IV. Methods

1. Isolation of plasmid DNA

1.1 Small-scale preparation of plasmid DNA [128]

Bacteria were grown overnight in 5 ml of LB medium, containing the necessary antibiotic. Bacterial cells from a 1.5 ml overnight culture were collected by centrifugation (2 minutes), and the cell pellet was fully resuspended in 150 µl of ice-cold solution I by vigorous vortexing. 150 µl of freshly prepared solution II were added, mixed with the solution by inverting the tube, and incubated for 5 minutes on ice. After addition of 150 µl of ice-cold solution III, the mixture was vortexed and the tube stored for another 5 minutes on ice. The cell debris and chromosomal DNA were precipitated by centrifugation for 5 minutes, and the supernatant was transfered to a new microtube containing a mixture of 900 µl L6 buffer and 50 µl Diatomaceous Earth solution. After vortexing the mixture, incubation for 1 minute at room temperature followed. The supernatant was removed through filtration, and the Diatomaceous Earth was washed with 2 ml wash buffer. The filter was shortly centrifuged to remove the rest of the wash buffer. To elute the Plasmid-DNA, 50 µl H_2O was added to the filter, and incubated for 5 minutes at room temperature. Plasmid-DNA were collected by centrifugation, and frozen at –20°C .

- **Solution I** 50mM Tris-HCl pH 8.0
  10 mM EDTA pH 8.0
  100 µg/ml RNase A
- **Solution II** 0.2 N NaOH
  1% SDS
- **Solution III** 3 M Na-acetate
-
- **L6- Buffer** 100 ml 0.1 M Tris-HCl pH 6.4
  8.8 ml EDTA pH 8.0
  13.2 ml H_2O
  2.6 ml triton
  120 g/l guanidinthiocyanate

- **Wash buffer** 50 mM NaCl
  10 mM Tris-HCl pH 7.5
  50% EtOH

- **Diatomaceous Earth solution**

  10 g diatomaceous earth
  50 ml H_2O
  500 µl HCl conc.

1.2 Large-scale preparation of plasmid DNA (Qiagen plasmid-Handbook, 1997)

Bacteria were grown overnight in 2 ml of antibiotic containing LB medium. 50 ml of fresh LB medium were inoculated with 1 ml of the overnight culture and grown again overnight. The bacterial cells were harvested by centrifugation at 6000 rpm in JA20-tube for 15 minutes at 4°C. The supernatant was removed, and the pellet were resuspended compeletely in 4 ml of ice-cold solution I. After addition of 4 ml solution II, the mixture was incubated 5 minutes on ice, and the addition of 4 ml solution III followed. The reaction was mixed throughly but gently by inverting the tube several times and then incubated on ice for 15 minutes. Cell debris and chromosomal DNA were precipitated by centrifugation (15000 rpm) at 4 °C for 30 minutes. After centrifugation, the supernatant was transfered immediately into a fresh tube, and applied to the Qiagen-tip 100 which was previously equilibrated with 4 ml of buffer QBT. The Qiagen-tip was washed 2 times with 10 ml QC-buffer, later the plasmid DNA was eluted by using 5 ml of buffer QF. The plasmid DNA was pelleted by addition of 3.5 ml isopropanol. After centrifugation (13000 rpm) for 30 minutes at 4°C, the plasmid DNA was washed with 5 ml of cold 70% EtOH, dried in a speed-vac, and dissolved in 50 µl H_2O.

- **Solution I, II, and III** prepared as described above.

- **QC buffer** 1.0 M NaCl
  50 mM MOPS
  15% EtOH

- **QF buffer** 1.25 M NaCl
  50 mM Tris-HCl
  15% EtOH.

- **QBT buffer** 750 mM NaCl
  50 mM MOPS
  15% EtOH
  0.15% triton X-100

2. Phenol treatment of DNA preparation

An equal volume of equilibrated phenol was added to the DNA containing solution, mixed and centrifuged. The upper aqueous phase, which contains the DNA, was placed into another centrifuge tube and supplemented with antibiotic equal volume of phenol:chloroform:isoamylalcohol (25:24:1). After mixing and centrifugation, the rest of phenol was removed by adding half the volume of chloroform:isoamylallcohol (24:1). Following centrifugation, the DNA (present in the upper aqueous phase) was precipitated with EtOH.

3. Ethanol precipitation of DNA

To precipitate the prepared DNA, sodium acetate at a final concentration of 0.3 M was added to the prepared DNA solution. 2.5 volumes of 96% EtOH were added and chilled to –80 °C for 30 minutes. After incubation, the DNA was precipitated by centrifugation at 13000 rpm for 10 minutes at 4 °C, and washed with 70% EtOH, centrifuged again and finally dried in a speed-vac and dissolved in the desired volume of H_2O.

