VI. Discussion:

Bacteria, which have limited capacities to alter their environment or to physically escape, are obliged to modulate themselves in order to meet changes in environmental conditions. It is known that the expression of many bacterial virulence factors is frequently influenced by various environmental stimuli [51, 140]. Successful pathogens have evolved the means for survival within the varied growth conditions encountered both inside and outside their respective hosts. This adaptive response includes the expression of proteins which facilitate survival under different growth conditions as well as for sensing environmental change and, in turn, regulating gene expression [141].

In this study the environmental regulation of the *ica* gene of *S. epidermidis*, coding for biofilm formation, a major virulence factor involved in *S. epidermidis* pathogenesis, was investigated. Therefore, a transcriptional P_{ica}-*lacZ* fusion was constructed and integrated into the chromosome of *S. epidermidis* 220. In consequence, the β-galactosidase production is equivalent to *ica* mRNA production and could be quantified as an indicator of the *ica* promoter activity. Thus, our system was proven to be suitable to monitor the *ica* expression and it could be used to investigate the influence of environmental factors on the expression of the *ica* operon. Integration of the P_{ica}-*lacZ* fusion into the chromosome of the wild type excludes undesirable multicopy effects which often counteract the regulatory events under question in plasmid system. This construction gave us the opportunity to understand the molecular regulation of the *ica* operon encoding the enzymes for biofilm formation, independently from other adherence mechanisms of *S. epidermidis* that can be mediated by expression of other factors like surface associated proteins [71], a 140-kilodalton extracellular protein [82], or by cell wall hydrophobicity [64, 142-144]. As a control strain for our experiments, pSK2 carrying a P_{ica}-*lacZ* fusion was introduced into the *ica* negative skin isolate *S. epidermidis* 195 and the laboratory strain *Bacillus subtilis* 168.

For β-galactosidase-measurement, a chemically defined medium was prepared. This medium is consists of 5 defined groups of micro-elements, amino acids, vitamins, nucleic acids and macroelements, respectively, in which the basal expression level of *ica* gene was higher than in other media described. The medium is not optimal for the bacterial growth in comparison to the growth in LB or BHI medium. Similar results were observed by loo, et al. [145] who investigated that *S. gordonii* Challis biofilm formation was enhanced in a minimal medium but not in a nutritionally rich environment which indicates that sessile growth may represent a survival strategy in a nutritionally limited environment.

In this study several new findings are presented:

- Expression of *ica* is growth phase dependent. The maximum *ica* expression occurs in the late logarithmic phase.
- Expression of the *ica* operon is temperature regulated, showing an optimum at 42°C.
- *ica* expression is sensitive to pH changes in the medium.
- The presence of glucose in chemically defined medium (CDM) is essential for the *ica* expression.
- The *ica* expression is influenced by osmotic stress, showing a strong induction at high osmolarities.
- The addition of sub-lethal concentrations of ethanol, H_2O_2, SDS and urea to the growth medium of the bacteria also induces the *ica* expression.
- Subinhibitory concentrations of some protein inhibitors induce strongly the expression of the *ica* operon.
- The classical two component regulatory system (*agr*) has no clear regulatory effect on the *ica* expression in the stationary growth phase.
- Finally, the expression of the *ica* operon in *S. aureus* is clearly regulated by the alternative sigma factor (*sigB*).

1. Biofilm expression is growth dependent and controlled by environmental factors

Over time, bacteria have evolved sophisticated regulatory circuits to modulate their gene expression in response to changing environmental factors [132]. Well known examples are the capsule production of serotype 5 and 8 in *S. aureus* which is greatly influenced by environmental and bacterial growth conditions [146, 147], or the toxic shock syndrome toxin 1 (TSST-1) which synthesized at the exponential growth phase [148].

Different environmental circumstances seems to induce the biofilm in *S. epidermidis*. Previous investigations showed that the *ica* operon is affected by bacterial growth phase [77]. The data we present demonstrate that slime production by *S. epidermidis* cultures typically start early in the logarithmic phase of growth, there after the rate of slime production increases rapidly, reaching a peak during the late logarithmic and the stationary phase of bacterial growth. Maximum expression occurred after 20 hours. The *ica* expression was reduced in the late stationary phase of the bacterial growth. This indicates that the *ica* expression is growth phase dependent.

Temperature is known as an environmental factor governing the expression of virulence genes in various bacterial pathogens [132]. The increase of the temperature up to 37°C was shown to be a signal for human pathogens to enter their host from the environment. Therefore, many virulence genes and regulators are optimally expressed at 37°C, including *E. coli* P [149] and S fimbriae [150], the virulence regulator *virR* of *Shigella flexneri* [151], *hla* gene of *S. aureus* wood 46 [126] and the *Bordetella pertusis bvg* regulon [152]. Others are preferentially expressed at a high level at low temperature, e. g. flagella of Legionella pneumophila [153], the genes of colonization, toxin production and bacterial survival within the host in *Vibrio cholerae* [154, 155], and the surface antigen Myf as well as the enterotoxin *Yst* in *Yersinia enterocolitica* [140, 156]. Hurst *et al*. [157] showed a thickened cell wall, irregularly sized clumps surrounded by capsular material, and abnormal cell morphology in *S. aureus* during incubation at 45°C in a medium containing 1 M NaCl. Moreover, temperature is also known to affect the resistance of bacteria to antibiotics, e.g. incubation of *S. epidermidis* at temperature less than 37°C increased the MICs of both ciprofloxacin and o-floxacin [158], and regulate adhesion of *S. aureus* to collagen [159].

