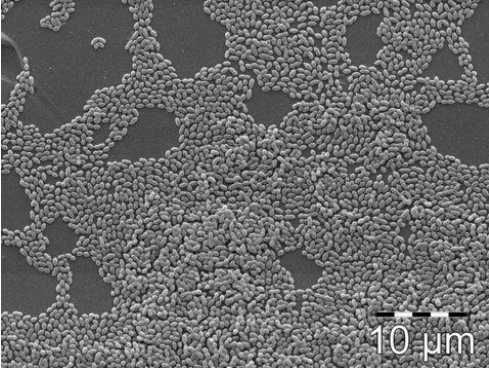
Streptomycin and spectinomycin enhanced biofilm formation in both the wild type and the *comC* deletion mutant SI009. A steady increase in biofilm formation, up to 15 fold, was associated with spectinomycin concentrations up to 100μg/mL. Above this concentration the increase effect was reduced to only 2 fold. A 5 fold increase in biofilm formation is observed with streptomycin up to 128μg/mL. The biofilm formation reduces to 2.5 fold above this concentration (Figures 6; a and b and Figure 7).

Figure 6: Biofilm formation and cell density at 7h (early growth phase) in the presence of (a) streptomycin and (b) spectinomycin

(a)
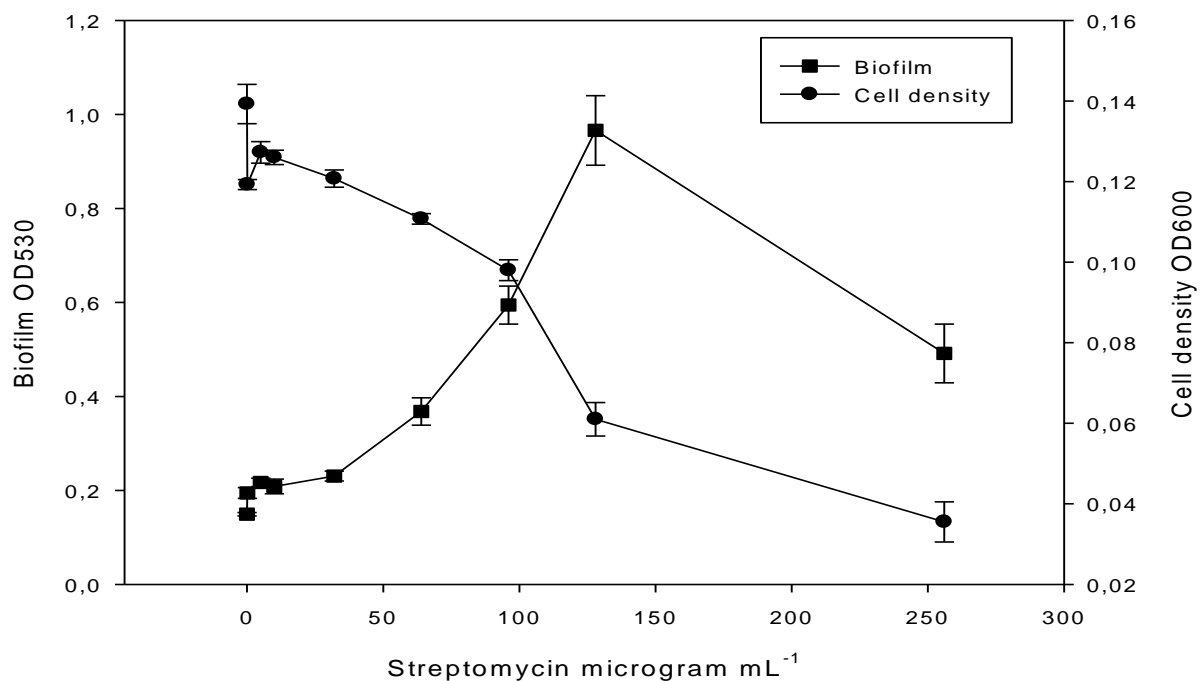
(b)
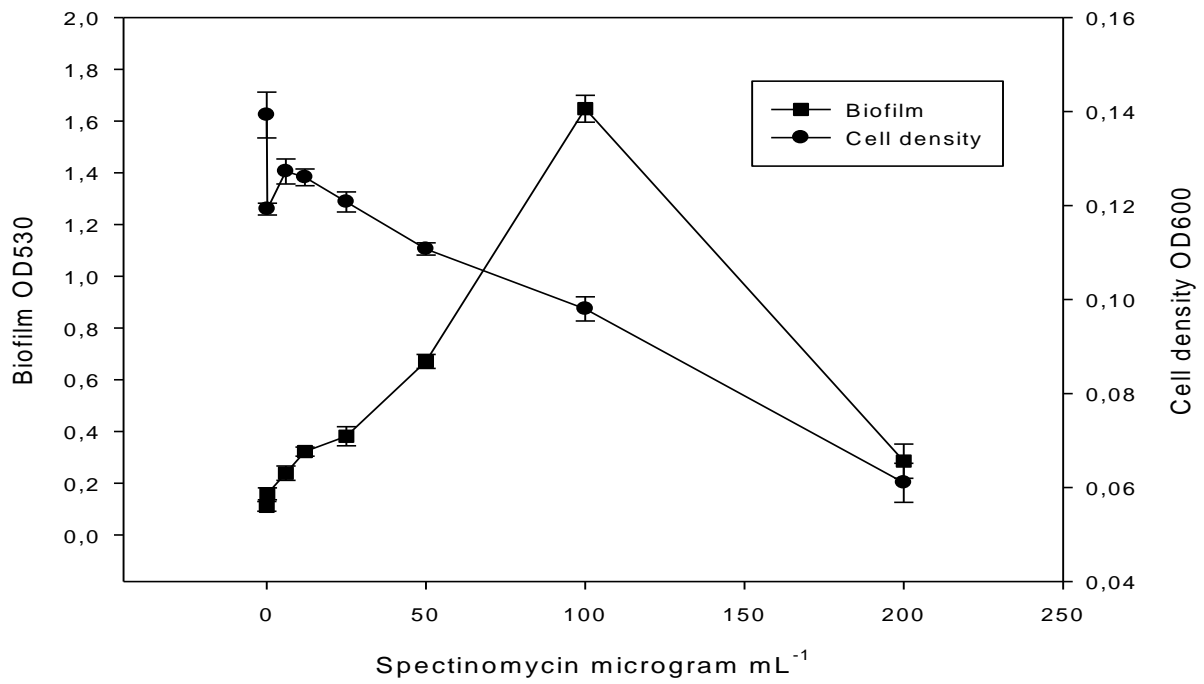
Figure 7: SEM images of wild type, SI009 and SI004 (a) in the absence or presence of streptomycin (128µg/mL), or (b) in the absence or presence of spectinomycin (50µg/mL).