4. DNA and RNA quantitation

For DNA and RNA amount quantitation, spectrophotometric measurement of the amount of UV irradiation absorbed by the bases was performed. Readings were taken at wavelengths of 260 nm and 280 nm. An OD of 1 corresponds to approximately 50 µg/ml of double-strand DNA, for RNA 40 µg/ml and for oligonucleotides 33µg/ml. The ratio between the reading

260 nm and 280 nm (OD_{260}/OD_{280}) provided an estimate of the purity of the prepared nucleic acid. Pure preparations of DNA and RNA have an OD_{260}/OD_{280} ratio of not less than 1.8 and 2.0 respectively.

5. Digestion of plasmid DNA with restriction enzymes

Reactions typically contain 0.2-1 µg of DNA in a volume of 20 µl or less. 1 unit of restriction enzyme was added and mixed by tapping the microtube. One unit of enzyme is usually defined as the amount required to digest 1 µg of DNA to completion in 1 hour in the recommended buffer and at the recommended temperature in a 20 µl reaction. Usually the restriction buffer of the company providing the restriction enzymes was used. Alternatively, universal restriction buffer was used. The reaction was stopped by the addition of 0.5 M EDTA (pH 7.5) to a final concentration of 10 mM.

- **Restriction digest**

  DNA, restriction buffer (1x), restriction enzyme (1 unit/µg DNA), H_2O

- **Universal restriction buffer**

  Tris-acetate (pH 7.9) (330mM), K-acetate (660 mM), Mg-acetate (100mM), DTT (5 mM), BSA (1 mg/ml), H_2O. The buffer was sterile filtered and stored at – 20 °C.

6. Agarose gel electrophoresis for separation of DNA fragments

The standard method used to separate, identify, and purify DNA fragments is electrophoresis through agarose gels. DNA molecules of different size migrate at different rates through the gel. Bands of DNA in the gel are stained with low concentrations of the fluorescent, intercalating dye ethidium bromide; as little as 1 ng of DNA can then be detected by direct examination of the gel in ultraviolet light. Different concentrations of agarose gels guarantee an efficient separation of DNA molecules.

The following amounts of agarose in gels were used to separate DNA molecules:

% agarose	Efficient range of separation of linear DNA molecules (kb)
0.3	60-5
0.6	20-1
0.7	10-0.8
0.9	7-0.5
1.2	6-0.4
1.5	4-0.2
2.0	3-0.1

The agarose gel loading buffer was added to the DNA samples before loading them onto the agarose gel. As DNA size standards, either a 1 kb or a 100 bp production DNA marker (MBI), were applied. Electrophoresis at approximately 100 volts followed until the DNA molecules were separated.

6.1 DNA-size marker (MBI)

Fragment (Nr.)	100 bp marker	1 kb marker
1	1000 bp	10000 bp
2	900 bp	8000 bp
3	800 bp	6000 bp
4	700 bp	5000 bp
5	600 bp	4000 bp
6	500 bp	3500 bp
7	400 bp	3000 bp
8	300 bp	2500 bp
9	200 bp	2000 bp
10	100 bp	1500 bp
11	80 bp	1000 bp
12		750 bp
13		500 bp
14		250 bp

7. Elution of DNA fragments from agarose gels (Gene Clean-Kit)

The desired DNA band was excised from the ethidium bromide stained gel with a razor blade under long-wave UV light as fast as possible. The approximate volume of the gel slice was determined by weight estimation of the agarose slice (0.1 g equal to approximately 100 µl) and then transfered to a plastic cap. 3 volumes of NaI (6M) were added to the agarose gel piece (the final concentration of NaI should be more than 4M), and the cap was incubated for 5 minutes at 45-55 °C until the agarose gel completely dissolved. 5-10 µl Glassmilk were added, mixed and chilled on ice for 5 minutes. The silica matrix (with the bound DNA) was pelleted by centrifugation for approximately 5 seconds at 13000 rpm. To wash the DNA, the pellet was resuspended in 200 µl "New wash" buffer, and centrifuged for a few seconds (this step was repeated two times). The rest of the "New wash" was discarded, and the pellet was dried by leaving the cap open for 5-10 minutes. The white pellet was resuspended in a volume equal to the volume of the Glassmilk used, and centrifuged for 30 seconds. The supernatant containing the eluted DNA was carefully removed, placed in a new eppendorf cap and stored at 20 °C.

8. Dephosphorylation of DNA fragments

The terminal 5´phosphate can be removed from DNA by treatment with calf intestinal alkaline phosphatase (CIP). 1 µg of restricted and purified vector DNA was dissolved in 180 µl of 10 mM Tris-HCl (pH 8.3) and supplemented with 20 µl of 10 x CIP dephosphorylation buffer. In the case of protruding 5´termini, 1 unit of CIP was added per 100 pmoles of 5´terminal residues and incubation followed at 37 °C for 30 minutes. In the case of blunt or recessed termini, 1 unit CIP was added per 2 pmoles, and followed by incubation at 37 °C for only 15 minutes. Then, another aliquot of CIP was added and incubation was continued for a further 45 minutes at 55 °C (2 µg of linearized plasmid DNA 5 kb in length contain approximately 1.4 pmole of 5´terminal phosphate residues).