The expression of the *ica* operon in *S. epidermidis* was clearly influenced by changes in the incubation temperature. Maximal expression of this gene was shown during incubation of the bacteria at 42°C. Expression of *ica* was reduced by incubation at 45°C or temperatures less than 37°C, while these conditions are not suitable for the bacterial growth. However, no data are available about the expression of heat or cold shock protein in *S. epidermidis* which could be involved in the regulation of bacterial adaptation to environmental conditions.

Bacteria respond to changes of internal and external pH by adjusting the activity and synthesis of proteins associated with many different processes, including protein translocation, amino acid degradation, adaptation to acidic or basic conditions and virulence. pH mediates changes in gene expression through changes in the amount of energy coupling, solute and ion transport (e.g. K^+, Na^+), metabolite transport, regulatory molecules (e.g. amino acid, sugar) transport, competence and motility. The macrophage phagolysosome present examples of low-pH environments created by this facultative intracellular parasite [160]. In *Salmonella typhimurium*, the shift of pH from 7.6 to 5.8 altered the transcriptional level of at least 18 proteins (12 increased and 6 decreased), and three invasion genes [161-163], and increased the synthesis of other proteins known to be induced by other stress factors like heat shock proteins, DnaK, GroEL, GroES and GrpE [164]. It has been demonstrated that pH in *S. aureus* plays an important role in RNAIII expression and can regulate indirectly the

expression of many virulence genes [50]. In addition, sensitivity of *S. aureus* cells to some antibiotics is increased at pH 6.0 or 9.0 compared to that at pH 7.4 [165].

Our data show that biofilm formation is very sensitive to any change of the environmental pH. High expression of *ica* was demonstrated at pH 7-7.5. Acidic (less than pH 6.0) or alkaline (more than 8.0) environment had dramatically repressed *ica* expression. This reduction of the *ica* gene expression is accompanied with the reduction of bacterial growth. Similar results were reported by Dunne and Burd 1992 [166] who observed a significant reduction in biofilm production in 5 strains of *S. epidermidis* during the growth in acidic medium (less than 6.0), and by Loo et al., 2000 [145] who showed a clear reduction in *Streptococcus gordonii* biofilm at pH levels below 6 or above 8 while growth was reduced only when the pH was below 6 or above 10.5. These data indicate that tolerance of *S. epidermidis* to acidic or alkaline environment plays an important role in the regulation of biofilm production.

Osmolarity regulates the expression of many bacterial virulence factors. Some factors are induced by high osmolarity including the PapA protein or *PmpC* gene in *E. coli* [167-169], *Yst* gene encoding heat stable enterotoxin and a surface antigen (*Myf*) in *Yersinia enterocolitica* [156], The *cpxA, invG* and *prgH* genes of *Salmonella typhi* responsible for type III secretion pathway of virulence proteins [170], six different invasion genes in *Salmonella typhimurium* [163], the genes of colonization, toxin production and bacterial survival within the host in *Vibrio cholera* [155] are also regulated positively by high osmolarity. Others are preferentially repressed during high osmolarity, e.g., ompF gene of *E. coli* [168], staphylococcal enterotoxin B and C [171-173], and transcription of the *hla* gene in *S. aureus* [126]. Generally, Staphylococci are more salt tolerant than other bacteria, they have a more rigid cell wall and a higher internal turgor pressure. Staphylococci as well as *E. coli* were shown to require choline or glycine betaine to achieve maximal salt tolerance. High concentrations of glycine betaine and K^+ in staphylococci even in the absence of osmotic stress, may explain in part their remarkable salt tolerance [174].

Our results show that the *ica* expression is osmotically regulated. Interestingly, the highest level of *ica* expression was observed when cultivation occurred in the presence of 3-5% NaCl. The same result was obtained by Northern (mRNA) blot analysis of the *ica* transcription, as well as in the quantitative adherence assay in *S. epidermidis* 220 wild type, in which the rate of slime production was strongly increased during addition of 3-5% NaCl to the growth medium. However, *S. epidermidis* 561 isolated from a catheter-related urinary tract infection which was noticed *in vitro* as non biofilm producer, although it was known to carry the entire intact *ica* gene cluster (Ziebuhr et al., unpublished), this bacteria started to produce a strong

biofilm when cultured in the growth medium with high osmolarity. The high *ica* promoter activity at high osmolyte concentrations may reflect a specific adaptation of *S. epidermidis* to NaCl, which is a common component of the habitat of this bacterium.

