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der Technischen Universität München

Nucleic Acid Based Detection and Separation of Bacteria Causing Nosocomial Infections Using Novel Types of Polynucleotide Probes

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Vollständiger Abdruck der von der Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt der Technischen Universität München zur Erlangung des akademischen Grades eines

Doktors der Naturwissenschaften

genehmigten Dissertation.

Vorsitzender:	Univ.-Prof. Dr. W. Höll
Prüfer der Dissertation:	1. Univ. Prof. Dr. K.-H. Schleifer, i.R. 2. Univ. Prof. Dr. S. Scherer

Die Dissertation wurde am 5. April 2007 bei der Technischen Universität München eingereicht und durch die Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt am 18. Juli 2007 angenommen.

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Abbreviations

A	adenine
aac(6´)	gene encoding an aminoglycoside 6´-*N*-acetyltransferase type II
A. baumannii	*Acinetobacter baumannii*
abs.	absolute
Ac	acetate
ampC	gene encoding an AmpC-type class C beta-lactamase
APS	ammoniumperoxodisulfate
bp	base pairs
B. cepacia	*Burkholderia cepacia*
$bla_{OXY\text{-}2\text{-}5}$	gene encoding an OXY-beta-lactamase
$bla_{SHV\text{-}1}$	gene encoding a SHV-beta-lactamase
BLAST	Basic Local Alignment Search Tool
°C	degree centigrade
C	cytosine
CIA	chloroform : isoamylalcohol = 24 : 1 (v/v)
cm	centimetre
Cy	cyanine dye
d	day(s)
dATP	deoxyadenosinetriphosphate
dCTP	deoxycytidinetriphosphate
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
ddNTP	2´,3´dideoxy-nucleoside-5´-triphosphate
dGTP	deoxyguanosinetriphosphate
dNTP	2´deoxy-nucleoside-5´-triphosphate
ds	double stranded
DSM	Deutsche Sammlung für Mikroorganismen und Zellkulturen, Braunschweig, Germany
dTTP	deoxythymidinetriphosphate
E	extinction
E. aerogenes	*Enterobacter aerogenes*

E. coli	*Escherichia coli*
EDTA	ethylendiaminetetraacetate
E. faecalis	*Enterococcus faecalis*
E. faecium	*Enterococcus faecium*
e.g.	exemplum gratiae
et al.	et alteri
etc.	et cetera
EtBr	ethidium bromide
$EtOH_{abs}$	100 % ethanol
FA	formamide
FACS	Fluorescence activated cell sorting
FISH	fluorescence *in situ* hybridisation
Fluos	5,(6)-carboxyfluorescein-N-hydroxysuccinimdiester
g	gram
G	guanine
GC	mol % guanine+cytosine
H_2O_{MQ}	ultra pure water (Millipore system)
h	hour(s)
H. influenzae	*Haemophilus influenzae*
HYCOMP	Hybridisation in coated microplates
kb	kilo bases
kDa	kilo dalton
K. oxytoca	*Klebsiella oxytoca*
K. pneumoniae	*Klebsiella pneumoniae*
l	litre
LB	Luria Bertani medium
LMG	Laboratorium voor Microbiologie Universiteit Gent, Belgien
M	molar
m	metre; milli (10^{-3})
MACS	Magnetic activated cell sorting
mecA	gene encoding a penicillin binding protein 2a (PBP2a)
min	minute(s)
M. morganii	*Morganella morganii*
mRNA	messenger RNA

MRSA	methicillin-resistant *Staphylococcus aureus*
µ	mikro (10^{-6})
n	nano (10^{-9})
NaAc	sodium acetate
N. canis	*Neisseria canis*
n.d.	no data, not determined
n.e.	not existing
n.r.	no result
n.t.	not tested
nt	nucleotide(s)
OD	optical density
o.n.	over night
p	pico (10^{-12})
p.	page
Pa	pascal
P. aeruginosa	*Pseudomonas aeruginosa*
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFA	paraformaldehyde
P. fluorescens	*Pseudomonas fluorescens*
pH	negative decadic logarithm of the proton concentration
poly-FISH	fluorescence *in situ* hybridisation using polynucleotide probes
pp.	pages
RNA	ribonucleic acid
RNase	ribonuclease
rDNA	ribosomal DNA
rpm	revolutions per minute
rRNA	ribosomal RNA
S. agalactiae	*Streptococcus agalactiae*
S. aureus	*Staphylococcus aureus*
SDS	sodiumdodecylsulfate
sec	second(s)
S. epidermidis	*Staphylococcus epidermidis*

S. haemolyticus	*Staphylococcus haemolyticus*
S. maltophilia	*Stenotrophomonas maltophilia*
S. pyogenes	*Streptococcus pyogenes*
SNP	single nucleotide polymorphism
sp	species (singular)
spp.	species (plural)
SSC	standard saline citrate
ssp	subspecies
ss	single stranded
T	thymine
TAE	tris-acetate-EDTA
Taq	*Thermus aquaticus*
TE	tris-EDTA
TEMED	*N,N,N´,N´*-tetramethylethylendiamine
T_m	melting temperature of nucleic acids
Tris	tris-(hydroxmethyl-)aminomethane
U	unit; uracil
UV	ultra violet
V	volt
vanB	gene involved in the vancomycin resistance mechanism
v/v	volume / volume
VRE	vancomycin-resistant enterococci
W	watt
w/v	weight / volume
X-Gal	5-chlor-4-brom-3-indolyl-β-D-galactosidase

A. Introduction

A.1. Identification of pathogens using FISH techniques

The fluorescence *in situ* hybridisation (FISH) is nowadays a standard technique to identify bacteria originating from different sources by hybridisation of a labelled probe with the complementary target region. In the meantime, the applicability of FISH is extended to detect bacteria in microbial communities in environmental samples, wastewater, food industry and drinking water control as well as in clinical diagnostics. In combination with other conventional detection methods, FISH offers many hints about the condition of the cells and allows the detection of bacteria prior to their cultivation. Furthermore, FISH is used for the determination of the position of several sequences within a chromosome (gene mapping) and for the detection of chromosomal abnormalities (chromosomal painting) (Raap, 1998). The target molecules used for hybridisations are often rRNA (16S and 23S), mRNA and intergenic spacer rRNA. The advantages of the single stranded ribonucleic acid as adequate target arise from its genetic stability, omnipresence among bacteria, archaea and eukarya and the high copy number existing in metabolically active and replicating cells (up to 10^5 ribosomes per cell). Particularly, the combination of highly conserved and variable regions within the rRNA provides the opportunity to design probes targeting different taxonomic levels, including species-specific probes. Followed by genus-, family-, order-, class- and phylum-specific probes (Woese, 1987; Göbel et al., 1984; Amann et al., 1995; Ludwig et al., 1994). The availability of public or commercial databases offering increasing sequence information improves specificity and sensitivity of FISH probes additionally.

A drawback of FISH analysis is the dependence on the ribosome content. On the one hand, cells containing high ribosome contents can be easily visualised by epifluorescence microscopy. On the other hand, bacteria in environmental or clinical samples contain few ribosomes and thus the microscopic detection is impeded. The major part of bacteria present in biological material, e.g. in environmental or clinical samples is not permanently replicating. Hence, the detection of bacteria in such specimens is hampered (DeLong, 1999). It is assumed that microbial cells persisting in a metabolically inactive state contain diverse amounts of rRNA molecules ranging from 10^3 to 10^8 (DeLong, 1989).

Many modifications of the FISH protocol or the applications of different fluorescent dyes were performed to assure a detection of these bacteria, too. One possibility is the use of a probe set targeting several regions of the 16S rRNA (Amann, 1990; Lee, 1993; Wallner, 1993). Another solution for signal amplification is the introduction of unlabelled helper-oligos, which bind near the target site and denature secondary structures of the rRNA to improve the interaction between target and probe (Fuchs et al., 2000). The treatment of target cells with the antibiotic chloramphenicol should enhance rRNA synthesis in bacteria in order to increase the content of target sites (Overney et al., 1997). Other methods concentrate the augmentation of signal intensity by enzyme-mediated signal amplification. TSA-FISH (Tyramide Signal Amplification; Schönhuber et al., 1997), also called CARD-FISH (Catalysed Reporter Deposition; Pernthaler et al., 2002) involves the deposition of fluorescently labelled tyramide by peroxidase activity. Another approach is the application of PNA-FISH (Polypeptide Nucleic Acids-FISH). PNA is a DNA analogue possessing a (2-aminoethyl)-glycine backbone instead of the deoxyribose-phosphate, which results in a stable duplex with the complementary DNA sequence (Corey, 1997). An additional approach concerning the augmentation of sensitivity is the application of multi-labelled RNA or DNA polynucleotide probes generated via *in vitro* transcription, nick translation or asymmetric PCR (Trebesius et al., 1994; DeLong et al., 1999; Pernthaler et al., 2002; Stoffels et al., 1999, Zimmermann et al., 2002; Zwirglmaier et al., 2004). Due to weak signals obtained in environmental samples with FISH, the development of multi-labelled single stranded RNA polynucleotide probes varying in length was introduced (DeLong, 1999). Hybridisations using polyribonucleotide probes in marine samples resulted in whole cell fluorescence of target cells.

After the application of FISH using polynucleotide probes targeting the domain III of 23S rRNA instead of an intense whole cell fluorescence signal, a strong signal in the periphery of the bacterial cell was observed. In further studies, this so-called "halo" was shown to be a typical result of FISH applying RNA polynucleotide probes targeting domain III of 23S rRNA. Trebesius et al. (1994) and Stoffels et al. (1999) described this phenomenon for the first time. Concerning the appearance of the ring-shaped signal, several theories and a hypothesis were proposed. Trebesius et al. (1994) and Stoffels et al. (1999) assumed that only a part of the 250 nucleotides long probe is anchored to its target while the remaining part of the probe molecule stands out of the cell spanning the cell envelope. Those fragments of biotin-labelled probes

can be used in further hybridisations for cell separation based on binding to streptavidin coated magnetic beads, also known as MACS (Magnetic Activated Cell Sorting) (Stoffels et al., 1999). Zwirglmaier et al. (2003) refined this theory and formulated the “network hypothesis”. Due to the ability of RNA to generate secondary structures, the protruding part of the polynucleotide probe binds to other protruding probe regions as well as to unbound probes forming a network within and around the cell envelope. Additionally to the MACS-technique, the appearance of the halo signal can be used for separations of bacteria in microplates after FISH using prolonged probes. Here the microplate cavities are coated with nucleic acids complementary to the polynucleotide probe. Target cells from different artificial or natural material (Zwirglmaier et al., 2004) can be immobilised due to the interaction of the probe and complementary nucleic acids in a subsequent hybridisation. In several experiments, the practicability of domain III targeted DNA polynucleotide probes was evaluated and confirmed (Zimmermann et al., 2001).

In previous studies, DNA was used as target site. Chromosomal painting was applied to determine eukaryotic chromosomes or chromosomal regions as well as gene mapping and recently karyotyping using differently labelled DNA probes (Lichter et al., 1988; Lawerence et al., 1990; Speicher et al., 1996). Based on this, bacterial chromosomal painting (BCP) was developed for the identification of bacteria (Lanoil and Giovannoni, 1997). However, these hybridisations did not lead to halo signals.

Due to the increased signal intensity obtained after FISH, the localisation of other genetic elements such as plasmid-mediated or chromosomally encoded genes is now possible using RNA polynucleotide probes. The RING-FISH method for the Recognition of Individual Genes was introduced by Zwirglmaier (2004) and led to the appearance of the typical halo signal after hybridisations on slides. Figure A.1 (p. 12) shows the typical appearances of halo and whole cell fluorescence occurring after hybridisations performed in the present study. In addition, the separation of cells via a second hybridisation in coated microplates can be accomplished with RING-FISH probes. Moreover, this immobilisation technique combines the separation of bacteria as well as further investigations of cells in one cavity and establishes many possibilities for characterisation of pathogens, e.g. by PCR or other molecular methods.