(a)

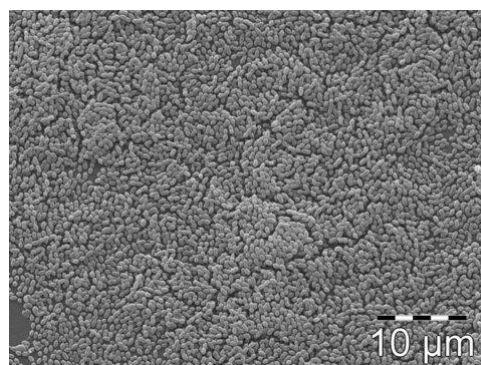NCTC 11324   NCTC 11324 + streptomycin
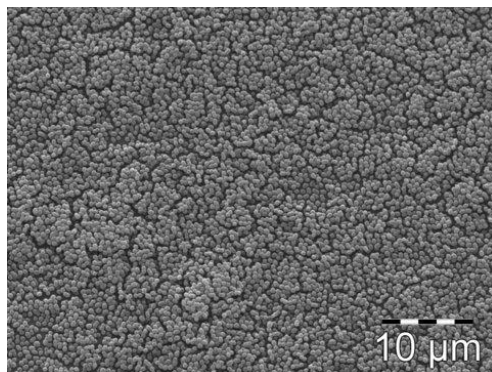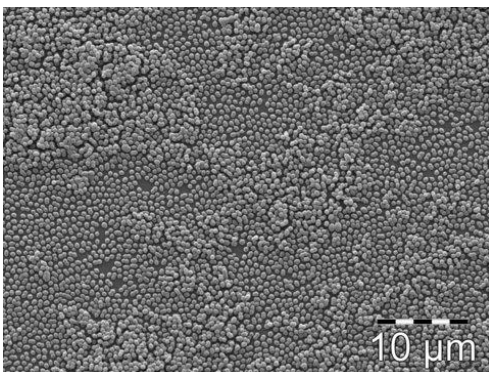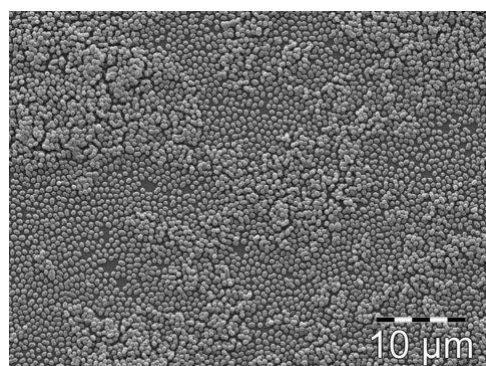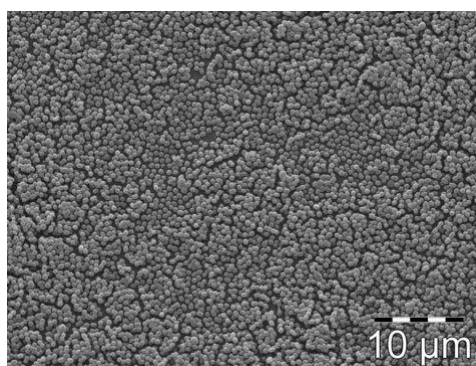
SI009
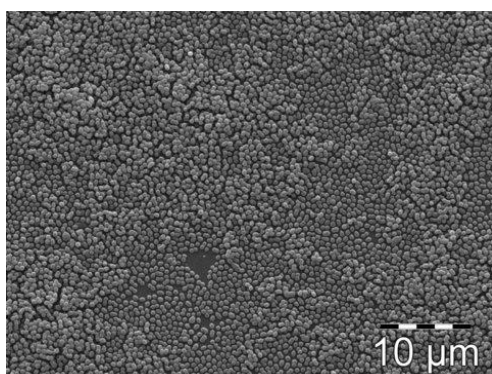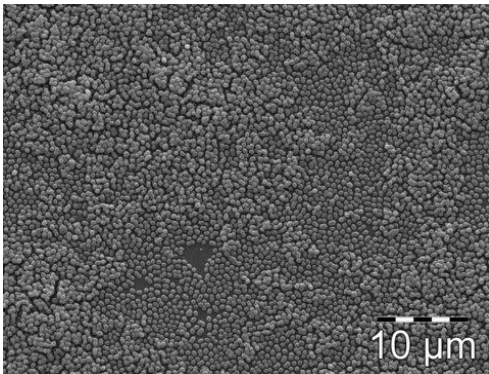
