V. Results

1. Construction of a P_{ica}-*lacZ* fusion and integration in the chromosome of *S. epidermidis* 220

To investigate the impact of different environmental factors on the regulation of slime production in *S. epidermidis*, we have constructed a chromosomal-encoded *ica*:::*lacZ* transcriptional fusion. A PCR fragment containing the initiation signal of *ica* expression was amplified, and ligated in a suitable cloning sites of a vector with *E. coli* β-galactosidase gene. The resulted P_{ica}-*lacZ*-transcriptional fusion was integrated in the chromosome of *S. epidermidis* 220 after cloning it in a temperature sensitive shuttle-vector system. The resulting strain of *S. epidermidis* 220-1 carried a copy of the β-galactosidase gene and under the control of the *ica* promoter. The production of β-galactosidase enzyme indicates the activity of the *ica*-promotor and can be measured by using a specific indicator substrate.

1.1 Cloning of the promotor region of the *ica* gene and fusion with the b-galactosidase gene from *E. coli*.

A 727-bp DNA fragment containing the promotor of the *ica* operon was amplified from *S. epidermidis* 220 by PCR using primer 1 (Pica-1) and primer 2 (Pica-2) containing an *Eco*RI and *Xba*I cleavage site, respectively. The PCR-primers bind at position 256 and 981 of the published *ica* sequence (accession U43366), respectively. The amplification conditions consisted of an initial denaturation step at 94 °C for 2 minutes followed by 30 cycles of 94°C for 60 s, 53°C for 60 s, and 72°C for 90 s. Following restriction endonuclease cleavage of PCR product with *Eco*RI and *Xba*I, the resulted 695 bp fragment was cloned into the *Eco*RI/*Xba*I restricted multiple cloning site of the pUC18 vector creating plasmid pSK1. *E. coli* DH5α was transformed with the ligation product by the $CaCl_2$ method, and plated onto a selective X-gal/IPTG-LB agar containing 100 µg/ml Ampicillin. The white colonies were selected and grown overnight in 2 ml LB medium containing 100 µg/ml Ampicillin, for plasmid isolation. The isolated plasmids were checked by restriction digest using *Eco*RI and *Xba*I to select the right plasmid with the cloned fragment. Further analysis was done by nucleotide sequencing of the cloned PCR fragment to check any mutation or a possible

mistakes during the amplification of the fragment. For construction of pSK2, the *hla* promotor of pKO10 was removed by restriction digest of the plasmid with *Eco*RI/*Hind*III, and the *ica* promotor fragment was cut from pSK1 by *Eco*RI/*Hind*III restriction cleavage and ligated into the *Eco*RI/*Hind*III restricted pKO10 shuttle vector containing a fusion between *spoVG*-ribosome binding site from *Bacillus subtilis* and a promotorless reporter-gene β-galactosidase from *E. coli* resulting in pKS2. Followed by the propagation in a *lacZ* negative *E. coli* MC4100 by electroporation of the bacteria with the ligation reaction, and plating onto the selective X-gal/IPTG-LB agar containing 100 µg/ml Ampicillin. The blue colonies were selected and grown overnight in 2 ml LB medium containing 100 µg/ml Ampicillin for plasmid isolation. The isolated plasmids were analysed with a restriction digest using *Eco*RI and *Hind*III to select the correct plasmid carrying the *ica* promotor fused to the reporter gene (figure 5).

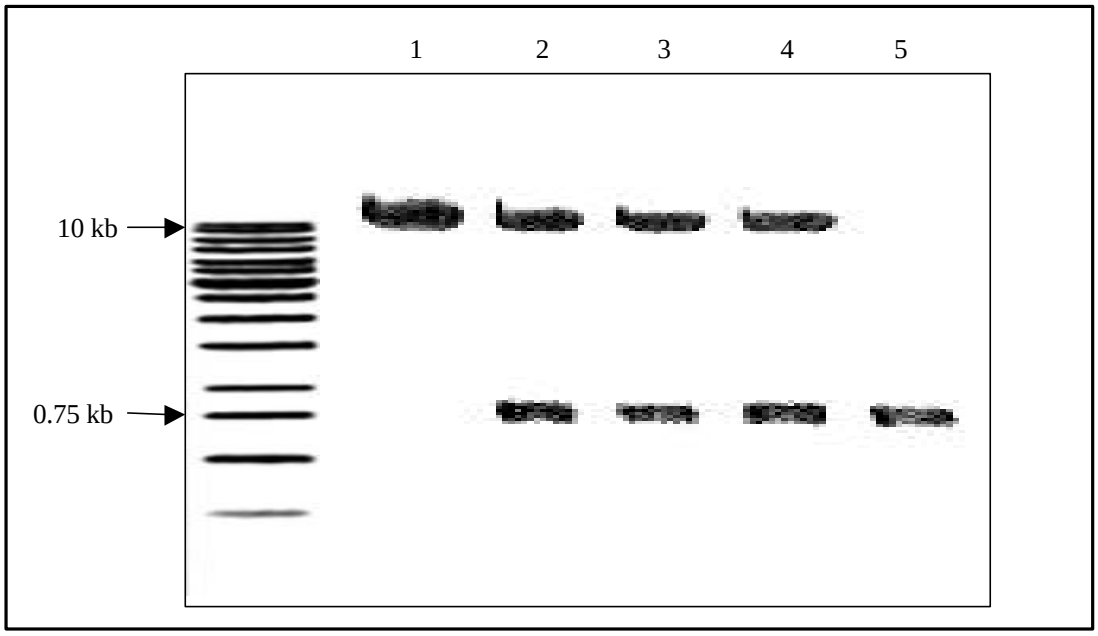

Figure 5: Restriction digest analysis of plasmid pSK10 using *Eco*RI und *Hind*III. Lane 1: *Eco*RI und *Hind*III restricted pKO10 shuttle vector without *hla*-promotor, lane 2, 3, and 4: constructed pSK2; lane 5: P_{ica} PCR-product.

1.2 Integration of the P_{ica}-*lacZ* fusion in the chromosome of *S. epidermidis* 220

Transformation of *S. epidermidis* with the shuttle vector will not be successful by a direct introduction of the vector isolated from *E. coli*, because of restriction systems present in Gram positive bacteria that will not accept DNA from gram negative species. To prevent restriction of the plasmid DNA introduced to the *S. epidermidis*, pSK2 was transformed into the restriction deficient strain *S. aureus* RN4220 electroporation. *S. aureus* RN4220 is a restriction negative mutant that can accept DNA isolated from *E. coli*, and 2 x 10^3 transformant cells/µg DNA were obtained as a result of electroporation method. Following the electroporation, the tranformants were grown overnight at 30°C in the presence of 10 µg/ml chloramphenicol, since as the vector carries a chloramphenicol resistance cassette and a temperature sensitive origin. The blue colonies were selected, and grown overnight in BHI medium containing 10 µg/ml chloramphenicol at 30°C to prevent any integration of the plasmid into the chromosome of the transformed *S. aureus* cells. The plasmid was isolated, and checked with a restriction digest using *Eco*RI and *Hind*III. Finally the *S. epidermidis* 220 cells were transformed with the isolated plasmid by electroporation, plated onto the DM3 agar plates containing X-gal and 10 µg/ml chloramphenicol, and the plates were incubated at 30°C overnight. The blue colonies were selected, grown overnight in LB medium with 1 % (vol/vol) glycine containing 10 µg/ml chloramphenicol, and incubated overnight at 30°C. The plasmid was isolated and checked by a restriction digest using *Eco*RI and *Hind*III. The correct clone was grown overnight at 30°C in the presence of 10 µg/ml chloramphenicol in BHI medium to generate a population of plasmid-bearing cells. Serial dilutions of this culture were plated onto BHI agar-plates with 10 µg/ ml chloramphenicol and incubated at the non-permissive temperature of 42°C. By a temperature shift to 42°C, the plasmid integrated by single cross over into the chromosome of the *S. epidermidis* 220 (figure 6). To determine whether pSK2 was integrated into the upstream sequence of the *ica* operon of the strain *S. epidermidis* 220, chloramphenicol resistance colonies were picked and the chromosomal DNA was examined by Southern hybridization.

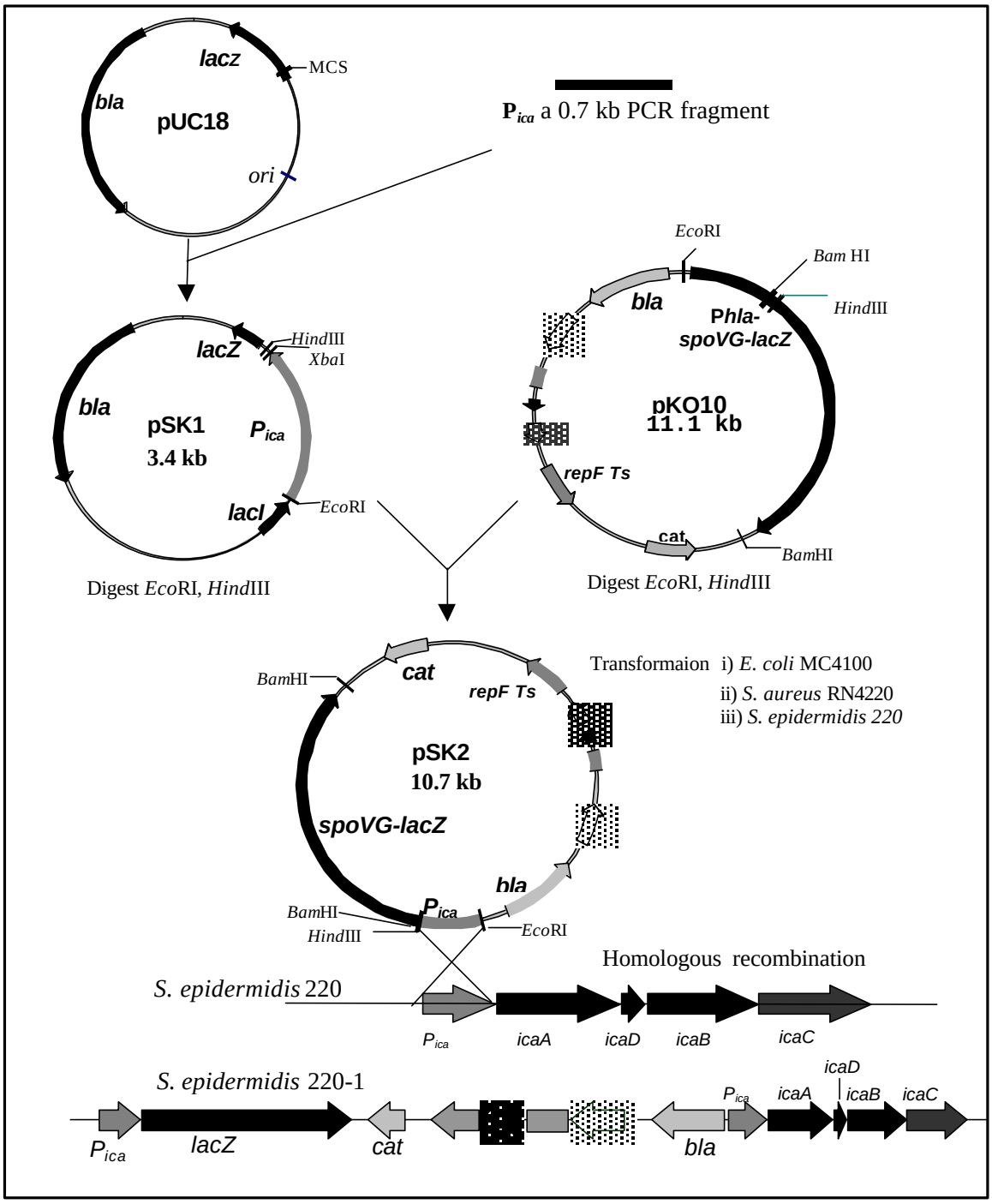

Figure 6: Construction of the chromosomal encoded *ica*::*lacZ* a transcriptional fusion in *S. epidermidis* 220-1. For detail see text.

1.3 Southern hybridization to determine the correct integration

The light blue chloramphenicol resistant clones were grown overnight in BHI medium, DNA was prepared and analyzed by Southern hybridization of *Kpn*I digested chromosomal DNA. In the wild type strain *S. epidermidis* 220, the P_{ica} fragment hybridizes on a 14 kb fragment.

After integration of the the vector pSK2 into the chromosome of the *S. epidermidis* 220, a band shift of about 24 kb was detected using the cloned *ica* promotor as a gene probe. The same shifted band on the same DNA blot was detected during hybridization with *lacZ*, as another gene probe (figure 7). The resulting strain, carrying a P_{ica}-*lacZ* fusion in the *ica* gene of the wild type strain 220 by a single crossing over was termed *S. epidermidis* 220-1 and used in further experiments. After recombination, no changes in the *ica* expression occurred, and the recombinant strain 220-1 remained biofilm positive. As controls, both *Bacillus subtilis* 168 and *S. epidermidis* 195 *ica* negative skin isolate were transformed with the pSK2 plasmid resulting in *Bacillus subtilis* 168-1 and *S. epidermidis* 195-1, respectively.

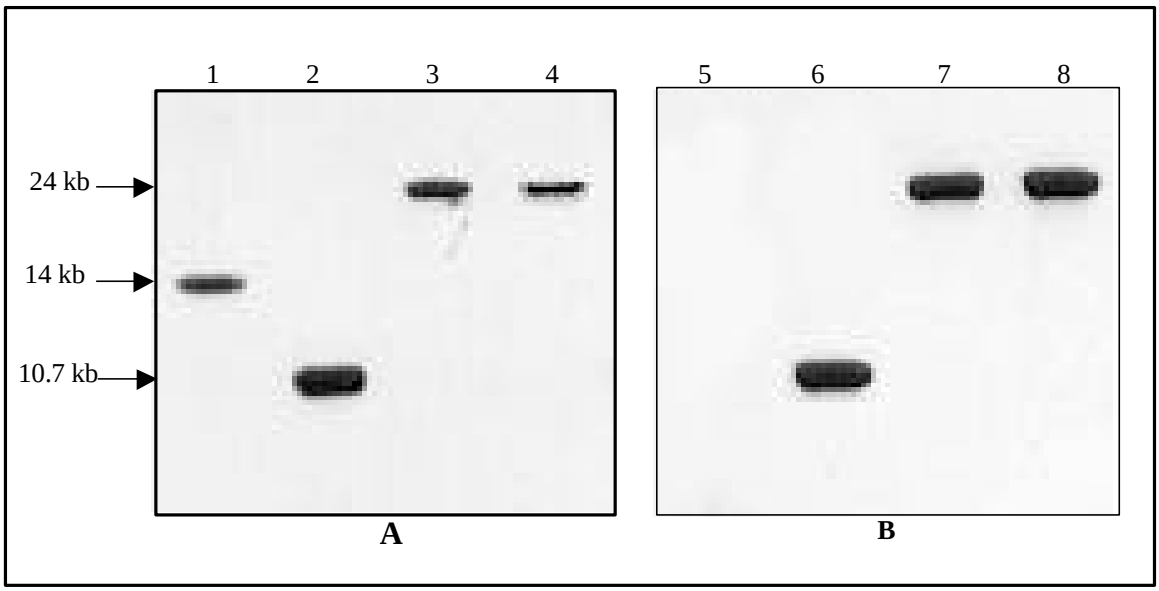

Figure 7: Southern blot analysis of *Kpn*I restriction digest of the chromosomal DNA of *S. epidermidis* 220 and 220-1 hybridized with the ECL labelled 0.7 kb P_{ica} (A), and the 1.2 kb *lacZ* fragment (B). Lane 1 and 5: *Kpn*I restricted chromosome of 220; lane 2 and 6: *Eco*RI restricted pSK2 plasmid; 3, 4, 7, and 8: *Kpn*I restricted chromosome of 220-1.

1.4 Sequence analysis of P_{ica} and *lacZ* in the recombinant strain 220-1

To examine the integration site between the *ica* promoter and the *lacZ* gene in *S. epidermidis* 220-1, the fusion site was amplified by PCR using primers pica-1 and lacZ-1. Figure 8 shows the nucleotide sequence of the fusion region. The 3´-end of the P_{ica} is cloned into the *Hind*III restriction site of the vector pK10 which followed by the *spoVG*-gene from *B. subtilis* and the

promoterless *lacZ* gene from *E. coli*. The strain 220-1 contains a chromosomal transcriptional fusion between the P_{ica} and a fusion construct of *spoVG*-ribosome binding site from *B. subtilis* and β-galactosidase gene from *E. coli* [126].

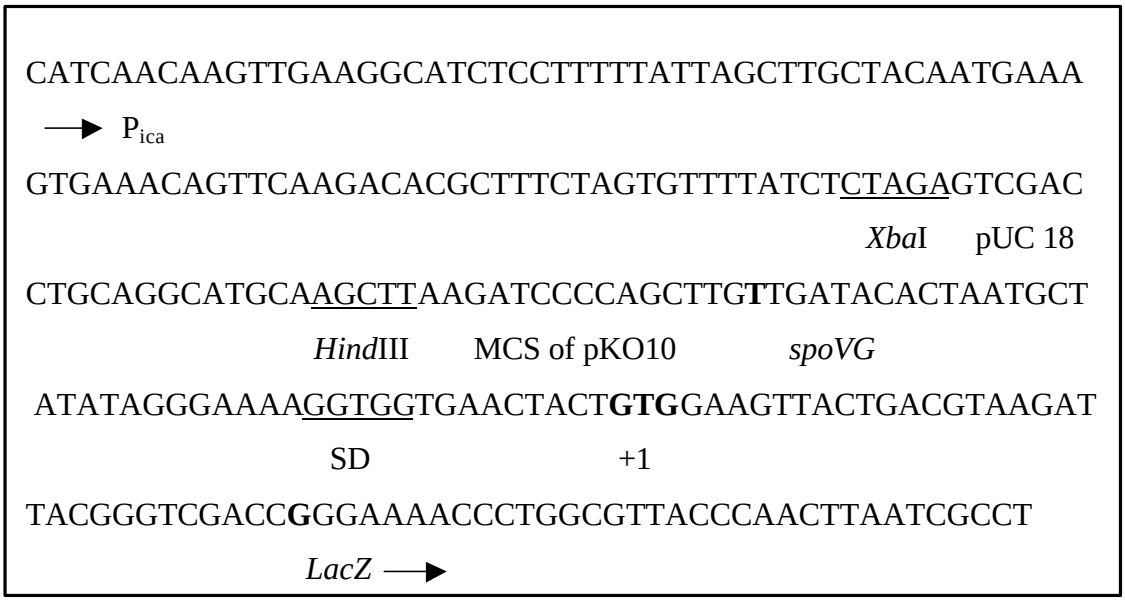

Figure 8: Nucleotide sequence of the fusion site between P_{ica} and β-galactosidase encoding gene (*lacZ*). SD (Shine Dalgarno) is the RBS signal of the *spoVG*-gene upstream the *lacZ*. +1 is the translation start of the *lacZ* (GTG).

2. Effect of environmental factors on the *ica* expression

Environmental factors play an essential role in the regulation of many virulence and virulence associated genes. We have constructed a chromosomal P_{ica}-*lacZ* fusion to study the impact of different environmental signals on the expression of the *ica* operon in *S. epidermidis*. In *S. epidermidis* 220-1, the production of β-galactosidase is controlled by the *ica* promoter. The amount of the produced β-galactosidase indicates directly the activity of the *ica* promoter. During the experiments, 10 µl of the cell lysates were tested for β-galactosidase activity using a luminometer with a 300 µl automatic injector and a 5-s integral.

2.1 Expression of the P_{ica}-*lacZ* during the growth phase in *S. epidermidis* 220-1

In order to study the β-galactosidase activity in *S. epidermidis* 220-1 as an indicator for the *ica* promoter activity, the bacteria were grown for 48 hours at 37°C and during the first 10 hours each hour, as well as after 15, 20, 24, 36, 48 hours, samples were taken for β-galactosidase activity measurement. Figure 9 demonstrates the expression of P_{ica}-*lacZ* during the bacterial growth curve. After 3 hours from beginning of the growth, a weak activity of β-galactosidase was investigated. The maximum expression of P_{ica}-*lacZ* was detected during the late logarithmic, and the beginning of the stationary phase of the bacterial culture. After 24 hours of the bacterial growth, a clear decrease in the *ica* expression was observed.

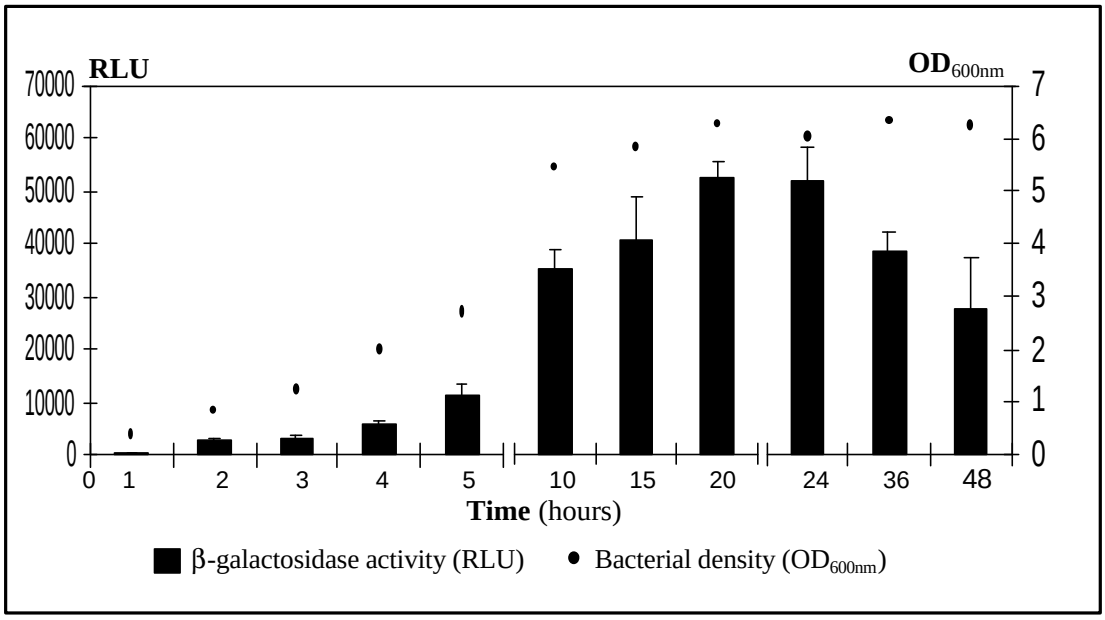

Figure 9: The P_{ica}-*lacZ* expression in *S. epidermidis* 220-1 during the growth phases.