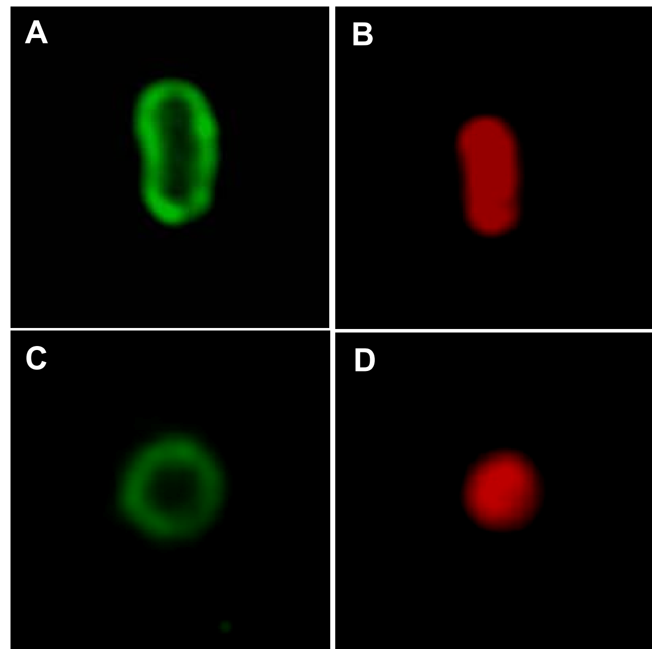


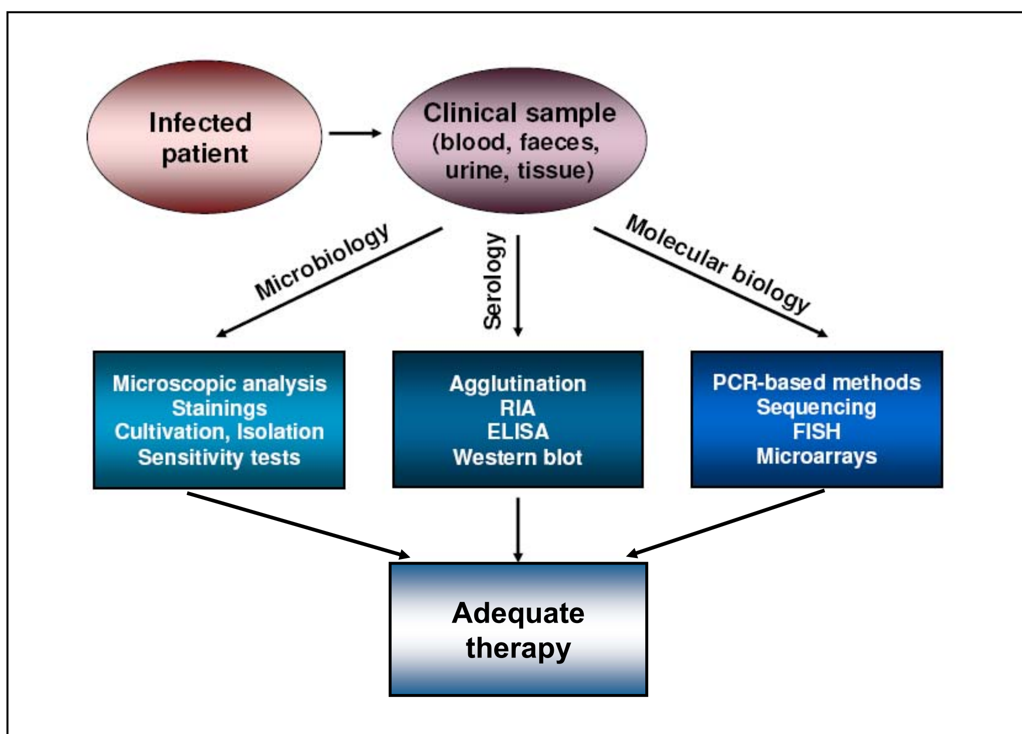
Figure A.1 Illustration of typical appearance of the signal using different FISH techniques
A: Halo signal observed after FISH using the fluorescein-labelled RING-FISH polynucleotide probe EaAmpC targeting *ampC* gene of **Gram-negative *Enterobacter aerogenes***. **B: Whole cell fluorescence** observed after FISH using the Cy3-labelled oligonucleotide probe Eae16S targeting the 16S rRNA of *E. aerogenes.* **C: Halo signal** observed after FISH using the fluorescein-labelled RING-FISH polynucleotide probe targeting streptokinase gene of **Gram-positive *Streptococcus pyogenes.*** **D: Whole cell fluorescence** observed after FISH using the Cy3-labelled oligonucleotide probe Spy467 targeting the 16S rRNA of *S. pyogenes.*

A drawback of this novel method are false positive signals obtained from some negative controls after hybridisations presumably due to the fact that polynucleotide probes are less specific than the oligonucleotide probes. Former studies on domain III of the 23S rRNA set up 78 % and 85 % sequence identity for positive/negative signals (Ludwig et al., 1994). Recent study (also focused on domain III) suggests a benchmark of 25-28 % sequence dissimilarity, which is necessary for the discrimination of non-target cells (Fichtl, 2005). Due to the length of up to 1000 nucleotides of RING-FISH probes, a specific identification comparable to species-specific detection is not possible when the positive and negative control harbour genes with high sequence similarity. Exact values are not known and the specificity has to be empirically evaluated. Additionally, the extended length of polynucleotide probes offers many possibilities for unspecific binding on non-target sites. A possibility to overcome this problem is the design of oligo-oligonucleotide probes. These novel types of polynucleotide probes combine the beneficial features of oligonucleotide (specificity) and polynucleotide probes (formation of secondary structures). Based on these advantages, oligo-oligonucleotide probes were designed and successfully applied in this thesis.

A.2. Clinical diagnostics in hospital laboratories

Nowadays, for the determination of an infection caused by pathogens many different identification technologies are available in laboratories, illustrated in figure A.2 (p. 13). In general, the approach consists of three main attempts: the conventional microbiological, the immunological and the molecular testing. This should enable the correct identification of the pathogen followed by the application of the adequate therapy. Depending on the infected tissue or fluid of the patient, the isolated samples could be analysed microscopically applying cell counting and diverse staining methods (Gram, Methylenblue or Giemsa). For further characterisation of the pathogen, cultivation on selective or differentiative media followed by isolation in pure culture and several tests can be performed. In contrast, the serological analyses rely on immunoassays using antibodies (agglutination, ELISA, RIA, western blot) (Janeway and Travers, 1997). In the last two decades genome targeted molecular tools allow a faster identification of pathogens in comparison to conventional procedures and contribute significantly to a rapid diagnosis of the infectious disease. Increasingly, PCR-based methods, gel electrophoresis, DNA microarrays, restriction endonuclease enzymes for plasmid or genomic DNA, sequencing and FISH techniques are well established in clinical laboratories (Mignard et al., 2006).

Figure A.2 Schematic illustration of laboratory procedures for the identification of pathogens



The modern technologies offer a detection of pathogens within hours but usually these novel methods are very expensive. Therefore, the diagnostic efficiency often depends on the hospital budget or medical standard of the respective country. Additionally, a long infection of a hospitalised patient increases the probability of pathogen transmission from sick to other sick or healthy persons (D´Costa et al., 2006). Rapid clinical diagnostics are urgent especially concerning the identification of antibiotic resistant bacteria. Hence, it is necessary to use reliable and low cost methods, e.g. FISH.

The nucleic acid hybridisation technology enables the specific detection of DNA or RNA sequences, which are characteristic for bacteria. The increased nucleic acid sequence information will be very helpful for treatment and surveillance of important pathogens. In comparison to classical approaches that require between one to several days the rapid determination of the bacteria leads to faster and precise drug treatment. Additionally, the higher heat and pH-value stability, the sensitivity and a good resistance to organic solutions and other chemicals is an advantage of nucleic acid probes. Hence, they are appropriate for the application in clinical material of different sources (blood, urine, stool or swabs) as well as for slowly growing or non-cultivable bacteria (Madigan, 2001).

Now, the automation, standardisation and miniaturisation of described methods (Figure A.2, p. 13) for practicability to high throughput and reduction of analysis time is strived for the detection of medically relevant pathogens. The development of several diagnostic proceedings was already transferred to microplates (Pettit et al., 2005) or DNA microarrays (Leinberger et al., 2005; Malanoski, 2006).

A.3. Bacteria associated with nosocomial infections and the PathoGenoMik project (Genome research on pathogenic bacteria)

Nosocomial infections, also called hospital-acquired or health-care associated infections, are diseases, which originate and appear after 48 hours or longer hospitalisation and are caused by bacterial, viral or fungal pathogens. The reasons for the emergence of nosocomial infections are manifold. These infections emerge due to invasive procedures (intubations or catherisations), pathogens present on hands of medical personnel, contamination of air-conditioning or water systems as well as individual risk factors (severity of illness, immunocompromised patients). Statistics estimate that worldwide nosocomial infections occur in 5 % of all acute care hospitalisations. No significant differences were observed concerning the incidence between genders but some infections are age-related (Nguyen, 2006). In 1996, the KISS (Krankenhaus-Infektions-Surveillance-System) was initiated in Germany based on the already existing NNIS (National Nosocomial Infections Surveillance System) in the USA. It collects reference data to control and prevent new tendencies concerning health-care associated diseases. Common nosocomial infections are sepsis, infections of the urinary or lower respiratory tract as well as gastrointestinal, skin, soft tissue and surgical-site infections. These are caused by *Escherichia coli* strains, coagulase-positive and -negative staphylococci, enterococci, *Enterobacter* and *Pseudomonas* species. Infections by *Acinetobacter baumannii* increase due to its augmented drug resistance against several antibiotics. In general, infections associated with hospitalisation are more dangerous and virulent in comparison to community-acquired infections because the pathogens often harbour antibiotic resistance genes (Jones et al., 2004).

The bacterial spectrum, which should be screened within the project, was selected by clinical microbiologists and is composed of Gram-negative and -positive bacteria that are divided in consideration of medical importance in five groups (non-fermenters, *Enterobacteriaceae*, enterococci, staphylococci and streptococci). The Gram-negative bacteria are composed of several non-fermenters (see below) and *Enterobacteriaceae.* The Gram-positive bacteria include enterococci, staphylococci and streptococci. The non-fermenter group contains aerobic *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens* and

Stenotrophomonas maltophilia, which are morphologically characterised as rod-shaped bacteria, apart from *A. baumannii* that can change to a coccoid form depending on used media.

A. baumannii is a common soil, water and sewage inhabitant but also found in food. This bacterium can occur on human skin without infectious effects for the chronic carrier but eventually it can be transmitted from human to human in the hospital environment. In the last 20 years, it increased its clinical importance because it affects especially immunocompromised patients and emergences as a major nosocomial pathogen worldwide of pneumoniae, sepsis, wound infections, urinary tract infections and meningitis. An additional problem of this organism is the ability to acquire increasingly multi-drug resistances to beta-lactams, fluoroquinolones and most aminoglycosides. The therapy is still possible by treatment with carbapenems, but imipenem-hydrolysing strains are known and were recently described (Lolans et al., 2006; Wareham and Bean, 2006).