Similar results were obtained by Loo *et al*. [145] who described that addition of 0.1 M NaCl induced biofilm formation in *Streptococcus gordonii*, however the molecular mechanism and regulation of biofilm in *S. epidermidis* differs from that in *Streptococcus gordonii*. It has been investigated that osmolarity increased the activity of several promoters in *S. aureus* as a result of an increase in the degree of negative supercoiling of plasmid DNA [175]. This might be also a possible mechanism for the effect of osmolarity on the *ica* expression. *S. epidermidis* cells can grow very well in BHI as well as other rich media even when supplemented with high concentration of NaCl while these environment contain enough osmoprotectant for the bacteria. Also, *S. aureus* is well adapted for living in high osmolarity medium and can actively accumulate the osmoprotectant glycin betain (GB) from the medium [176]. The *S. epidermidis* 220 produces high amount of biofilm in chemically defined medium (CDM) while it contains no osmoprotectant, and biofilm production may acts as an alternative mechanism to protect the bacteria from this type of stress. Vijaranakul *et al.* [177] showed that the grown cells of *Staphylococcus aureus* in a defined medium under conditions of high ionic stress (2.5 M NaCl) were significantly larger than cells grown under unstressed conditions, even though the cells grew much more slowly under stressed conditions, and analysis of the structure of peptidoglycan from these stressed cells showed a shorter interpeptide bridge than in peptidoglycan from unstressed cells. They showed that glycine betaine (osmoprotectant) inclusion in the high-NaCl medium resulted in cells with sizes and interpeptide bridges similar to those of cells grown under unstressed conditions. Moreover, our data showed inhibition of the *ica* expression in *S. epidermidis* during the growth in more than 5% (wt/vol) NaCl when the bacterial growth was strongly reduced. Similar data were obtained by Akiyama *et al*.[178], who showed that attachment of *S. aureus* cells isolated from dermatitis lesion to plastic tissue culture coverslips is suppressed in the presence of 10% (wt/vol) NaCl.

Our data suggest that addition of 3-5% (wt/vol) NaCl to the growth medium induced the *ica* expression in the skin isolate *S. epidermidis* 195-1 as well as in *Bacillus subtilis* 168-1 carrying the pSK2. These results might indicate the possibility of the presence of similar regulatory pathway controlling the P_{ica}-*lacZ* expression in both *S. epidermidis* and *Bacillus subtilis* under high osmolarity conditions.

Utilized carbon sources are essential components for the bacterial growth, and have an important role in the regulation of different virulence factors. The presence of rapidly metabolizable carbon sources, especially glucose, results in a co-ordinated change of metabolic functions in many bacteria. Regulation is achieved by altering the activity of a number of proteins, which then lead to the differential expression of operons encoding metabolic enzymes, a process termed carbon catabolite repression [179]. For example, addition of glucose to the growth medium of *S. xylosus* reduced the transcription initiation of *lacPH* gene [180], or inhibited the activity of the *exp* gene product in *S. aureus* [181]. The expression of extracellular enterotoxin C [182], *agr* gene [183], and *hla* gene [126] in *S. aureus* as well as 60 general stress proteins and expression of *xylA* gene in *Bacillus subtilis* [184-186], were clearly inhibited during addition of glucose.

In contrast, glucose limitation reduces toxin yields of Clostridium up 20- to 100-fold in defined media [187]. The hemagglutinin expression in *S. epidermidis* which has a strong association with adherence and biofilm production is also induced by the addition of glucose to the growth medium, but it is markedly inhibited by beta-lactose and its monosaccharide [188].

Our data indicated that carbon sources play an important role in *ica* gene regulation in *S. epidermidis*-220. We tested the effect of different sugars on the *ica* expression. The addition of glucose clearly stimulated the *ica* expression. Maximum β-galactosidase activity was detected by the addition of 1.5-2% glucose to the growth medium of *S. epidermidis* 220-1. These data were confirmed by Northern blot analysis of the *ica* transcription which indicates that high transcription appeared in the presence of 1.5-2% glucose in the medium, as well as in the quantitative adherence assay, in which an increase in biofilm production was investigated under the same conditions. The addition of 1-2% (wt/vol) glucose to the growth medium of the skin isolate *S. epidermidis* 195-1 carrying the pSK2 with P_{ica}-*lacZ* caused a week induction of the *ica* expression. But no induction of *ica* expression was observed when glucose was added to the growth culture of the *Bacillus subtilis* 168-1 carrying the plasmid pSK2. Moreover, expression of *ica* during the bacterial growth curve changed when 1.5-2% glucose was added to the growth medium. Maximum expression occurred during the beginning or middle of the logarithmic phase (10 hours after inoculation) when medium contained sufficient glucose. This result indicates that the *ica* expression is active during the logarithmic phase as long as no glucose starvation started. Glucose limitation in the stationary or late stationary phase leads to the inhibition of the *ica* expression. It is conceivable that the presence of glucose might inhibit a negative regulator of the *ica* expression which is subject to

catabolite repression. However, such regulators remain yet to be elucidated. The same result was reported by Deighton and Balkau, 1990 [189] who reported that, adherence of *S. epidermidis* is enhanced by addition of glucose to the growth medium. Also Mack *et al.* [190] showed a 32-64-fold increase in the amount of the PIA antigen in bacterial extract of *S. epidermidis* cells parallel to increased biofilm and adherence to plastic tissue culture plate, when the cells were grown in TSB supplemented with glucose compared to the extracts of cells grown in TSB lacking glucose.