2.2 Effect of incubation temperature on the P_{ica}-*lacZ* expression in *S. epidermidis* 220-1

The expression of many bacterial genes is influenced by temperature [132]. The impact of the growth temperature on the expression of the P_{ica}-*lacZ* in *S. epidermidis* 220-1 was investigated by incubating the bacteria in temperatures ranging from 28 - 45°C. The P_{ica}-*lacZ* expression was determined during the late logarithmic phase of the growth. Figure 10 demonstrates the expression of the *ica* operon under the effect of different incubation temperatures. Our results showed that 42°C was an optimal temperature for *ica* gene

expression, and the expression of the *ica* operon was inhibited at temperatures higher than 42°C or below 37°C.

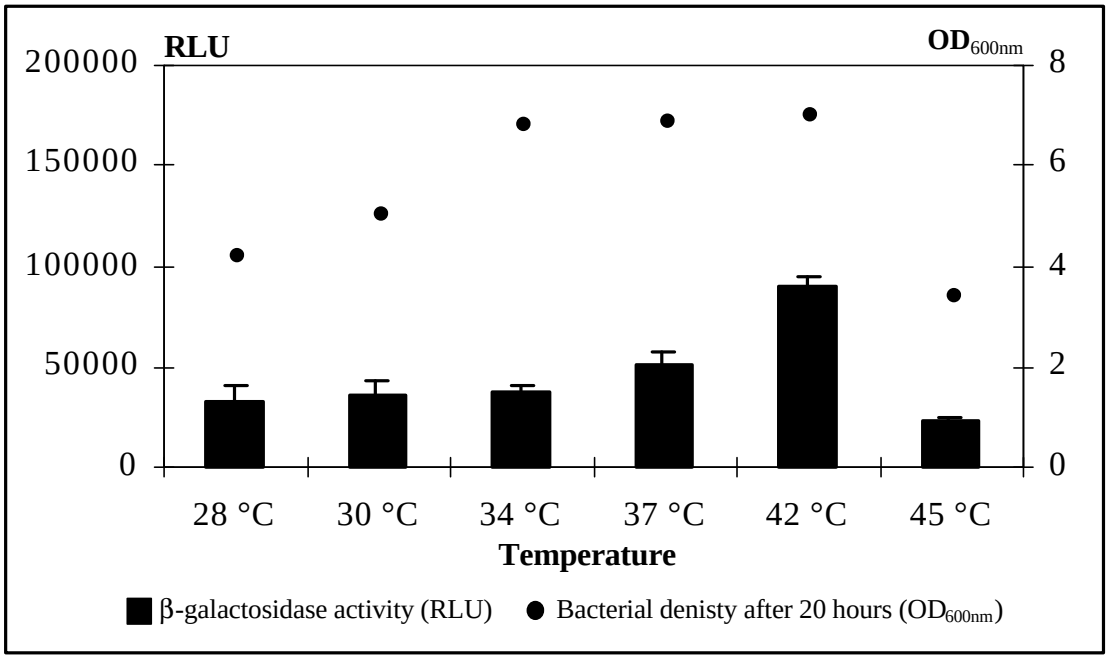

Figure 10: Effect of the growth temperature (28, 30, 34, 37, 42, and 45°C) on the P_{ica}-*lacZ* expression following growth in CDM for 20 hours.

2.3 Effect of pH on the P_{ica}-*lacZ* expression in *S. epidermidis* 220-1

S. epidermidis can survive in a wide range of pH values. In order to investigate, whether or not, the pH plays a role in the regulation of the *ica* gene expression, the β-galactosidase activities in *S. epidermidis* 220-1 were measured during the growth of the bacteria in CDM. The pH of the growth medium was adjusted to different pH values (5-9). In this series of experiments, the adjusted starting values were kept constantly throughout the whole growth period by addition drops of 1 N NaOH or 1 N HCl. Within the pH range of 7 and 7.5, a maximum expression of the β-galactosidase was determined, the pH values below or under the optimal pH inhibited the *ica* gene expression (Figure 11).

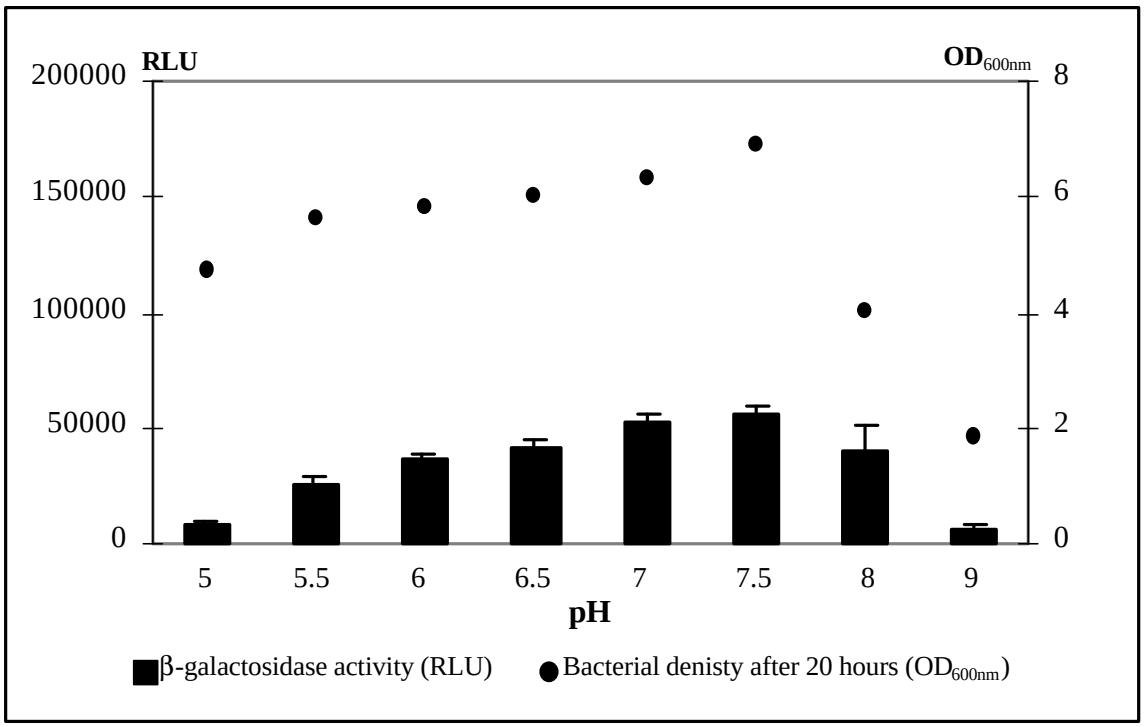

Figure 11: Expression of the P_{ica}-*lacZ* in *S. epidermidis* 220-1 following growth in CDM with different pH values (5-9).

2.4 Effect of the osmolarity on the *ica* expression in *S. epidermidis*.

2.4.1 Effect of the osmolarity on the P_{ica}-*lacZ* expression in *S. epidermidis* 220-1

Osmolarity is an important factor for the regulation of different virulence genes in bacteria (Dorman, 1992). The impact of the osmolarity on the *ica* gene expression in *S. epidermidis* 220-1 was investigated using the CDM as growth medium supplemented with different concentrations of NaCl (1-5 %). The bacteria were grown for 20 hours in the medium with different osmolarities and β-galactosidase activity was determined. Figure 12 demonstrates that the presence of 1-5 % NaCl in the medium induced the *ica* expression 2-5-fold more than the control. The addition of more than 5% NaCl inhibited the growth of the bacteria which was reflected by the inhibition of the β-galactosidase activity.

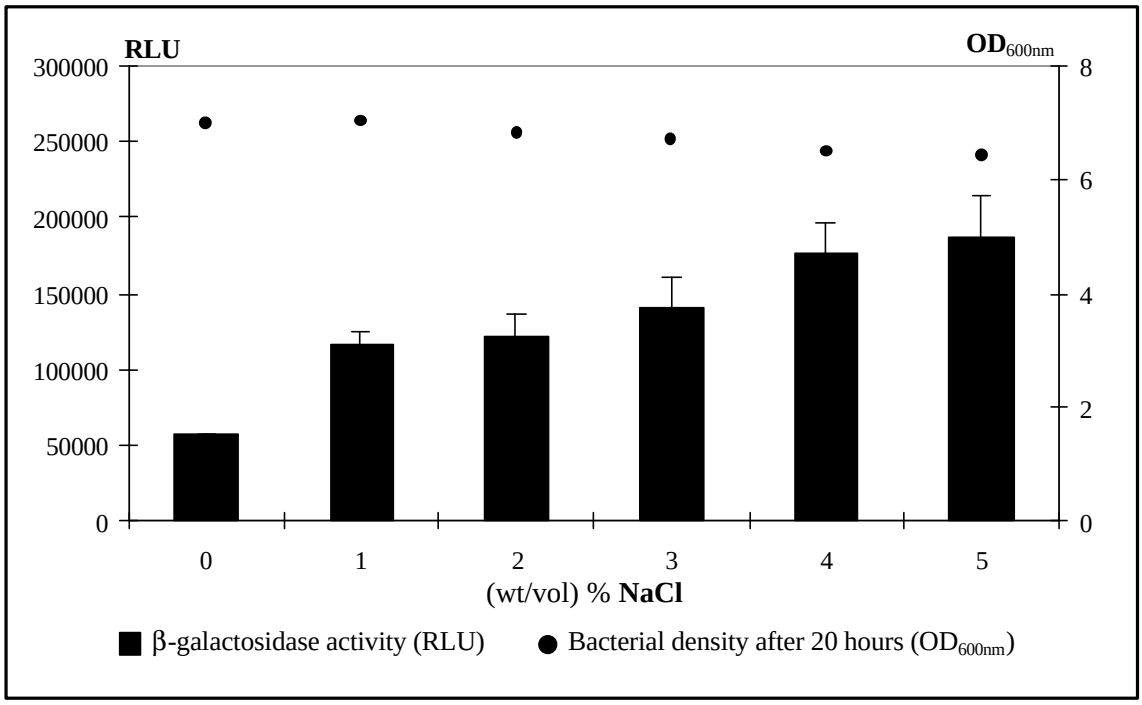

Figure 12: Effect of the osmolarity on the P_{ica}-*lacZ* expression in *S. epidermidis* 220-1 following growth in CDM supplemented with 0-5 % NaCl.

Also, the impact of the osmolarity on the P_{ica}-*lacZ* expression in the biofilm negative skin isolate *S. epidermidis* 195-1 was investigated. The addition of 3-5 % NaCl into the growth medium of *S. epidermidis* 195-1 induced the P_{ica}-*lacZ* expression 4-5-fold in comparison to the control medium. The same effect of the osmolarity on the P_{ica}-*lacZ* expression was observed in *Bacillus subtilis* 168–1. It has been investigated, that the addition of 2-4 % NaCl increased the P_{ica}-*lacZ* expression 2.5-fold, but more than these concentrations inhibited the growth of the bacteria significantly and subsequently the expression of the P_{ica}-*lacZ* (Figure 13).

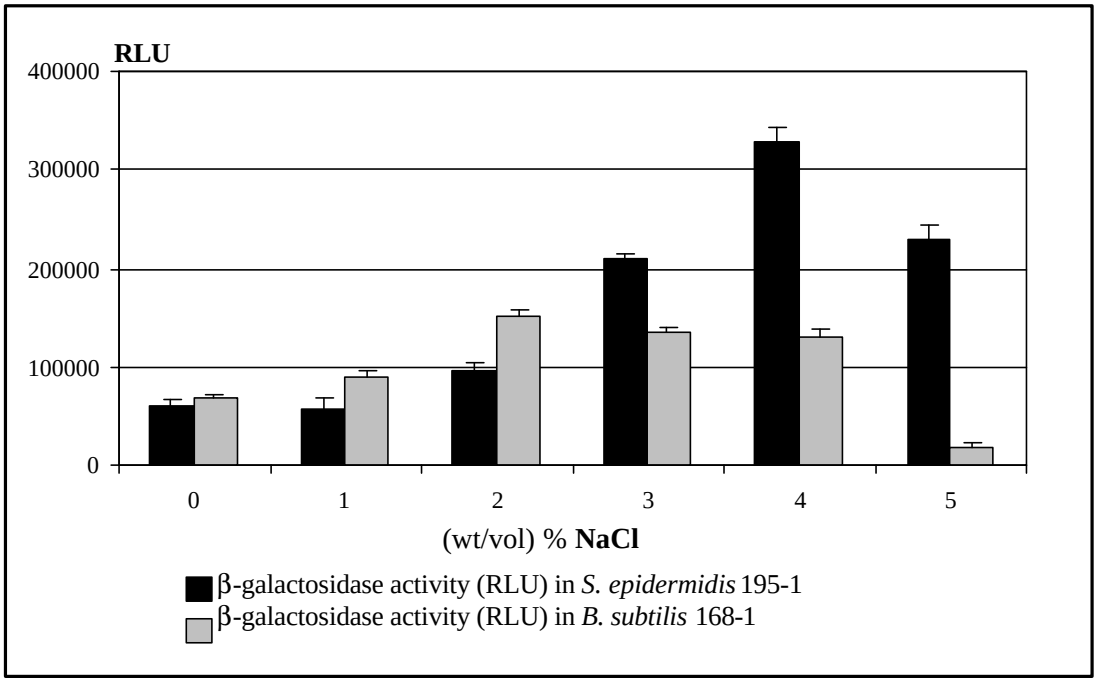

Figure 13: Effect of the osmolarity on the P_{ica}-*lacZ* expression in *S. epidermidis* 195-1 and *Bacillus subtilis* 168-1, following growth in CDM supplemented with 1-5 % NaCl.

2.4.2 Effect of the osmolarity on the biofilm production in *S. epidermidis* 220 wild type strain

The impact of osmolarity on the slime production in the biofilm positive *S. epidermidis* 220 was studied by the quantitative adherence assay. Following growth for 20 hours in a medium supplemented with 1-5 % NaCl, the biofilm production was tested using an ELISA-reader and a wavelength of 490 nm as described in materials and methods. The addition of 1-5 % NaCl to the growth medium induced the biofilm production compared to the biofilm production in the medium that lacks NaCl. In addition, the effect of the osmolarity on the biofilm production in *S. epidermidis* 561 strain, isolated from a patient suffering from a catheter-related urinary tract infection, was studied. This strain carries a copy of the *ica* operon, but it is described *in vitro* as biofilm negative when grown in TSB medium. The presence of 1-6 % NaCl in the medium, clearly induced the biofilm production. The maximum biofilm production in this strain was investigated by applying 2-3 % of NaCl to the medium (figure 14).

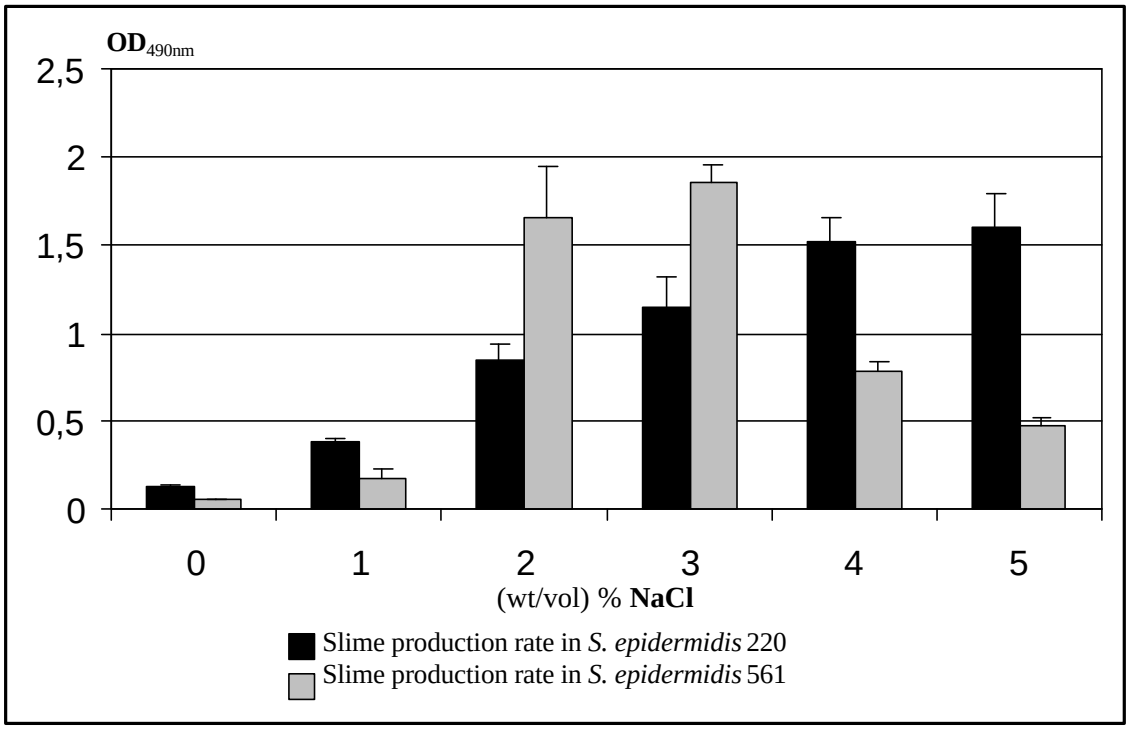

Figure 14: Influence of the osmolarity on the biofilm production in *S. epidermidis* 220 and 561 by applying different concentrations of NaCl to the growth medium.

2.4.3 Effect of the osmolarity on the *ica* transcription in *S. epidermidis* 220

In order to examine whether or not, the results of the P_{ica}-*lacZ* expression under the influence of high osmolarity conditions are compatible with the *ica*-transcription level in the wild type *S. epidermidis* 220, a Northern analysis of the *ica* transcription was performed. The *S. epidermidis* 220 cells were grown in CDM supplemented with 1-5 % NaCl. Following the growth at 37°C, the RNA was isolated and a Northern hybridization was done by using an *ica*-specific DNA probe. Figure 15 indicates that the presence of 3-4 % NaCl in the growth medium, enhanced the transcription of the *ica* operon, which is consistent with the results of the β-galactosidase reporter gene fusion experiments described above.

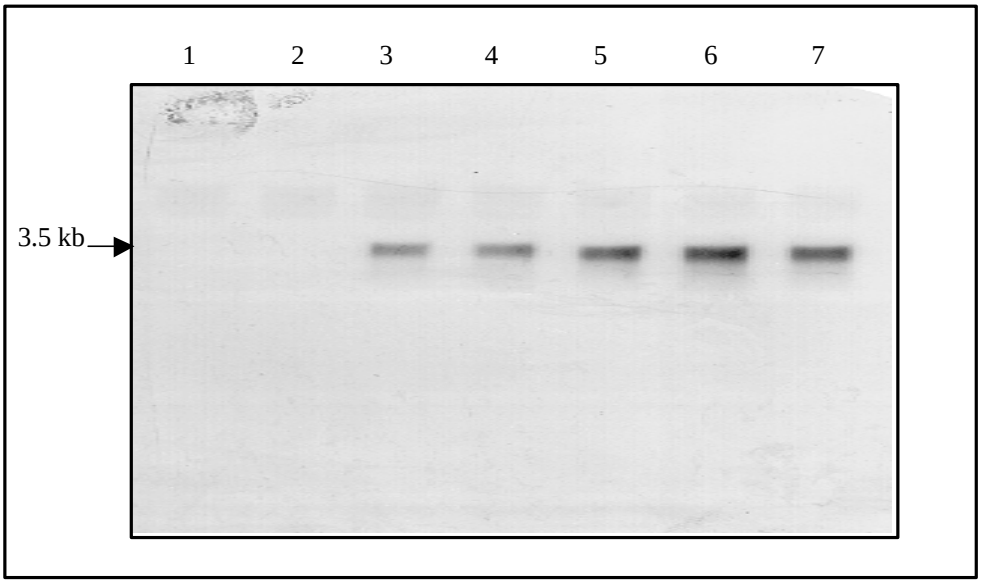

Figure 15: Northern analysis of the *ica* transcription hybridized with ^{32}P-labelled *ica*A gene explains the effect of NaCl and glucose on *ica* expression in *S. epidermidis* 220 wild type. Lane 1: control; lane 2, 3, and 4: *ica* transcripts under the effect of 1, 1.5, and 2% glucose respectively; lane 5, 6, and 7: *ica* transcripts in CDM supplemented with 3, 4, and 5 % NaCl.

2.5 Effect of different carbon sources on the *ica* expression in *S. epidermidis*

2.5.1 Effect of carbon sources on the P_{ica}-*lacZ* expression

The influence of utilized carbon on the expression of the P_{ica}-*lacZ* was examined by growing the *S. epidermidis* 220-1 in CDM, supplemented with different sources of carbon, (e.g. fructose, glucose, mannose and sucrose,) in different concentrations (0.5-2 %). The P_{ica}-*lacZ* expression of the bacteria was examined after incubation for 20 hours. Figure 16 demonstrates that only glucose in concentrations of 1-2 % increased the expression of the *ica* operon in *S. epidermidis* 220-1. The addition of 1.5-2 % of fructose and mannose to the growth medium inhibited both the growth and the P_{ica}-*lacZ* expression upto 50% in comparison to the control. Sucrose in all concentrations used did not alter the P_{ica}-*lacZ* expression.