2 µl of 0.5 M EDTA (pH 8.0), 10 µl of 10% SDS and 0.5 µl protienase K (20 mg/ml) were added and incubation was continued for another 30 minutes at 56 °C. The reaction was cooled to room temperature and extracted once with phenol:chloroform:isoamylalcohol (25:24:1) and once with chloroform:isoamylalcohol (24:1). Then, the DNA was precipitated with EtOH as described above.

- **10 x CIP dephosphorylation buffer**

$ZnCl_2$ (10 mM), $MgCl_2$ (10mM), Tris-HCl (pH 8.3) (100 mM), H_2O

9. Production of blunt end DNA fragments

1 µg of DNA, from a restriction digest or a PCR reaction, was dissolved in 13 µl H_2O. 4 µl of Chase mixture, 2 µl of 10 x Klenow buffer and 5 units Klenow DNA polymerase were added and incubated for 20 minutes at 25 °C. Then the DNA was phenolysed and ethanol-precipitated as described above.

- **10 x Klenow buffer**

  Tris-HCl (pH 7.2) (50 mM), $MgSO_4$ (10mM), DTT (0.1mM), BSA (50 mg/ml), H_2O

- **Chase mixture**

  0.5 mM of each nucleotide, dATP, dCTP, dGTP, and dTTP

10. DNA ligation

200 ng of linearized, purified vector DNA and a threefold molar excess of the purified fragment to be subcloned were mixed and precipitated with ethanol as described above. The DNA was dissolved in 7 µl H_2O, then, 2 µl of 5 x ligation buffer and 1 µl of T4-DNA-ligase were added. Incubation followed overnight at 14 °C (in the case of sticky end fragments ligation), or at 16-20 °C (in the case of blunt end fragments ligation).

11. Preparation of competent bacterial cells

11.1 Preparation of competent bacterial cells for transformation by the $CaCl_2$ procedure

Bacterial cells were grown overnight. Susequently, 50 ml of a 1% inoculum of the overnight culture were incubated until an OD_{600} of 0.5 was reached, and chilled on ice. 35 ml of the culture were centrifuged at 5000 rpm at 4 °C and the cell pellet was resuspended in 17.5 ml of

ice-cold 0.1 M $CaCl_2$ and incubated on ice for 30 minutes. After centrifuging at 5000 rpm for 10 minutes at 4 °C, the cell pellet was resuspended in 2.5 ml of ice-cold 0.1 M $CaCl_2$ and either used immediately for transformation or prepared for freezing at –80 °C by adding 1.5 ml of 50% glycerol.

11.2 Preparation of competent bacterial cells for transformation by electroporation

50 ml of a 1% inoculum of the overnight culture were grown to an OD_{600} of 0.5. 40 ml of the culture was centrifuged at 5000 rpm for 10 minutes at 4 °C, and the cell pellet was resuspended in 40 ml of 10% glycerol. After centrifugation, the cells were resuspended in 20 ml of 10% glycerol, centrifuged and resuspended in 10 ml of 10% glycerol. After the final centrifugation, the cells were resuspended in 1 ml of 10% glycerol and stored in 40 µl aliquots at –80 °C.

11.3 Preparation of *Staphylococcus* competent cells

1 ml of overnight culture was added to 100 ml LB-medium and grown to an OD_{600} of 0.7. The bacterial cells were then centrifuged at 6000 rpm for 10 minutes at room temperature, and the cell pellet was washed twice in one volume sterile H_2O. After centrifugation (10 minutes, 6000 rpm, room temperature), another two wash steps with 20% volumes and 10% volumes of 10% glycerol were done. After the pellet was resuspended in 10% glycerol, the mixture was incubated at room temperature for 15 minutes. Then the cells were centrifuged and resuspended in 1 ml of 10% glycerol, and stored in 70 µl aliquots at –80 °C. The end concentration of the cells should not be less than $1x\ 10^{10}$ cells/ml.

11.4 Preparation of *Bacillus subtilis* competent cells

Bacterial cells were grown in LB-medium overnight at 30 °C. Subsequently, the overnight culture were diluted 100 fold into LB-medium and incubated at 30 °C until an OD_{600} of 0.7 was reached. The cells were harvested by centrifugation, resuspended in ice-cold sterile distilled H_2O, and centrifuged (6000 rpm) for 10 minutes at 4 °C. The cell pellet was resuspended in 10% volumes of ice-cold electroporation buffer (0.5% glucose and 15%

glycerol). After centrifugation, the cells were resuspended in 1 ml of the electroporation buffer and stored in 100 µl aliquots at –80 °C.

12. Transformation of competent cells

12.1 Transformation by the $CaCl_2$ procedure

50 µl of competent bacterial cells were stored on ice and 1-100 ng of plasmid DNA was added. After incubation on ice for 30 minutes, the tubes were incubated at 42 °C water bath for 90 seconds. 800 µl of pre-warmed (37 °C) LB medium was added and incubation followed at 37 °C for 2 hours. After centrifugation, the cell pellet was resuspended in 50 µl of LB medium and plated onto selective, antibiotic containing LB agar.