The genus *Pseudomonas* includes motile, long or slightly curved rods ubiquitously present in almost all natural habitats. They occur associated with plants and animals either as normal flora or as pathogen. *Pseudomonas* spp. can generate food spoilage of milk and dairy products. In the environment, they play an important role in global decomposition, biodegradation and in the carbon cycle. They possess the ability to form viscous biofilms and produce lipopolysaccharides as a component of their outer cell wall (Drenkard et al., 2002). *P. aeruginosa* inhabits terrestrial and aquatic habitats as well as plants, fruits, food and colonise the intestinal tract of humans and animals. While mostly harmless for healthy people, this bacterium is clinically relevant especially in wound, respiratory and urinary tract infections, pneumonia and sepsis for hospitalised patients. In addition to lipopolysaccharides intra- and extracellular virulence factors (flagellum, adhesins, proteases and haemolysins) aggravate an infection. Especially, people suffering from cystic fibrosis or AIDS are at risk to die of pneumonia caused by this pathogen. Its severity is due to biofilm formation by impeding the access of drugs or macrophages. *P. aeruginosa* is often resistant against many metallo-beta-lactams and aminoglycosides (Pseudomonas genome database; Henrichfreise et al., 2005). *P. fluorescens* secretes a soluble pigment called fluorescein, inhabits soil and water and is rather

known as a plant inhabitant or food spoiler as an infectious bacterium. A *P. fluorescens* strain, which possesses enzymes conferring resistance to the aminoglycoside gentamicin, was described in a former study (Lambert et al., 1994).

A further representative of non-fermenters is the motile bacterium *Stenotrophomonas maltophilia*, which was deemed an opportunistic bacterium ubiquitously present in soil, water, animals and plants or as commensal of the human skin flora. Due to e.g. contact infection, a colonisation in wounds, mucosa or fluids like blood is possible. It is also associated with cystic fibrosis or present as colonizer of the respiratory tract of immunosuppressed patients. In the last years, *S. maltophilia* became increasingly important as a nosocomial pathogen and intrinsic virulence factors (fibrinolysin, lipase, protease or elastase) contribute to its pathogenicity. This pathogen is naturally resistant against some beta-lactams. The additional ability to become insensitive to aminoglycosides is the result of conformational changes of the outer membrane at 30°C leading to decreased permeability. This inhibits the accessibility of antimicrobial substances to their targets and makes this pathogen unique (Waters and Ratjen, 2006; Anderson et al., 2006).

The rod-shaped and motile representatives of the family *Enterobacteriaceae* are *Enterobacter aerogenes*, *Escherichia coli*, *Klebsiella oxytoca* as well as *Klebsiella pneumoniae* used in this study are found in almost all natural habitats. They colonise the gut flora of humans and animals and can be associated with infections of the intestinal tract.

Enterobacter aerogenes is part of the human intestinal flora. Due to acquired antibiotic resistances it became a nosocomial pathogen frequently causing different infections (sepsis, infection of the respiratory or urinary tract, skin and soft tissue infections and endocarditis) and it is especially dangerous in intensive care units. Chromosomal-mediated resistance of this bacterium to inducible beta-lactams is known (Wiegand, 2003). Additionally, the alteration of its endotoxin inactivates carbapenems and contributes to the intrinsic virulence common in *E. aerogenes*. Furthermore, resistances against aminoglycosides, trimethoprim-sulfamethoxazole and quinolones are widely spread (Leying et al., 1991).

Escherichia coli colonises as a commensal the lower intestinal tract of mammals and contributes to the production of vitamin K in the gut. It is an important indicator of faecal contamination of drinking water and food. Several strains cause diseases concerning the intestinal or the urinary tract and become manifest inter alia in diarrhoea or kidney damage. Based on virulence properties the enteric human relevant *E. coli*-strains are divided in enterotoxigenic (ETEC), enteropathogenic (EPEC), enteroinvasive (EIEC), enteroaggregative (EAggEC), neonatal meningitis (NMEC) and enterohaemorhaggic *E. coli* (EHEC). EHEC includes the strain O157:H7 that possesses the Shiga-like toxin and causes severe diarrhoea. Hospital-acquired infections associated with *E. coli* are sepsis, meningitis, infections of the urinary tract or intestines and mastitis. Although many antimicrobial substances are still effective in last years extended-spectrum beta-lactamases (ESBLs) produced by *E. coli* emerge increasingly conferring resistance to broad-spectrum antibiotics like cephalosporins (Hanson, 2003; Mulvey et al., 2004).

Klebsiella spp. are non-motile, encapsulated rods found in soil. They are able to fix nitrogen and are common in the natural flora of the mouth, skin and intestines. *K. oxytoca* differs from *K. pneumoniae* in several biochemical reactions. However, both are opportunistic pathogens involved in antibiotic-associated diarrhoea and in nosocomial infections (infections of the urinary or lower respiratory tract and of wounds). Ampicillin resistance is mediated by chromosomal class A beta-lactamases. Additionally, acquired ESBLs contribute to the development of further drug resistances (Beaugerie and Petit, 2004). The danger of strains producing ESBLs or other drug resistances is the possibility to spread genes on plasmids or mobile genetic elements to susceptible strains. Especially in hospitals, the transmission of resistances is higher where resistant pathogenic strains exist side by side with other bacteria and humans.

The Gram-positive representatives of the genera *Enterococcus*, *Staphylococcus* and *Streptococcus* are coccoid and non-motile. *Enterococcus* including *E. faecalis* and *E. faecium* occurring in pairs are lactic acid bacteria usually found in food e.g. cheese and raw sausage. They are also commensal organisms of human and animal intestinal tract. Beside their positive effects on the alimentary system, particularly *E. faecalis* can cause infections of immunocompromised people. In recent years,

these bacteria became medically important causing about 15 % of nosocomial diseases including urinary tract infections, bacteraemia, endocarditis and meningitis. Sensitive strains could be treated with ampicillin and vancomycin. Some strains are inherently resistant to beta-lactams and many aminoglycosides. Problematically, in the last 20 years, vancomycin-resistant enterococci (VRE) emerged in the USA. Vancomycin was once considered as the drug of last resort (Ribeiro et al., 2006).

Staphylococcus spp. are widespread in the natural environment. These cocci are present on skin and mucosa of humans or animals and are clinically differentiated in coagulase-positive and -negative strains. They produce a toxin, which is responsible for food poisoning or invasion and can cause different infections. The coagulase-positive pathogenic *S. aureus* can infect wounds and is responsible for the toxic shock syndrome by releasing several toxins into the blood stream. Alarming is the increased occurrence of methicillin-resistant *Staphylococcus aureus* (MRSA) resistant to all known penicillins including methicillin. Methicillin is insensitive to beta-lactamases (penicillinases) produced by bacteria. MRSA is often a synonym for oxacillin-resistant and multi-resistant *S. aureus* usually involved in hospital-acquired infections. Currently apart from hospital-acquired MRSA, community-acquired strains of MRSA were also described in the USA. In general, the medical treatment was performed with vancomycin but some vancomycin-resistant *S. aureus* currently emerged. *S. aureus* is one of the most frequent isolates from hospital-acquired diseases of the blood stream as well as from infections of the respiratory tract and wounds (Bressler et al., 2005).
The coagulase-negative *S. epidermidis* can be a problematic pathogen after nidation in immunocompromised patients who carry indwelling catheters or pace makers. It can form biofilms and secrete slime, thus impeding the accessibility of drugs. Penicillin-resistant strains that also are insensitive to methicillin were described but can still be combated with vancomycin in combination with rifampicin or aminoglycosides (Barberis et al., 2001).

The genus *Streptococcus* includes pathogenic and non-pathogenic bacteria, which appear in pairs or chains and colonise human and animal tissues or mucosa of the mouth, nares or pharynx. The members *S. agalactiae* and *S. pyogenes* are beta-haemolytic streptococci. They hydrolyse blood cells completely and are responsible

for several cases of pharyngitis, rheumatic fever, scarlet fever and acute glomerulonephritis, meningitis, endocarditis and sepsis. Additionally, some of them produce exotoxins as well as enzymes (streptolysine or hyaluronidase), which enable the colonisation and persistence of the host with an intact immune system. Macrolide resistances occurring in *Streptococcus* species were described (Cascone et al., 2002).

The actual study was part of the PathoGenoMik (Genome research on pathogenic bacteria) project within the scope of “Rapid diagnosis of antibiotic resistances in medicine”, which was financed by the German federal Ministry of Education and Research (Bundesministerium für Bildung und Forschung, BMBF).

Novel types of taxon-specific oligo-oligonucleotide probes and additional polynucleotide probes targeting resistance genes of pathogens should be designed. Afterwards, these probes should be evaluated in hybridisations for the identification and separation of pathogens from clinical material with the object of suitability for diagnostic screening. Furthermore, antibiotic resistance genes should be analysed and their presence verified on DNA microarrays developed by project partners to be useable after immobilisation of target cells from clinical samples.

A.4. Aims of this study

The main part of this study was focused on the development and application of mono- and multispecific oligo-oligonucleotide probes combining the advantages of oligonucleotide probes (high specificity) and polynucleotide probes (halo signals essential for enrichments). The hybridisations using these novel types of probes should be evaluated for their applicability in separation experiments of target cells in microplates. Further investigations on identity, virulence or presence of resistance genes of pathogens should be applicable for hospital laboratory diagnostics subsequent to the immobilisation of bacteria from different clinical material.
Hence, the design and generation of novel types of polynucleotide probes was necessary as well as modifications of existing protocols and the introduction of additional steps. This approach implied the evaluation of taxon-specific oligonucleotide probes, additional pretreatment methods of several samples and the adaptation to Gram-positive bacteria. The time-reducing procedure and the applicability in real clinical material were considered in the development of an appropriate protocol.
The next objective was the development of further antibiotic resistance genes targeted RING-FISH polynucleotide probes to enlarge the spectrum of detectable resistance genes and their applicability in immobilisation experiments. Therefore, RING-FISH probes of variable length were synthesised and special preparation methods for several clinical material as well as pretreatments on additional target organisms were required. Finally, for the diagnostic screening in laboratories, the hybridisation and enrichment protocol using polynucleotide probes targeting resistance genes should be also evaluated.

B. Material and Methods

B.1. Bacterial strains and sample material

Table B.1 Gram-negative bacteria in pure culture and needed cultivation conditions.

Bacteria	Origin	Cultivation conditions
Acinetobacter baumannii	AF 438775	Nutrient 37°C aerob
Burkholderia cepacia	RVB strain2	Nutrient 37°C aerob
Enterobacter aerogenes	AF 427068	Nutrient 37°C aerob
Escherichia coli	AF 441612	Nutrient 37°C aerob
Haemophilus influenzae	AF 484476	Nutrient 37°C aerob
Klebsiella oxytoca	AF 479983	Nutrient 37°C aerob
Klebsiella pneumoniae	AF 429457	Nutrient 37°C aerob
Morganella morganii	DSM 30164	Nutrient 37°C aerob
Neisseria canis	LMG 8383T	LB 37°C aerob
Pseudomonas aeruginosa	AF 426470	Nutrient 30°C aerob
Pseudomonas fluorescens	AF 603085	Nutrient 30°C aerob
Stenotrophomonas maltophilia	AF 484133	Nutrient 37°C aerob

AF: number of internal clinical classification of patient samples and organism isolated from clinical specimen; organisms and sample material were fixed and kindly provided by staff members of Prof. Dr. Cornelius Knabbe, Department of Clinical Chemistry and Laboratory Medicine, Robert Bosch hospital, Stuttgart; Nutrient- and LB-medium see B.2, p. 25.
DSM: DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany: http://www.dsmz.de
LMG: BCCM/LMG-Bacteria Collection, Laboratorium voor Microbiologie, Gent, Belgium: http://www.belspo.be/bccm/index.htm

Table B.2 Gram-positive bacteria in pure culture and needed cultivation conditions.