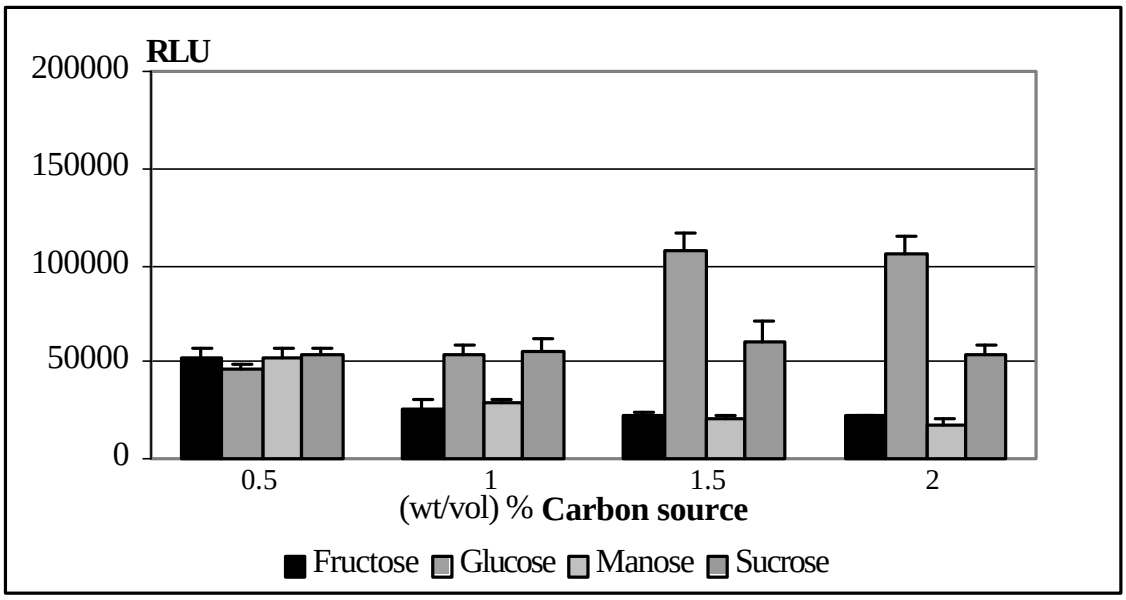

Figure 16: Influence of different carbon sources on the P_{ica}-lacZ expression following incubation in CDM supplemented with 0.5-2% fructose, glucose, manose, and sucrose. RLU indicates the β-galactosidase activity.

Moreover, the addition of glucose altered the *ica* expression during the bacterial growth phase, when the bacteria were grown in CDM supplemented with 1.5 and 2% glucose. In addition to a general increase in the P_{ica}-*lacZ*, glucose accelerates the P_{ica}-*lacZ* expression during the logarithmic phase more than the in the stationary growth phases (Figure 17).

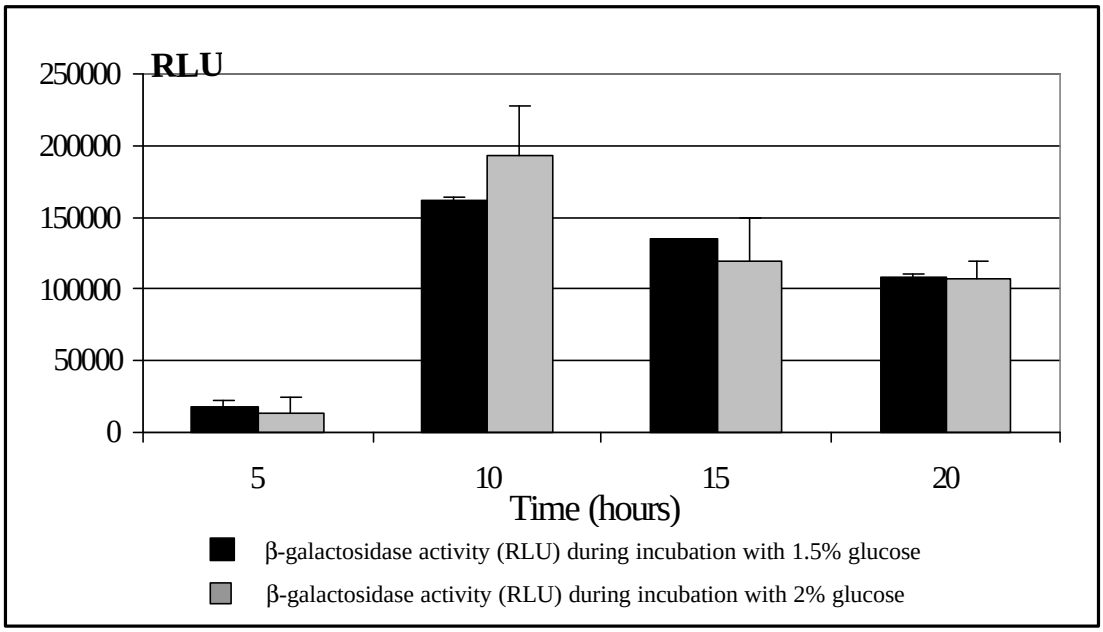

Figure 17: Influence of glucose on the *ica* expression during the growth phases by incubation of the *S. epidermidis* 220-1 in CDM supplemented with 1.5 and 2% glucose.

In addition, the P_{ica}-*lacZ* expression was determined in *S. epidermidis* 195-1 and *Bacillus subtilis* 168-1 after addition of glucose to the growth medium. It was investigated that the β-galactosidase activity is weakly induced in *S. epidermidis* 195-1 during the addition of 1-1.5 % glucose into the bacterial growth culture. β-galactosidase activity is weakly inhibited in *Bacillus subtilis* 168-1 during the presence of 1.5 and 2 % glucose (Figure 18).

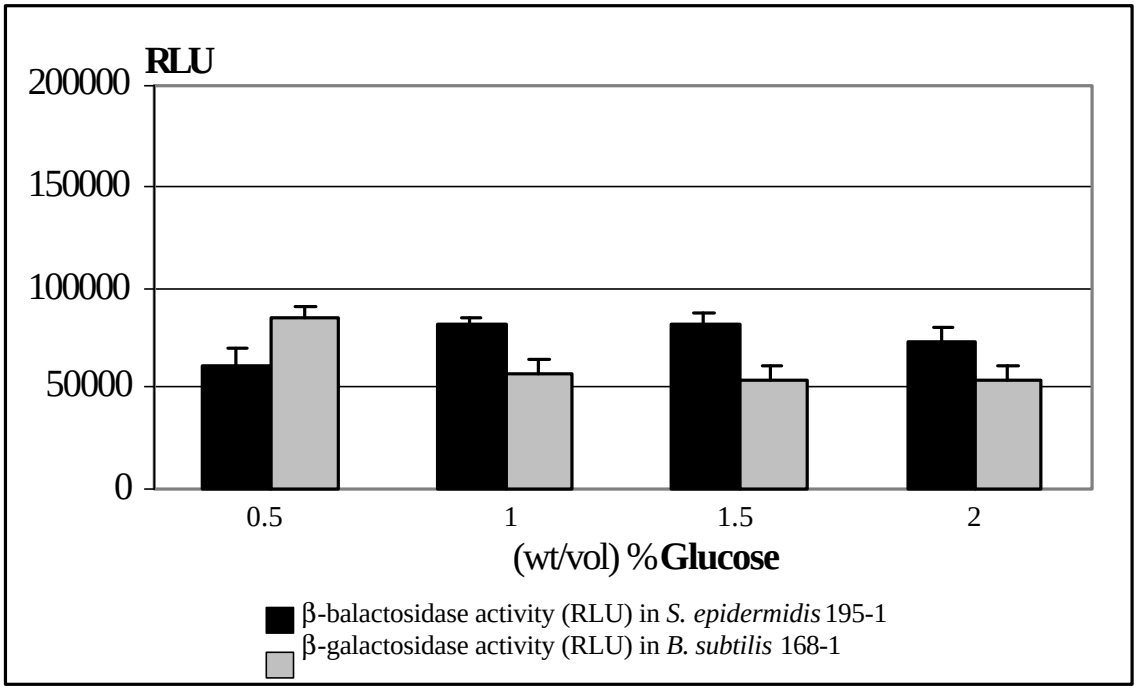

Figure 18: Influence of glucose on the P_{ica}-*lacZ* expression in *S. epidermidis* 195-1 and *Bacillus subtilis* 168-1 following growth in CDM supplemented with 0.5-2 % glucose.

2.5.2 Effect of glucose on the biofilm production in *S. epidermidis* 220

In order to investigate whether or not, glucose enhances the slime production also in the biofim positive *S. epidermidis* 220 wild type strain. The quantitative adherence assay was used by growing the bacteria in CDM supplemented with 0.5-3% glucose. The biofilm production was clearly increased by the addition of 1.5 –2 % glucose in comparison to the control medium, In contrast, addition of 2.5 and 3 % glucose to the medium inhibited the biofilm production (Figure 19).

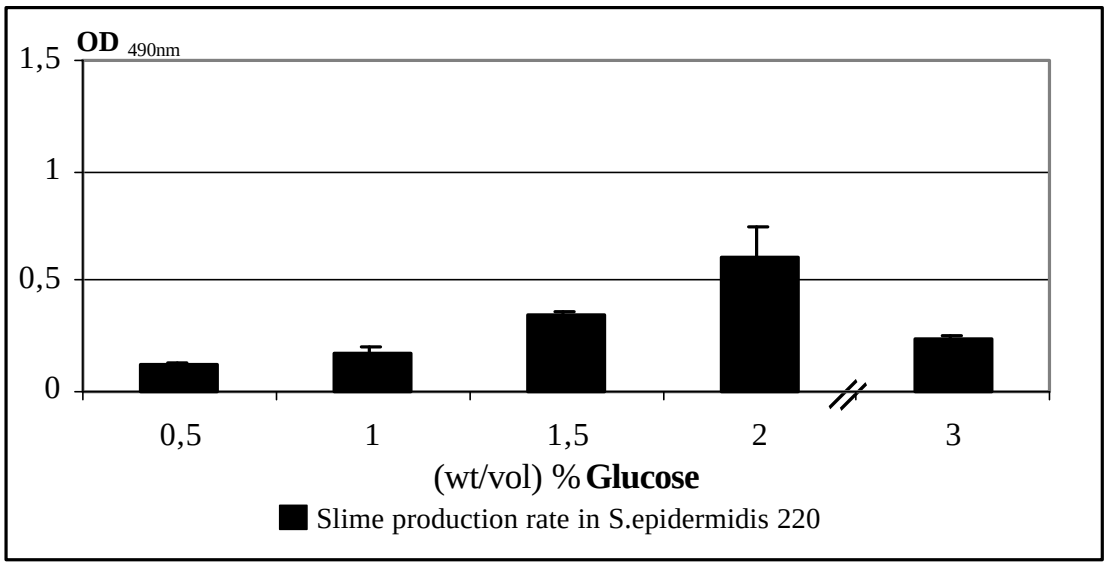

Figure 19: Influence of glucose on the biofilm production in *S. epidermidis* 220, by addition of 0.5-3 % (wt/vol) glucose into the growth medium (CDM).

2.5.3 Influence of glucose on the *ica* transcription in *S. epidermidis* 220

The previous results have indicated that P_{ica}-*lacZ* expression can be induced by the addition of glucose to the bacterial growth medium. After the growth in CDM supplemented with 0.5–2 % glucose, the total RNA of *S. epidermidis* cells was isolated and a Northern blot analysis was performed to evaluate the effect of glucose on the *ica* transcription. Figure 15 shows that the growth of the bacteria in a medium supplemented with 1.5 and 2 % glucose increased the *ica* transcription in comparison to the control medium.

2.6 Effect of ethanol, H_2O_2, SDS and urea on the biofilm production in *S. epidermidis* 215

In order to elucidate the effect of ethanol, H_2O_2, sodiumdodecyl sulfate, and urea on biofilm production in *S. epidermidis* 215, a biofilm positive blood culture isolate, sub-lethal concentrations (0.015-5%, 0.005-0.08%, 0.0003-0.02% and 0.007-0.2%) of ethanol, H_2O_2, SDS and Urea respectively, were added to the growth medium of *S. epidermidis* 215. Following incubation at 37°C, the biofilm production rates were measured by the quantitative adherence assay. High expression of biofilm was detected by the addition of 0.005 and 0.01%

(wt/vol) SDS to the growth medium. The biofilm production was 20 fold higher compared to that in the control medium (figure 20).

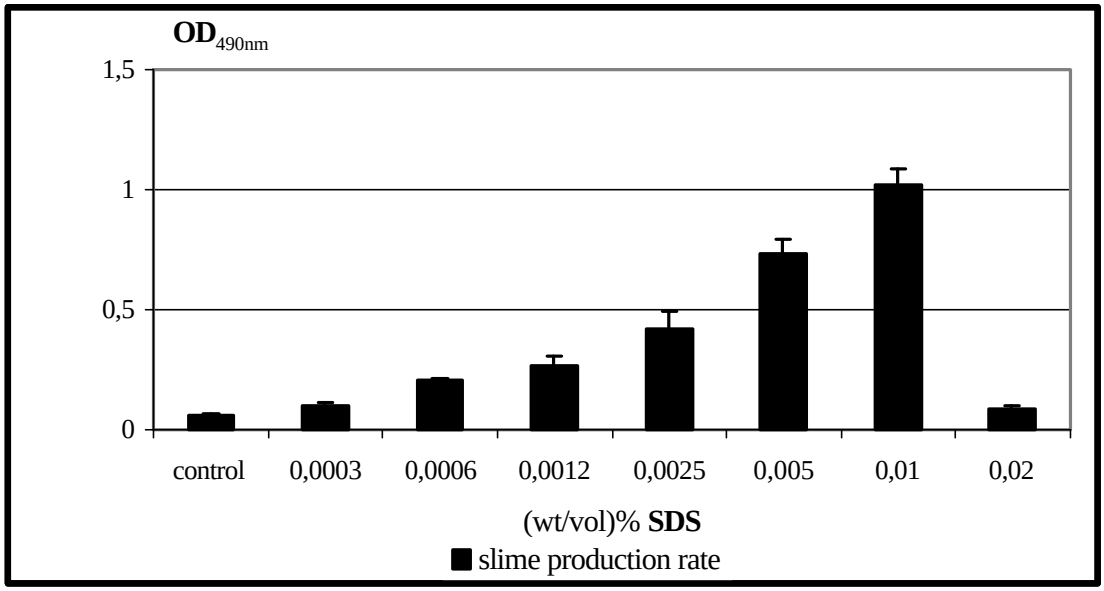

Figure 20: Effect of SDS on the biofilm production in *S. epidermidis* 215 by addition of the sub-lethal concentrations (0.0003-0.02%) of SDS to the growth medium. The biofilm measurement was done by the quantitative adherence assay (OD_{490nm}).

Likewise, the addition of 0.007-0.5% (wt/vol) urea increased the biofilm expression 8-fold in comparison to the control, while concentrations more than this inhibited the biofilm expression (figure 21).

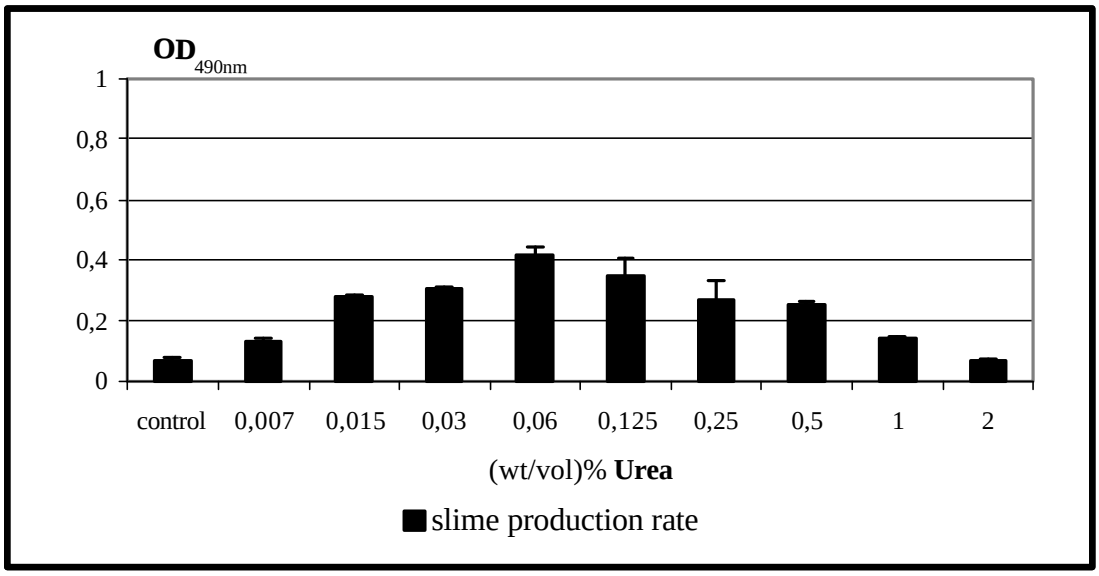

Figure 21: Effect of Urea on the biofilm production in *S. epidermidis* 215 by addition of 0.007-2% urea to the growth medium and using the quantitative adherence assay for the biofilm measurement

Incubation of the bacteria with ethanol in concentrations ranging from 0.0015 to 3% (vol/vol), increased the biofilm production 8-fold compared to that in the control medium (figure 22).

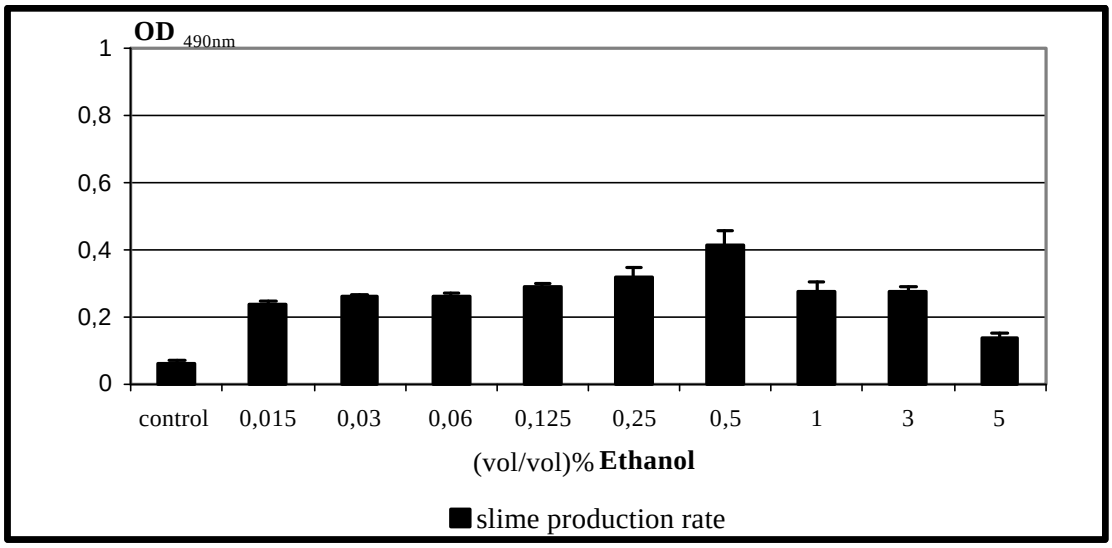

Figure 22: Effect of different concentrations (0.015-5%) of ethanol on the biofilm production in *S. epidermidis* 215 using the quantitative adherent assay.

0.005-0.08% (vol/vol) H_2O_2 was added to the growth culture of the bacteria. Following incubation at 37°C, the biofilm formation was measured. This experiment revealed that biofilm expression was also increased during the presence of 0.005-0.04% H_2O_2 in the growth medium (figure 23).

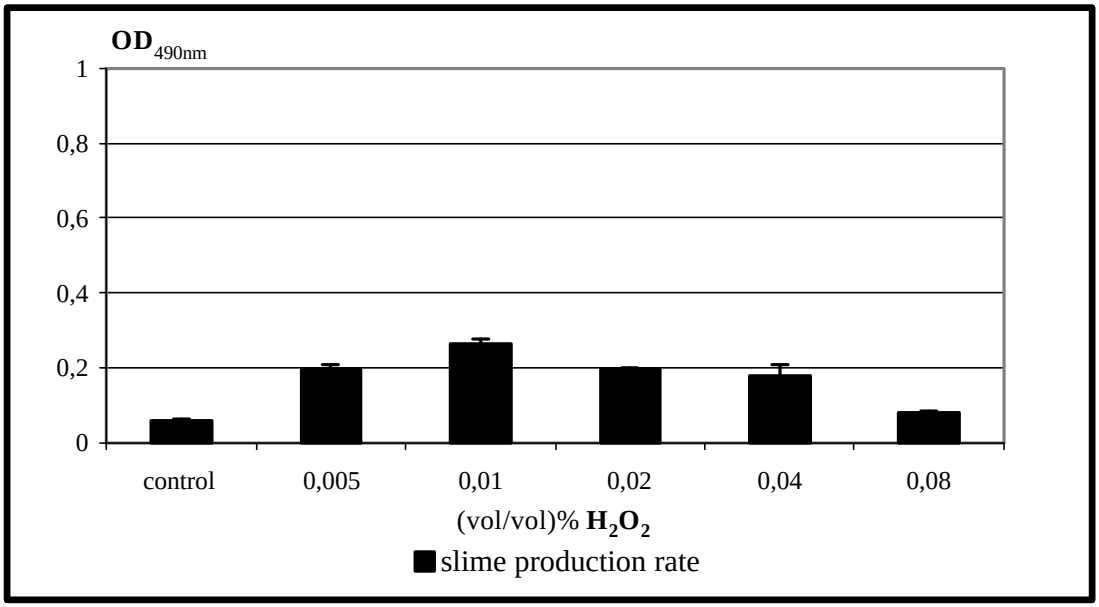

Figure 23: Effect of H_2O_2 on the biofilm expression in *S. epidermidis* 215 by addition of 0.005-0.08% H_2O_2 to the growth medium. Biofilm production was measured by the quantitative adherent assay.