12.2 Transformation by the electroporation method

One aliquot (70 µl) of competent cells was thawed at room temperature, and mixed with 0.5 µg plasmid-DNA by tapping the tube or by swirling the cells with the pipettor. The DNA and the cells were applied into a “Gene pulser” cuvette with an electrode gap of 0.1 cm, and incubated for 15 minutes at room temperature. The cells were electroporated with a capacitor of 25 µf and 2.5 kv, and 100 ohms by using the Genepulser transfection apparatus (Bio-Rad). After 10 minutes incubation on ice, 900 µl $SMMP_{75}$-medium was added to the electroporated cells and incubated at 37 °C for 2 hours in a rotary shaker at 250 rpm. Suitable dilutions were plated onto DM-3 agar containing a suitable antibiotic for selection.

For *S. epidermidis* cells that are difficult to be transformed by the standard electroporation method, the improved electroporation method was used. One aliquot of 70 µl competent cells was thawed at room temperature, centrifuged and the pellet was resuspended in 1 ml 0.5 M Sucrose. 20-30 µl of Lysostaphin (2 mg/ml) was added and chilled on ice for 20-30 minutes. After centrifugation (6000 rpm) for 5 minutes, the pellet was gently resuspended in 70 µl of 0.5 M sucrose. 0.5-1 µg plasmid was added to the competent cells and incubated at room temperature for 30 minutes. Then the cells were transferred into a “Gene pulser” with an electrode gap of 0.2 cm, and electroporated with a capacitor of 25 µf and 1.5 kV, and 200 ohms. After 10 minutes incubation on ice, 900 µl $SMMP_{75}$-medium was added to the

electroporated cells and incubated at 37 °C for 2 hours in a rotary shaker at 50 rpm. Suitable dilutions were plated onto DM-3 agar containing antibiotics for selection.

12.3 Transformation of *Bacillus subtilis* competent cells

100 µl of the competent cells were mixed with a plasmid-DNA, put on ice and electroporated as follows:

Electrode gap:	0.2 cm
Voltage:	2.5 kV
Capacitor:	25 µF
Pulse controller setting:	200 ohms
Field strength:	1.5 kV

After 10 minutes incubation on ice, 900 µl LB-medium was added to the electroporated cells and incubated at 37 °C for 2 hours in a rotary shaker at 50 rpm. Suitable dilutions were plated onto LB agar containing antibiotics for selection.

13. DNA sequencing

Nucleotide sequencing was performed by using the dideoxy chain termination method in the automatic sequencing system (MWG), by using infrared-dye-labelled primers. The sequencing reactions were carried out by using the Thermo Sequenase™ fluorescence-labbelled primer cycle sequencing kit. 1 µg of plasmid DNA was supplemented in a total volume of 50 µl with 1 µl RNase A (10 mg/ml) and incubated at 37 °C for 30 minutes. After phenolysation and ethanol-precipitation, the dried DNA was resuspended in 12 µl H_2O and mixed with 1 pmol of a labelled primer. 4 PCR caps were prepared, each of which contained 3 µl of the reaction mixture as well as 1 µl of either A-, C-, G-, or T-mix, and each were overlayed with one drop of light mineral oil. The amplification was carried out in either “Eppendorf” or “Techne” thermocycler. The amplification conditions consisted of an initial denaturation step at 94 °C for 2 minutes followed by 30 cycles of 94 °C for 30 s, annealing temperature (varied depending on the primer sequence) for 30 s, and 70 °C for 30 s. After thermocycling has been completed, 3 µl “stop buffer” was added, and 1.5 µl of each reaction

were loaded on the fluorescent sequencing gel. Data analysis were done by the sequencer machine with a special software provided for this purpose.

A-, C-, G-, and T- reagents

Tris-HCl (pH 9.0), $MgCl_2$, Tween™20, Nonidet™ , 2-mercapto-ethanol, dATP, dCTP, 7-deaza-dGTP, dTTP, Thermstable pyrophosphatase, thermo Sequenase DNA-Polymerase and one of Didesoxynicleotide (ddATP, ddCTP, ddGTP or ddTTP).