Bacteria	Origin	Cultivation conditions
Enterococcus faecalis	Staph/Strep28	BHI 37°C anaerob
Enterococcus faecium	AF 458387	BHI 37°C anaerob
Staphylococcus aureus	AF 433088	BHI 37°C anaerob
Staphylococcus epidermidis	MRSE/ S:23	BHI 37°C anaerob
Staphylococcus haemolyticus	Nr unknown	BHI 37°C anaerob
Streptococcus agalactiae	AF 616311	BHI 37°C anaerob
Streptococcus pneumoniae	AF 674837	BHI 37°C anaerob
Streptococcus pyogenes	AF 611333	BHI 37°C anaerob

AF: number of internal clinical classification of patient samples and organism isolated from clinical specimen; organisms and sample material were fixed and kindly provided by staff members of Prof. Dr. Cornelius Knabbe, Department of Clinical Chemistry and Laboratory Medicine, Robert Bosch hospital, Stuttgart; BHI-medium see B.2, p. 25.

Table B.3 Clinical samples spiked with target bacteria originating from Robert Bosch hospital, Stuttgart, Germany.

Bacteria	AF number	Sample material *
Acinetobacter baumannii	AF 438775	EDTA blood, plasma, urine
Burkholderia cepacia	RVB strain2	Urine
Enterobacter aerogenes	AF 427068	EDTA blood, plasma, urine
Escherichia coli	AF 441612	EDTA blood, plasma, urine
Haemophilus influenzae	AF 484476	EDTA blood, plasma, urine
Klebsiella oxytoca	AF 479983	EDTA blood, plasma, urine
Klebsiella pneumoniae	AF 429457	EDTA blood, plasma, urine
Pseudomonas aeruginosa	AF 426470	EDTA blood, plasma, urine
Pseudomonas fluorescens	AF 603085	EDTA blood, plasma, urine
Stenotrophomonas maltophilia	AF 484133	EDTA blood, plasma, urine
Enterococcus faecalis	Staph/Strep28	EDTA blood, plasma, urine
Enterococcus faecium	AF 458387	EDTA blood, plasma, urine
Staphylococcus aureus	AF 433088	-
Staphylococcus epidermidis	MRSE/S:23	EDTA blood, plasma, urine
Staphylococcus haemolyticus	Nr unknown	Urine
Streptococcus agalactiae	AF 616311	EDTA blood, plasma, urine
Streptococcus pyogenes	AF 611333	EDTA blood, plasma, urine

AF: number of internal clinical classification of patient samples and organism isolated from clinical specimen; organisms and sample material was fixed and kindly provided by staff members of Prof. Dr. Cornelius Knabbe, Department of Clinical Chemistry and Laboratory Medicine, Robert Bosch hospital, Stuttgart; * Sample material was fixed with PFA as described under B.3, p. 26.

Table B.4 Real clinical samples containing target bacteria as well as other coexistent organisms, collected at Robert Bosch hospital, Stuttgart, Germany.

Target bacteria and other organisms present in real clinical samples	**number**	**AF number**	**Real clinical sample ***
Enterococcus faecium	**1**	403250	Anus-/ Rectal smear
Enterococcus faecium	**3**	492806	Blood culture
***Enterococcus faecalis* +** *Enterobacter cloacae*	**4**	501779	Urine
Enterococcus faecalis	**7**	405607	Urine
***Enterococcus faecalis* +** *E. coli*	**15**	405639	Urine
Escherichia coli	**2**	478499	Vaginal smear
Escherichia coli	**6**	405059	Blood culture
***E. coli* +** *Klebsiella terrigena*	**10**	682242	Wound smear
***E. coli* +** *Candida albicans*	**12**	682396	Urethra smear
***E. coli* +** *Enterococcus faecalis*	**15**	405639	Urine
Klebsiella pneumoniae	**9 (KF)**	41162	Tracheal swab
Pseudomonas aeruginosa	**3 (KF)**	625811	Wound smear
Pseudomonas aeruginosa	**4 (KF)**	492106	Tracheal swab
Pseudomonas aeruginosa	**5**	416431	Blood culture
Pseudomonas aeruginosa	**16**	416990	Urine
Pseudomonas aeruginosa	**17**	506985	Blood culture
***Staphylococcus aureus* MRSA**	**8**	678848	Wound smear, sacral
***Staphylococcus aureus* +** other Gram-positive bacteria	**11**	683711	Swab i.op., rump
***Streptococcus agalactiae* +** few Gram negative rods	**14**	403174	Anus/Rectum
Streptococcus pyogenes	**13**	677365	Wound smear, perianal

AF: number of internal clinical classification of patient samples and organism isolated from clinical specimen; organisms and sample material was fixed and kindly provided by staff members of Prof. Dr. Cornelius Knabbe, Department of Clinical Chemistry and Laboratory Medicine, Robert Bosch hospital, Stuttgart; * Sample material was fixed with PFA as described under B.3, p.26.
KF: real clinical samples, which were fixed by K. Fichtl at Robert Bosch hospital in March 2004; number: sample number of real clinical specimens

B.2. Cultivation of organisms

The organisms listed in Tables B.1 and B.2 (p. 22) were cultivated under given conditions. Culture media were prepared with H_2O_{MQ}, sterilised in an autoclave for 20 min at 121°C and a pressure of one bar (1013 hPa). For solid media, 12 g/l of agar (Difco Bacto Agar, Gibco BRL, Eggenstein, Germany) were added.

Culture media:

LB (Luria Bertani) medium

tryptone (tryptic digest of casein peptone)	10.0 g	
yeast extract	5.0 g	
NaCl	5.0 g	
dH_2O	ad 1000 ml	pH 7.0-7.2

Nutrient medium

peptone	5 g	
beef extract	3 g	
dH_2O	ad 1000 ml	pH 7.0

BHI (Brain-Heart-Infusion) medium

(ready made medium, Oxoid, Wesel, Germany)	37 g
dH_2O	ad 1000 ml

B.3. Cell fixation of bacteria from pure culture and sample material with PFA

The cell fixation leads to an increased permeability of the cell envelope for oligo- or polynucleotide probes and impedes RNA or DNA degradation by denaturing proteins and enzymes. For standard fixation a 4 % PFA solution was used as described below. For several Gram-negative and Gram-positive bacteria, pre-/postfixational protocols were applied as described under B.9.1.2. (p. 39), B.9.2.2. (p. 43), B.9.3.2. (p. 45), B.11.2.2. (p. 59) and C.5.1. (p. 114).

Material:

PBS (phosphate buffered saline): 130 mM NaCl
1.5 mM KH_2PO_4
8.0 mM Na_2HPO_4
2.7 mM KCl

$EtOH_{abs}$

PFA (paraformaldehyde): 4 % (w/v) in PBS, pH 7.0

Preparation of PFA solution:
Heat PBS to 60°C and dissolve PFA by slowly adding NaOH. Titrate to pH 7.0

Procedure for fixation of bacterial cells in pure culture:

- harvest cells during exponential growth phase (OD_{600} 0.6 -0.8) and centrifuge at 5000-12000 rpm for 2-10 min (depending on culture volume)
- resuspend cell pellet in PBS (dependent on cell pellet volume: 100-300 µl) and add 3 volumes of PFA 4 % (300-900 µl)
- incubate at 4°C for 0.5-12 h
- centrifuge at 5000-12000 rpm for 2-10 min
- wash with appropriate amount of PBS (300-500 µl)
- resuspend in appropriate amount (100-500 µl) of PBS: $EtOH_{abs}$ in ratio 1:1 and store at -20°C up to 1 year

Procedure for fixation of clinical material containing bacteria:

- centrifuge the sample material at 5000-12000 rpm for 2-10 min (depending on sample volume)
- resuspend cell pellet in PBS (dependent on cell pellet volume: 100-500 µl) and add 3 volumes of PFA 4 % (300-1500 µl)
- incubate at 4°C for 0.5-12 h
- centrifuge at 5000-12000 rpm for 2-10 min
- wash with appropriate amount of PBS (500 µl)
- resuspend in appropriate amount (250-500 µl) of PBS: $EtOH_{abs}$ in ratio 1:1 and store at -20°C up to 1 year

B.4. Purification of nucleic acids

B.4.1. Purification of genomic DNA (Wisotzkey et al., 1990 modified)

Enzyme solutions were freshly prepared. Buffers and other solutions were made with water from a Milli-Q-plus-system (Millipore, Bradford, Mass., USA) and autoclaved at 121°C and 1bar excess pressure for 20 min unless stated otherwise.

<u>Material:</u>

Saline-EDTA	0.15 M NaCl 0.01 M EDTA, pH 8.0
SSC, 20x (standard saline citrate)	3 M NaCl 0.3 M trisodiumcitrate, pH 7.0
Tris-HCl	10 mM Tris-HCl, pH 8.5
NaAc	5 M sodium acetate
SDS	25 % (w/v) sodiumdodecylsulfate; not autoclaved
CIA	chloroform: isoamylalcohol 24:1 (v/v)

lysozyme solution	10 mg/ml in 10 mM Tris-HCl
proteinase K	10 mg/ml proteinase K (Roche, Mannheim, Germany) in H_2O_{MQ}
RNase A	0.5 mg/ml in 2xSSC
$EtOH_{abs}$	
EtOH 70 %	

Procedure

- centrifuge 5-30 ml overnight culture at 12000 rpm for 5 min
- remove medium and resuspend cell pellet in 0.5-3 ml Saline-EDTA
- add 20-120 µl of lysozyme solution and incubate at 37°C for 30 min
- add 10-60 µl of RNase A and incubate at 37°C for 30 min
- add 10-60 µl of proteinase K and incubate at 37°C for 30 min
- add 40-200 µl of 25 % SDS and incubate at 60°C for 10 min
- add 745-4470 µl CIA and 180-1080µl NaAc, shake carefully and centrifuge 10 min at 12000 rpm
- transfer upper phase into a new reaction tube, discard lower phase
- add 2 vol of $EtOH_{abs}$ and store at -20°C for 2 h to precipitate DNA
- centrifuge at 12000 rpm for 15 min, wash pellet with EtOH 70 %
- resuspend DNA in 50-100 µl in and store at -20°C H_2O_{MQ}

B.4.2. Purification of plasmid DNA

The isolation of plasmid DNA was performed with QIAprep Spin Miniprep Kit (QIAGEN, Hilden, Germany) according to the manufacturer´s instructions.

B.5. Photometric measurement of nucleic acid concentration in solution

Due to their aromatic ring system, nucleic acids absorb light at certain wavelengths. The maximum absorption of light for DNA and RNA occurs at 260 nm. To determine the concentration, an aliquot of nucleic acids was measured in a spectral photometer (Spekol 1300, Analytik Jena, Germany) at 260 nm. The determination of the concentration is derived from the following approximated values.

Double stranded (ds) DNA:	1 OD 260 nm ≈ 50 µg/ml
Single stranded (ss) DNA:	1 OD 260 nm ≈ 37 µg/ml
(www.amershambiosciences.com)	
Single stranded (ss) RNA:	1 OD 260 nm ≈ 40 µg/ml

Potential contaminations of DNA can be estimated using the quotient between E260 nm/E230 nm and E260 nm/E280 (Marmur, 1961). The quotient E260/E230 < 2.0 indicates that the solution could be contaminated with RNA, a quotient of E260/280 < 1.8 shows a possible impurity with proteins and aromatic substances e.g. phenol.

B.6. Agarose Gel Electrophoresis

Agarose gel electrophoresis is a common method to analyse size, purity and concentration of nucleic acids. Due to their negative charge, they migrate to the positive pole in an electric field depending on their size and conformation (circular, linear or supercoiled). For the visualisation of DNA/RNA under UV light (302 nm) an intercalating substance: ethidium bromide was used. To identify the fragments and concentration of the sample a standard size marker was used.