However, in our experiments, addition of other carbon sources like fructose and maltose did not support bacterial growth; therefore, the *ica* expression was clearly inhibited. Neither *ica* expression, nor growth rate alterations were observed during addition of sucrose to the growth medium.

The effect of ethanol, hydrogen peroxide, sodiumdodecyle (SDS), and urea on biofilm production was studied. Previous studies indicated that ethanol shock can increase the expression of some bacterial genes, e. g. enhancement of 8 different cytoplasmic proteins including HSP60 and HSP10 [191], the Asp23 protein [123] in *S. aureus* and 60 general proteins in *Bacillus subtilis* [186], respectively. Ethanol shock in gram negative bacteria plays also a major role in gene regulation. Stress imposed by a continuous feed of high ethanol shock increased a phage shock protein A (PsoA) [167], *acrA* and *acrB* in *E. coli* [192] and antibiotic production in *Pseudomonas fluorescens* which is mediated by the autoinducer (*N*-acyl-homoserine lacton) [193].

Our data indicate that biofilm production in *S. epidermidis* is significantly increased by exposure of the cells to ethanol. Maximum level of biofilm production was observed by the addition of 0.5% ethanol. It is not clear how ethanol shock stimulates the induction of the *ica* operon, but it is known that hydrophobic organic solvent molecules intercalate into biological membranes, and as a result, lipid interaction is weakened and the membrane structure is disturbed [169]. This will due to stress for the bacteria, and probably in turn, causes an induction of the *ica* expression.

Hydrogen peroxide (H_2O_2) is reactive superoxide which can cause damage and death for bacterial cells. The H_2O_2 effect may be mediated via the generation of hydroxyl radicals, alternatively, it may act indirectly, perhaps by causing membrane damages [194]. Superoxide itself probably does not play an important role in host-mediated killing of *S. aureus*, as *S. aureus* is resistant to externally generated superoxide [176]. The inducible resistance to oxidative stress in *Bacillus subtilis* is caused by the specific induction of genes of the peroxide regulon, including the genes encoding vegetative catalase (KatA), alkyl hydroperoxide

reductase (AhpCF), and DNA-binding protecting protein (MrgA) [195]. When bacteria aggregate in the form of a biofilm, catalase is extremely effective in protecting bacteria from damage by H_2O_2 [196].

To verify the effect of H_2O_2 on biofilm production in *S. epidermidis*, sublethal concentrations of H_2O_2 was added to the growth culture of the biofilm forming *S. epidermidis* 215. Addition of 0.01% (vol/vol) H_2O_2 to the growth medium increased the biofilm production 5-fold compared to that in the medium lacking H_2O_2. This might indicate that biofilm can act as a mechanism to withstand this unfavourable condition and prevents the bacteria from oxidative stress [197].

Detergents have a lethal effect on bacterial cells by damaging the bacterial cell membrane. It has been reported that the rates of synthesis of 45 and 34 proteins in *Enterococcus faecalis* were enhanced after treatment with bile salts and SDS, respectively. Despite of the overlap of 12 polypeptides, the protein profiles induced by the two detergents were different, suggesting that these detergents trigger different responses in *E. faecalis* [198]. It was unknown whether or not detergents alter the biofilm production in *S. epidermidis*. We added sublethal concentrations (0.0012-0.02% (wt/vol)) of SDS to the growth culture of *S. epidermidis* 215. A 8-fold induction of biofilm production was determined by the quantitative adherence assay when 0.01% (wt/vol) SDS was added to the growth medium of the bacteria. It is not clear how SDS enhanced the biofilm production, but it seems that the bacteria produce more biofilm as a result of SDS stress and as a barrier to reduce the lethal effect of the detergent.

It has been investigated that the presence of urea also positively regulates the biofilm production in *S. epidermidis*. It is possible that the presence of urea may create a pH environment which is more favourable for growth and gene expression.

It is suggested that the multicellular organization in biofilms is a crucial mechanism for bacteria to withstand unfavourable external conditions [199, 200]. Cellular adaptation can be achieved by the activation of regulatory networks as well as through changes in the genetic material [201]. The induction of biofilm formation by external factors, especially by detergents, ethanol and H_2O_2 which are common disinfectants, might explain the high survival rate of Staphylococci in the hospital environment.