SI009 + streptomycin
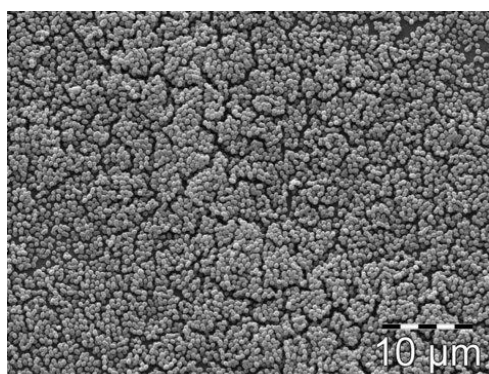
SI004
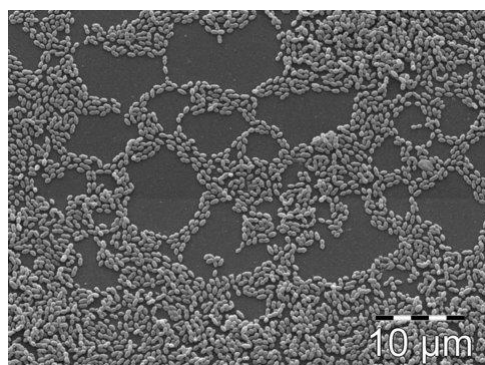
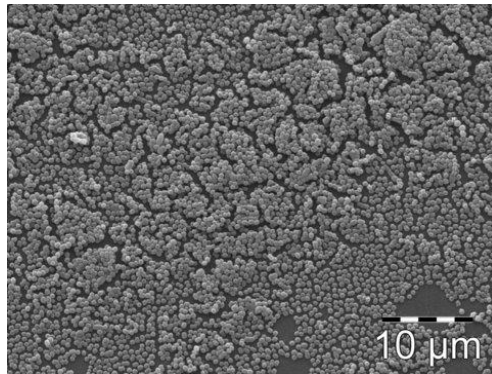SI004 + streptomycin

(b)

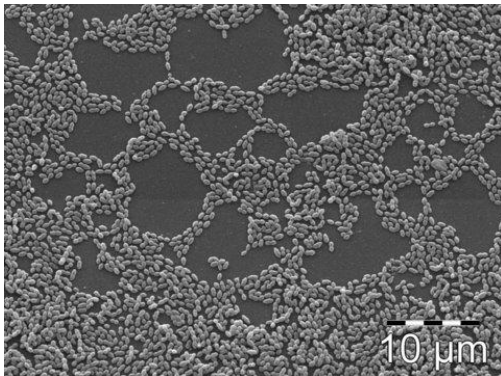NCTC 11324

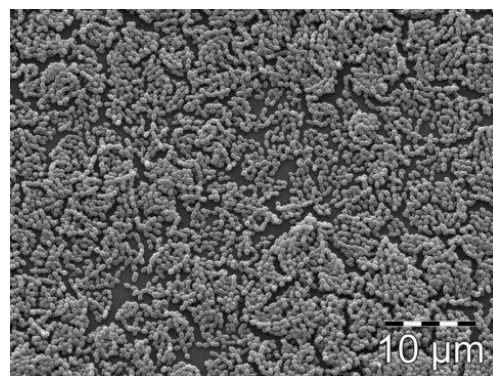NCTC 11324 + spectinomycin
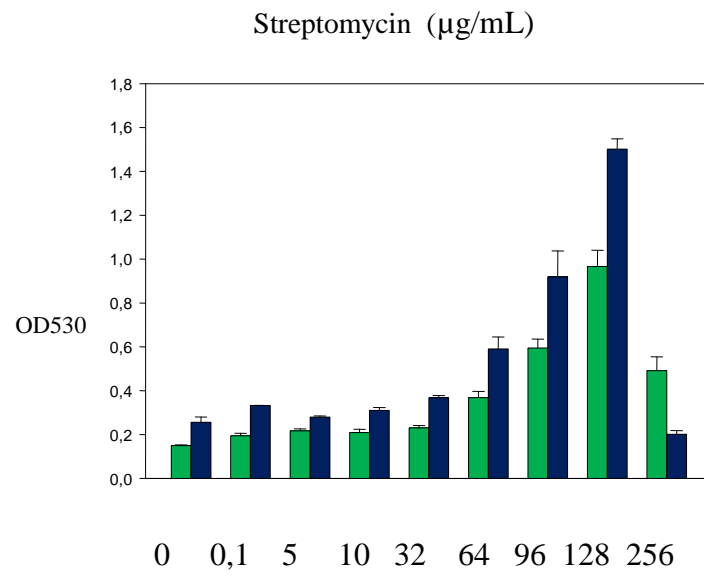
SI009