14. Polymerase Chain Reaction (PCR) [129]

This method was used for analysis of specific DNA sequences and creation of a specific restriction sites. 100 ng of template-DNA was added to a PCR cap containing a mixture of 10 µl of 10x of Taq polymerase-buffer, 1µl of each primers, 1 µl of (50 mM) $MgCl_2$, 4 µl of (20 mM) dNTP-mix, and 1 µl of Taq-polymerase. The cap was filled to 100 µl with H_2O. The denaturation of the DNA was done for 2 minutes at 94°C, followed 30 cycles of 30 seconds at 94°C, annealing of the primers (temperature varied depending on the primers sequences) for 30 seconds, and primer extension at 72°C for 1 minute. After completion of the PCR reaction, the reaction was stopped and applied on an agarose gel. The primer annealing temperature was calculated according to the following formula:

Tm = 69.3 + 0.4 (% GC content) – 650/ the long of the oligonucleotide

15. Cloning of the PCR-fragments

15.1 "SureClone®" ligation kit

This kit is designed for the rapid and reliable cloning of PCR fragments directly into a vector of choice. The kit eliminates the adenine nucleotides at the 3´ ends of PCR products. The protocol utilizes a Klenow digestion by addition of 2 µl of 10 x blunting/kinasing buffer, 1 µl of Klenow-fragment and 1 µl of Polynucleotide kinase into a cap containing 1-16 µl PCR product. H_2O was added to a volume of 20 µl. The reaction was incubated at 37°C for 30

minutes, and phenolisation was done by addition of 20 µl Phenol/chloroform to the reaction. After centrifugation, the upper, aqueous layer was collected and purified by using a Sepharyl® S-200-MicroSpin column. Followed by cloning of the PCR product into a dephosphorylated blunt end vector as follows:

PCR product (blunt end)	2 µl
50 ng of dephosphorylated vector	1 µl
2 X ligation buffer	10 µl
DTT solution	1 µl
T4 DNA ligase	1 µl
Sterile distilled H_2O	to 20 µl
Total volume	20 µl

After incubation for 1-2 hours at 16 °C, *E. coli* competent cells were transformed with a portion of 5 µl of the reaction as described previously.

15.2 pGEM®-T Easy vector system

This kit is a convenient system for the direct cloning of a PCR product. A linearized pGEM®-T Easy vector with 3´ terminal thymidine at both ends is provided which improves the efficiency of ligation of a PCR product with adenine nucleotides at the 3´ ends generated by certain thermostable polymerases. The pGEM®-T Easy vector contains T7 and SP6 RNA polymerase promoters flanking a multiple cloning site with the α-peptide coding region of the enzyme β-galactosidase. Insertion inactivation of the α-peptide allows recombinant clones to be directly identified by color screening. White colonies of *E. coli* grown on LB-X-Gal-Ampicillin-plates indicate a positive cloning of the PCR product. The cloning reaction was prepared follows:

2 x T4 DNA ligation buffer	5 µl
pGEM®-T Easy vector (50 ng)	1 µl
PCR product	1-3 µl
T4 DNA ligase	1 µl
H_2O to final volume of	10 µl

The reaction was mixed, and incubated for 1 hour at room temperature or overnight at 4°C.

16. Isolation of the *Staphylococcus* chromosomal DNA

The bacterial cells were grown in LB-medium overnight at 37°C. The overnight culture was diluted 100 fold into 5 ml LB-medium with 1% glycine and incubated at 37°C for 5 hours. The grown cells were centrifuged and resuspended in 100 µl of 10 mM Tris-HCl, 25% sucrose pH 7.5. 15 µl of 500 mM EDTA pH 8.0, 10 µl Lysostaphin (2 mg/ml)(Sigma, Deisenhofen, Germany), and 1 µl of RNase A (20 mg/ml) was added and incubated at 37 °C until the mixture became viscous. After cell lysis, 375 µl TE buffer, and 225 µl 10% SDS were added, and the mixture was incubated for 5 minutes at 37 °C. 10 µl of protienase K (20 mg/ml) was added and incubated at 55°C for at least 30 minutes. After incubation for 5 minutes at room temperature with 150 µl of 5M $NaClO_4$, the mixture was extracted with phenol: chloroform: isoamylalcohol (25:24:1), and homogenized by horizontal shaking for 90 minutes at room temperature. The suspension was centrifuged in an eppendorf-tube for 10 minutes at 4°C, the upper, aqueous layer was collected in a fresh tube and precipitated by addition of 2.5 volumes EtOH. The collected DNA was washed with 70% EtOH, airdried and resuspended in 50 µl H_2O.

17. Southern-hybridization

The procedure is specifically designed for blotting an agarose gel onto an uncharged or positively charged nylon membrane. 10 µg of chromosomal DNA was digested with a distinct restriction enzyme, and loaded onto the 1% TPE agarose gel. After electrophoresis was completed, the ethidium bromide stained gel was photographed to record the electrophoretic separation of the loaded DNA fragments. Then it was covered with fresh depurination solution and shaken by agitation for 15 minutes until the bromophenol blue dye has turned completely yellow. After depurination, the DNA strands were separated by rinsing the gel in H_2O to remove excess depurination solution, and covering with denaturation solution until the bromophenol indicator return to blue colour. The gel was then neutralized by soaking it for 30 minutes in neutralization solution at room temperature with constant gentle shaking. 3 mm Whatman paper was then wrapped around a stack of glass plates and put into a large baking dish. The dish was filled with 10 x SSC almost to the top of the glass plates. Then the gel was placed upside down on the 3 mm paper. A nylon membrane was soaked in H_2O for 5 minutes, 3 minutes in 20 x SSC and placed on top of the gel as well as 2 pieces of 3 mm paper, also