Alternative sigma factors of the RNA polymerase complex are known to be involved in the regulation of gene expression in many bacteria [27, 42, 48, 49, 202, 203]. Extensive studies in

B. subtilis, *E. coli*, *P.aeroginosa* and *S. aureus* give evidence that the stress response of the bacterial cell is controlled by alternative transcription factors. They function as global regulators which also influence the expression of many virulence-associated genes, including biofilm formation [202]. Our data on the biofilm expression in *S. epidermidis* suggest that the generation of biofilm in this organism might be associated with the expression of the alternative transcription factor *sigB*. Thus, Northern blot analysis revealed that the *sigB* transcription is induced under external stress conditions. The same conditions were shown to induce the formation of biofilm in *S. epidermidis*. In both *S. epidermidis* 215 and 567 the *sigB* transcription was growth phase dependent, with a maximum *sigB* transcription during the stationary growth phase. Moreover, *sigB* transcription was strongly induced when the bacteria were grown in high osmolarity environment. These conditions strongly induced the *sigB* transcription even in the logarithmic phase of growth compared with that in cells grew in a low osmolarity environment.

2. Alteration of the *ica* expression by subinhibitory concentration of antibiotics

Effective antibiotic treatment of biofilm forming bacteria is difficult because bacteria organized in biofilm exhibit an inherent higher resistance to antibiotics than planktonic cells [204, 205]. For example, in a study on twenty *P. aeroginosa* strains and nineteen *S. aureus* strains isolated from burn wounds, an enhanced resistance against bacterial action of the applied antibiotics was observed when bacterial cells were attached to polystyrene surface [206].

Under clinical conditions, during antibiotic treatment, micro-organisms often grow at subinhibitory concentrations. This may lead to altered adhesive cell surface properties and to a disruption of the indigenous microflora. In addition, biofilm production might be favoured. Previous studies showed that subinhibitory antibiotics concentrations induced the expression of some bacterial virulence factors such as epidermolytic toxin A protein in *S. aureus*, which is strongly induced when the bacterial cells are grown in the presence of subinhibtory concentrations of novobiocin, a DNA gyrase inhibitor [175], the induction of α-toxin in *S. aureus* by the addition of subinhibitory concentrations of methicillin to the bacterial culture [122], or the induction of PspA synthesis in *E. coli* by globomycin, an inhibitor of the lipoprotein processing [207]. It has been reported that oncostatic chemotherapeutics and non-

antibiotic antimicrobial agents play an important role in *S. epidermidis* adhesion to intravascular catheters and plastic materials [197, 208, 209].

In this study we have analyzed the influence of subinhibtory antibiotic concentrations on the *S. epidermidis* biofilm formation. Our result clearly indicate that the expression of the *ica* operon can be strongly enhanced by the streptogrmin mixture pristinamycin and tetracycline. Whereas tetracycline is rarely used for the treatment of staphylococcal infections, pristinamicin is a promising new substance which has proven to act very efficiently in the treatment of serious infections caused by multiresistant gram positive cocci [136, 210, 211]. Recent studies give unequivocal evidence that the substance is also active against *S. aureus* and *S. epidermidis* cells which are organized in biofilm [212, 213]. These finding seems to be in contrast to the data presented here indicating that pristinamycin has a positive effect on the staphylococcal biofilm formation. The explanation for this is that our approach differ markedly from that of the studies mentioned above. In the experiments performed by Berthaud and Desnottes [212] and by Hamilton-Miller and Shah [213] bacteria were initially grown in antibiotic free medium until a staphylococcal biofilm has formed. Subsequently, the adherent bacteria were treated by inhibitory dosage of pristinamycin which led to an efficient killing of the bacteria within the biofilm. In contrast, we exposed the bacteria to very low dosages of antibiotics (1/70 to 1/2 of the MIC) during the growth and the development of the biofilm. Under these subinhibitoy concentrations, the expression of the *ica* operon was found to be induced on the transcriptional level and the formation of biofilms was strongly enhanced. According the results of the studies from above, no *ica* induction was observed when the antibiotic concentrations were increased to higher levels (> 1/2 of the MIC) where bacterial growth inhibition occurred.

The induction of *ica* expression in *S. epidermidis* 220-1 was observed by applying sub-MICs of tetracycline, pristinamycin or quinupristin and dalfopristin, separately, to the growing cells of biofilm-forming *S. epidermidis*. All these substances are protein synthesis inhibitors which act at the bacterial ribosome. These data were confirmed by Northern blot analysis of the mRNA transcription level of the *ica* operon and by a quantitative adherence assay for biofilm formation in both *S. epidermidis* 220 and *S. epidermidis* 561. It has been investigated that P_{ica}-*lacZ* expression in *S. epidermidis* 195-1 was induced by the MICs of these antibiotics, while no induction in the P_{ica}-*lacZ* expression was noticed when *Bacillus subtilis* 168-1 cultured with MICs of the mentioned antibiotics. This might indicate that the regulatory pathway for the *ica* induction by antibiotics in *S. epidermidis* is differ from that in *Bacillus subtilis*. Moreover, a strong P_{ica}-*lacZ* expression in *S. epidermidis* 220-1 as well as a strong biofilm

production in *S. epidermidis* 220 was investigated by the short exposure of the bacterial cells to high concentrations of pristinamycin, tetracycline, dalfopristin, and quinupristin. This may be important during the clinical treatment with pristinamycin since the elimination half-live of dalfoprstin and quinupristin in blood is 0.4-0.5 and 1 hour, respectively [214].