SI009 + spectinomycin

SI004

SI004 + spectinomycin

The effect of spectinomycin and streptomycin on biofilm formation was not related to the presence or absence of exogenous CSP. CSP could probably have an additive effect on biofilm formation. Spectinomycin and streptomycin stimulated biofilm formation both in 11324 and SI009 indicating that competence was not a key factor (Figures 8, 9, 10 and 11).

Figure 8: Showing biofilm formation with and without CSP addition. The blue columns represent *S.intermedius* with CSP addition and the green ones without CSP: (a) wild type (b) SI009
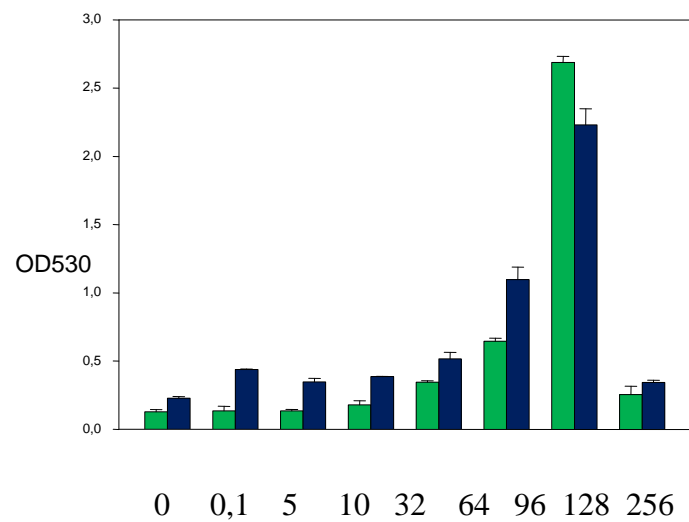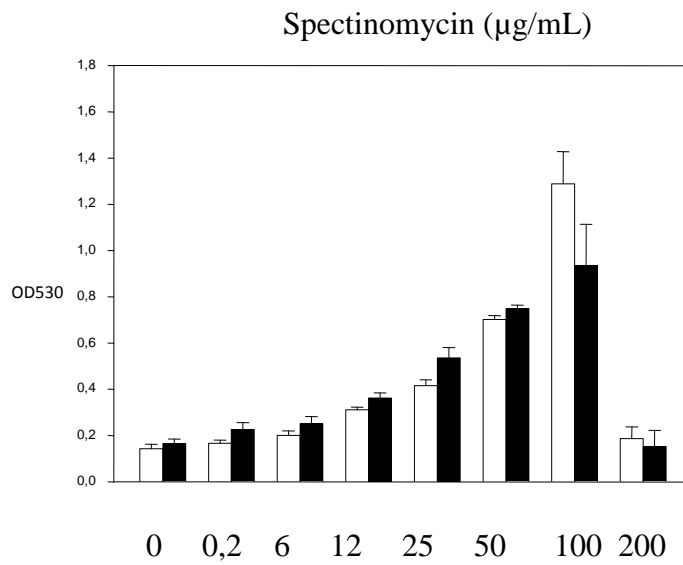
(a)

Streptomycin (µg/mL)

OD530

0   0,1   5   10   32   64   96   128   256

(b)