Material:

100xTAE	4.0 M Tris
	2.0 M acetic acid
	0.2 m EDTA, pH 8.0

Agarose gel	1-2 % agarose (Gibco/BRL, Eggenstein, Germany) Boiled in 1xTAE
Loading buffer	10 mM EDTA 5 % (w/v) Ficoll (Sigma-Aldrich, Steinheim, Germany) 0.05 bromphenol blue 0.05 xylene cyanol
Ethidium bromide	1 µg/ml ethidium bromide
Standard marker 1	1 µg 1kb standard (Invitrogen, Carlsbad, Ca., USA)
Standard marker 2	1 µg 100bp standard (Invitrogen, Carlsbad, Ca., USA)
Gel chamber	gel electrophoresis chamber (Gibco/BRL, Eggenstein, Germany) Type H3: 11x14 cm, 100ml gel volume
Gel documentation	UV transilluminator; 302 wavelength (Bachofer, Reutlingen, Germany) Cybertech CS1 image Documentation (Cybertech, Berlin, Germany)

Procedure

- load agarose gel with a mixture of 1 vol sample and 1 vol loading buffer
- start electrophoresis in 1xTAE and 90-160mA
- stain gel in ethidium bromide solution for 20 min and wash with water
- visualisation using transilluminator

B.7. Primers

Table B.5 Primers for amplification of *ampC* gene of *Acinetobacter baumannii* AF438775 using the sequence AY178996 from NCBI as reference; V: forward primer, R: reverse primer, the underlined sequence represents the T3 promoter as binding region for the RNA polymerase

Name	Sequence 5→3´	T_D [°C]	GC [%]
AbAmpCV	ATTTATGCGGGCAATACACC	50	45
AbAmpCVT3	ATAGGTATTAACCCTCACTAAAGATTTATGCGG GCAATACACC	65	40
AbAmpCR	CAACCTTTCCAAATAGGCCA	50	45
AbAmpCRT3	ATAGGTATTAACCCTCACTAAAGCAACCTTTCCA AATAGGCCA	65	40

Table B.6 Primers for amplification of *ampC* gene of *Enterobacter aerogenes* AF 427068 using the sequence AJ544161 from NCBI as reference; V: forward primer, R: reverse primer, the underlined sequence represents the T3 promoter as binding region for the RNA polymerase

Name	Sequence 5´→3´	T_D [°C]	GC [%]
EaAmpCV1	GCGAGAACCGATAATGGAAA	50	45
EaAmpCVT3	ATAGGTATTAACCCTCACTAAAGGCGAGAACCG ATAATGGAAA	60	40
EaAmpCR	GCGGAATAAAAGCCACGTAA	50	45
EaAmpCRT3	ATAGGTATTAACCCTCACTAAAGGCGGAATAAA AGCCACGTAA	60	40

Table B.7 Primers for amplification of *ampC* gene of *Escherichia coli* AF441612 using the sequence AB108683 from NCBI as reference; V: forward primer, R: reverse primer, the underlined sequence represents the T3 promoter as binding region for the RNA polymerase

Name	Sequence 5´→3´	T_D [°C]	GC [%]
EcAmpCV	ACCGCTAAACAGTGGAATGG	52	50
EcAmpCVT3	ATAGGTATTAACCCTCACTAAAGACCGCTAAACA GTGGAATGG	66	42
EcAmpCR	TTGCCAGTAGCGAGATTGTG	52	50
EcAmpCRT3	ATAGGTATTAACCCTCACTAAAGTTGCCAGTAGC GAGATTGTG	66	42

Table B.8 Primers for amplification of *ampC* gene of *Klebsiella oxytoca* AF479983 using the sequence D84548 from NCBI as reference; V: forward primer, R: reverse primer, the underlined sequence represents the T3 promoter as binding region for the RNA polymerase

Name	Sequence 5´→3´	T_D [°C]	GC [%]
KoAmpCV	TGGTCTGGAGTCCGATTACC	54	55
KoAmpcVT3	ATAGGTATTAACCCTCACTAAAGTGGTCTGGAG TCCGATTACC	67	44
KoAmpCR	TGATCTTCCGGCCAGATAAC	52	50
KoAmpCRT3	ATAGGTATTAACCCTCACTAAAGTGATCTTCCG GCCAGATAAC	66	42

Table B.9 Primers for amplification of *bla*$_{OXY2\text{-}5}$ gene of *Klebsiella oxytoca* AF479983 using the sequence AY303806 from NCBI as reference; V: forward primer, R: reverse primer, the underlined sequence represents the T3 promoter as binding region for the RNA polymerase

Name	Sequence 5´→3´	T_D [°C]	GC [%]
KoCef1V	TAAGCAGGCGCTGGAAGGAC	61	60
KoCef1VT3	ATAGGTATTAACCCTCACTAAAGTAAGCAGGCGC TGGAAGGAC	70	46
KoCef1R	CACATGGCAAAACGCTCGTC	59	55
KoCef1RT3	ATAGGTATTAACCCTCACTAAAGCACATGGCAAA ACGCTCGTC	70	44
KoCef2V	GACGAGCGTTTTGCCATGTG	60	55
KoCef2VT3	ATAGGTATTAACCCTCACTAAAGGACGAGCGTTT TGCCATGTG	70	44
KoCef2R	ACCAGCGGAGCGTGATCTTC	61	60
KoCef2RT3	ATAGGTATTAACCCTCACTAAAGACCAGCGGAGC GTGATCTTC	70	46

Table B.10 Primers for amplification of *bla*$_{SHV-1}$ gene of *Klebsiella pneumoniae* AF 429457 using the sequence AF124984 from NCBI as reference; V: forward primer, R: reverse primer, the underlined sequence represents the T3 promoter as binding region for the RNA polymerase

Name	Sequence 5´→3´	T_D [°C]	GC [%]
KpPip1V	TCGGCCCTCACTCAAGGATG	61	60
KpPip1VT3	ATAGGTATTAACCCTCACTAAAGTCGGCCCTCAC TCAAGGATG	70	46
KpPip1R	CCGTCGGCAAGGTGTTTTTC	59	55
KpPip1RT3	ATAGGTATTAACCCTCACTAAAGCCGTCGGCAAG GTGTTTTTC	70	44
KpPip2V	CAGATCGGCGACAACGTCAC	61	60
KpPip2VT3	ATAGGTATTAACCCTCACTAAAGCAGATCGGCGA CAACGTCAC	70	46
KpPip2R	CCTTTGACCCGGTTCACCAC	61	60
KpPip2RT3	ATAGGTATTAACCCTCACTAAAGCCTTTGACCCG GTTCACCAC	70	46

Table B.11 Primers for amplification of *aac(6´)* gene of *Pseudomonas fluorescens* AF 473825 using the sequence L06163 from NCBI as reference; V: forward primer, R: reverse primer, the underlined sequence represents the T3 promoter as binding region for the RNA polymerase

Name	Sequence 5´→3´	T_D [°C]	GC [%]
PsflGTMV	GCCGTAGAAGAGCAAGG	59	55
PsflGTMR188	ACAGTTTACGAACCGAACAGG	57	47
PsflGTMR188T3	ATAGGTATTAACCCTCACTAAAGACAGTTTA CGAACCGAACAGG	71	40
PsflGTMR282	TAACAAGCCATGAAAACCGCC	57	47
PsflGTMR282T3	ATAGGTATTAACCCTCACTAAAGTAACAAGC CATGAAAACCGCC	71	40
PsflGTMR409	CTTTGTTTTAGGGCGACTGC	57	50
PsflGTMR409T3	ATAGGTATTAACCCTCACTAAAGCTTTGTTTT AGGGCGACTGC	71	41

Table B.12 Primers for amplification of *vanB* gene of *Enterococcus faecalis* Staph/Strep 28 and *Enterococcus faecium* AF 458387 using the sequence AF786179 from NCBI as reference; V: forward primer, R: reverse primer, the underlined sequence represents the T3 promoter as binding region for the RNA polymerase

Name	Sequence 5´→3´	T_D [°C]	GC [%]
EcfmVan_VP	CAGGGGCTGTTTGTATTG	53.7	50
EcfmVan_VPT3	ATAGGTATTAACCCTCACTAAAGCAGGGGCTG TTTGTATTG	70.4	41.5
EcfmVan_RP	GATGGATGCGGAAGATAC	53.7	50
EcfmVan_RPT3	ATAGGTATTAACCCTCACTAAAGGATGGATGC GGAAGATAC	70.4	41.5

Table B.13 Primers for amplification of *mecA* gene of *Staphylococcus aureus* AF 678848 using the sequence AY786579 from NCBI as reference; V: forward primer, R: reverse primer, the underlined sequence represents the T3 promoter as binding region for the RNA polymerase

Name	Sequence 5´→3´	T_D [°C]	GC [%]
SauMec_VP	ATCTTGGGGTGGTTACAACG	57.3	50
SauMec_VPT3	ATAGGTATTAACCCTCACTAAAGATCTTGGGG TGGTTACAACG	71.3	41.9
SauMec_RP	GTTCTGCAGTACCGGATTTG	57.3	50
SauMec_RPT3	ATAGGTATTAACCCTCACTAAAGGTTCTGCAG TACCGGATTTG	71.3	41.9

B.8. Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) including three major steps, which are repeated for 30 to 40 times, is an enzymatic technique to amplify defined DNA fragments. An automated cycler is used, which can rapidly heat and cool and enables the amplification of the fragment. The PCR comprises following steps: denaturation, annealing and elongation, which are done at different temperatures depending on the enzyme and primers.

Denaturation: thermal denaturation of the double stranded template DNA

Annealing: reduction of the temperature followed by specific binding of the oligonucleotide primers to their complementary sequence

Elongation: elongation of the primers in 5´-3´direction by a thermostable DNA polymerase

Repeated heating and cooling cycles duplicate the target DNA exponentially. 10^6 fold amplification of the template DNA is possible under optimal conditions of 30 cycles (Saiki et al., 1988).

The annealing temperature depends on the dissociation temperature T_D of chosen primers. An approximate value +/- 2°C for T_D can be calculated with the following formula (Suggs et al., 1981):

$$T_D\,[°C] = 2 \times (A+T) + 4 \times (G+C)$$

(A, C, G, T: number of nucleotides)

All PCR reactions were carried out in a Primus 96plus Thermal Cycler (MWG, Ebersberg, Germany) or Eppendorf Mastercycler Gradient Cycler (Eppendorf, Hamburg, Germany) using the following reagents:

TaKaRa ExTaq™ system (TaKaRa Shuzo Co., Otsu, Japan)

Reaction mixture per 100 µl PCR reaction:

Buffer (10x)	10 µl
dNTP-mix (2.5 mM each)	8 µl
Primer forward (50 pmol/µl)	1 µl
Primer reverse (50pmol/µl	1 µl
DNA	100 ng
ExTaq™	0.8 µl
H_2O_{MQ}	ad 100 µl

BioTaq™ DNA Polymerase system (Bioline, Luckenwalde, Germany)

Reaction mixture per 100 µl PCR reaction:

NH_4 Reaction Buffer (10x)	10 µl
dNTP-mix (2.5 mM each)	10 µl
50 mM $MgCl_2$	8 µl
Primer forward (50 pmol/µl)	0.5 µl
Primer reverse (50pmol/µl)	0.5 µl
DNA	100 ng
BioTaq™	0.5 µl
H_2O_{MQ}	ad 100 µl

Unless stated otherwise, the following standard PCR programme was used:

Table B.14 Standard PCR programme; x: T_D of primer; y: depending on fragment length, approximately 1 min per 1kb fragment length

Reaction	T	Time	Number of cycles
Initial denaturation	94°C	5 min	1
Denaturation	94°C	45 s	30
Annealing	x °C	1 min	
Elongation	72°C	y min	
Final elongation	72°C	5 min	1

B.9. Fluorescence *in situ* hybridisations (FISH) on slides

Hybridisations are subjected to several parameters. For the calculation of T_m following formulas can be considered as indication. However, to obtain a successful hybridisation resulting in reproducible specific signals of the probe, it is required to determine empirically optimal conditions for each probe.