2.7 Identification of the *sigB* gene in *S. epidermidis* and its expression in response to the biofilm inducing conditions.

To elucidate the presence of the *sigB* gene in *S. epidermidis*, a *sigB* gene from *S. aureus* was used as a probe for hybridization. Following the growth of the bacteria at 37°C, chromosomal DNA of *S. epidermidis* 220, 215 and 567 was isolated, digested with *Hind*III and a Southern DNA analysis was performed to identify the *sigB* gene. *S. aureus sigB* gene was amplified by PCR using primers sigB-1 and sigB-2, labelled with ECL as described previously and used as a gene probe in this experiment. It was investigated that the *sigB* gene is conserved throughout different *S. epidermidis* and *S. aureus* strains. Under stringent hybridization conditions, a band of 4.5 kb in *S. epidermidis* 220 and 215 carrying the *sigB* gene strongly cross-hybridized with the *sigB* probe of *S. aureus*. In *S. epidermidis* 567 two bands (6.5 kb and 4.5 kb) bands were observed by using this probe (figure 24).

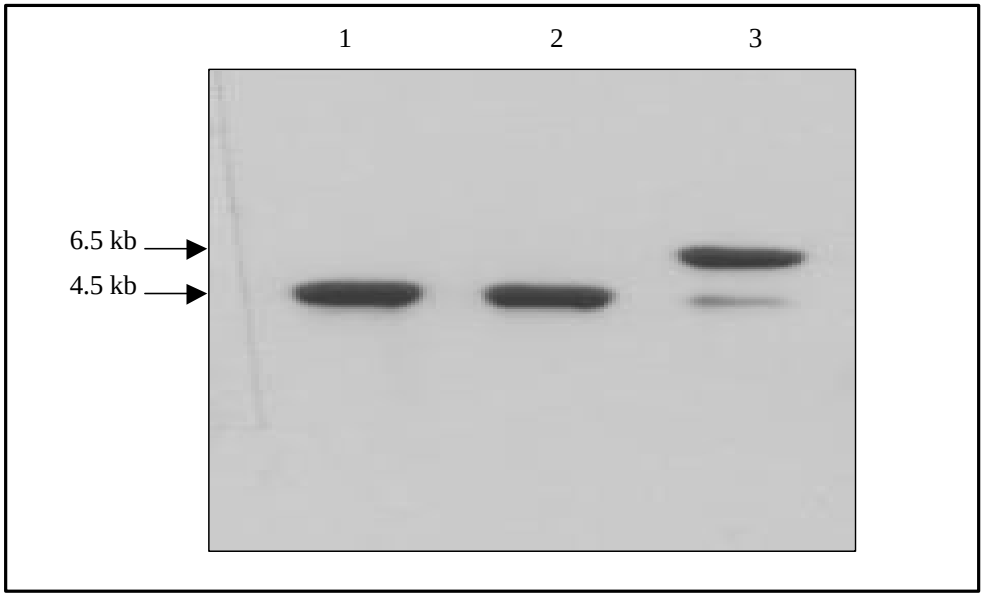

Figure 24: Southern blot analysis of the *sigB* gene in *S. epidermidis* 215, 220 and 567 (lanes 1, 2 and 3 respectively) cross-hybridized with the ECL-labelled *sigB* gene of *S. aureus*.

The transcription of the *sigB* gene in *S. epidermidis* 215 during the growth phase was tested by preparation of total cellular RNA during the lag phase, logarithmic phase, stationary phase and late stationary phase. In addition, the transcription of the *sigB* gene under the environmental stress was examined by addition of 3%, 4% and 5% NaCl to the growth medium of both *S. epidermidis* 215 and 567. Following the growth at 37°C, RNA was isolated

and a Northern blot was performed using a ^{32}P-labelled *sigB* as gene probe for hybridization. Figure 25 shows a high transcription of *sigB* (1.5 kb and 2.6 kb transcript) during the stationary and late stationary phases of *S. epidermidis* 215. Moreover, the *sigB* transcription was strongly increased during the logarithmic phase by addition of 3%, 4% and 5% (wt/vol) NaCl to the growth medium of both *S. epidermidis* 215 and 567.

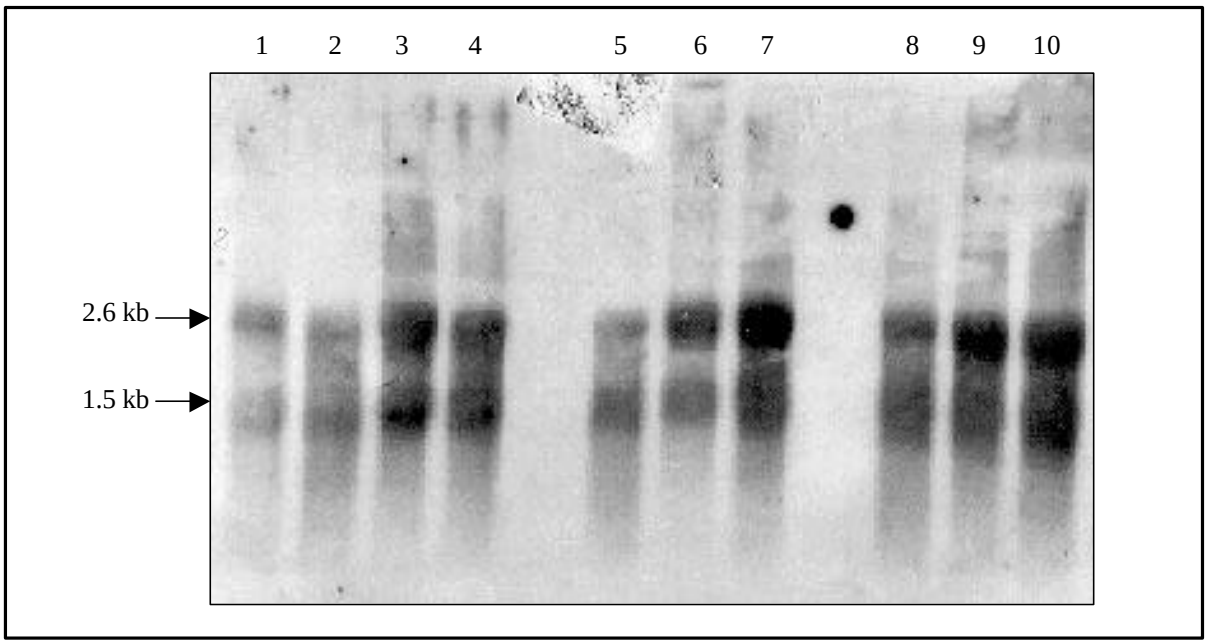

Figure 25: Northern blot analysis of the *sigB* transcription (1.5 kb and 2.6 kb transcript) during different phases of the bacterial growth (hybridized with the ^{32}P-labelled *sigB* gene of *S. aureus*). Lane1: lag phase; lane 2: logarithmic phase; lane 3: stationary phase; lane 4: late stationary phase. Lanes 5, 6 and 7: logarithmic phase under the influence of 3%, 4% and 5% NaCl in *S. epidermidis* 215. Lanes 8, 9 and 10: logaritmic phase under the influence of 3%, 4% and 5% NaCl in *S. epidermidis* 567.

3. Influence of subinhibitory concentrations of antibiotics on the *ica* expression

In addition to growth inhibition, it has been shown that subinhibitory concentrations of some antibiotics can influence adherence, phagocytosis resistance or toxin production in many bacteria [122, 133]. The previous data indicated that the *ica* expression can be influenced by some environmental factors. The second part of this study is directed on the investigation of the influence of subinhibitory concentrations of antibiotics on the *ica* expression. We started first with determination of the minimal inhibitory concentrations (MIC) of the antibiotics used in this study. Table 8 describes the MIC values of the antibiotics that are inhibit the growth of *S. epidermidis* 220 except erythromycin and chloramphenicol, to which *S. epidermidis* 220-1 is resistant. Later, concentrations ranging from 1/70 to 1/2 of the MIC of these antibiotics were added into growth medium of the bacteria to study whether or not these antibiotics play any role in alteration of P_{ica}-*lacZ* expression.

Table 8: MIC values and the mechanism of function for some antibiotics against *S. epidermidis* 220-1.

Antibiotics	Function	MIC µg/ml
Chloramphenicol	Inhibition of protein synthesis	64
Clindamycin	Inhibition of protein synthesis	0.5
Erythromycin	Inibition of protein synthesis	> 128
Fusidic acid	Inhibition of protein synthesis	0.25
Gentamicin	Inhibition of protein synthesis	> 1
Ofloxacin	Inhibition of DNA-gyrase	0.5
Oxacillin	Inhibition of cell wall	0.125
Penicillin G	Inhibition of cell wall	0.03
Quinupristin/Dalfopristin	Inhibition of protein synthesis	0.5
Dalfopristin (RP 54476)	Inhibition of protein synthesis	0.5
Quinupristin (RP 57669)	Inhibition of protein synthesis	0.5
Teicoplanin	Inhibition of cell wall synthesis	0.25
Tetracycline	Inhibition of protein synthesis	0.5
Vancomycin	Inhibition of cell wall	0.5
Viriginiamycin	Inhibition of protein synthesis	0.5

3.1. Influence of pristinamycin (quinupristin/dalfopristin), tetracycline and viriginiamycin on the biofilm production

The effect of subinhibitory concentraions of these antibiotics on the P_{ica}-*lacZ* expression in *S. epidermidis* 220-1, *S. epidermidis* 195-1 and *Bacillus subtilis* 168-1, as well as biofilm production in both wild types 220 and 561 was tested. Pristinamycin is a protein synthesis inhibitor antibiotic belonging to the streptogramin group which consists of a combination of dalfoptistin and quinupristin. It is successfully used as an effective bactericidal substance acting against many multiresistant gram positive bacteria [134-137]. Tetracycline is also a protein synthesis inhibitor, and despite the increase of resistant strains, it still plays an important role in the clinical treatment of many bacterial infections.

3.1.1 Influence of pristinamycin and tetracycline on the P_{ica}-*lacZ* expression

S. epidermidis 220-1 was incubated in CDM supplemented with subinhibitory concentrations (0.0035-0.25 µg/ml) of pristinamycin and (0.007-0.25 µg/ml) tetracycline for 20 hours, Following the growth, the β-galactosidase activitiy was measured as described previously. Figure 26 indicates that both antibiotics significantly increase the *ica* expression. The addition of 0.007-0.125 µg/ml pristinamycin altered the P_{ica}-*lacZ* expression in *S. epidermidis* 220-1. 0.06 µg/ml pristinamycin induced the expression 10 fold compared to the expression in the medium without antibiotic. The concentrations more than 0.6 µg/ml inhibited both the growth and the P_{ica}-*lacZ* expression. Also, tetracycline influenced the *ica* expression. The addition of 0.015-0.125 µg/ml of tetracycline strongly enhanced the β-galactosidase activity. Supplementation of the medium with 0.06 µg/ml of this antibiotic increased the β-galactosidase activitiy 10 fold compared to that in the control medium, but at concentrations more than this value inhibited both the growth and the P_{ica}-*lacZ* expression (figure 26).

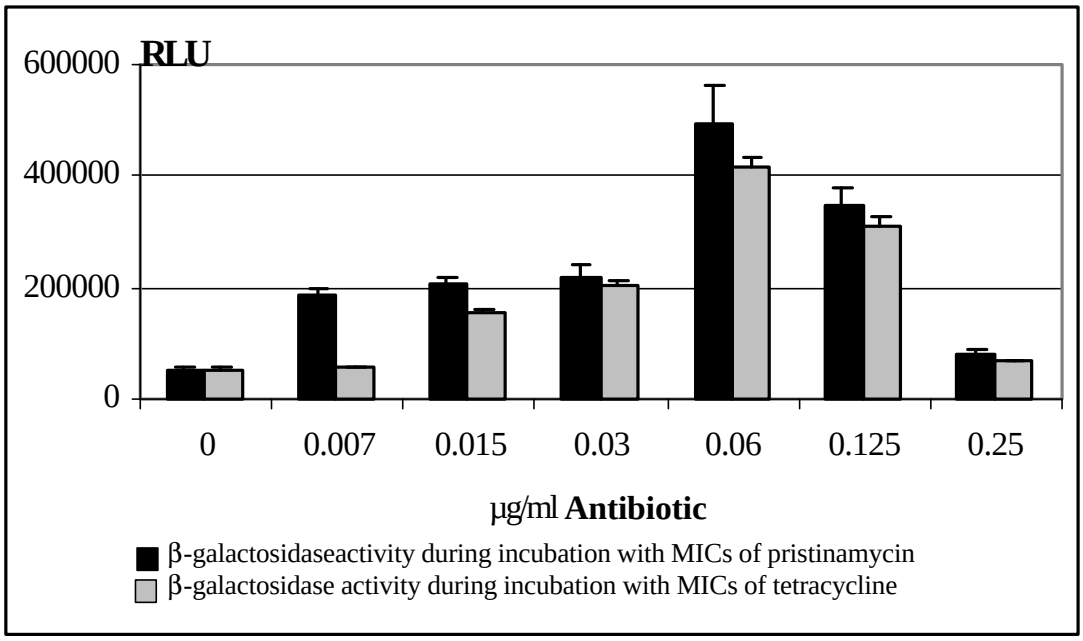

Figure 26: Effect of of pristinamycin and tetracycline on the P_{ica}-*lacZ* expression in *S. epidermidis* 220-1 by addition of subinhibitory concentrations of the antibiotics into the growth medium.

To define the effect of dalfopristin and quinupristin separately on the P_{ica}-*lacZ* expression, 0.25-0.015 µg/ml from both substances were added to the growth medium of *S. epidermidis* 220-1. Following the growth at 37°C for 20 hours, β-galactosidase activity was measured. Figure 27 describes that 0.125-0.015 µg/ml dalfopristin and 0.25-0.03 µg/ml quinupristin increased dramatically the P_{ica}-*lacZ* expression.

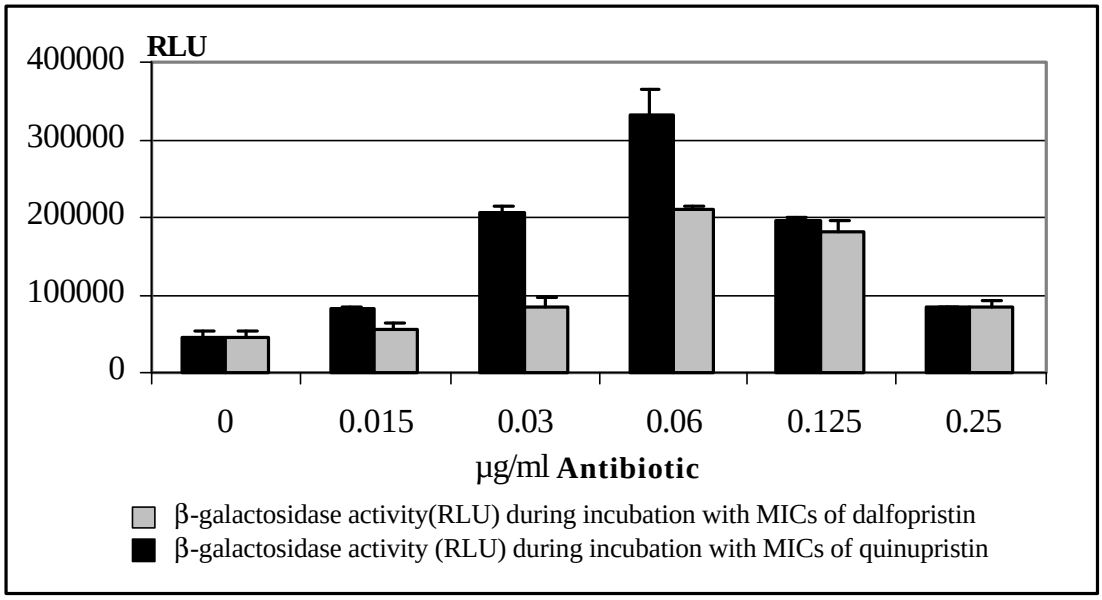

Figure 27: Effect of subinhibitory concentrations of dalfopristin and quinupristin on the P_{ica}-*lacZ* expression in *S. epidermidis* 220-1.

As a control, the effect of subinhibitory concentrations of both pristinamycin and tetracycline on the P_{ica}-*lacZ* expression was tested in *S. epidermidis* 195-1 and *Bacillus subtilis*-1 by incubation of the bacteria with 0.03-0.125 µg/ml and 0.0035-0.015 µg/ml of both pristinamycin and tetracycline, respectively. Addition of 0.06 µg/ml of pristianmycin and 0.015 µg/ml tetracycline into the growth medium of *S. epidermidis* 195-1, induced the β-galactosidase activity more than 3 and 8 times, respectively, compared to the control medium (Figure 28).

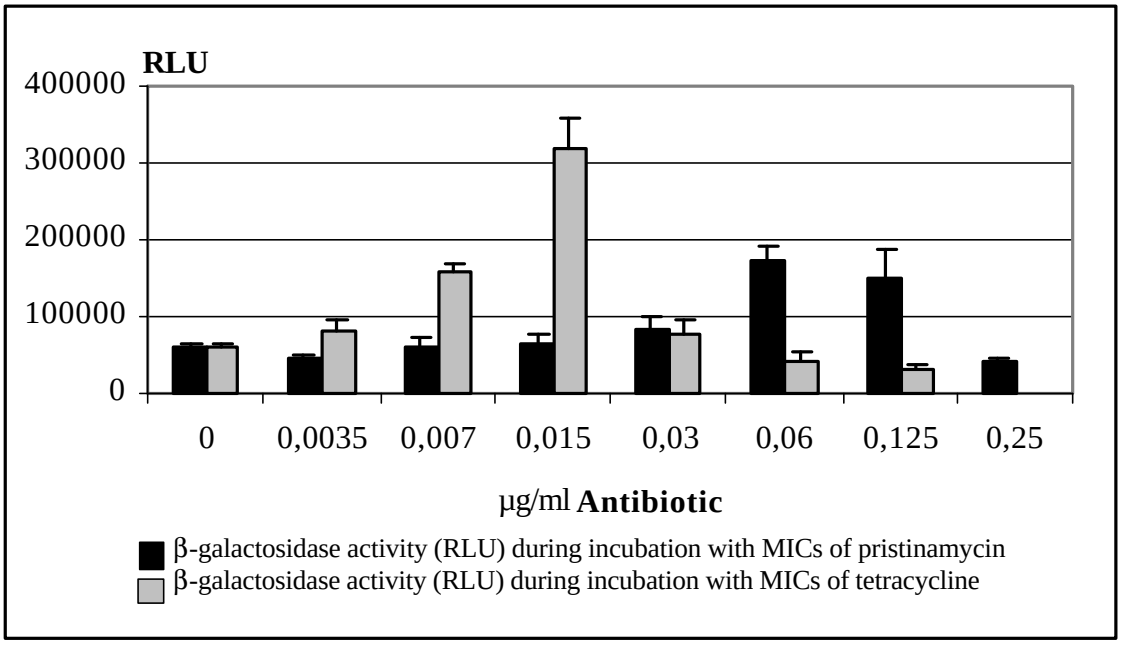

Figure 28: Effect of pristiamycin and tetracycline on the P_{ica}-*lacZ* expression in *S. epidermidis* 195-1, by incubation of the bacteria in CDM supplemented with subinhibitory concentrations of the antibiotics.

In contrast, in *Bacillus subtilis*, subinhibitory concentrations of pristinamycin and tetracycline did not alter the β-galactosidase activity, and the addition of more than 0.25 µg/ml of both antibiotics inhibited both growth and P_{ica}-*lacZ* expression (Figure 29).