OD530

0   0,1   5   10   32   64   96   128   256

Figure 9: Biofilm formation in wild type and SI009 in the presence of (a) spectinomycin (0,2-200 µg/mL) and (b) streptomycin (0,1-256 µg/mL) with no CSP addition. The white columns represent wild type and the black ones represent SI009.
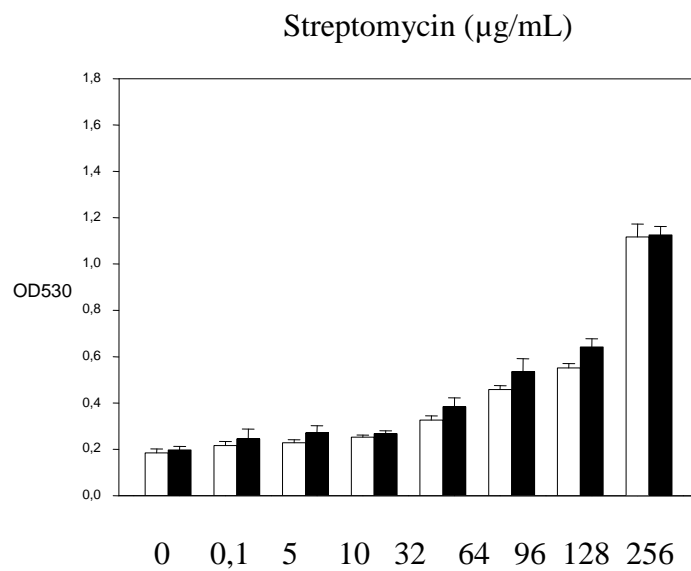
(a)

(b)

Figure 10: Biofilm formation in wild type in the presence or absence of CSP, grown in the presence of spectinomycin (0,2-200µg/mL). The blue columns represent wild type with CSP addition, and the green columns in the absence of CSP.

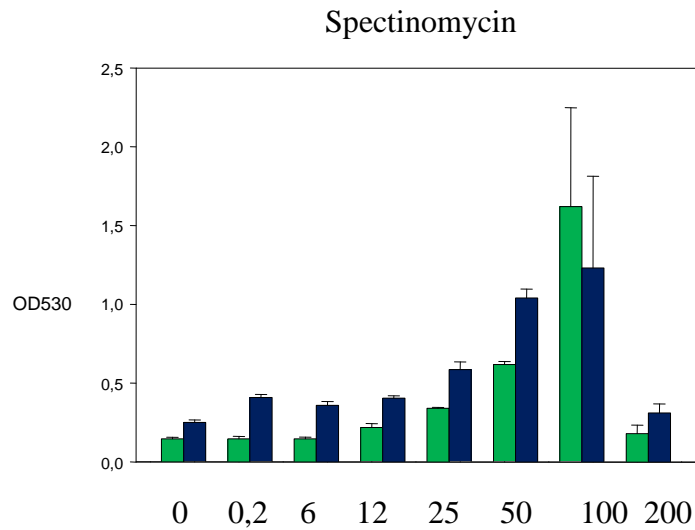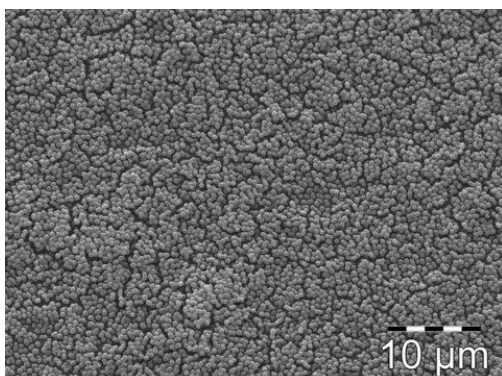
Spectinomycin

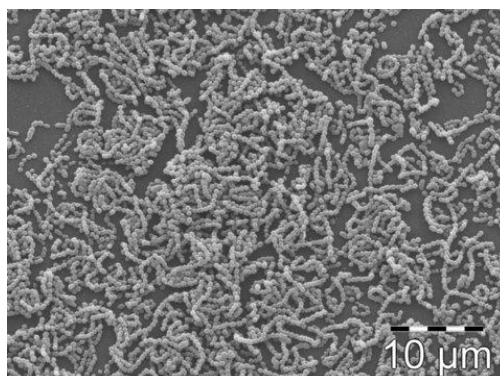*Figure 11*: SEM images of wild type, SI009 and SI004 in the presence or absence of CSP (a) streptomycin (128µg/mL) and (b) spectinomycin: (50µg/mL).
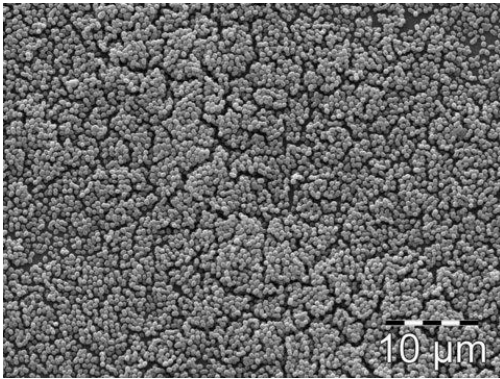
(a)