soaked in 20 x SSC. A stack of paper towels, a glass plate and a 500 g weight were placed on top. The DNA was allowed to transfer onto the filter membrane for 12-24 hours. Paper towels were replaced as they became completely wet. After transfer, the gel was discarded and the filter membrane was soaked in 0.4 M NaOH for 1 minute, and in 0.25 M Tris-HCl (pH 7.0) for 1 minute. The filter was dried in room temperature and the DNA fixation was done by UV radiation using Gene linker or alternatively the membrane was backed in a vacuum-oven for 30 minutes at 80°C. The nylon membrane was wetted with 2x SSC and then incubated with prehybridization solution at 42°C.

Depurination solution	0.25 N HCl
Denaturation solution	0.5 M NaOH, 1.5 M NaCl, H_2O
Neutralisation solution	1.5 M NaCl, 0.5 M Tris-HCl pH 7.5, H_2O
20 x SSC	300 mM Na-citrate pH 7.0, 3M NaCl, H_2O

18. Labelling of the gene probes

18.1 Radioactive labelling method

The “Random Primers DNA Labelling System” of GIBCO BRL was used. Approximately 25 ng of DNA was dissolved in 20 µl H_2O and denaturated by incubation for 10 minutes at 100°C. The denaturated DNA was immediately put on ice and the following solutions were added: 2 µl of each dATP, dGTP and dTTP solutions, 15 µl random primers buffer mixture, 5µl of α-^{32}P-dCTP and 3 µl H_2O. After mixing briefly, 1 µl Klenow fragment was added and incubation followed for 1 hour at 25°C. Then the reaction was stopped with 5 µl stop buffer. The reaction was diluted with TE buffer and boiled again for 5 minutes before use. In a hybridization tube, 20 ml of hybridization buffer was prepared and pre-heated to 42°C, the blot was put into the hybridization tube and pre-hybridized by incubation for 4 hours at 42°C in hybridization oven. Following the pre-hybridization, the labelled probe was added to the pre-hybridization tube, and incubation continued overnight at 42°C. After hybridization finished, the filter was washed twice with the pre-heated primary wash buffer for 20 minutes at 56°C. Later the blot was transferred into the secondary wash buffer, and washed twice for 30 minutes at 56°C. The blot was then placed side up in the film cassette, and a sheet of radiography x-ray film was put on the top of the blot and exposed for 4-5 days at -20°C.

Hybridization buffer	43.6 ml H_2O
	25 ml 20 x SSC
	20 ml deionised formamid
	5 ml 1 M Tris-HCl pH 7.5
	5 ml 100 x Denhardt`s reagent
	1 ml 10% SDS
	0.4 ml 0.25 M EDTA pH 8.0
	750 µl denaturated Herringsperm-DNA (10 mg/ml)
100 x Denhardt`s reagent	2 g Polvvnyl-Pyrrolidon 360
	2 g Ficoll 400
	2 g BSA
	H_2O to 100 ml
Primary wash buffer	2 x SSC/0.1% SDS
Secondary wash buffer	0.1 x SSC/0.1% SDS

18.2 Non radioactive labelling method "ECL-kit"

This system involves a direct labelling of DNA or RNA with the enzyme horseradish peroxidase, and a detection system based on enhanced chemiluminescence. Once the probe was labelled, it can be used in hybridization with target DNA or RNA immobilized on a nylon membrane and produces a blue-light that can be detected with blue-light sensitive film. 0.1-1 µg of DNA probe in 10 µl H_2O was denaturated by heating the sample for 5 minutes in a boiling water bath, and immediately cooled by incubating for 5 minutes on ice. An equivalent volume of DNA labelling reagent was added to the cooled DNA, and mixed thoroughly. 10 µl of glutaraldehyde solution was added to the mixture, mixed well, and briefly centrifuged to collect the content at the bottom of the tube, then the mixture was incubated for 10 minutes at 37°C. Following pre-hybridization of DNA or RNA blot, the labelled probe was added to the pre-hybridization buffer, mixed gently and incubated overnight at 42°C. After hybridization, the filter was washed as follows:

2 x 10 minutes at 50°C in 0.5 x SSC/0.4 SDS

2 x 5 minutes at room temperature in 2 x SSC

The blot was put in a clean container, and an equal volume of detection reagent 1 with detection reagent 2 were mixed and added to the washed blot and incubated for 1 minute at room temperature. The excess detection reagents were discarded, and the blot was placed side up in the film cassette. Hybridization was detected by exposing a sheet of Hyperfilm-ECL for 5-30 minutes at room temperature.