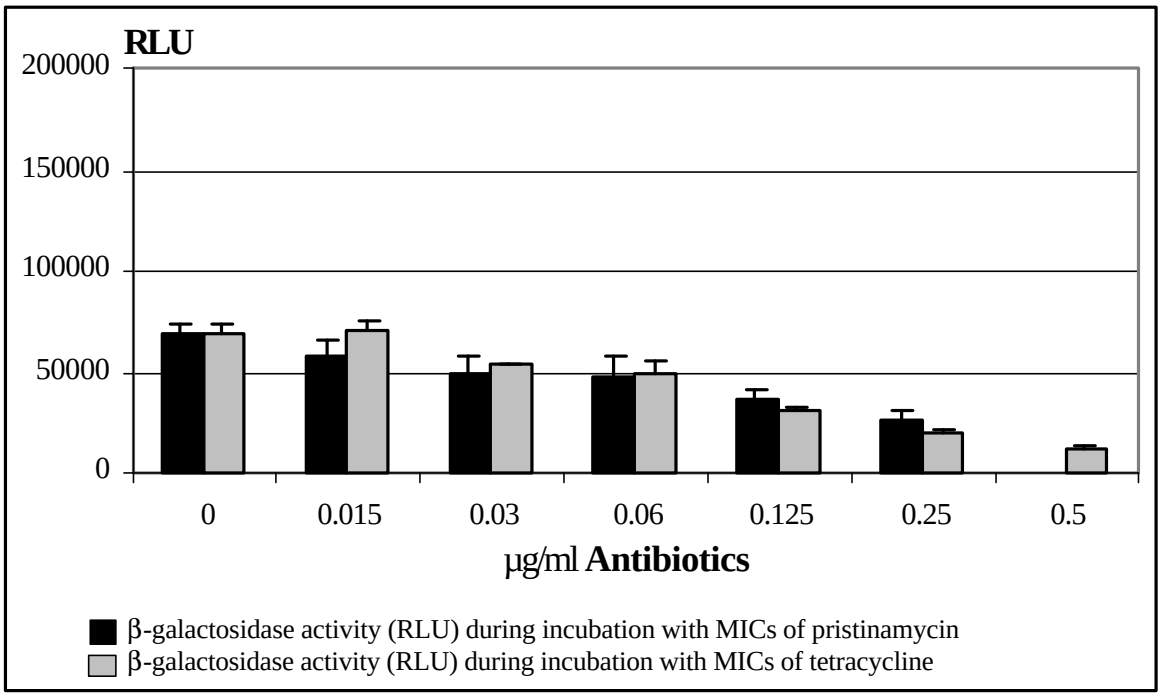

Figure 29: Influence of pristinamycin and tetracycline on the P_{ica}-*lacZ* expression in *Bacillus subtilis*-1 by addition of subinhibitory concentrations of the antibiotics into the growth medium.

3.1.2 Influence of pristinamycin and tetracycline on the biofilm production in *S. epidermidis* 220

The impact of subinhibitory concentrations of antibiotics on slime production in the biofilm positive *S. epidermidis* 220 was studied by the quantitative adherence assay. The same ranges of subinhibitory concentrations of pristinamycin and tetracycline as tested in *S. epidermidis* 220-1 were added to the growth medium of *S. epidermidis* 220 wild type. This experiment revealed that slime production can be strongly induced by addition of 0.125-0.015 µg/ml pristinamycin or 0.125-0.007 tetracycline to the growth medium. The addition of 0.06 µg/ml of both pristinamycin and tetracycline to the growth medium increased the biofilm production more than 10 times compared to the control without antibiotics (Figure 30).

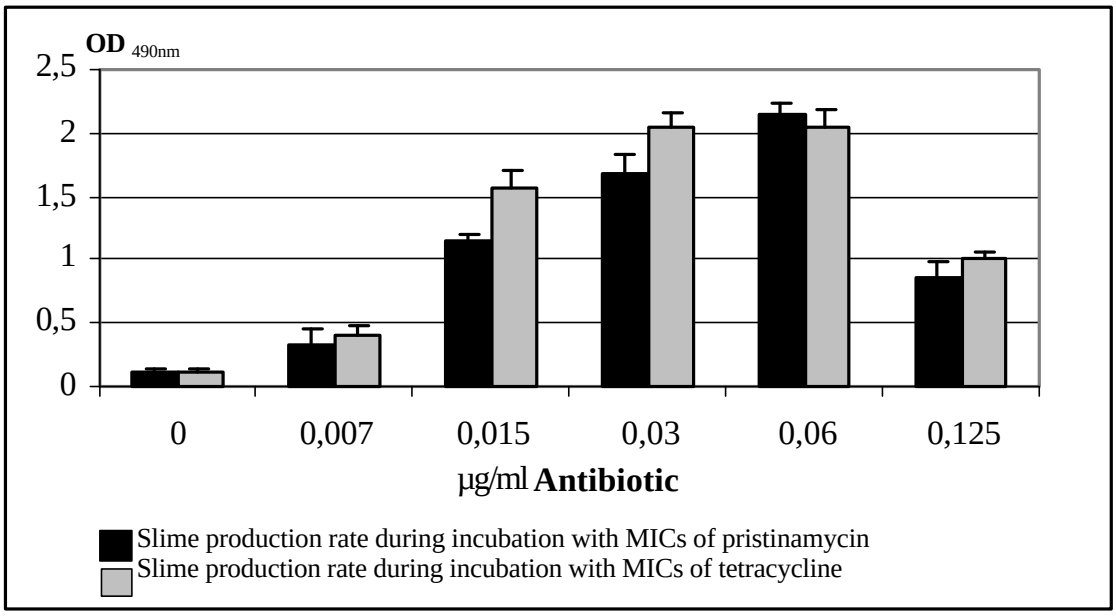

Figure 30: Effect of pristinamycin and tetracycline on the biofilm production in *S. epidermidis* 220 using the quantitative adherence assay, by addition of subinhibitory concentrations of the antibiotics into the growth medium.

Also, in *S. epidermidis* 561, a strain obtained from a urinary tract infection, the addition of pristinamycin to the growth medium induced the biofilm formation. However, in this isolate, tetracycline did not significantly influence the *ica* expression (Figure 31).

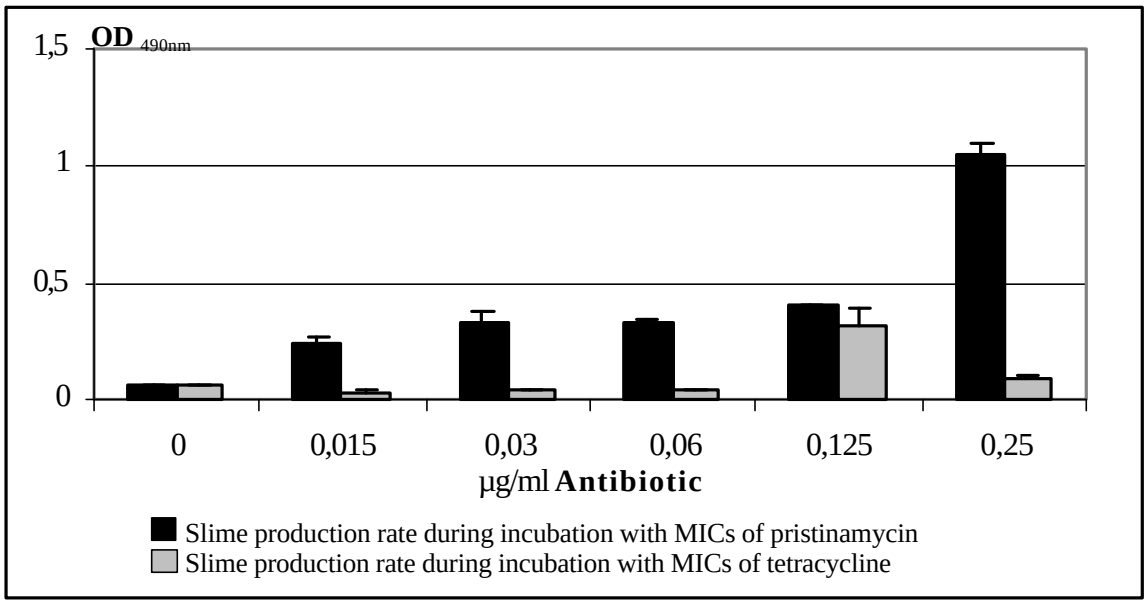

Figure 31: Effect of pristinamycin and tetracycline on the biofilm formation in *S. epidermidis* 561 using the quantitative adherence assay, by addition of subinhibitory concentrations of the antibiotics into the growth medium.

3.1.3 Influence of pristinamycin and tetracycline on the *ica* transcription

The above mentioned experiments indicated that the *ica* expression in *S. epidermidis* 220-1 and the slime production in *S. epidermidis* 220 as well as in *S. epidermidis* 561 were induced by the effect of subinhibitory concentrations of pristinamycin and tetracycline. To examine the influence of these antibiotics on the *ica* gene transcription, a Northern blot analysis was performed. The *S. epidermidis* 220 cells were grown in CDM supplemented with subinhibitory concentrations of both antibiotics. Following the growth at 37°C, the total RNA were isolated and the transcription level of *ica* gene under the effect of the antibiotics was analyzed. Figure 32 shows that 0.03 and 0.06 µg/ml of pristinamycin and tetracycline in the bacterial growth medium, induced strongly the *ica* transcription.

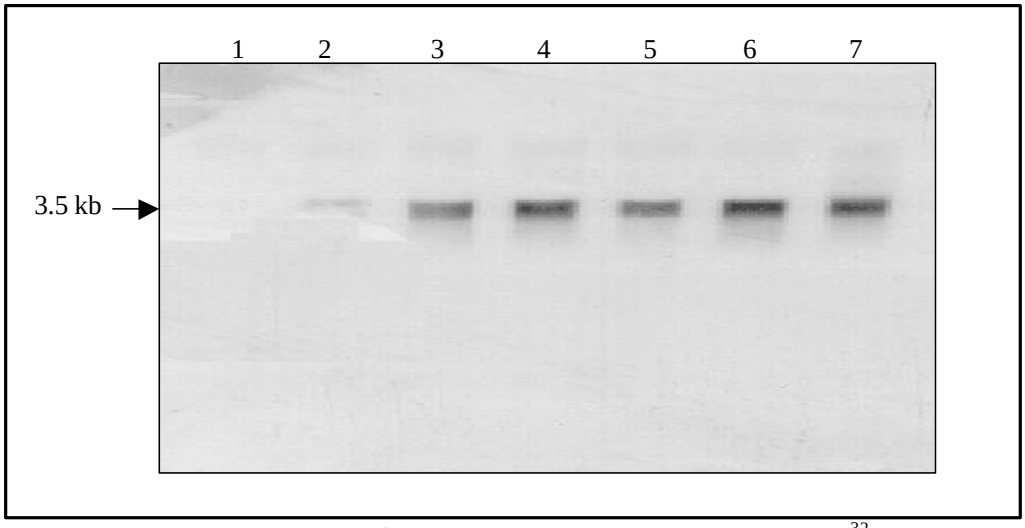

Figure 32: Northern blot analysis of the *ica* transcription hybridized with the ^{32}P-labelled *ica*A gene, indicates the positive effect of subinhibitory concentrations of pristinamycin and tetracycline on the *ica* transcription. Lane1: control; lanes 2, 3, and 4: 0.015, 0.03, and 0.06 µg/ml pristiamycin respectively; and lanes 5, 6, and 7: 0.015, 0.03, and 0.06 µg/ml tetracycline respectively.

3.1.4 Post-exposure effects of pristinamycin and tetracycline on the P_{ica}-*lacZ* expression in *S. epidermidis* 220-1

In order to investigate whether or not the P_{ica}-*lacZ* expression in *S. epidermidis* 220-1 can be influenced by short exposures of the bacteria to high concentrations of pristinamycin and tetracycline, the bacteria were grown in CDM to an OD_{600} of 1.0. 5 µg/ml of the antibiotics were then added to the growth culture and incubated for 5 minutes. The cells were washed

twice with CDM, completely resuspended and incubated to an OD_{600} of 7.0. Following the growth, the β-galactosidase activity was tested. Figure 33 demonstrates that a short exposure to a concentration of 5 µg/ml of pristinamycin, dalfopristin, quinupristin, or tetracycline had the same effect like subinhibitory concentrations. The *ica* expression was increased 6-8 fold compared to the control.

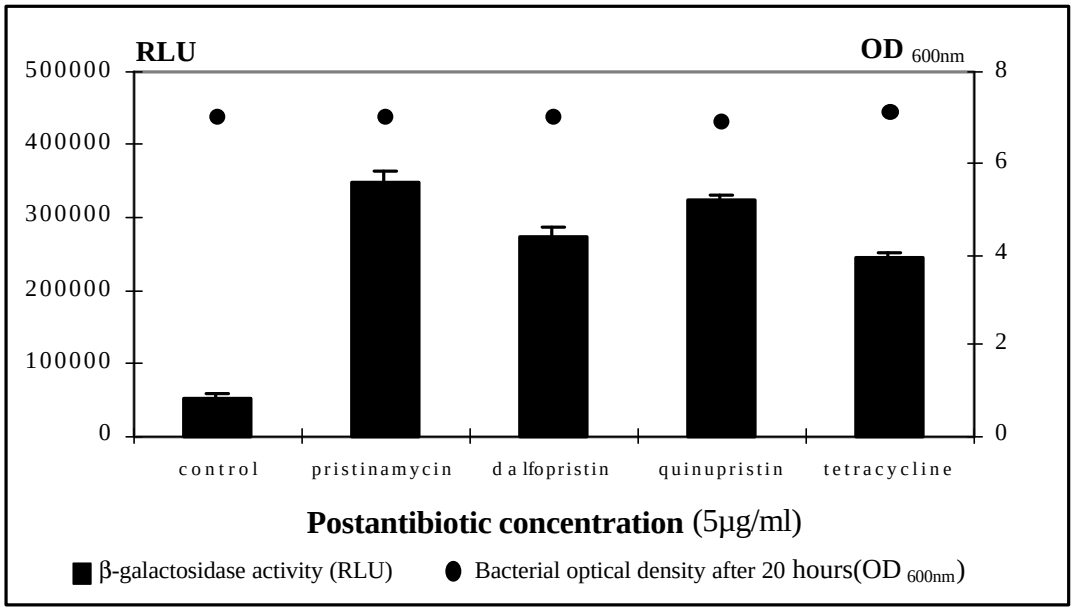

Figure 33: Post-exposure effects of pristinamycin, dalfopristin, quinupristin, or tetracycline on the P_{ica}-*lacZ* expression in *S. epidermidis* 220-1.

3.1.5 Post-exposure effects of pristinamycin, tetracycline, and dalfopristin and quinupristin separately, on the biofilm production in *S. epidermidis* 220.

The impact of short exposure to high concentrations of pristinamycin dalfopristin, quinupristin and tetracycline on slime production in the biofim positive *S. epidermidis* 220 wild type strain was studied (Figure 34). The bacterial cells were grown to an OD_{600} of 1.0, and exposed shortly (5 minutes) to the 5 µg/ml of each antibiotic respectively, Then the cells were washed twice, resuspended in the same medium and flat-bottomed tissue culture plates were filled with 200 µl aliquots of the washed bacteria. The plates were incubated at 37 °C and biofilm production was measured spectroscopically as described previously. As result, it was noticed that short exposure of the bacteria to the antibiotics induced significantly the slime production in *S. epidermidis* 220.

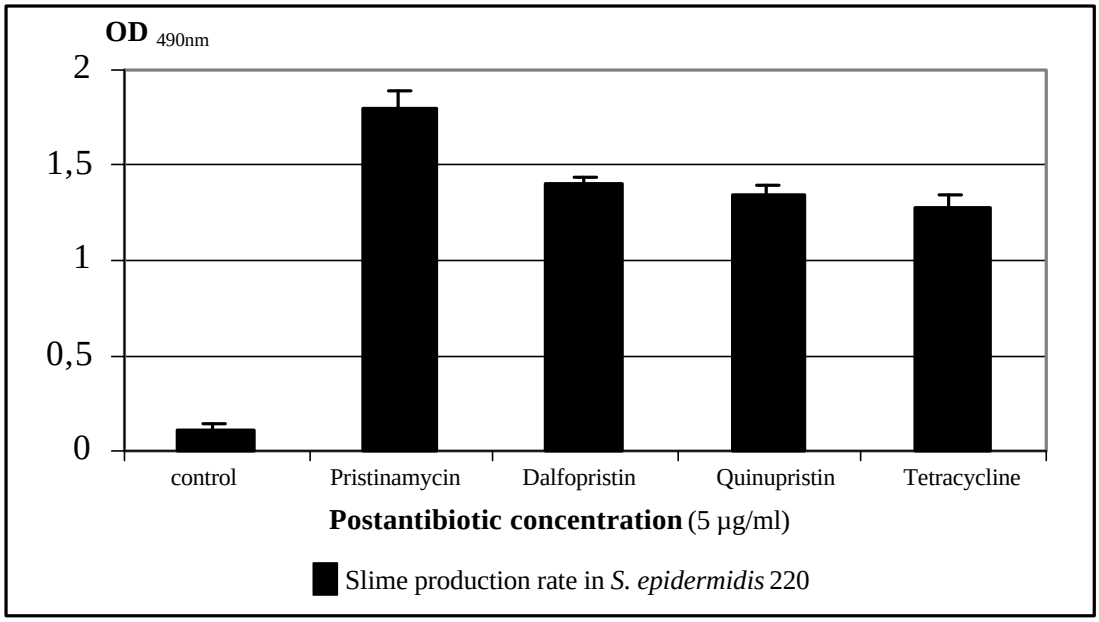

Figure 34: Influence of short exposure to high concentrations of pristinamycin, dalfopristin, quinupristin, and tetracycline on the biofilm production in *S. epidermidis* 220 by short exposure of the bacteria to 5 µg/ml of each antibiotic.

3.1.6 Influence of subinhibitory concentraions of viriginiamycin on the *ica* expression in *S. epidermidis* 220.

A clear alteration in the P_{ica}-*lacZ* expression was investigated during the incubation of the bacteria with subinhibitory concentrations of viriginiamycin, another protein inhibitor belonging to the streptogramin group. The addition of 0.015-0.06 µg/ml of the antibiotic induced the P_{ica}-*lacZ* expression approximately 4-5 fold compared to the control. The concentrations more than 0.06 µg/ml did not affect the *ica* expression, but clearly inhibited the growth of the bacteria (figure 35). The level of slime production in *S. epidermidis* 220 similarly to the β-galactosidase in *S. epidermidis* 220-1 was clearly increased by the addition of subinhibitory concentrations of this antibiotic to the growth medium of the bacteria.

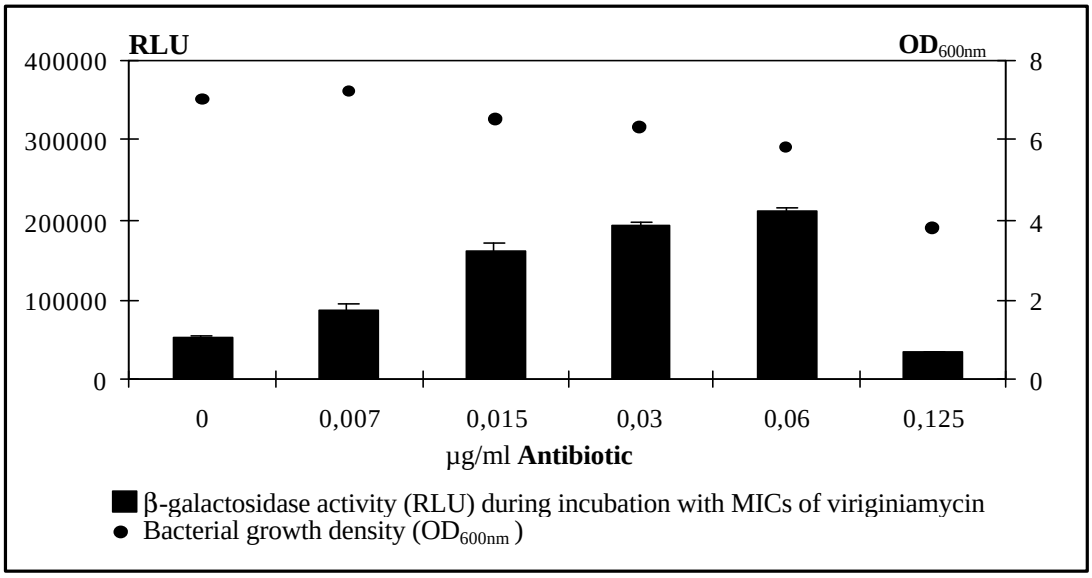

Figure 35: Influence of viriginiamycin on the P_{ica}-*lacZ* expression in *S. epidermidis* 220-1, following incubation of the bacteria in CDM supplemented with subinhibitory concentrations of the antibiotic.

Figure 36 demonstrates a higher production of biofilm by the addition of 0.007-0.06 µg/ml viriginiamycin to the growth medium of the bacteria compared to the control medium. Maximum biofilm production was recorded by addition of 0.06 µg/ml of the antibiotic, which increased the slime production levels 6-fold. The addition of more than 0.06 µg/ml viriginiamycin inhibited the slime production by inhibition of the bacterial growth.

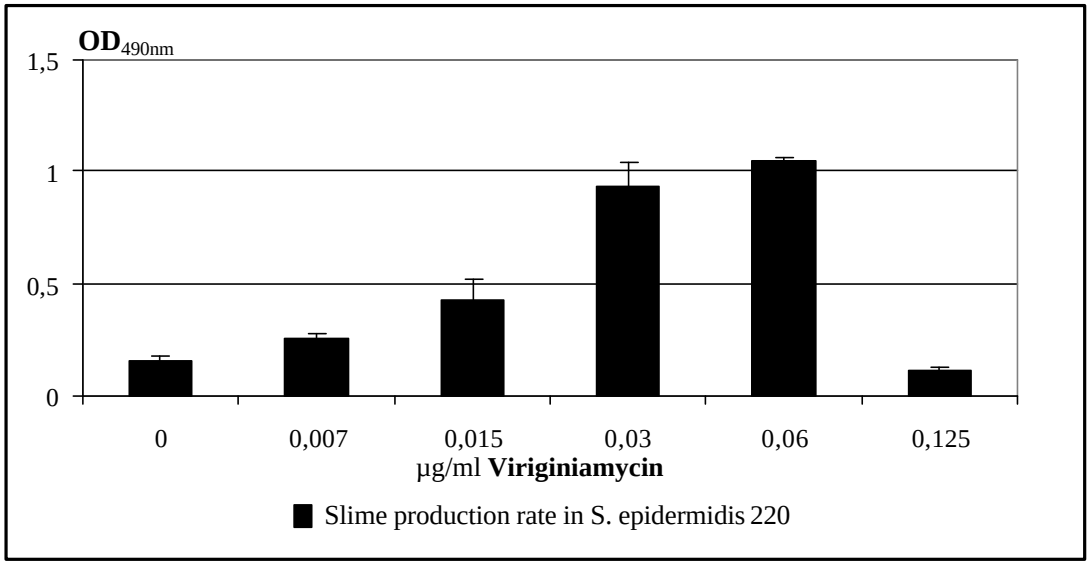

Figure 36: Influence of viriginiamycin on the biofilm production in *S. epidermidis* 220, following incubation of the bacteria with subinhibitory concentrations of the antibiotic.