Viriginiamycin , a streptogramin compound which is used as growth promoter in husbandry, was also able to induce the *ica* expression in *S. epidermidis* 220-1 to a similar extend as quinupristin/dalfopristin, and the same effect on *S. epidermidis* 220 biofilm formation was investigated by quantitative adherence assay. These observation suggest that the activating process might depend on structural feature of the streptogramin compounds. Apart from erythromycin and chloramphenicol, which had a weak induction effect, other protein synthesis inhibitors as clindamycin, fusidic acid or gentamicin did not enhanced the the *ica* expression. Except for the effects ofloxacin as a DNA-gyrase inhibitor and penicillin, which had a weak inhibition effect, other cell wall synthesis inhibitors like oxacillin, teicoplanin and vancomycin did not altered the *ica* expression.

Our results are in agreement with the previous observations on the effect of clindamycin, fusidic acid, gentamicin, ofloxacin, penicillin, teicoplanin and vancomycin on *S. epidermidis* adherence to plastic materials [143, 215-218]. These studies indicated that there is no change in staphylococcal adherence to plastic material during the growth in presence of these antibiotics. However, none of the reports investigated the *ica* expression in detail.

The data here are *in vitro* results and it is temping to speculate whether or not they will have clinical consequences. Pharmacokinetic studies show that pristinamycin reaches sufficiently high concentrations in most of the tissues analyzed [214]. However, the substance does not enter the central nervous system and the concentrations measured there would match the sub-MICs which can activate the *ica* expression [214]. This could play a role in patients carrying intrathecal shunt system who are endangered by line-associated staphylococcal infections [219]. In this respect, it is notworthy that also a short exposure of the bacteria to high antibiotic concentrations can induce the *ica* expression and, therefore, trigger the staphylococcal biofilm formation. At present there are no reports suggesting that the use of quinupristin/dalfopristin or tetracycline would lead to more infections by biofilm forming Staphylococci. Nevertheless, the data presented here emphasize that antibiotics should be adequately used in order to avoid low subinhibitory concentrations as well as unnecessary high concentrations without inhibitory effects.

3. The role of the *agr*–quorum sensing system in the regulation of the *ica* expression in *S. epidermidis*

Bacteria coordinate their colonization and association with higher organisms by intercellular communication systems (denoted quorum sensing systems). They function via small diffusible signal molecules. In gram negative bacteria, the signal molecules are synthesized from precursors by a synthetase protein I , and they interact with a transcriptional activating R protein to induce the expression of different target gens [220-223].

The expression of most virulence factors in *S. aureus* and *S. epidermidis* is controlled by the global regulator *agr* . The *agr* locus is consists of two divergent transcriptional units (RNAII and RNAIII). The RNA III is the effector molecule of the operon, which regulates the expression of many exoproteins (e.g., toxic shock syndrom toxin 1, alpha-toxin, and tissue-degrading enzymes), surface proteins (e.g., protein A, coagulase, and fibronectin-binding proteins), and capsular polysaccharides in a growth phase dependent manner [224-231].

The transcription of RNAIII is highly dependent on the activation of the *agr* genes (*agrA*, *agrB*, *agrC*, and *agr*D) encoding RNAII. It has been shown that *agrB* and *agrD* encode small octa-peptide pheromones which are the signal for the quorum-sensing system [25, 93]. *AgrA* shows sequence homologies to response regulators, while *agrC* represents a histidine kinase signal tranducer [26] of a classical two-component regulatory system [232]. AgrC is thought to bind the octapeptides and subsequently phosphorylates AgrA. The respective promoters for RNAII and RNAIII (P2 and P3) are both thought to be autocatalytically activated by phosphorylated AgrA [26]. Both promoters are also activated by a second regulatory locus, *sar* [34, 233]. However, *sar* influences the expression of virulence factors not only via *agr* but also by independent mechanisms [234].

As in *S. aureus*, the *agr* system in *S. epidermidis* appears to be responsible for the upregulation of many exoproteins and downregulation of many surface bounded and surface associated proteins in the stationary growth phase, although a small number of proteins seems to be under the opposite control [235]. The deletion mutant of *agr* gene in *S. epidermidis* TüF38 showed a clear reduction in both proteolytic and lipolytic activity , but generally, the amount of surface proteins and surface-associated proteins was higher in the mutant than in the wild type [235].