19. Isolation of total RNA from *Staphylococcus* cells

19.1 RNA isolation by "RNeasy™"-kit

A bacterial colony, grown on LB agar, was inoculated in 3 ml of LB medium and grown to an OD_{600} of 1.0. 1 x 10^9 cells were harvested by centrifugation of the bacterial culture at 6000 rpm for 5 minutes at 4°C. Then the cells were resuspended completely in 100 µl TE containing lysostaphin (200 µg/ml), and incubated for 5-10 minutes at 37°C. 350 µl RTL lysis buffer was added and vortexed vigorously for 2 minutes. The mixture was applied to the RNeasy spin column, centrifuged at 10000 rpm for 15 seconds, and the spin column then placed in a new 2 ml collection tube. The column was washed twice by addition of 500 µl RPE washing buffer and centrifuged at 13000 rpm for 2 minutes at room temperature. The spin column then transferred into a new 1.5 ml collection tube and the RNA was eluted by addition of 40 µl DEPC-H_2O. The concentration and purity of RNA were determined by measuring the absorbance at 260 nm and 280 nm in a spectrophotometer, and the collected RNA was saved at –20°C.

19.2 RNA isolation by "FastRNA"-kit

The cellular RNA from *S. epidermidis* was isolated by using the FastRNA kit (√Bio 101). 1 x 10^9 *S. epidermidis* cells grown (OD_{600} of 1.0) in TSB medium supplemented with 3 % NaCl,

were harvested by centrifugation at 5000 rpm for 10 minutes at 4°C. The pellet was resuspended in 200 µl DEPC-H_2O, and transfered into a FastRNA BLUE tube containing 0.5 ml of 0.1 mm-diameter Ziriconia/Silica beads, 100 µl CIA, 500 µl CRSR-BLUE and 500 µl PAR reagents. The cells were disrupted by shaking rapidly in a high-speed reciprocating homogenizer (FastaPREP apparatus) for 40 seconds. The mixture was centrifuged at 13000 rpm for 15 minutes. The aqueous layer was collected in a fresh tube, extracted with 500 µl CIA and the upper phase was collected in a fresh tube. For RNA precipitation, 500 µl of DIPS was added to the tube, mixed, and incubated at room temperature for 2 minutes. The mixture was centrifuged for 5 minutes, the pellet was washed with 500 µl SEWS, air dried for 5-10 minutes. Later the pellet was dissolved in 50 µl DEPC-H_2O.

20. Northern hybridization analysis

When working with RNA it is important to keep all equipment and reagent solutions free from RNase contamination. Gloves should be worn and all the solutions must be treated with DEPC-H_2O, except the solutions containing Tris. DEPC-H_2O was produced by adding 0.1 % (vol/vol) DEPC (diethyl pyrocarbonate) to distilled water, incubated overnight at 37°C, and then autoclaved for 30 minutes to remove the trace of DEPC.

20.1 RNA gel electrophoresis

A 1.2% agarose/Formaldehyde-gel was prepared by mixing 4.2 g agarose and 304.5 ml DEPC-H_2O. The agarose was dissolved by heating in a microwave, mixed well, and cooled to 60°C. In a fume hood, 16.2 ml of 37% formaldehyde, and 35 ml MOPS buffer were added, mixed well by swirling, and poured into the gel former. The RNA samples were denaturated as follows:

RNA	30 µg
MOPS	1 x
Formamid	50 %
Formaldehyde	7 %
DEPC-H_2O to final volume	50 µl

The mixture was incubated for 15 minutes at 55°C, and 10 µl of RNA loading buffer was added. The RNA probes were loaded onto the prepared formaldehyde gel in 1 x MOPS running buffer at 5-7.5 V/cm for 2-3 hours, or until the bromophenol blue dye has migrated three-quarter of the way down the gel. After electrophoresis was completed, the gel was rinsed in DEPC-H_2O for 30 minutes, stained with ethidium bromide and photographed to record the electrophoretic separation of the loaded RNA.

20.2 RNA-blotting

RNA transfer can be done either by the vacuum transfer or by the capillary transfer. RNA transfer using the capillary blot, was done as described previously for Southern analysis except that RNA transfer does not need depurination, denaturation, and neutralization steps. The vacuum transfer of RNA was done by cutting a piece of nylon membrane just larger than the RNA, and rinsing it 5 minutes in H_2O then 5 minutes in 20 x SSC. Then the nylon membrane was put on the membrane holder of the vacuum blotter. Later, the RNA gel was placed on the nylon membrane. The transfer was started by loading 50-60 mbar for 2 hours after covering the top of the gel with 20 x SSC. Later, the membrane was washed with 2 x SSC, and dried at room temperature. The RNA fixation was done by UV radiation using the Gene linker or alternatively the membrane was backed in a vacuum oven for 30 minutes at 80°C. The nylon membrane was prepared for hybridization and the hybridization probe was labelled like as described for Southern hybridization analysis.

10 x MOPS-buffer

41.8 g of MOPS was dissolved in 800 ml DEPC-H_2O, pH was adjusted to 7.0 with NaOH. 16.6 ml of 3 M Na-Acetate and 20 ml of 0.5 M EDTA pH 8.0 were added. The volume was adjusted to 1 liter with DEPC-H_2O, filtered and kept in room temperature.