3.2 Influence of other protein inhibitors on the P_{ica}-*lacZ* expression in *S. epidermidis* 220-1

The effect of subinhibitory concentrations of other protein inhibitor antibiotics on the P_{ica}-*lacZ* expression in *S. epidermidis* 220-1 was evaluated by addition of 10-40 µg/ml chloramphenicol, 10-70 µg/ml erythromycin, 0.0017-0.06 µg/ml fusidic acid, and 0.015-0.5 µg/ml gentamicin to the growth medium of *S. epidermidis* 220-1. Following the incubation for 20 hours, the cells were harvested and the β-galactosidase activity was estimated. No significant alterations in β-galactosidase activity were observed by the addition of subinhibitory concentrations of clindamycin, fusidic acid, and gentamicin to the growth medium of the bacteria (Figure 37).

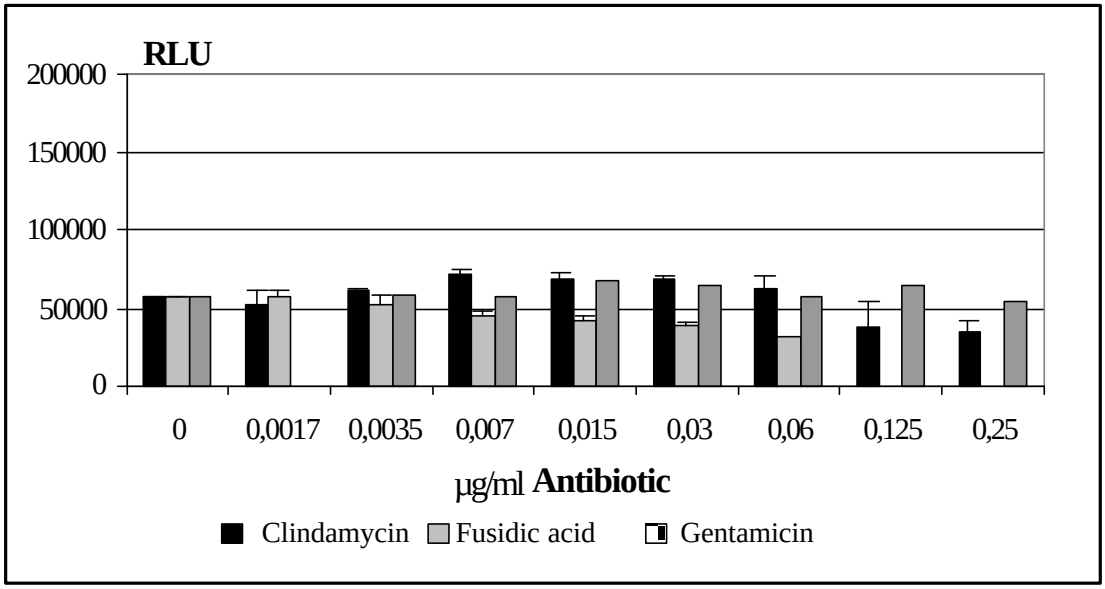

Figure 37: Influence of clindamycin, fusidic acid, and gentamicin on the P_{ica}-*lacZ* expression in *S. epidermidis* 220-1, following incubation of the bacteria in the CDM supplemented with subinhibitory concentrations of the antibiotics.

A weak induction of the P_{ica}-*lacZ* expression was investigated by the addition of 20 or 30 µg/ml chloramphenicol to the growth medium, and in the presence of 30 µg/ml erythromycin. Under these conditions, the P_{ica}-*lacZ* expression was increased two fold compared to the *ica* expression without antibiotics (Figure 38).

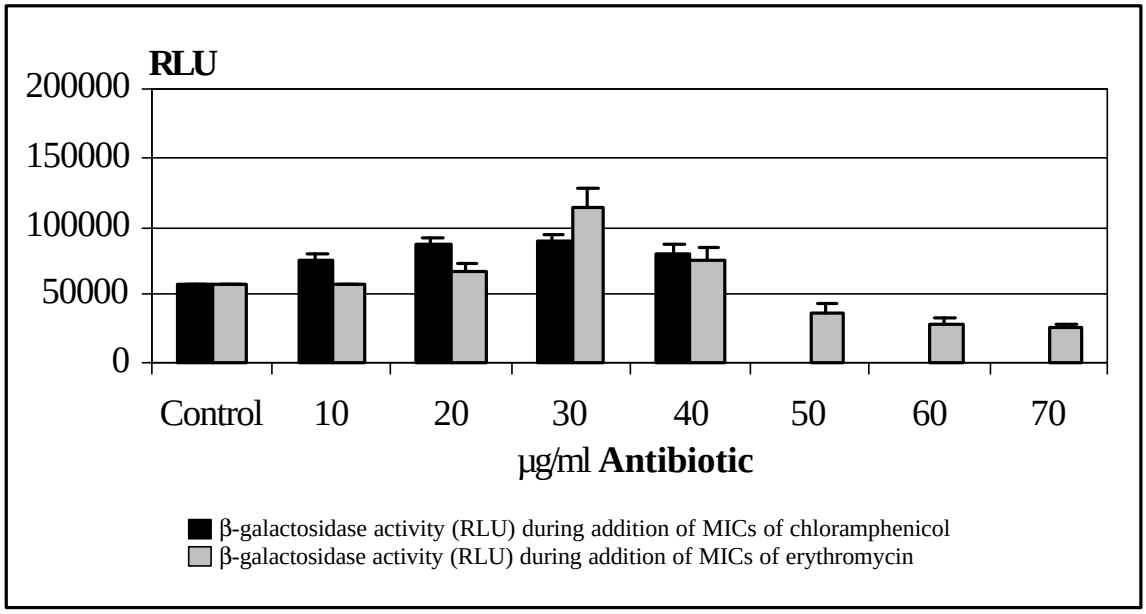

Figure 38: Influence of chloramphenicol and erythromycin on the P_{ica}-*lacZ* expression in *S. epidermidis* 220-1, by addition of subinhibitory concentrations of the antibiotics into the growth medium.

3.3 Influence of cell wall and DNA-gyrase inhibitor antibiotics on the *ica* expression

3.3.1 Influence of cell wall and DNA-gyrase inhibitor antibiotics on the P_{ica}-*lacZ* expression in *S. epidermidis* 220-1

In order to test the impact of other groups of antibiotics for which *S. epidermidis* 220 is sensitive, the MICs of oxacillin, penicillin G, teicoplanin, and vancomycin as examples for cell wall inhibitors, and ofloxacin as an exapmle for a DNA-gyrase inhibitor were added to the growth medium of *S. epidermidis* 220-1. Following incubation for 20 hours, the P_{ica}-*lacZ* expression was tested by measurement of the β-galactosidase activity. No clear differences in β-galactosidase activity were investigated by addition of subinhibitory concentrations (0.007-0.125, 0.0017-0.125, 0.015-0.125 µg/ml) of oxacillin, teicoplanin, and vancomycin respectively to the growth culture of the bacteria (figure 39) .

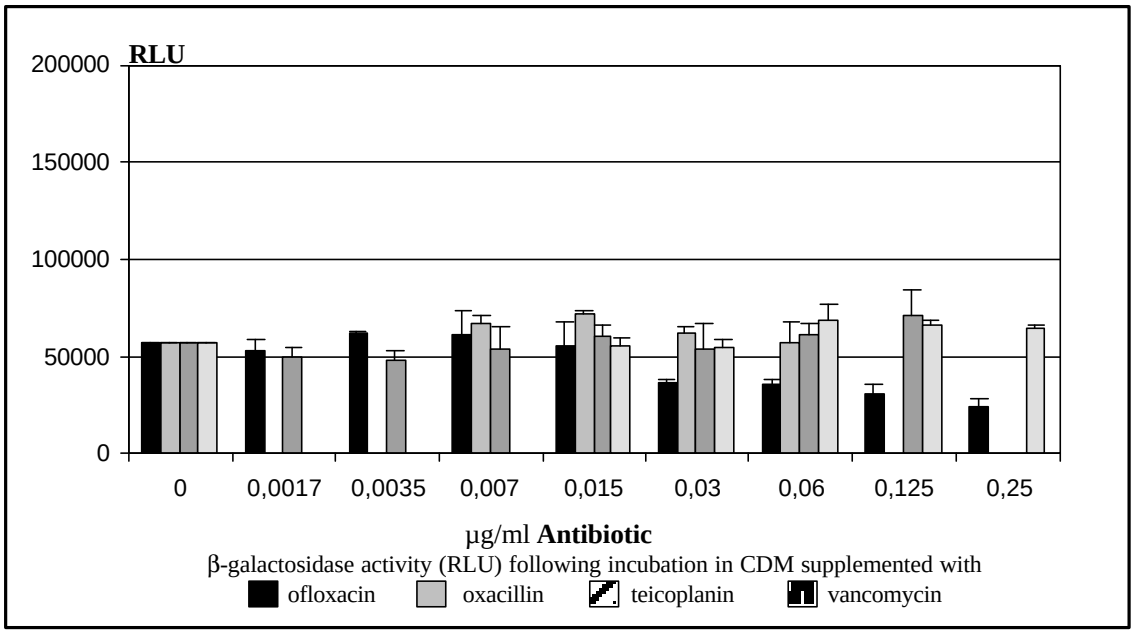

Figure 39: Influence of ofloxacin, oxacillin, teicoplanin, and vancomycin on the P_{ica}-*lacZ* expression in *S. epidermidis* 220-1, following incubation of the bacteria 20 hours with subinhibitory concentrations of the antibiotics.

The β-galactosidase activity was weakly inhibited by addition of 0.03-0.125 and 0.0006-0.005 µg/ml ofloxacin and penicillin respectively, and this inhibition was not accompanied with growth inhibition. But, addition of more than these concentrations (0.25 µg/ml, and 0.01 µg/ml O-floxacin and penicillin respectively) inhibited both the bacterial growth and the P_{ica}-*lacZ* expression (figure 39 and 40) .

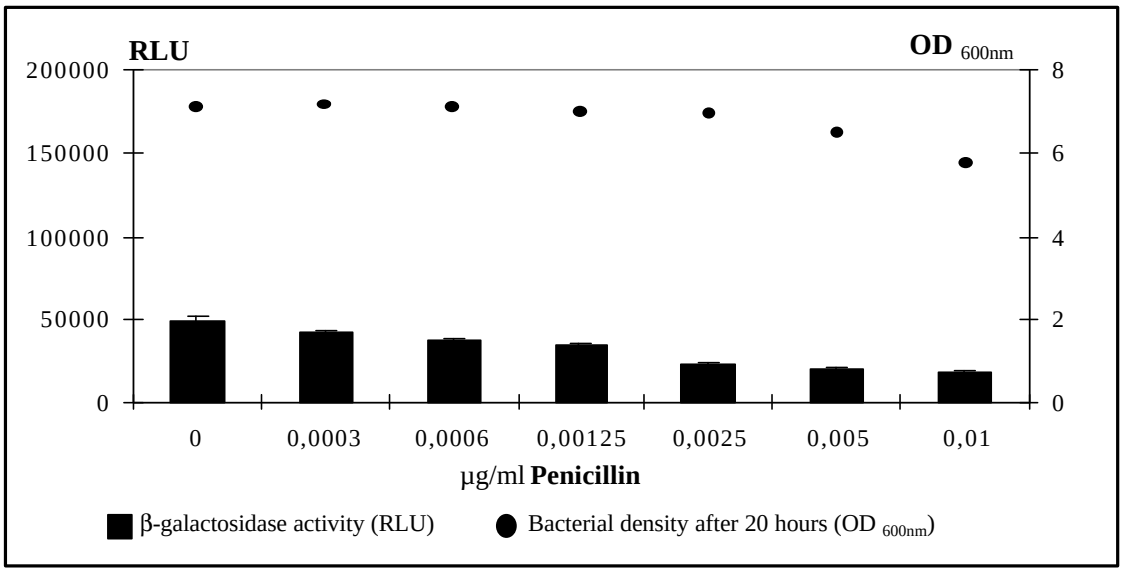

Figure 40: Influence of penicillin on the P_{ica}-*lacZ* expression in *S. epidermidis* 220-1, following incubation of the bacteria in the CDM supplemented with subinhibitory concentrations of the antibiotic.

3.3.2 Influence of penicillin on the biofilm production in *S. epidermidis* 220 wild type strain

The above described results indicated that penicillin as an example for a cell wall inhibitor repressed the P_{ica}-*lacZ* expression in *S. epidermidis* 220-1. In order to elucidate the negative effect of this antibiotic on slime production, first, we had to increase the basal level of slime production in the bacteria by addition of 1.5 % glucose to the growth culture of the bacteria, then 0.0006-0.01 µg/ml penicillin was added to the medium, finally, the slime production was measured by the quantitative adherence assay. The same result as in the P_{ica}-*lacZ* expression assay of *S. epidermidis* 220-1 was observed. The addition of 0.01-0.0006 µg/ml penicillin to the growth culture of the bacteria decreased (2-3 times) the slime production in *S. epidermidis* 220 compared to the slime production in the same medium without antibiotic (figure 41).

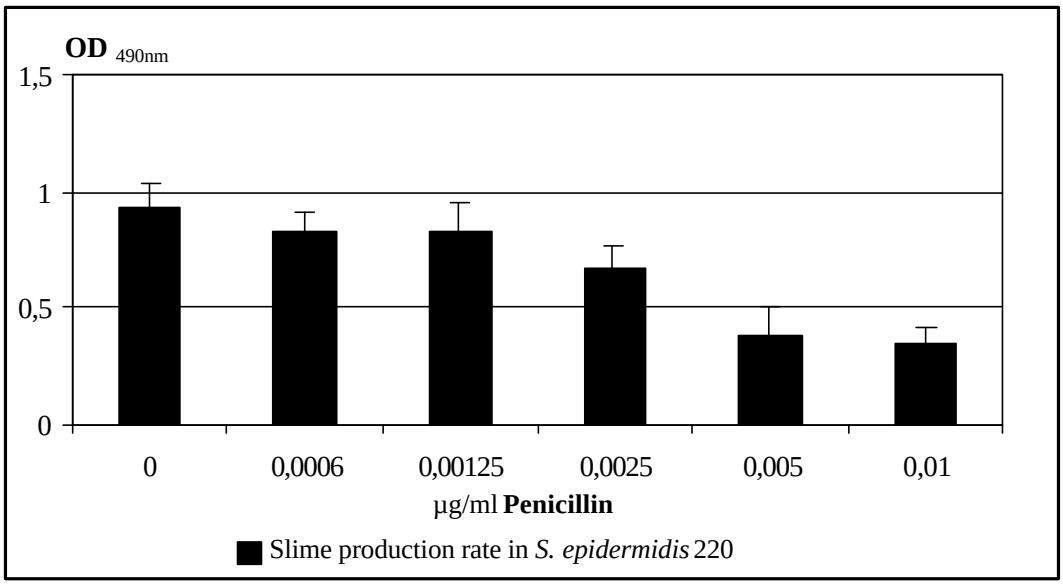

Figure 41: Effect of penicillin on the biofilm production in *S. epidermidis* 220 using the quantitative adherence assay, by addition of subinhibitory concentrations of the antibiotic to the growth medium.

Summary

- Environmental factors including growth phase, pH, temperature and carbon source influenced clearly the expression of *ica* operon.
- High osmolarity stress induced the *ica* expression in *S. epidermidis* as well as in *B. subtilis*.
- The biofilm expression is induced by the effect of SDS, ethanol, H_2O_2, and urea.
- Southern blot analysis of the *S. epidermidis* chromosome cross-hybridized with *sigB* of *S. aureus* indicates the presence of *sigB* in *S. epidermidis*.
- Northern blot analysis of the *sigB* transcription in *S. epidermids* indicates the induction of the *sigB* expression in response to the biofilm inducing conditions.
- Subinhibitory concentrations of some antibiotics induced significantly the expression of the ica operon in *S. epidermidis*.
- Sub-MICs as well as post-exposure effect of streptogramins including pristinamycin, both dalfopristin and quinupristin influenced significantly the expression of *ica* operon in *S. epidermidis* but not in *B. subtilis*.
- Sub-MICs as well as post-exposure effect of tetracycline induced significantly the expression of *ica* operon in *S. epidermidis* but not in *B. subtilis*.
- Other protein inhibitors, cell wall inhibitors, and DNA-gyrase inhibitors did not clearly alter the expression of *ica* operon.

4. The role of the accessory gene regulator (*agr*) gene in the regulation of the biofilm production in *S. epidermidis*

The accessory gene regulator (*agr)* of Staphylococci controls the expression of several virulence factors [32]. While the transcription of RNAIII in *S. epidermidis* 567 as well as the *ica* gene was induced during the presence of 3 and 4% NaCl, it has been speculated that the *agr* gene might be involved in the regulation of *ica* operon in *S. epidermidis.* In order to elucidate its role, inactivation of the *agr* gene in *S. epidermidis* 567 was done by double cross-over replacement of the *agr* gene with an *agr*::*ermB* fragment (figure 42). Then the level of slime production in the *agr* mutant strain was compared to that in the wild type.

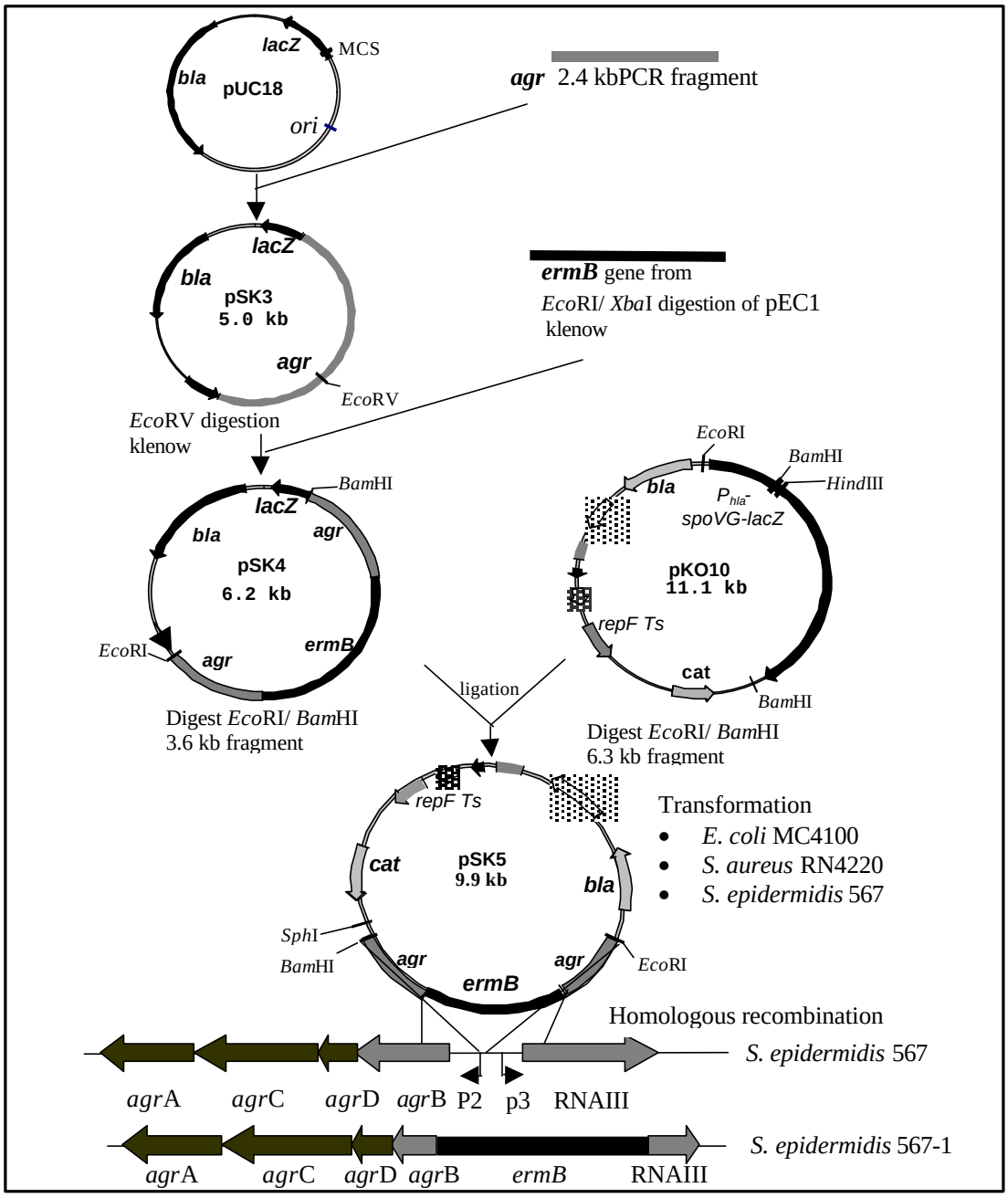

Figure 42: Construction of the pSK5 plasmid (carrying the *agr::ermB* fragment) and inactivation of the *agr* gene in *S. epidermidis* 567 by double cross over creating the *agr* minus *S. epidermidis* 567-1. For detail see text.

4.1 Effect of the osmolarity on the RNAIII transcription.

The RNA molecule RNAIII is the effector molecule of the *agr* operon, which exhibits negative or positive regulatory functions [138]. In order to elucidate the effect of osmolarity on RNAIII, *S. epidermidis* 567 was grown in CDM medium supplemented with 3 and 4% (wt/vol) NaCl. Following incubation at 37°C, the total cellular RNA was isolated and Northern blot was performed using a P^{32}-labelled RNAIII-specific DNA probe (amplified with PCR using primers agr-3 and agr-4) for hybridization. Figure 43 indicates that the transcriptional level of RNAIII is positively regulated during the presence of 3 and 4% NaCl.