In our study, we investigated the transcription of the effector molecule of the *agr* system (RNAIII) under conditions which are known to induce the *ica* expression. It is known that both the transcription of RNAIII and biofilm production is induced during stationary growth

phase, and we showed by Northern RNA analysis of RNAIII transcription that the addition of 3 and 4% (wt/vol) NaCl to the growth medium increased both the transcriptional level of the RNAIII and the *ica* transcription. In order to investigate whether or not the *agr*-quorum sensing system controls the production of biofilm in *S. epidermidis* 220, we constructed an *agr* mutant by deletion of 478 bp including a 150 bp of *agr*B, the entire P2 and P3, and a 121 bp of RNAIII. The *agr* gene was replaced by an erythromycin cassette by homologous recombination with a temperature sensitive plasmid pSK5 carrying an *agr::ermB* fragment. Correct insertion was proven by direct sequencing of the flanking regions, and by Southern hybridization. The *S. epidermidis* 567-1 *agr* deletion mutant showed no clear alteration in biofilm production compared to that of the isogenic strain *S. epidermidis* 220. This indicates that the *agr*-quorum sensing does not significantly influence the biofilm production during the stationary growth phase in this particular *S. epidermidis* strain. Additional studies are needed to identify other regulatory systems that control the biofilm production in *S. epidermidis*. However, this result does not exclude minor effects of this important regulator on the *ica* expression. Thus, it is conceivable that the *agr*-system might act only in the exponential growth phase or in association with distinct external growth conditions. But to substantiate this suggestion, more studies are needed.

4. The role of the alternative transcription factor *sigB* in the *S. aureus* biofilm production

S. aureus is frequently isolated in association with peripheral intravascular catheters, endotracheal and tracheotomy tubing, peritoneal dialysis tubing, corneal infections related to contact lens wear, prosthetic joint and vascular graft infections [236-242]. *S. aureus* also has the ability to produce biofilm on prosthetic biomaterials [80, 243, 244]. Recently, the presence of the *ica* operon and the molecular mechanism of PIA production in *S. aureus* were described by Cramton *et al.* [83]. There are numerous studies on the virulence and the ability of *S. aureus* to produce biofilms, but the knowledge on biofilm regulation is limited.

Our results on biofilm regulation by high osmolarity indicate, that biofilm expression is clearly increased during the addition of 3% (wt/vol) NaCl to the growth medium of the *S. aureus* MA12. It is well established, that the transcription factor *sigB* which is known to play a crucial role in the regulation of gene expression upon major changes in the environment is induced by the high osmolarity [245, 246]. In our study we constructed a *sigB* mutant by

insertion of an erythromycin cassette by homologous recombination with the plasmid pSK8 carrying a *sigB::ermB* fragment and a temperature sensitive origin. The correct mutant was checked by Southern DNA analysis of the *sigB* specific fragment, Northern RNA analysis of *asp*23 transcription which possess a SigB dependent promoter (the transcription failed in *sigB* mutational strain MA12.2), and PCR analysis of the flanking regions. Moreover, the mutant-strain 12.2 lost its yellow pigmentation and showed enhanced hemolysis on rabbit erythrocyte agar plates. Both are phenotypical features of *sigB* minus strains.

Our data showed a dramatically inhibition of the *ica* transcription level in the *sigB* mutant *S. aureus* MA12.2 compared to the parental strain MA12. These data were confirmed by a quantitative adherence assay for the biofilm formation using a 96 well, tissue culture plate, which showed a clear decrease in biofilm formation in the *sigB* negative *S. aureus* MA12.2. The complementation of the *sigB* gene in *S. aureus* MA12.2 *sigB* mutant strain was done by introducing the plasmid pSK9 carrying a functional *sigB* gene into the *sigB* minus strain. The complementation of the *sigB* was evaluated by the Northern RNA analysis of *asp23* (restored), and both yellow pigmentation (restored) and hemolysis on rabbit erythrocyte containing agar plates (inhibited). Our results showed a positive *ica* transcription as well as a positive biofilm in *sigB* complemented strain *S. aureus* MA12.2SK to the same extend as in the parental strain MA12. Moreover, biofilm induction by 3% (wt/vol) NaCl was only investigated in both wild type and the *sigB* complemented strains. These data indicate that the expression of *ica* gene in *S. aureus* is controlled at least by the alternative transcription *sigB*. These observations are consistent with the results obtained by Adams and McLean, 1999 [202], who reported an inhibition of biofilm production in *E. coli* by deletion of the stationary sigma factor *rpoS*. Finally, additional studies will be required to completely elucidate the detail molecular mechanism(s) of *ica* regulation by *sigB* or other possible regulatory systems.