DNA-DNA-hybridisations

Oligonucleotides (max. 50 nt) (Suggs et al., 1981):

$$T_D = 2 \times (A+T) + 4 \times (G+C)$$

Polynucleotides (Howley et al., 1979):

$$T_m = 81.5 + 16.6 \times \log M + 0.41 \times (\%GC) - 0.7 \times (\%\ FA)$$

RNA-RNA-hybridisations

(max. 500 nt) (Wetmur, 1991; Wahl et al., 1987):

$$T_m = 78 + 16.6 \log \frac{M}{1.0 + 0.7 \times M} + 0.41 \times \%\ GC - \frac{500}{D} - P - 0.35 \times \%\ FA$$

RNA-DNA-hybridisations

Oligonucleotides (max. 50nt) (Lathe, 1985; Wahl et al., 1987):

$$T_D = 81.5 + 16.6 \times \log M + 0.4 \times \%\ GC - 820/n - 0.5 \times \%\ FA$$

Polynucleotides (50 –max. 500nt) (Wetmur, 1991; Wahl et al., 1987):

$$T_m = 67 - 16.6 \times \log 10 \frac{M}{1.0 + 0.7 \times M} + 0.8 \times \%\ GC - \frac{500}{D} - P - 0.5 \times \%\ FA$$

T_m : melting temperature [°C] (polynucleotides)

T_D : dissociation temperature [°C] (oligonucleotides)

M:	molar concentration of sodium ions in hybridisation buffer
% GC:	percent guanine and cytosine
D, n:	length of duplex
% FA:	percent formamide in hybridisation buffer
P:	percent of mismatches

B.9.1. FISH using oligonucleotide probes

B.9.1.1. Protocol for FISH with Gram-negative bacteria using oligonucleotide probes

Material:

Hybridisation buffer	NaCl	900 mM
	Tris-HCl	20 mM pH 8.0
	SDS	0.01 %
	Formamide	x %
Washing buffer	NaCl	x mM (Table B.15)
	Tris-HCl	20 mM
	SDS	0.01 %
	EDTA	

Table B.15 Composition of washing buffer for oligonucleotide FISH

% formamide in hybridisation buffer	Na^+-concentration [mM] in washing buffer	µl 5M NaCl for 50 ml washing buffer
0	900	9000
5	636	6360
10	450	4500
15	318	3180
20	225*	2150*
25	159*	1490*
30	112*	1020*
35	80*	700*
40	56*	460*
45	40*	300*
50	28*	180*
55	20*	100*
60	14*	40*
65	10*	-*

* add 5 mM Na_2EDTA to washing buffer using more than 20 % formamide in the hybridisation buffer

Procedure

- apply 4 µl of a suspension of PFA fixed cells per microscope slide well (Marienfeld, Germany)
- dry for 5-10 min at 60°C
- dehydrate cells in increasing ethanol series (50 %, 80 %, 100 %) for 3 min each
- put 9 µl of hybridisation buffer on each well, add 1 µl probe solution (30 ng/µl) and mix carefully
- place tissue paper (moisturised with the hybridisation buffer) into a hybridisation tube, put slide into the tube and close it
- incubate 1.5-2 h at 46°C
- rinse slide with dH_2O
- take slide in tube filled with washing buffer (Table B.15, p. 38) for 15 min at 48°C
- rinse slide again with dH_2O and air dry
- for microscopy analysis: embed with mounting medium (Vectashield)

B.9.1.2. Protocol for FISH with Gram-positive bacteria using oligonucleotide probes

Due to the robust cell wall of Gram-positive bacteria, a respective permeabilisation of the cell wall prior to hybridisations is necessary to enable the target-probe hybrid. Therefore, different enzymatic and heat pretreatment methods were applied without degrading the cell structure.

Lysozyme	10 mg/ml in 50 mM Tris-HCl
Tris-HCl	50 mM
Mutanolysin	20 U/ml in H_2O_{MQ}
Lysostaphin	1 U/ml H_2O_{MQ}

Table B.16 Pretreatments of Gram-positive bacteria recommended for FISH using oligonucleotide probes on slides

Microorganism	1st EtOH series	Pretreatment method	2nd EtOH series
Enterococci	+	Lysozyme 10 min at 0°C	+
Staphylococci	+	Lysostaphin 13-20 min 37°C	+
Streptococci	+	Lysozyme 5 min at 0°C	+

Procedure

- apply 4 µl of a suspension of PFA fixed cells per microscope slide well (Marienfeld, Germany)
- dry for 5-10 min at 60°C
- dehydrate cells in increasing ethanol series (50 %, 80 %, 100 %) for 3 min each
- use respective enzyme or other pretreatment method (Table B.16, p. 40) recommended for the permeabilisation of the cell wall
- rinse slide with H_2O and dry under airflow
- dehydrate slide in second increasing ethanol series (50 %, 80 %, 100 %) for 3 min each
- put 9 µl of hybridisation buffer on each well, add 1 µl probe solution (30 ng/µl) and mix carefully
- place tissue paper (moisturised with the hybridisation buffer) into a hybridisation tube, put slide into the tube and close it
- incubate 1.5-2 h at 46°C
- rinse slide with dH_2O
- take slide in tube filled with washing buffer (Table B.15, p. 38) for 15 min at 48°C
- rinse slide again with dH_2O and air dry
- for microscope analysis: embed with mounting medium (Vectashield)

Table B.17 Oligonucleotide probes targeting 16S or 23S rRNA used for FISH.

Bacteria	Probe name	Probe sequence 5´→ 3´	rRNA	T_D [°C]	GC [%]
A. baumannii	Aba88[1]	TCCGCCGCTAGGTCCGGT	16S	62	72.2
	Aba79[1]	TAGGTCCGGTAGCAAGCT	16S	56	55.6
B. cepacia	Burcep[2]	CTGTGCGCCGGTCTTTCT	16S	53	61.1
E. aerogenes	Eae16S[3]	CGAGTAACGTCAATCGCC	16S	56	55.6
	Ent16S[1]	CCCCCTCTTTGGTCTTGC	16S	58	61.1
E. coli	Eco271[1]	CACACACTGATTCAGGCT	23S	54	50
H. influenzae	Hin343[1]	GTACCACAATATGGTTTTT	23S	50	31.6
K. oxytoca	Kox458[1]	GAATAAGGTTATTAACCTC	16S	50	31.6
K. pneumoniae	Kpn1701[1]	CCTACACACCAGCGTGCC	23S	60	66.7
P. aeruginosa	PseaerB[2]	TCTCGGCCTTGAAACCCC	23S	58	61.1
P. fluorescens	Ps-Ag1[4]	GATAACTCGTCATCAGCTC	23S	56	47.4
S. maltophilia	Sma1158[1]	AAGTTATGCACCCCAAG	23S	50	47.1
	Sma471[1]	GGTACCGTCATCCCAACC	16S	58	61.1
E. faecium	Efi58[5]	TGACTCCTCTTCAGACTT	23S	52	44.4
E. faecalis	Efe286[1]	TCCTTCAACTATACTAAC	23S	48	33.3
	Efe1473[1]	TAACTCTACTCAAGACTCAT	16S	54	35
S. aureus	Sau69[1]	GAAGCAAGCTTCTCGTCCG	16S	60	57.9
	Sau227[1]	AATGCAGCGCGGATCCAT	16S	56	55.6
S. epidermidis	Sep16S[3]	GTCAGAGGAGCAAGCTCC	16S	54	61.1
	Sep1498[1]	TCAGCCTTATGAGTGCCG	23S	56	55.6
S. haemolyticus	Sha307[1]	CTTCTTTGATTCAACTTTC	23S	50	31.6
S. agalactiae	Sag210[1]	CCATCTCACAGTGAAGCA	16S	54	50
S. pyogenes	Spy467[1]	CCGTCACTTGGTGGATTT	16S	54	50

[1] Santos, 2002, unpublished; [2] Hogardt et al., 2000; [3] Sandjong, 2003, unpublished; [4] Boye et al., 1995; [5] Behr, 2002;

B.9.2. FISH using RNA polynucleotide probes targeting antibiotic resistance genes - RING-FISH

Hybridisation buffer:

NaCl	75 mM
Tris-HCl, pH 8.0	20 mM
SDS	0.01 %
Formamide	0-10 %

B.9.2.1. Protocol for RING-FISH with Gram-negative bacteria using RING-FISH probes

Hybridisation on slides (Marienfeld, Germany)**:**

- adjust temperature of hybridisation oven to 80°C for the denaturation of RNA polynucleotide probes and another oven to 53°C for the hybridisation
- apply 4 µl of fixed bacterial cell or sample material per microscope slide well
- dry 5 -10 min at 60°C
- dehydrate slide in increasing ethanol series (50 %, 80 %, 100 %) for 3 min each
- put 10 µl of hybridisation buffer onto each well, add 4-5 µl probe solution and mix carefully
- put slide into the hybridisation tube lined with hybridisation-buffer-moisturised tissue and close it
- denature RNA polynucleotide probes at 80°C for 20 min
- hybridise at 53°C for 17-25 h
- rinse slide with dH_2O and air dry
- for microscope analysis: embed with mounting medium (Vectashield) or store at -20°C

B.9.2.2. Protocol for RING-FISH with Gram-positive bacteria using RING-FISH probes

Table B.18 Pretreatments of Gram-positive bacteria recommended for RING-FISH on slides.

Bacteria	1st EtOH series	Enzymatic pretreatment at 37°C	Pretreatment at 200°C	2ndt EtOH series
Enterococci	+	Lysozyme 10 min	5 min	+
Staphylococci	+	Lysostaphin 10 min	7 min	+
Streptococci	+	Mutanolysin 5 min	1 min	+

- apply 4 µl of a suspension of PFA fixed cells per microscope slide well (Marienfeld, Germany)
- dry for 5-10 min at 60°C
- dehydrate cells in increasing ethanol series (50 %, 80 %, 100 %) for 3 min each
- use respective enzyme or other pretreatment method (Table B. 18, p. 43) recommended for the permeabilisation of the cell wall
- rinse slide with H_2O and dry under airflow
- dehydrate slide in second increasing ethanol series (50 %, 80 %, 100 %) for 3 min each
- put 10 µl of hybridisation buffer onto each well, add 4-5 µl probe solution and mix carefully
- put slide into the hybridisation tube lined with hybridisation-buffer-moisturised tissue and close it
- denature RNA polynucleotide probes at 80°C for 20 min
- hybridise at 53°C for 17-25 h
- rinse slide with dH_2O and air dry
- for microscope analysis: embed with mounting medium (Vectashield) or store at -20°C

B.9.3. FISH using RNA oligo-oligonucleotide probes targeting rRNA

Hybridisation buffer:

NaCl	75 mM
Tris-HCl, pH 8.0	20 mM
SDS	0.01 %
Formamide	0-40 %

B.9.3.1. Protocol for FISH with Gram-negative bacteria using oligo-oligonucleotide probes

Hybridisation on slides (Marienfeld, Germany)**:**

- adjust temperature of hybridisation oven to 80°C for the denaturation of RNA polynucleotide probes and another oven to 53°C for the hybridisation
- apply 4 µl of fixed bacterial cell or sample material per microscope slide well
- dry 5 -10 min at 60°C
- dehydrate slide in increasing ethanol series (50 %, 80 %, 100 %) for 3 min each
- put 10 µl of hybridisation buffer onto each well, add 4-5 µl probe solution and mix carefully
- put slide into the hybridisation tube lined with hybridisation-buffer-moisturised tissue and close it
- denature RNA polynucleotide probes at 80°C for 25-30 min
- hybridise at 53°C for 3-6 h
- rinse slide with dH_2O and air dry
- for microscope analysis: embed with mounting medium (Vectashield) or store at -20°C

B.9.3.2. Protocol for FISH with Gram-positive bacteria using oligo-oligonucleotide probes

Table B.19 Pretreatments recommended for FISH using RNA oligo-oligonucleotide probes on slides

Bacteria	1st EtOH series	Enzymatic pretreatment at 37°C	Pretreatment at 200°C	2nd EtOH series
Enterococci	+	Lysozyme 10 min	5 min	+
Staphylococci	+	Lysostaphin 10-15 min	7 min	+
Streptococci	+	Mutanolysin 5 min	1 min	+