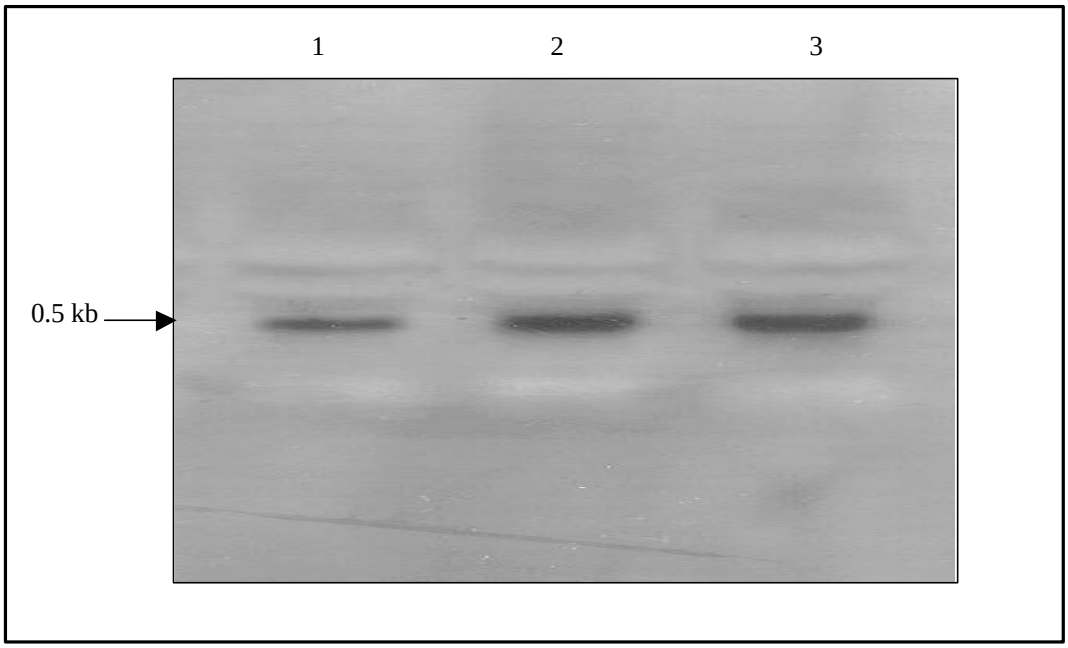

Figure 43: Northern blot analysis of the RNAIII transcription in *S. epidermidis* 567, hybridized with a ^{32}P-labelled 0.5 kb PCR fragment of the RNAIII. Lane 1: RNAIII transcription during growth of the bacteria in CDM (control) and lanes 2 and 3: RNAIII transcription during growth in CDM supplemented with 3 and 4% NaCl respectively.

4.2 Construction of an *agr* mutant in *S. epidermidis* 567

A 2.4 kb fragment containing the 429 bp *agr*C gene, the complete genes of *agr*D, *agr*B, P2, P3, RNAIII, and a 458 bp fragment downstream of the *agr* region was amplified by PCR by using the primers agr 1 and agr 2, with a *Bam*HI and *Eco*RI cleavage site, respectively. The

PCR-primers bind at position 1836 and 4227 of the published *agr* sequence (accession AF012132), respectively. The amplification conditions consisted of an initial denaturation step at 94°C for 2 minutes followed by 30 cycles of 94°C for 60 s, 54°C for 60 s, and 72°C for 90 s. The resulted fragment was cloned into the multiple cloning sites of the pGEM®-T Easy vector (digested with *Eco*RV and T added to both 3´-ends) creating the plasmid pSK3. *E. coli* DH5α was transformed by introducing the ligation product, using the $CaCl_2$ method, and plated onto a selective X-gal/IPTG-LB agar with 100 µg/ml Ampicillin. For plasmid isolation, the white colonies were selected and grown overnight in 2 ml LB medium containing 100 µg/ml Ampicillin. The isolated plasmids were analysed by a restriction digest using *Eco*RI and *Bam*HI to select the correct plasmid with the cloned fragment. PSK3 plasmid was digested with *Eco*RV and *Nhe*I to excise a 487 bp including 150 bp of *agr*B, P2, P3, and 121 bp of RNAIII of the cloned fragment and inactivating the *agr* expression. A 1.7 kb *ermB* gene conferring resistance to erythromycin was obtained by digesting the pEC1 plasmid using *Hind*III and *Xba*I and blunt end cloned into the linearized blunt ended *Eco*RV and *Nhe*I restricted pSK3, yielding the plasmid pSK4. The resulted plasmid was introduced into *E. coli* DH5α by $CaCl_2$ transformation, and plated onto LB agar containing 100 µg/ml of both erythromycin and ampicillin. A pSK5 was constructed by cloning a 3.6 kb fragment from *Eco*RI/*Bam*HI restricted plasmid pSK4 into the clonning sites of the 6.3 kb *Eco*RI/*Bam*HI restricted pKO10 plasmid. *E. coli* DH5α was transformed with the ligation product, plated onto LB plates containing 100 µg/ml both ampicillin and erythromycin, and the grown colonies were incubated overnight in LB medium containing 100 µg/ml ampicillin and erythromycin for plasmid isolation. The isolated plasmids were analysed with a restriction digest using *Eco*RI/*Bam*HI to select the correct plasmid carrying the *agr*::*ermB* (Figure 44). The resulted plasmid (pSK5) was subsequently transferred into the restriction deficient *S. aureus* RN4220 by electroporation and the transformant cells were selected on BHI agar plates containing 10 µg/ml of both chloramphenicol and erythromycin. The plasmid was isolated and finally transferred into the *S. epidermidis* 567 by electroporation as described previously.

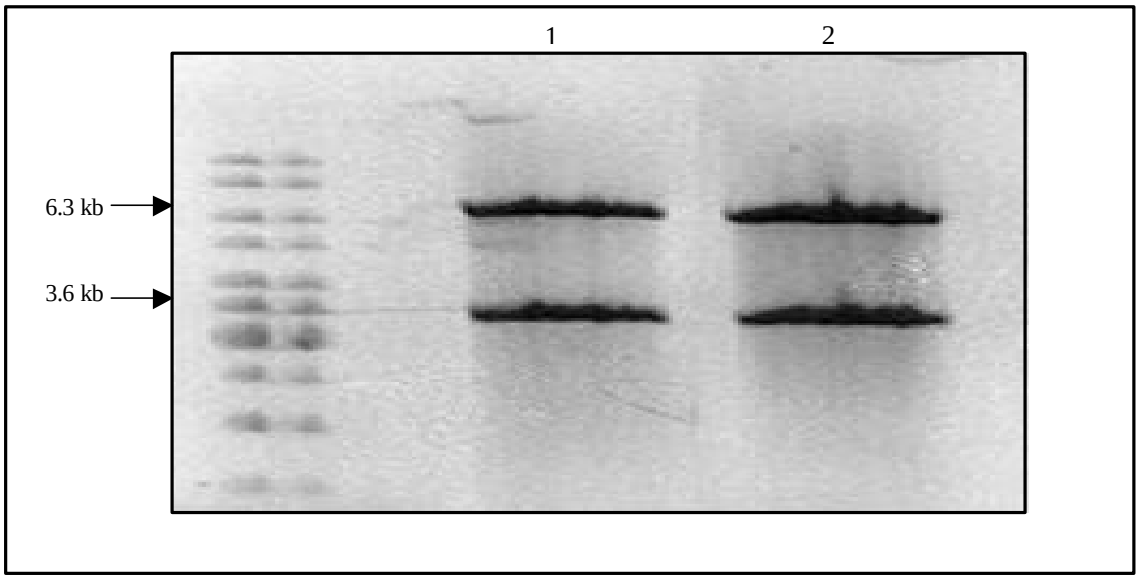

Figure 44: Restriction digest of the pSK5 by using *Eco*RI and *Bam*HI (lanes 1 and 2). The 3.6 kb fragment represents the *agr::ermB* obtained from plasmid pSK4, and the 6.3 kb fragment of the *Eco*RI /*Bam*HI digested pKO10, a temperature sensitive shuttle vector.

4.3 Inactivation of the *agr* gene in *S. epidermidis* 567

The gene replacement in *S. epidermidis* 567 was performed as described by Brückner [125]. Following transformation of *S. epidermidis* 567 with the plasmid pSK5, the recombinant strains were grown in LB medium containing 10 µg/ml chloramphenicol and erythromycin at 30°C to late-stationary phase so as to generate a population of plasmid-bearing cells. Subsequently, the 30°C culture was diluted 1:100 into 500 ml fresh LB medium containing 2.5 µg/ml erythromycin. The second culture was incubated at 40°C until it reached the stationary phase. A third round of growth followed exactly the conditions of the previous culture. The fourth culture was grown like the preceding ones, except that erythromycin was omitted. Appropriate dilutions of the last culture were spread on LB agar plates supplemented with 2.5 µg/ml erythromycin and incubated at 37°C. The colonies from these plates were patched onto 2.5 µg/ml erythromycin and 10 µg/ml chloramphenicol containing agar plates. Chloramphenicol sensitive, erythromycin resistant colonies indicate successful replacement recombination and creating an *agr* mutant strain *S. epidermidis* 567-1.

4.4 Southen blot analysis to determine the *agr* gene replacement in *S. epidermidis* 567-1

Southern blot analyses were performed to evaluate the mutational replacement of the *agr* gene in *S. epidermidis* 567-1. In the *Eco*RI digested chromosome of the wild type strain *S. epidermidis* 567, the *agr* gene is located on a 8 kb fragment. After double cross-over and gene replacement, a band shift of 9.3 kb was detected by using the 2.4 kb amplified *agr* operon as a gene probe. The same shifted bands were identified by hybridization with the 1.7 kb *ermB* gene as a gene probe (Figure 45).

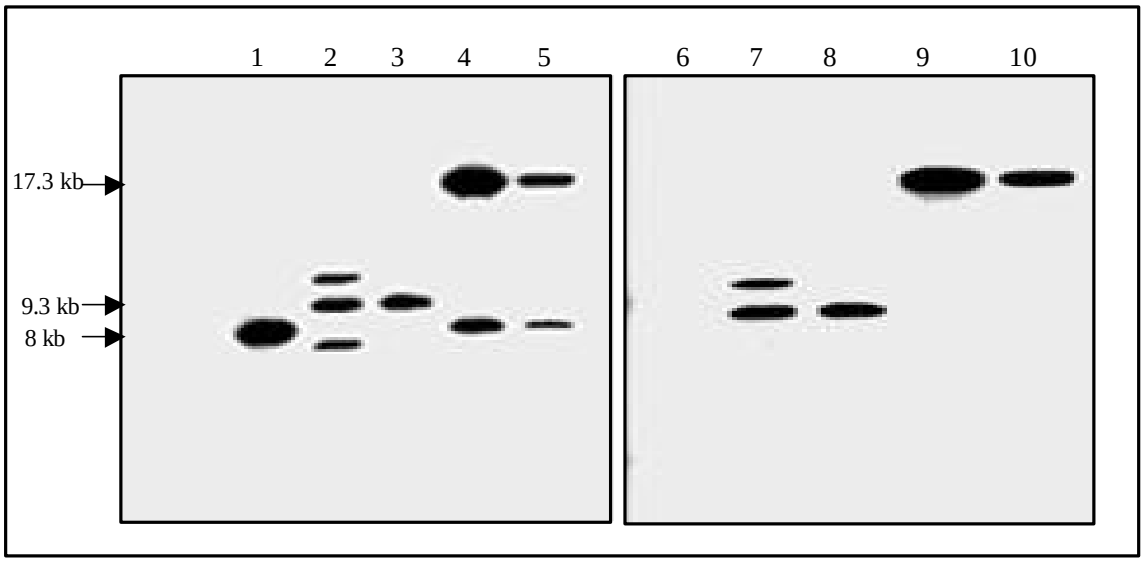

Figure 45: Southern blot analysis of *Eco*RI restriction digest of the chromosomal DNA of *S. epidermidis* 567 and 567-1, hybridized with an ECL labelled 2.4 kb PCR fragment of the *agr* gene (A), and ~1.8 kb *ermB* gene (B). Lanes 1 and 6: the *Eco*RI restricted chromosome of *S. epidermidis* 567, and lanes 2, 3, 4, 5, 7, and 8: the *Eco*RI restricted chromosome of *S. epidermidis* 567-1. Lanes 4, 5, 9, and 10: resulted from a single cross-over between the entire plasmid and the *agr* gene of *S. epidermidis* 567.

4.5 Sequence analysis of the *agr::ermB* site in the recombinant strain 567-1

In order to evaluate the sequence of the *agr::ermB* region in *S. epidermidis* 567-1, the fusion site was amplified by PCR using primers Agr-eryth-1, and Agr-eryth-2 resulting in a 1.9 kb fragment. A 228 bp fragment was obtained during the use of wild type chromosomal DNA. The nucleotide sequence of the fragment comprising the *agr::ermB* region was analyzed by nucleotide sequencing (figure 46).

AA TTA AAA AAT AAC TTA AAT GAG TTA AAA GTG TGT AAA AGA AAG TAT

⟶ **a*grB*** operon

GAA AGA TTA TTG CTA GAG------***ermB*** **gene**-------TCGACC TGC AGG CAT GCA

pUC18 MCS ***Pst*I** ***Sph*I**

ATC ATT TCT ACA ATC GGT GAT TTA GTA AAA TGG ATT ATC GAT ACA G

⟶ **RNAIII**

TTA AAT AAA TTC

Figure 46: Nucleotide sequence analysis of the *agr::ermB* region in the chromosomal DNA of *S. epidermidis* 567-1.

4.6 Biofilm production in the *agr* mutant *S. epidermidis* 567-1

The levels of slime production in the stationary phase of *S. epidermidis* 567 and the *agr* minus 576-1 were measured during the normal, and under the influence of the factors affecting the slime production in *S. epidermidis*, including; 0.06 µg/ml Pristinamycin, and 4 % NaCl. The biofilm production was determined by the quantitative adherence assay using 96-well, flat-bottomed tissue culture plates as described previously. The test revealed that there is no effect of the *agr* inactivation on the *ica* expression in *S. epidermidis* 567-1 during the normal bacterial growth as well as under the effect of pristinamycin and osmolarity.(Figure 47).

Figure 47: Effect of the *agr* inactivation on the biofilm production in *S. epidermidis* 567. The biofilm production in the wild type was compared to that in the *agr* mutant strain 567-1 under the normal condition, and under the effects of pristinamycin and high osmolarity.

5. The role of the alternative sigma factor (*SigB*) in the regulation of biofilm production.

In bacteria, alternative sigma factors of the RNA polymerase are known to play a crucial role in regulating gene expression and bacterial metabolisms in response to environmental stress and during the stationary phase [46, 139]. Since *S. aureus* carries the *ica* locus [83], the role of the *sigB* gene in biofilm regulation was tested in a biofilm *S. aureus*. First, a *sigB* mutant was constructed in bioflm positive *S. aureus* MA12, and the biofilm production in the *sigB* mutant strain was compared with that in the wild type strain. Later, the mutant was complemented with the functional *sigB* gene to restore the *sigB* activity.

5.1. Construction of a *sigB* knock out mutant

A *sigB* insertional mutant was constructed by replacing the wild type *sigB* gene with a non functional *sigB* gene carrying the erythromycin resistance determinant (*ermB*) inside the gene. The replacement was achieved by double cross-over integration. The plasmid pSK8 containing the *ermB* determinant flanked by a 0.5 kb upstream *sigB* region and a 0.5 kb downstream *sigB* region was initially constructed in *E. coli* DH5α and then transformed into the restriction negative *S. aureus* strain RN4220. For this purpose the erythromycin resistant cassette (*ermB*) was cloned into the pSK7. Transformants were selected on LB agar plates containing chloramphenicol and erythromycin. The plasmid pSK8 was isolated and in the second transformation step introduced into *S. aureus* MA12. Transformants were again selected on LB agar plates containing erythromycin and chloramphenicol at 30°C. As pSK8 has a temperature sensitive replication origin for *S. aureus*, transformants were first propagated at 30°C and then shifted to 42°C to block replication favouring homologous recombination. A double cross-over event between the mutated *sigB* gene and the wild-type *sigB* gene was then obtained by selecting erythromycin-resistant but chloramphenicol-sensitive colonies (figure 48).

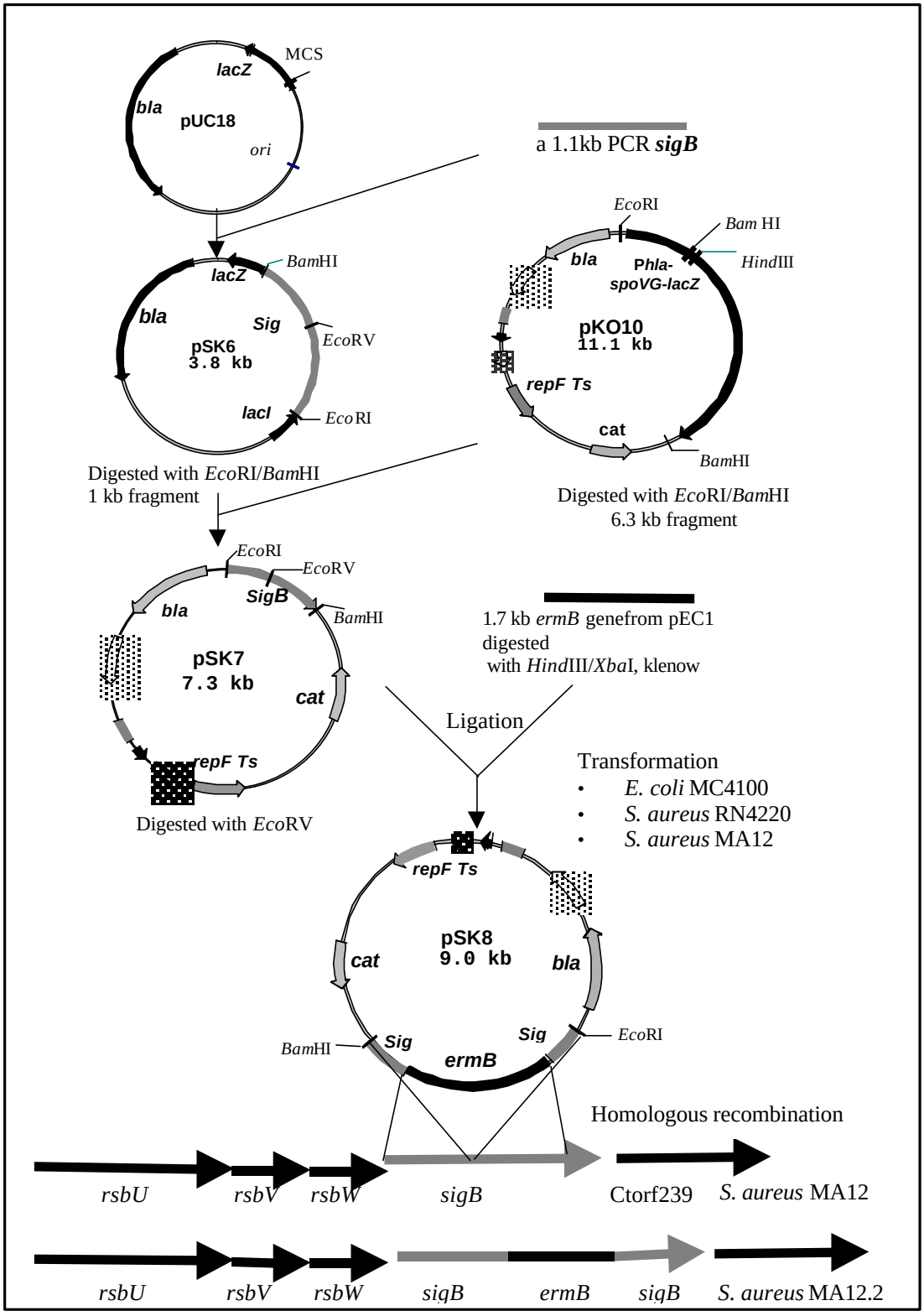

Figure 48: Construction of the pSK8 and the *sigB* mutant *S. aureus* MA12 by insertion of the *ermB* gene into the *sigB* operon by double cross over with the plasmid pSK8. For detail see text.

5.1.1 Construction of the *sigB* knock out mutator plasmid pSK8

A 1.1 kb *sigB* fragment was amplified by PCR using the primers 5` CGG GAT CCG GTG TGA CAA TCA GTA TGA C 3` and 5` CGG AAT TCG CGA CAT TTA TGT GGA TAC AC 3`. Within the primer sequence *EcoR*I and *BamH*I restriction sites were introduced, respectively. The 1.1 kb fragment was then ligated into vector pUC18, forming pSK6, and fidelity of the cloned fragment was checked by DNA-sequencing. Following *EcoR*I/*BamH*I digestion of pSK6, the 1.1 kb *sigB* fragment was ligated into *EcoR*I/*BamH*I digested shuttle vector pKO10 forming pSK7. The *sigB* gene was then digested with EcoRV, dephosphorylated by shrimp alkaline phosphatase (Pharmacia, Freiburg, Germany) and ligated with blunt ended *ermB* gene obtained from pEC1 to form pSK8. The *ermB* gene was digested with *Xba*I and *Hind*III and filled in by the Klenow fragment (Pharmacia) of DNA polymerse resulting in blunt ended *ermB*. This new plasmid contains a mutated *sigB* with an integral copy of the erythromycin resistance cassette *ermB* (figure 49).