From experiments in *B. subtilis* and *S. aureus* it is known that SigB recognizes and binds to distinct promoter sequences infront of SigB dependent genes. However, neither in *S. aureus* nor in *S. epidermidis* the *ica* promoter has obvious similiarities to the defined sequences analyzed so far. Therefore, it is tempting to speculate that the *ica* activation is not directely mediated by SigB, but might be indirectely controlled by other SigB-dependent regulatory elements which remain to be elucidated. Figure 56 suggests a model for possible regulatory pathways influencing the expression of the *ica* operon arising from the data of this study. Taken together, both environmental stress and subinhibitory concentrations of distinct antibiotics can trigger the staphylococcal biofilm formation. However, these signals obviously activate the *ica* expression by the mediation of different regulatory elements.

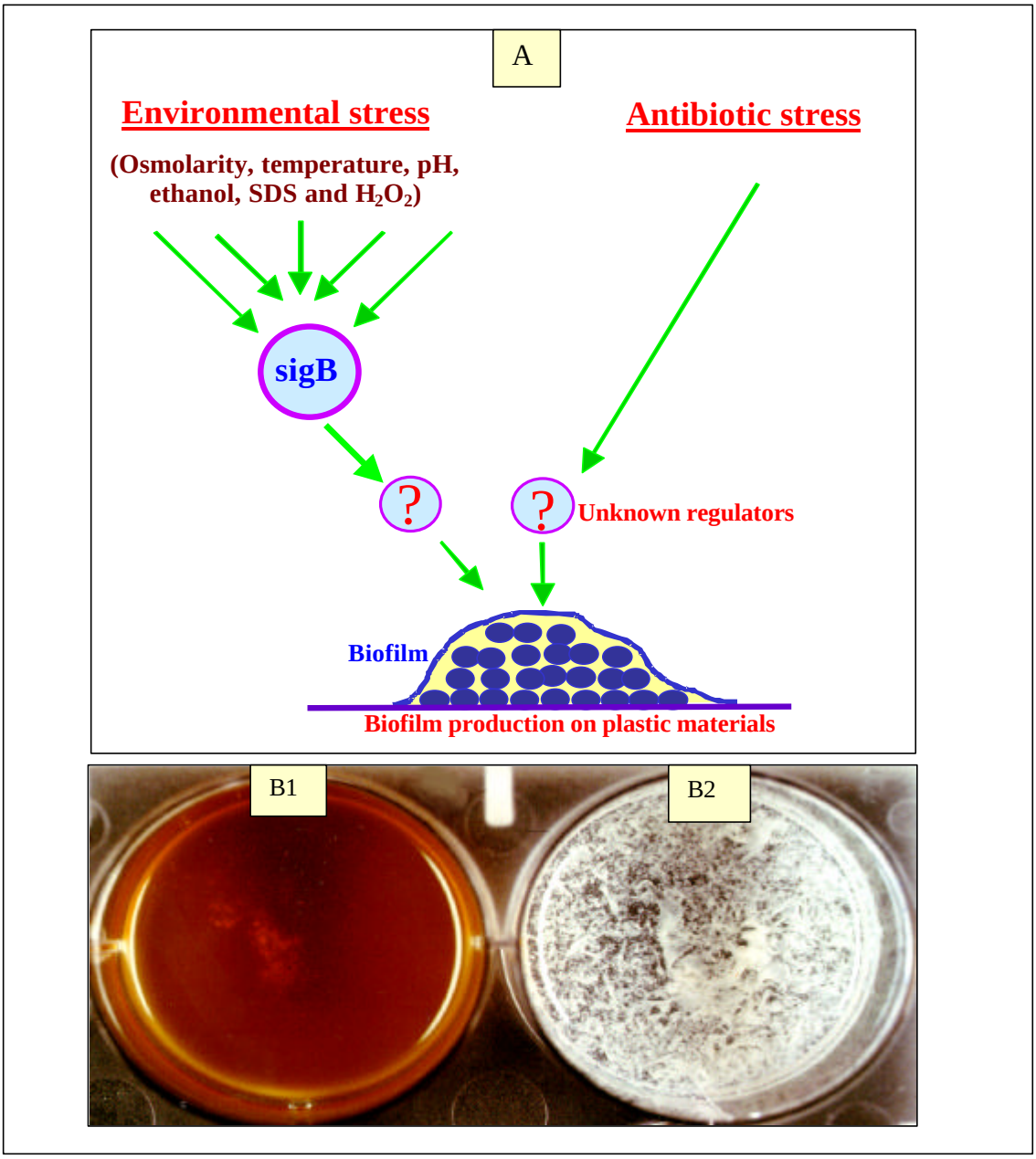

Figure 56: (A) a model for possible regulatory pathways controlling the expression of the *ica* operon in staphylococci. A weak biofilm production (B1) by incubation of the bacteria in BHI medium compared to a strong production (B2) by incubation of the bacteria in chemically defined medium supplemented with 4% NaCl.

However, these signals obviously activate the *ica* expression by the mediation of different regulatory elements. One of these factors is the alternative transcription factor SigB which seems to play a key role in the induction of biofilm formation in response to environmental stress. The detailed investigation of the other elements involved in this process will represent an interesting scientific field which could contribute to the development of new antiadhesive therapeutic strategies for the treatment of staphylococcal infections.