Hybridisation on slides (Marienfeld, Germany)**:**
- adjust temperature of hybridisation oven to 80°C for the denaturation of RNA polynucleotide probes and another oven to 53°C for the hybridisation
- apply 4 µl of a suspension of PFA fixed cells per microscope slide well (Marienfeld, Germany)
- dry for 5-10 min at 60°C
- dehydrate cells in increasing ethanol series (50 %, 80 %, 100 %) for 3 min each
- use respective enzyme or other pretreatment method (Table B.19, p. 45) recommended for the permeabilisation of the cell wall
- rinse slide with H_2O and dry under airflow
- dehydrate slide in second increasing ethanol series (50 %, 80 %, 100 %) for 3 min each
- put 10 µl of hybridisation buffer onto each well, add 4-5 µl probe solution and mix carefully
- put slide into the hybridisation tube lined with hybridisation-buffer-moisturised tissue and close it
- denature RNA polynucleotide probes at 80°C for 25-30 min
- hybridise at 53°C for 3-6 h
- rinse slide with dH_2O and air dry
- for microscope analysis: embed with mounting medium (Vectashield) or store at -20°C

B.9.4. FISH using DNA oligo-oligonucleotide probes targeting rRNA

Material:

Hybridisation buffer	NaCl	900 mM
	Tris-HCl	20 mM pH 8.0
	SDS	0.01 %
	Formamide	x %
Washing buffer	NaCl	x mM (Table B.15, p. 38)
	Tris-HCl	20 mM
	SDS	0.01 %
	EDTA	

Hybridisation on slides (Marienfeld, Germany)**:**

- adjust temperature of hybridisation oven to 80°C for the denaturation of RNA polynucleotide probes and another oven to 46°C for the hybridisation
- apply 4 µl of fixed bacterial cell or sample material per microscope slide well
- dry 5 -10 min at 60°C
- dehydrate slide in increasing ethanol series (50 %, 80 %, 100 %) for 3 min each
- put 10 µl of hybridisation buffer onto each well, add 4-5 µl probe solution and mix carefully
- put slide into the hybridisation tube lined with hybridisation-buffer-moisturised tissue and close it
- denature RNA polynucleotide probes at 80°C for 30 min
- hybridise at 53°C for 3-6 h
- rinse slide with dH_2O and air dry
- for microscope analysis: embed with mounting medium (Vectashield) or store at -20°C

B.10. Generation of labelled polynucleotide probes

B.10.1. Generation of RING-FISH polynucleotide probes via *in vitro* transcription

To generate RING-FISH probes targeting antibiotic resistance genes, primers were specifically designed and fragments of genes of interest were amplified. After cloning and sequencing, the correct PCR-fragments serve as template for the synthesis of RNA polynucleotide probes. One of the primers contains the T3 promoter sequence as binding site for the T3 RNA-polymerase. The addition of the promoter sequence to the sense-strand primer leads to a template for transcription of the sense-strand of the amplified fragment. For the generation of an antisense probe, the promoter sequence has to be added to the 5´ end of the antisense primer. During the transcription, fluorescein-12-UTPs or biotin-16-UTPs were incorporated to synthesize a directly/indirectly labelled RNA probe in a ratio of 0.65/0.35 labelled/unlabelled UTP. Higher amounts of labelled UTP reduce the yield, whereas lower amounts result in an insufficient labelling (Stoffels et al., 1999).

<u>Material:</u>

rNTP-Mix (Roche, Mannheim, Germany)	
ATP [100mM]	8 µl
CTP [100mM]	8 µl
GTP [100mM]	8 µl
UTP [100mM]	2.8 µl
Fluorescein-12-UTP [10mM] or	
Biotin-16-UTP [10mM]	25 µl
T3-RNA polymerase (Roche, Mannheim, Germany)	
RNase-Inhibitor (Roche, Mannheim, Germany)	
DNase I, RNase free (Roche, Mannheim, Germany)	
TE-buffer	Tris-HCl 10 mM, pH 8.0
	EDTA 1 mM, pH 8.0

Ammoniumacetate	NH_4-Acetate 10 M
EDTA	0.2 M, pH 8.0
EtOH $_{abs.}$	
EtOH 70 %	

Reaction mix pipetted at 24°C:

Template DNA	0.5-4 µg
rNTP-Mix	4 µl
Transcription buffer (10x)	3 µl
RNase-Inhibitor	1.5 µl
T3-RNA polymerase	3 µl
H_2O_{MQ}	ad 30 µl

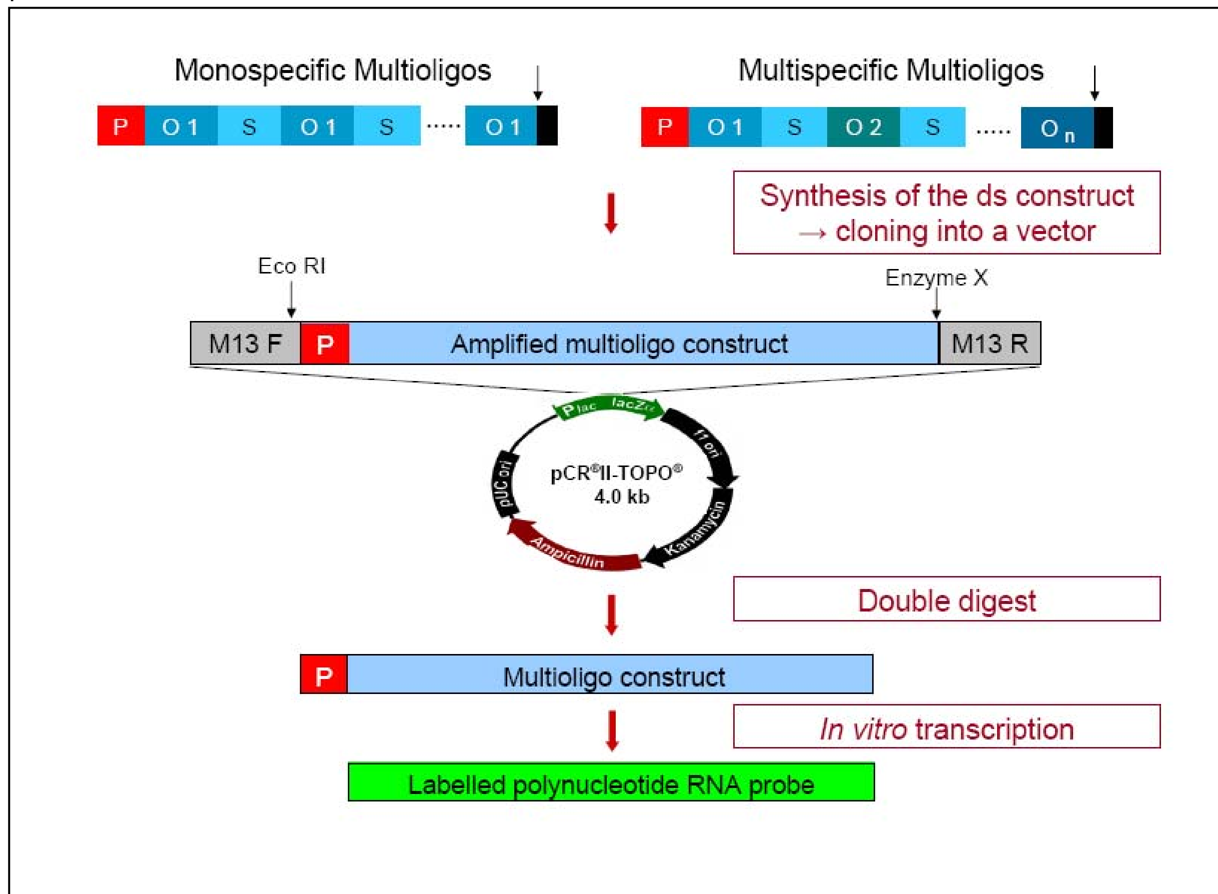
Procedure

- incubate reaction mix for 3 h or longer at 37 °C in a water bath
- add 3 µl DNase I for the degradation of the template DNA to avoid binding to the target region during hybridisation and incubate 15 min at 37°C
- add 3 µl EDTA 0.2 M
- add 16 µl NH_4-Acetate 10 M and 156 µl $EtOH_{abs.}$ for precipitation and incubate 2 h at -80°C or o.n. at -20°C
- centrifuge 15 min at 4°C and 14,000 rpm, discard supernatant
- wash with 100 µl EtOH 70%
- centrifuge 15 min at 4°C and 14,000 rpm, discard supernatant
- resuspend the pellet in 50 µl TE-buffer, add 1 µl RNase-Inhibitor to protect the probe against degradation by RNases
- run agarose gel to control the quality of the probe (5 µl transcript probe + 5 µl formamide + 5 µl loading buffer)
- store at -20°C

B.10.2. Generation of RNA oligo-oligonucleotide probes via *in vitro* transcription

The main project of this study was focused on the development and application of a novel type of polynucleotide probes, so-called oligo-oligonucleotide probes. These probes should be tested in hybridisations on slides and in enrichment experiments in microplate cavities. For the design of these oligo-oligonucleotide probes, at first taxon-specific oligonucleotide probes targeting 16S and 23S rRNA of bacteria causing hospital-acquired infections were tested. The sequences of these oligonucleotide probes are listed in table B.17 (p. 41). The single stranded construct template consists of an alternating sequence of repetitive specific oligonucleotide sequences and spacer regions. The T3 promoter is localised at the 5´end and an endonuclease recognition site at the 3´end. The synthesis of the second strand of the DNA construct was done by the Klenow enzyme. After gel purification, all double stranded templates were cloned and sequenced to ensure the correct sequence and length. After a double digestion using two different enzymes simultaneously (EcoRI and a specific one for each probe) the generation of directly or indirectly labelled RNA polynucleotide probes via *in vitro* transcription (see B. 10.1., p. 47) could be performed. In this study, monospecific and multispecific construct probes were developed and applied. Monospecific probes contain a repetition of a species-specific oligonucleotide probe, whereas multispecific probes contain different species-specific oligonucleotide probes. Figure B.1 (p. 50) illustrates schematically the proceeding concerning the development of both monospecific and multispecific oligo-oligonucleotide probes.

Figure B.1 Schematic illustration of the proceeding for the development of oligo-oligonucleotide probes



P	Position of the promoter for the T3 RNA polymerase
O1	Position of the oligonucleotide fragment
S	Position of the spacer region
↓	Restriction endonuclease site
M13 F/R	Position of the forward / reverse primer within the vector
Enzyme X	Restriction endonuclease site of the respective restriction enzyme

Tables B.20-B.22 (pp. 51 and 52) show the sequences of single stranded DNA oligo-oligonucleotide constructs from which oligo-oligonucleotide probes were synthesised. Templates for monospecific probes for Gram-negative bacteria are shown in table B.20 (p. 51). Table B.21 (p. 52) shows designed monospecific probes for Gram-positive bacteria. Table B.22 (p. 52) shows the templates for both multispecific oligo-oligonucleotide probes targeting five different Gram-negative strains.