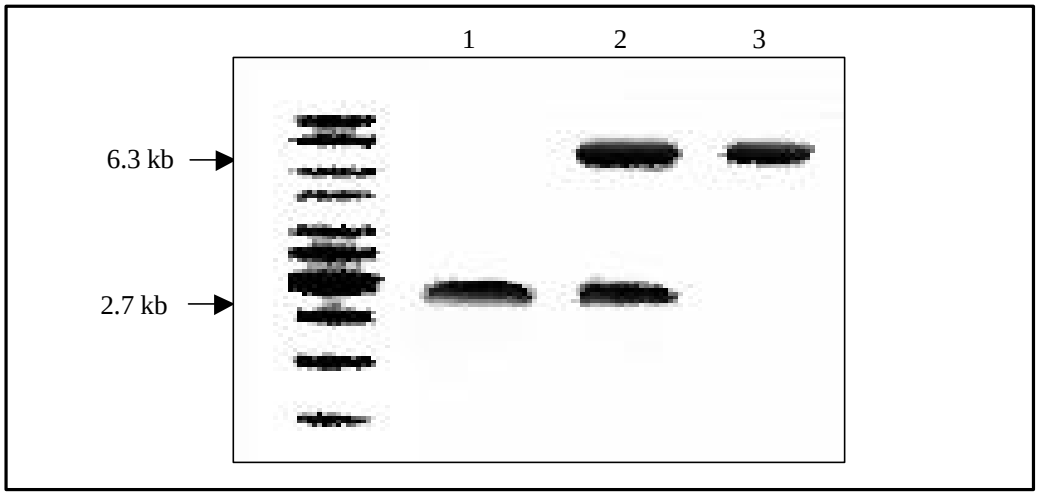

Figure 49: *EcoR*I/*BamH*I restriction digest of the pSK8. Lane 1: a 2.7 kb *ermB* gene inserted into the *sigB* gene, lane2: pSK8 consists of the shuttle vector (6.3 kb) and the 2.7 kb mutated *sigB* fragment, and lane 3: the shuttle vector (6.3 kb).

5.1.2 Isolation of the *sigB* knock out mutants

The plasmid pSK8 was isolated from *E. coli* DH5α and first electroporated into restriction negative *S. aureus* strain RN4220. From transformants grown on LB-agar plates containing 10µg/ml erythromycin and 7.5µg/ml chloramphenicol at 30°C plasmid integrity was analyzed

and DNA was transformed into MA12. Transformants were again selected on plates containing erythromycin and chloramphenicol and plasmid integrity was checked. One transformant was grown overnight in 20ml LB broth containing erythromycin (10µg/ml) and chloramphenicol (7.5µg/ml) at 30°C for plasmid amplification. A 500µl aliquot of that culture was inoculated into fresh LB broth containing 4µg/ml erythromycin and incubated overnight at 42°C. A 500µl of that culture was again inoculated into fresh LB broth containing 4µg/ml erythromycin and incubated overnight at 42°C. Following dilution of the culture cultivation was done without antibiotics overnight at 42°C. A 100µl aliquots of this culture were plated onto LB plates containing 4µg/ml erythromycin and incubated at 37°C. Single colonies were replica plated onto LB plates containing 7.5µg/ml chloramphenicol. Colonies sensitive to chloramphenicol but resistant to erythromycin were selected and putative mutants of the *sigB* gene were confirmed by PCR and Southern blotting. The isogenic mutant derivative of MA12 was designated MA12.2.

5.1.3 PCR analysis of the *sigB* knock out mutant

The insertion of the *ermB* gene into the *sigB* was further evaluated by PCR analysis of DNA of the wild-type and the mutant. Using primers specific for *sigB* upstream (sigB-1) and downstream (sigB-2) regions, respectively an 1.1 kb PCR product was obtained using template isolated from wild-type DNA and a 2.8 kb PCR product was obtained when the same PCR amplification reaction was performed using DNA from the *sigB* mutant as template. These PCR results are consistent with the ~1.7 kb *ermB* fragment integrated into *sigB*. The sequencing of the 2.8 kb PCR product verified the insertion of *ermB* into the *sigB* gene (figure 50).

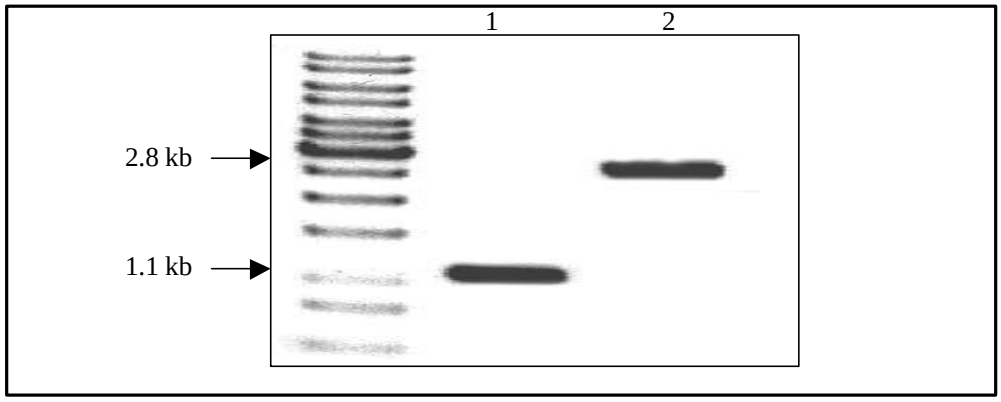

Figure 50: PCR analysis of the *sigB* mutant region. Lane 1: a 1.1 *sigB* fragment amplified by PCR using the chromosome DNA of *S. aureus* MA12. Lane 2: a 2.8 kb fragment of the mutated *sigB* using the chromosome DNA of *S. aureus* MA12.2 as a template.

5.1.4 Southern blot analysis of the *sigB* mutant

To confirm the genetic status of the putative *sigB* mutant in *S. aureus* MA12.2, *Eco*RI digested total DNA from wild-type and mutant strains was analyzed by Southern hybridization. The blot was first hybridized with probes specific for the *sigB* gene and in a second hybridization step with a probe specific for the *ermB* gene. As shown in Figure 51, a ~8kb EcoRI fragment from wild-type strain MA12 hybridized with the *sigB*-specific probe, however, no hybridizing fragment of this size was found in the mutant strain MA12.2. Instead, a hybridizing band of ~9.8 kb was observed with DNA from the mutant strain. Hybridization with an *ermB* specific probe revealed no signal in the wild-type strain but a hybridizing band of ~9.8 kb in the mutant strain. This signal is of the same size as the signal detected after hybridization with the *sigB* specific probe indicating a double cross-over event leading to insertion of *ermB* into the *sigB* gene.

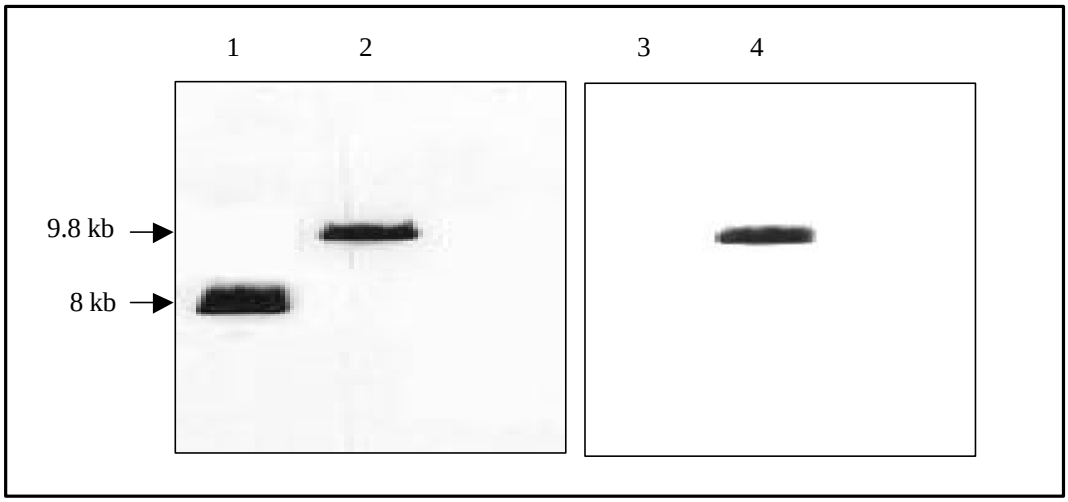

Figure 51: Southern analysis of the *sigB* gene hybridized with the ECL labelled (A) *sigB* gene and the (B) *ermB* gene. Lanes 1 and 3: the *Eco*RI digested chromosome of *S. aureus* MA12 wild type strain; and lanes 2 and 4: the *Eco*RI digested chromosome of *sigB* negative *S. aureus* MA12.2.

5.1.5 Evaluation of the *sigB* dependent *asp*23 expression

To confirm the functional inactivation of the *sigB* gene by insertion of *ermB*, the expression of the *sigB* dependent gene *asp*23 was analyzed by Northern analysis. A*sp*23 has been shown to possess a *sigB* dependent promoter and expression failed in *sigB* mutational strains [46,

123]. Strain MA12 and MA12.2 were cultivated to early stationary phase and *asp*23 expression was induced by adjustment of the pH of the growth medium to pH 8.5. For control purposes the same procedure was carried out with strain COL and isogenic *sigB* mutant COL. Analysis of the transcription profile revealed a strong *asp*23 specific signal in both wild-type strains MA12 and COL, respectively. On the other hand no signal was detected from the mutant strains MA12.2 and COL ρ*sigB*, respectively (figure 52). These data show that strain MA12.2 is a functional *sigB* mutant. Moreover, mutant-strain 12.2 lost yellow pigmentation and showed enhanced hemolysis on rabbit erythrocyte containing agar plates, both are phenotypical features of *sigB* minus strains.

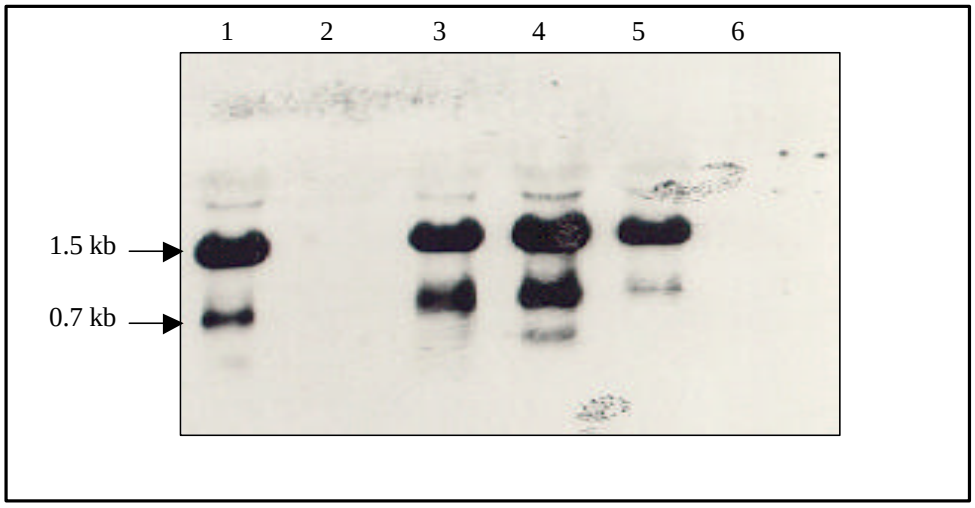

Figure 52: Northern blot analysis showing both transcripts (1.5 kb and 0.7 kb) of the *asp*23 gene, hybridized with an ECL-labelled PCR fragment of the *asp*23 gene. Lane 1: Wild type strain MA12, Lane 2: *sigB* mutant strain MA12.2; lanes 3 and 4: *sigB* complemented strain MA12.2SK; and lanes 5 and 6: The COL and the Δ *sigB* COL strains respectively.

5.2 Complementation of the *sigB* mutant *S. aureus* MA12.2 with a functional *sigB* gene

For complementation of the mutant *sigB* gene in *S. aureus* MA12.2, the pSK6 carrying a functional *sigB* gene was digested with *Eco*RI/*Bam*HI. The 1.1 kb *sigB* fragment resulting from this digestion was cloned into *Eco*RI/*Bam*HI digested shuttle vector pHPS9 under the control of the lactococcal Wg2-derived promoter 59 resulting the pSK9 (figure 53). The plasmid was then isolated from *E. coli* DH5α and electroporated into the restriction negative *S. aureus* strain RN4220. From transformants grown on LB-agar containing 7.5 µg/ml chloramphenicol , plasmid integrity was analyzed and DNA was transformed into MA12.2. resulting in MA12.2SK. Transformants were selected on LB-agar plates containing 7.5 µg/ml

chloramphenicol and 10 µg/ml erythromycin. Finally, the plasmid integrity was checked, and the resulting strain was analyzed for both *sigB* complementation and biofilm production (figure 52 and 54, respectively).

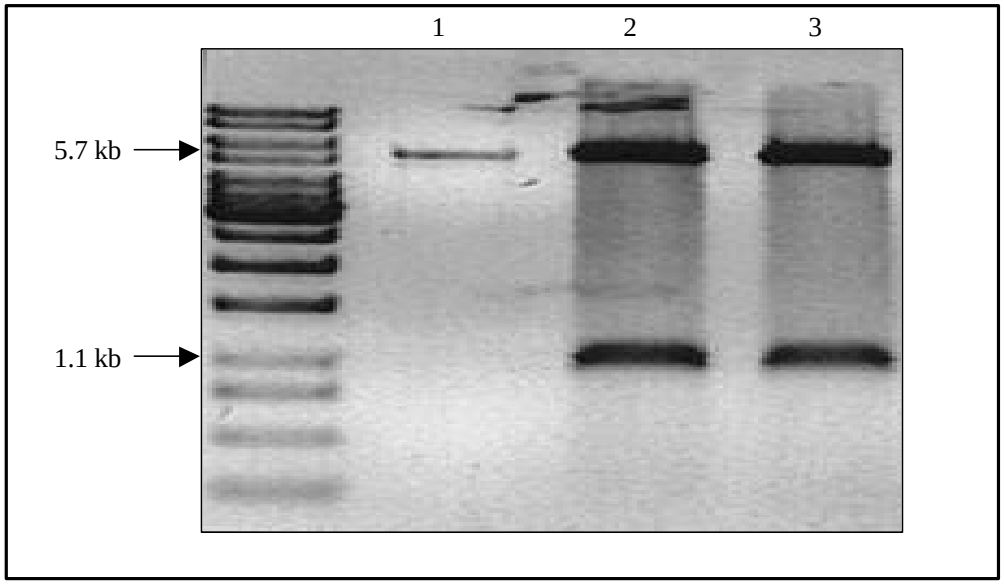

Figure 53: Lane 1 is the *Eco*RI/*Bam*HI restricted pHPS9 (shuttle vactor); and lanes 2 and 3 are the *Eco*RI/*Bam*HI restriction digest of the pSK9 resulting in a 1.1 kb *sigB* gene and a 5.7 kb of the pHPS9.

5.3 Influence of *sigB* inactivation on the ica expression in *S. aureus* MA12.2

The quantitative adherence assay was used as an indirect measurement for slime production as described previously. It has been investigated that *sigB* minus strain MA12.2 has lost its ability to form a biofilm compared to the biofilm positive *S. aureus* MA12 wild type. The *sigB* mutant strain had restored its ability to produce biofilm by complementation with plasmid pSK9 carrying a functional *sigB* gene. Addition of 3% NaCl to the growth culture, increased the biofilm production rate in both MA12 and MA12.2SK strains. No effect of NaCl on the *ica* expression in MA12.2 was detected (figure 54).

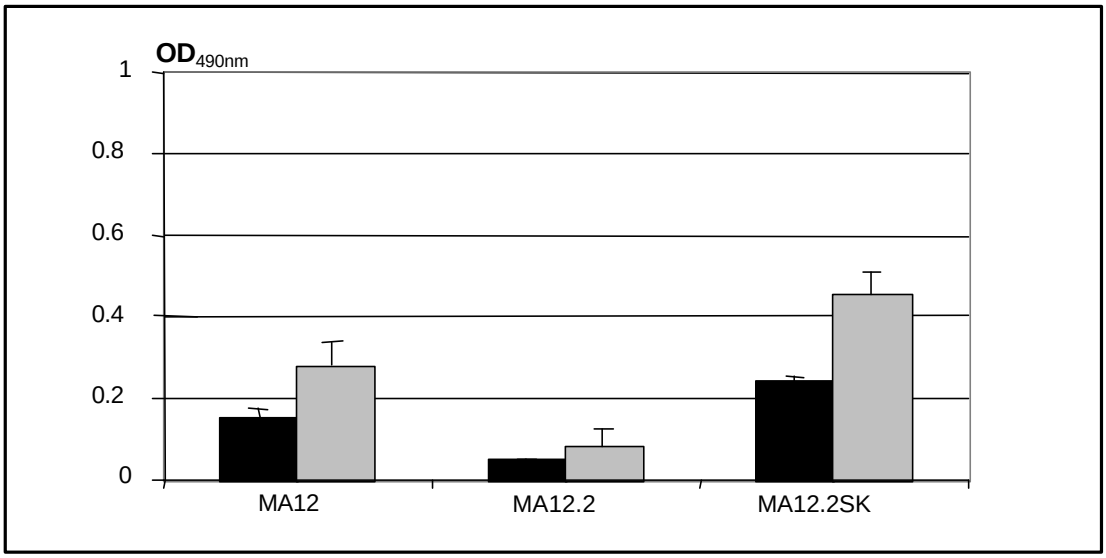

Figure (54): Biofilm production in *S. aureus* MA12, MA12.2 and MA12.2SK during incubation in ■ TSB medium and in ■ TSB supplemented with 3% NaCl using the quantitative adherence assay ($OD_{490\,nm}$).

To analyze the *ica* transcription, a Northern blot analysis was performed by growing the bacterial cells of MA12, MA12.2 and MA12.2SK at 37°C to the mid log phase in TSB supplemented with 3% sodium chloride. After harvesting, RNA extraction was done using the FastRNA kit, Blue (BIO101, Vista) and the FP120 FastaPrep cell disrupter apparatus (Savant instruments) as described in materials and methods. Thirty micrograms of RNA of each bacterial strains was applied to a 1.5% agarose-2.2 M formaldehyde gel in MOPS (morpholinepropanesulphonis acid) running buffer. RNA was blotted onto Nylon membrane, UV cros-linked, hybridized with a ^{32}P-labelled *icaA* probe in 50% formamid at 42°C overnight [118]. The *ica*A probe was amplified by PCR using the primers TAC CGT CAT ACC CCT TCT CTG and GAC AAG AAG TAC TGC TGC GT and DNA of the MA12 as a template. The Northern RNA analysis showed that an *ica* transcript of 3.5 kb was detectable in the wild type as well as in *S. aureus* MA12.2SK complemented with the pSK9. While, a very weak *ica* transcript was identified in the *sigB* mutant *S. aureus* MA12.2 (figure 55).

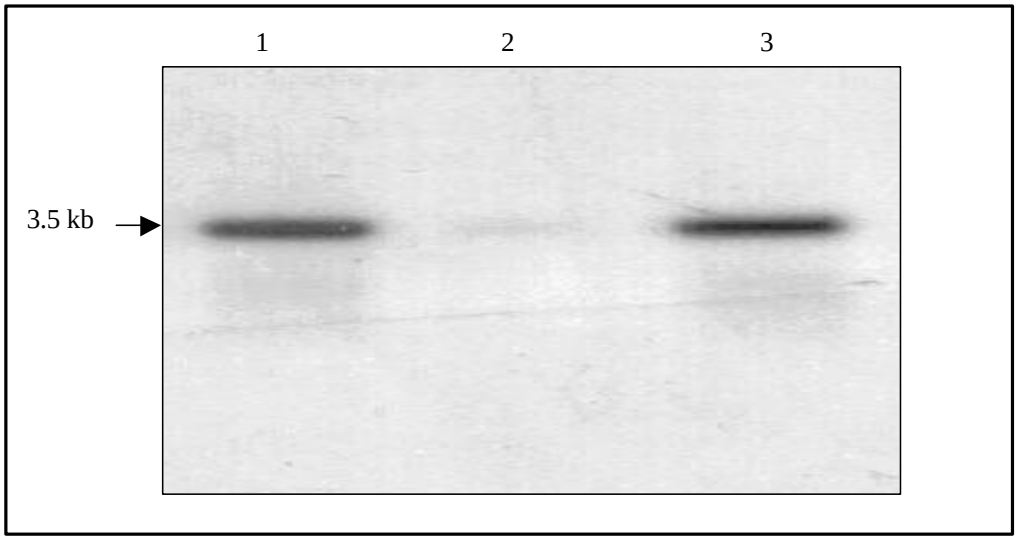

Figure 55: Northern blot analysis of *ica* gene transcription. Lane 1: the wild type strain (*S. aureus* MA12), lane 2: the *sigB* mutant (*S. aureus* MA12.2) and lane 3: the *sigB* complementant strain (*S. aureus* MA12.2SK).

Summary

- The expression of RNAIII of the *agr* operon in *S. epidermidis* is induced by high osmolarity conditions (3% and 4% NaCl).
- Inactivation of the *agr* operon in *S. epidermidis* was done by double cross-over replacement of the *agr* gene with an *agr::ermB* fragment.
- No clear effect of the *agr* inactivation on the biofilm production during the stationary growth phase was observed.
- We constructed a *sigB* mutant by insertion of an erythromycin cassette into the *sigB* operon of a biofilm positive *S. aureus*.
- Our data showed a dramatically inhibition of the *ica* expression in the *sigB* mutant *S. aureus* MA12.2 compared to the parental strain MA12.
- Complementation of the *sigB* mutant with a functional *sigB* gene restored the phenotype of the wild type.