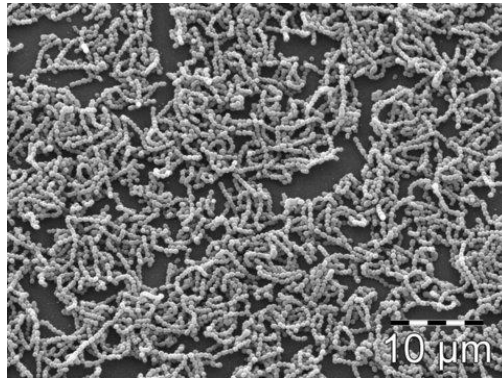NCTC 11324 - CSP + streptomycin   NCTC 11324 + CSP + streptomycin
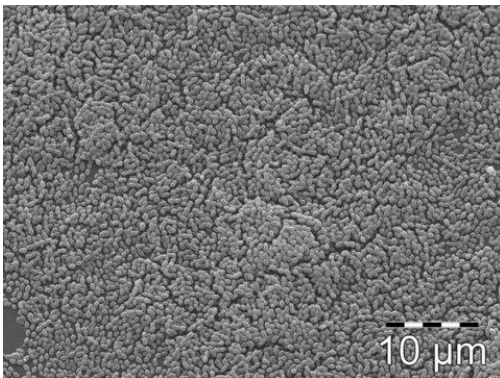
SI009 - CSP + streptomycin　　　　　SI009 + CSP + streptomycin

SI004 - CSP + streptomycin　　　　　SI004 + CSP + streptomycin
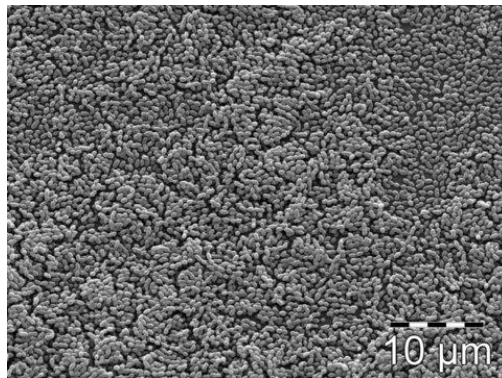
(b)

NCTC 11324 - CSP + spectinomycin　　NCTC 11324 + CSP + spectinomycin
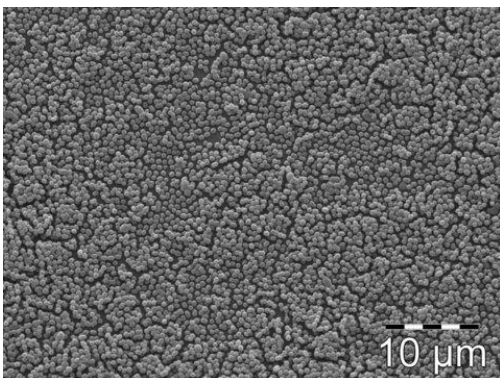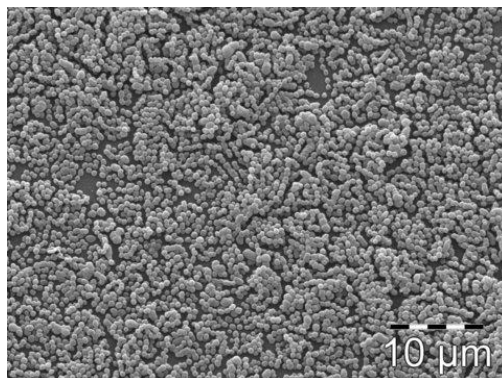
SI009 - CSP + spectinomycin   SI009 + CSP + spectinomycin
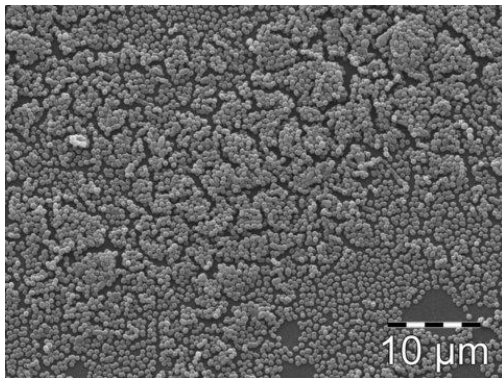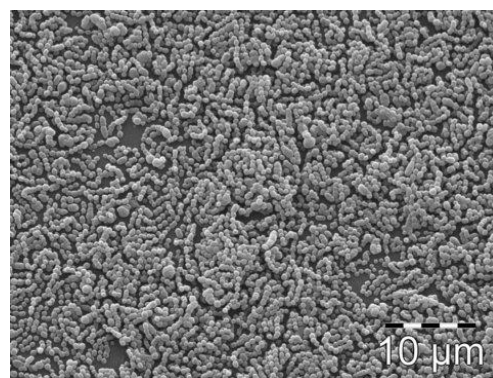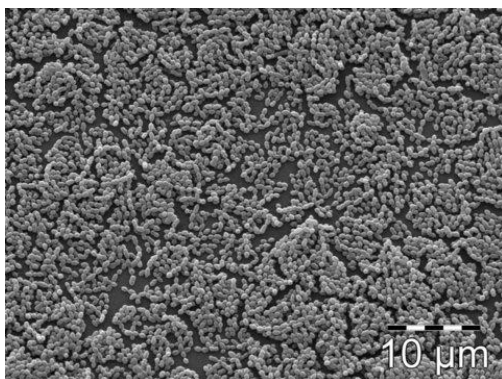
SI004 - CSP + spectinomycin   SI004 + CSP + spectinomycin
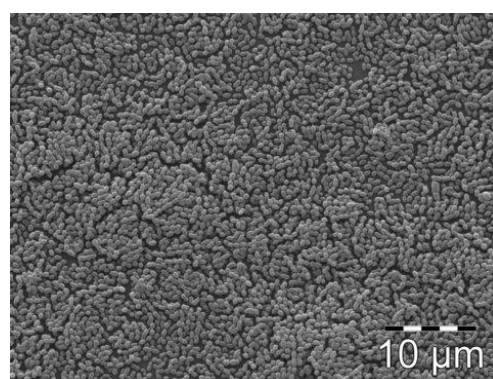
Discussion:

Competence is a specific mechanism in bacterial communication. Among the streptococci, the *S. pneumoniae* competence system is the one that has been described in more detail. In *S. pneumoniae*, strains lacking the competence stimulating signal CSP, as well as the CSP receptor ComD, or the cognate response regulator ComE are unable to communicate via this system. This is also the case for the oral *S. gordonii*, which similar to *S. pneumoniae*, belongs to the mitis group of streptococci. (Havarstein, Hakenbeck et al. 1997)

In the anginosus group of streptococci, which includes among other members *S. intermedius*, much less is known about the competence system. The identification and partial sequencing of the *comCDE* locus in the anginosus group indicate, however, that *comCDE* plays a central role in competence in this group as well. Supporting this notion, CSP has been shown to induce both competence and biofilm formation in *S. intermedius*. (Petersen, Pecharki et al. 2004)

To investigate whether this effect is mediated by *comCDE*, deletion mutants lacking the *comC*, *comDE*, or *comE* genes were constructed. We found out that the *comCDE* system was involved in *S. intermedius* biofilm formation. Induction of biofilm formation by CSP was dependent on the presence of ComDE, whereas in the *comC* deletion mutant, the defect in biofilm formation was restored to near wild type levels by addition of exogenous CSP.

We tested further the hypothesis that antibiotics, by inducing the competence system, may lead to increased biofilm formation. *S. intermedius* wild type and the *comC* deletion mutant exposed to increasing streptomycin or spectinomycin concentrations in the presence or absence of CSP resulted in more than 40 % increase in biofilm formation. It is important to note that this effect was reversed with higher concentrations of antibiotics (from 128µg/mL in streptomycin and 100µg/mL in spectinomycin), probably indicating fewer cells in the culture due to the killing effects of antibiotics and CSP. The finding that the *comC* and *comE* deletion mutants showed a response similar to the wild type in the absence of CSP, indicated that the effect of antibiotics on biofilm formation was not dependent on the CSP response. This was also supported by the additive effect of CSP and antibiotics on biofilm levels. It was interesting to find out, however, that most of the subinhibitory streptomycin and spectinomycin concentrations used in this study enhanced biofilm formation. This adds to the list of antibiotics, including tetracycline, ampicillin, and ciprofloxacin, which have been

shown to also induce *S. intermedius* biofilm formation at subinhibitory concentrations. (Ahmed, Petersen et al. 2009)

Such an effect may be associated to the autoinducer 2 communication system, but it may also involve a general response to concentrations that slow down *S. intermedius* growth rate.

The classical SOS-response mediated by LexA and RecA, which favors bacterial survival by reducing the effect of DNA damaging agents, has not been identified in streptococci. It has been recently proposed that the competence system would possibly function as a SOS-alternative system in streptococci. This hypothesis is supported by the finding that the DNA damaging agent mitomycin, as well as several antibiotics targeting DNA or protein synthesis, including streptomycin, induce *S. pneumoniae* genes upregulated in the presence of CSP. Such genes include ssbB and recA, involved in DNA processing and recombination, respectively. The requirement for an intact CSP exporter system has also been described. Studies in the laboratory where the current study was conducted are underway to determine whether the S. intermedius competence system may also be induced by antibiotics and other DNA damaging agents. The possibility that S. intermedius may use alternative pathways in the SOS-response may, however, not be excluded. The ongoing genome sequencing of *S. intermedius* will certainly contribute to future studies aiming at elucidating the role of regulatory pathways, such as the competence system, in *S. intermedius* virulence and survival to stress, such as antibiotic attack. The finding that antibiotics at subinhibitory concentrations may stimulate biofilm formation is of particular clinical relevance, as it may be involved in infections recalcitrant to antibiotic treatment. Understanding the mechanisms involved in biofilm formation may lead to novel strategies to fight biofilms.

Acknowledgments:

References:

Ahmed, N. A., F. C. Petersen, et al. (2009). "AI-2/LuxS is involved in increased biofilm formation by Streptococcus intermedius in the presence of antibiotics." Antimicrob Agents Chemother **53**(10): 4258-4263.

Aragao, D., S. Macedo, et al. (2003). "Reduced hybrid cluster proteins (HCP) from Desulfovibrio desulfuricans ATCC 27774 and Desulfovibrio vulgaris (Hildenborough): X-ray structures at high resolution using synchrotron radiation." J Biol Inorg Chem **8**(5): 540-548.