5 X RNA loading buffer:

Deionised formamid	3084 µl
0.5 M EDTA pH 8.0	80 µ
37 % formaldehyde	720 µl
Bromophenol-blue (solution)	16 µl
86 % glycerin	2 ml

MOPS	4 ml
DEPC-H_2O to final volume of	10 ml

21. b-galactosidase-test "Galacto-Light™"

Galacto-Light Plus™ is a chemiluminescent reporter assay system (Tropix, Bedford, Mass) designed for the rapid, sensitive, and non isotopic detection of β-galactosidase in bacterial cell lysates. Galacton™ chemiluminescent substrate has a half-life of light emission of approximately 4.5 minutes after the addition of light emission accelerator. It is suited for use with luminometer in which light emission measurement can be taken within a short period of time and calculated as relative light unit (RLU). For our experiments, an overnight culture of *S. epidermidis* was diluted 1:100 with CDM supplemented with the appropriate concentrations of glucose, sodium chloride, or antibiotics, and incubated at 37°C overnight in a shaker at 180 rpm. After harvesting the bacterial cells, the pellet was washed and resuspended in 0.9 % NaCl. Cell density was adjusted to an OD_{600} of 1.0. A 200 µl volume of this suspention was centrifuged at 13000 rpm for 10 minutes, and the cell pellet was resuspended in 200 µl lysis solution containing 50 µg/ml lysostaphin and incubated at 37°C for 10 minutes. 10 µl of the supernatant was mixed with a 1:100 diluted Galacton™ in Galacto-light™ reaction buffer, and incubated in room temperature for 30 minutes. Following incubation, the mixture was transfered into a luminometer cuvette and added in a luminometer. The β-galactosidase activity was measured after 5 seconds following the injection of 300 µl of light emission accelerator using the LB 9051 luminometer (Berthold, Wildbad, Germany).

Lysis buffer	Potassium phosphate pH 7.8	100mM
	Triton x-100	0.2 %
Galacto-light™ reaction buffer	Sodium phosphate pH 8.0	100 mM
	Magnesium chloride	1 mM

22. Determination of minimal inhibitory concentration of antibiotics (MIC)

A 4 hours (MH)-Bouillon culture of 5 x 10^7 CFU (Mc Farland 0.5) was prepared by dilution of an overnight culture of *S. epidermidis* 1:100 in (MH)-Bouillon medium. Then the culture was again diluted 1:100 with the same medium. A serial dilution of antibiotic concentration was prepared. An antibiotic solution of 256 µg/ml was prepared, and 12 test tubes were numbered and to each 0.5 ml of culture medium was added. 0.5 ml of the antibiotic solution was added to the tube number 2, mixed well and 0.5 ml of the dilution from tube number 2 was added to the tube number 3. The dilution was continued until the test tube number 12, and the test tube number 1 remained without antibiotic as a growth control. 0.5 ml of the prepared bacterial inoculum to each of the 12 was added, and followed by incubation for 18 hours at 37°C. After incubation, the minimal inhibitory concentration of the used antibiotic was determined. The MIC is a minimal concentration of an antibiotic which is enough to inhibit the growth of a bacteria.

23. Quantitative adherance assay [130]

Quantitative adherence assay was used as an indirect measure of the slime production in Staphylococci. The basis of the assay is that slime-positive cells adhere to polystyrene and to each other, forming a biofilm whose density is measured spectroscopically after staining with crystal violet. The *S. epidermidis* cells were grown in CDM-medium overnight at 37°C. Then the overnight culture was diluted 1:100 with fresh CDM-medium, or CDM/LB (1:1) to inhibit the basic level of biofilm production and prevent the biofilm rate out of reading scale during the *ica* induction. The individual wells of a sterile, polystyrene, 96 well, flat-bottom tissue culture plate were filled with 200 µl aliquots of the diluted culture. The tissue culture plate was incubated for 18 hours at 37°C. *S. epidermidis* RP62A was used as a positive control, and *S. carnosus* TM 300 as a negative control. Following incubation, the content of each well were gently aspirated by a low vacuum, then the wells were washed four times with 0.2 ml phosphate buffer saline (pH 7.0). Adherent bacteria were fixed by addition of 50 µl 2.5 % glutaraldehyde to each well and incubation for 10 minutes at room temperature. The wells were washed with H_2O, and stained with Hucker crystal violet. Excess stain was rinsed off by placing the plate under running tap water, then the plate was dried and the ODs of stained adherent bacterial film were measured with an ELISA-reader (BioRad) using a wavelength of

490 nm. Adherence measurement were performed in quadruplicate, and the values were averaged. If the ODs were equal or less than 0.12, the organism was then described as non-adherent, ODs between 0.12 and 0.24 as weakly adherent, and more than 0.24 as strongly adherent bacteria.

24. Statistics

For all the experiments data, the average (x) and standard deviation were calculated as described by Cavalli-Sforza [131].