Brown, M. R., D. G. Allison, et al. (1988). "Resistance of bacterial biofilms to antibiotics: a growth-rate related effect?" J Antimicrob Chemother **22**(6): 777-780.

Brown, M. R. and A. O. Lea (1988). "FMRFamide- and adipokinetic hormone-like immunoreactivity in the nervous system of the mosquito, Aedes aegypti." J Comp Neurol **270**(4): 606-614.

Cirz, R. T., J. K. Chin, et al. (2005). "Inhibition of mutation and combating the evolution of antibiotic resistance." PLoS Biol **3**(6): e176.

Claverys, J. P., B. Martin, et al. (2007). "Competence-induced fratricide in streptococci." Mol Microbiol **64**(6): 1423-1433.

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

Costerton, J. W., P. S. Stewart, et al. (1999). "Bacterial biofilms: a common cause of persistent infections." Science **284**(5418): 1318-1322.

Havarstein, L. S., R. Hakenbeck, et al. (1997). "Natural competence in the genus Streptococcus: evidence that streptococci can change pherotype by interspecies recombinational exchanges." J Bacteriol **179**(21): 6589-6594.

Kirthi, N., B. Roy-Chaudhuri, et al. (2006). "A novel single amino acid change in small subunit ribosomal protein S5 has profound effects on translational fidelity." RNA **12**(12): 2080-2091.

Kleerebezem, M., L. E. Quadri, et al. (1997). "Quorum sensing by peptide pheromones and two-component signal-transduction systems in Gram-positive bacteria." Mol Microbiol **24**(5): 895-904.

Lewin, C. S. and S. G. Amyes (1991). "The role of the SOS response in bacteria exposed to zidovudine or trimethoprim." J Med Microbiol **34**(6): 329-332.

Mah, T. F. and G. A. O'Toole (2001). "Mechanisms of biofilm resistance to antimicrobial agents." Trends Microbiol **9**(1): 34-39.

Marsh, P. D. (1999). "Microbiologic aspects of dental plaque and dental caries." Dent Clin North Am **43**(4): 599-614, v-vi.

Miller, C., L. E. Thomsen, et al. (2004). "SOS response induction by beta-lactams and bacterial defense against antibiotic lethality." Science **305**(5690): 1629-1631.

Moazed, D. and H. F. Noller (1987). "Interaction of antibiotics with functional sites in 16S ribosomal RNA." Nature **327**(6121): 389-394.

Nickel, J. C., I. Ruseska, et al. (1985). "Tobramycin resistance of Pseudomonas aeruginosa cells growing as a biofilm on urinary catheter material." Antimicrob Agents Chemother **27**(4): 619-624.

Perry, J. A., M. B. Jones, et al. (2009). "Peptide alarmone signalling triggers an auto-active bacteriocin necessary for genetic competence." Mol Microbiol **72**(4): 905-917.

Petersen, F. C., D. Pecharki, et al. (2004). "Biofilm mode of growth of Streptococcus intermedius favored by a competence-stimulating signaling peptide." J Bacteriol **186**(18): 6327-6331.

Petersen, F. C., L. Tao, et al. (2005). "DNA binding-uptake system: a link between cell-to-cell

 communication and biofilm formation." J Bacteriol **187**(13): 4392-4400.

Power, E. G. and I. Phillips (1993). "Correlation between umuC induction and Salmonella mutagenicity assay for quinolone antimicrobial agents." FEMS Microbiol Lett **112**(3): 251-254.

Schauder, S. and B. L. Bassler (2001). "The languages of bacteria." Genes Dev **15**(12): 1468-1480.

Scheie, A. A. and F. C. Petersen (2004). "The Biofilm Concept: Consequences for Future Prophylaxis of Oral Diseases?" Crit Rev Oral Biol Med **15**(1): 4-12.

Stewart, P. S. (1996). "Theoretical aspects of antibiotic diffusion into microbial biofilms." Antimicrob Agents Chemother **40**(11): 2517-2522.

Summanen, P. H., M. C. Rowlinson, et al. (2009). "Evaluation of genotypic and phenotypic methods for differentiation of the members of the Anginosus group streptococci." Eur J Clin Microbiol Infect Dis **28**(9): 1123-1128.

Tetz, V. V. and G. V. Tetz (2010). "Effect of Extracellular DNA Destruction by DNase I on Characteristics of Forming Biofilms." DNA Cell Biol **29**(8): 399-405.

Varhimo, E., K. Savijoki, et al. (2007). "Identification of a novel streptococcal gene cassette mediating SOS mutagenesis in Streptococcus uberis." J Bacteriol **189**(14): 5210-5222.

Xavier, K. B. and B. L. Bassler (2003). "LuxS quorum sensing: more than just a numbers game." Curr Opin Microbiol **6**(2): 191-197.

Xu, K. D., P. S. Stewart, et al. (1998). "Spatial physiological heterogeneity in Pseudomonas aeruginosa biofilm is determined by oxygen availability." Appl Environ Microbiol **64**(10): 4035-4039.