Table B.20 Synthetic single stranded DNA templates, which were developed for Gram-negative bacteria and used for the synthesis of a double stranded template by Klenow enzyme,
T3 promoter region: red sequence at 5´end; oligonucleotide fragment: blue bold sequence;
GCGCG or AAAAA-spacer: black bold italics; endonuclease recognition site: black box at 3´end

Bacteria and AF number	Whole sequence of the template 5´→ 3´ direction	GC [%]
A. baumannii 438775	ATAGGTATTAACCCTCACTAAAG**TCCGCCGCTAGGTCCGGT*GCGCG*TCCGCCGCTAGGTCCGGT*GCGCG*TCCGCCGCTAGGTCCGGT*GCGCG*TCCGCCGCTAGGTCCGGTACC**	69.9
A. baumannii 438775	ATAGGTATTAACCCTCACTAAAG**TCCGCCGCTAGGTCCGGT*AAAAA*TCCGCCGCTAGGTCCGGT*AAAAA*TCCGCCGCTAGGTCCGGT*AAAAA*TCCGCCGCTAGGTCCGGTACC**	55
E. aerogenes 427068	ATAGGTATTAACCCTCACTAAAG**CGAGTAACGTCAATCGCC*GCGCG*CGAGTAACGTCAATCGCC*GCGCG*CGAGTAACGTCAATCGCC*GCGCG*CGAGTAACGTCAATCGCCTTAGC**	58
E. coli 441612	ATAGGTATTAACCCTCACTAAAG**CACACACTGATTCAGGCT*GCGCG*CACACACTGATTCAGGCT*GCGCG*CACACACTGATTCAGGCT*GCGCG*CACACACTGATTCAGGCTCCGGA**	55.8
K. pneumoniae 429457	ATAGGTATTAACCCTCACTAAAG**CCTACACACCAGCGTGCC*GCGCG*CCTACACACCAGCGTGCC*GCGCG*CCTACACACCAGCGTGCC*GCGCG*CCTACACACCAGCGTGCCTTAGC**	65.2
P. aeruginosa 426470	ATAGGTATTAACCCTCACTAAAG**TCTCGGCCTTGAAACCCC*GCGCG*TCTCGGCCTTGAAACCCC*GCGCG*TCTCGGCCTTGAAACCCC*GCGCG*TCTCGGCCTTGAAACCCCGGG**	63.2
S. maltophilia 484133	ATAGGTATTAACCCTCACTAAAG**AAGTTATGCACCCCAAG*GCGCG*AAGTTATGCACCCCAAG*GCGCG*AAGTTATGCACCCCAAG*GCGCG*AAGTTATGCACCCCAAGCT**	53.1

Table B.21 Synthetic single stranded DNA templates, which were developed for Gram-positive bacteria and used for the synthesis of a double stranded template by Klenow enzyme,
T3 promoter region: red sequence at 5´end; oligonucleotide fragment: blue bold sequence; GCGCG-spacer: black bold italics; endonuclease recognition site: underlined sequence at 3´end

Bacteria and AF number	Whole sequence of the template 5´→ 3´ direction	GC [%]
E. faecium 458387	ATAGGTATTAACCCTCACTAAAG**TGACTCCTCTTCAGACTT*GCGCG*TGACTCCTCTTCAGACTT*GCGCG*TGACTCCTCTTCAGACTT*GCGCG*TGACTCCTCTTCAGACTT*GCGCG*TGACTCCTCTTCAGACTTCGAA**	51.1
E. faecalis Staph/Strep 28	ATAGGTATTAACCCTCACTAAAG**TCCTTCAACTATACTAAC*GCGCG*TCCTTCAACTATACTAAC*GCGCG*TCCTTCAACTATACTAAC*GCGCG*TCCTTCAACTATACTAAC*GCGCG*TCCTTCAACTATACTAACCCGGG**	46
S. aureus 433088	ATAGGTATTAACCCTCACTAAAG**AATGCAGCGCGGATCCAT*GCGCG*AATGCAGCGCGGATCCAT*GCGCG*AATGCAGCGCGGATCCAT*GCGCG*AATGCAGCGCGGATCCAT*GCGCG*AATGCAGCGCGGATCCATTAAT**	56.9
S. epidermidis MRSE / S:23	ATAGGTATTAACCCTCACTAAAG**GTCAGAGGAGCAAGCTCC*GCGCG*GTCAGAGGAGCAAGCTCC*GCGCG*GTCAGAGGAGCAAGCTCC*GCGCG*GTCAGAGGAGCAAGCTCC*GCGCG*GTCAGAGGAGCAAGCTCCTTAGC**	61.6
S. agalactiae 616311	ATAGGTATTAACCCTCACTAAAG**CCATCTCACAGTGAAGCA*GCGCG*CCATCTCACAGTGAAGCA*GCGCG*CCATCTCACAGTGAAGCA*GCGCG*CCATCTCACAGTGAAGCA*GCGCG*CCATCTCACAGTGAAGCATATG**	54
S. pyogenes 611333	ATAGGTATTAACCCTCACTAAAG**CCGTCACTTGGTGGATTT*GCGCG*CCGTCACTTGGTGGATTT*GCGCG*CCGTCACTTGGTGGATTT*GCGCG*CCGTCACTTGGTGGATTT*GCGCG*CCGTCACTTGGTGGATTTAAA**	53.7

Table B.22 Synthetic single stranded DNA templates, which were developed for Gram-negative bacteria and used for the synthesis of a double stranded template by Klenow enzyme; T3 promoter region: red sequence at 5´end; oligonucleotide fragment: differently coloured bold sequences; GCGCG-spacer: black bold italics; endonuclease recognition site: underlined sequence at 3´end; The order of the microorganisms corresponds to the arrangement of respective oligonucleotide sequences within the template

Bacteria and AF number	Whole sequence of the template 5´→ 3´direction	GC [%]
A. baumannii 438775 *S. maltophilia* 484133 *E. aerogenes* 427068 *K. pneumoniae* 429457 *E. coli* 441612	ATAGGTATTAACCCTCACTAAAG**TCCGCCGCTAGGTCCGGT*GCGCG*AAGTTATGCACCCCAAG*GCGCG*CGAGTAACGTCAATCGCC*GCGCG*CCTACACACCAGCGTGCC*GCGCG*CACACACTGATTCAGGCTCCGGA**	69.9
E. aerogenes 427068 *A. baumannii* 438775 *E. coli* 441612 *S. maltophilia* 484133 *K. pneumoniae* 429457	ATAGGTATTAACCCTCACTAAAG**CGAGTAACGTCAATCGCC*GCGCG*TCCGCCGCTAGGTCCGGT*GCGCG*CACACACTGATTCAGGCT*GCGCG*AAGTTATGCACCCCAAG*GCGCG*CCTACACACCAGCGTGCCTTAGC**	69.9

Klenow reaction:

- denature every template for 10 min at 94°C

Klenow Fragment (DNA polymerase I Large Fragment, (Fermentas GmbH, St. Leon-Rot, Germany)

Reaction mixture per 100 µl PCR reaction:

Reaction Buffer (10x)	10 µl
dNTP-mix (2.0 mM each)	5 µl
DNA (50 pmol/µl)	10µl
Klenow Fragment	5 µl
H_2O_{MQ}	ad 100 µl

- incubate the reaction mixture for 5 h or over night at 37°C
- inactivate the Klenow enzyme for 15 min at 65°C

The templates produced during the Klenow reaction can be directly applied for *in vitro* transcription as described under B.10.1. (p. 47). Due to the low amount of the double stranded templates after the Klenow reaction, all constructs were polyadenylated and cloned (see B.12., p. 62) followed by a double digestion of the plasmid containing the templates. If necessary, gel extraction steps using the QIAquick® Gel Extraction Kit (Qiagen, Hilden, Germany) should be performed, according to the manufacturer. To enable the ligation with the pCR®II-TOPO® 4.0 vector during the cloning procedure, a polyadenylation takes place:

Polyadenylation:

Double stranded template	7 µl
dATP (2 mM)	1 µl
r-Taq buffer	1 µl
r-Taq	1 µl

- incubate 30 min at 70°C in a water bath and use for cloning described in B.12. (p.62)

After cloning of the fragments, an isolation of plasmid-DNA and a control-PCR using M13 primers was performed (see B.12.2., p. 63) followed by a double digestion from the control-PCR product to obtain the template for *in vitro* transcription:

Material:

All enzymes and buffer Tango^{TM} were ordered from MBI Fermentas (Fermentas GmbH, St. Leon-Rot, Germany)

Reaction mix:

Double stranded DNA template (obtained after the control-PCR)	8 µl
Tango^{TM} buffer	1 µl
Restriction enzyme 1	1 µl
Restriction enzyme 2	1 µl

- incubate over night at 37°C in a water bath and deactivate the enzymes for 15 min at 65°C or as recommended by the manufacturer.

Table B.23 Restriction enzymes and respective restriction sites used in double digestion of monospecific templates

Bacteria and AF number	Enzyme 1	Enzyme 2	Buffer	Restriction site 5´→ 3´direction
A. baumannii 438775	EcoRI	BspLI (NlaIV)	Tango^{TM}	GGN↓NCC CCN↑NGG
E. aerogenes 427068	EcoRI	Bpu10I	Tango^{TM}	CC↓TNAGC GGANT↑CG
E. coli 441612	EcoRI	Kpn2I (BspMII)	Tango^{TM}	T↓CCGGA AGGCC↑T
K. pneumoniae 429457	EcoRI	Bpu10I	Tango^{TM}	CC↓TNAGC GGANT↑CG
P. aeruginosa 426470	EcoRI	SmaI	Tango^{TM}	CCC↓GGG GGG↑CCC
S. maltophilia 484133	EcoRI	AluI	Tango^{TM}	AG↓CT TC↑GA
E. faecium 458387	EcoRI	Bsp119I (AsuII)	Tango^{TM}	TT↓CGAA AAGC↑TT
E. faecalis Staph/Strep 28	EcoRI	Cfr9I	Tango^{TM}	C↓CCGGG GGGCC↑C
S. aureus 433088	EcoRI	VspI	Tango^{TM}	AT↓TAAT TAAT↑TA
S. epidermidis MRSE / S:23	EcoRI	Bpu10I	Tango^{TM}	CC↓TNAGC GGANT↑CG
S. agalactiae 616311	EcoRI	NdeI	Tango^{TM}	CA↓TATG GTAT↑AC
S. pyogenes 611333	EcoRI	DraI (AhaIII)	Tango^{TM}	TTT↓AAA AAA↑TTT

Table B.24 Restriction enzymes and respective restriction sites used in double digestion of multispecific templates

Bacteria and AF number	enzyme 1	enzyme 2	buffer	Restriction site 5´→ 3´direction
A. baumannii 438775 *S. maltophilia* 484133 *E. aerogenes* 427068 *K. pneumoniae* 429457 *E. coli* 441612	EcoRI	Kpn2I (BspMII)	Tango™	T↓CCGGA AGGCC↑T
E. aerogenes 427068 *A. baumannii* 438775 *E. coli* 441612 *S. maltophilia* 484133 *K. pneumoniae* 429457	EcoRI	Bpu10I	Tango™	CC↓TNAGC GGANT↑CG

- after double digest: run on an agarose gel to control the digestion result and exercise the respective gel fragments from the gel according to the manual QIAquick® Gel Extraction Kit (Qiagen, Hilden, Germany)
- generate RNA probes from the extracted fragments by *in vitro* transcription (see B.10.1., p. 47)
- run agarose gel to control the quality of the probe (5 µl transcript probe + 5 µl formamide + 5 µl loading buffer)

B.10.3. Generation of DNA oligo-oligonucleotide probes via asymmetric PCR

An approach to obtain single stranded DNA probes is based on asymmetric PCR. This allows production of the sense or antisense probe due to application of only one of the primers in the reaction mix resulting in a linear amplification of the product. Simultaneously, labelled probes can be achieved by incorporating fluorescein-dUTPs (Zimmermann, 2002).
The double stranded Klenow-reaction product (see B.10.2., p. 49) of the synthetic constructs serves as template for the asymmetric PCR. The template did not contain the T3 promoter at the 5´end.

Reaction mixture per 100 µl asymmetric PCR reaction:

Reaction Buffer (10x)	10 µl
dNTP-mix (1 mM each)	8 µl
Fluorescein-12-dUTP (0.35 mM)	2 µl
Forward primer (50 pmol/µl)	5 µl
DNA	50-100 ng
Ex-Taq Polymerase	0.4 µl
H_2O_{MQ}	ad 100 µl

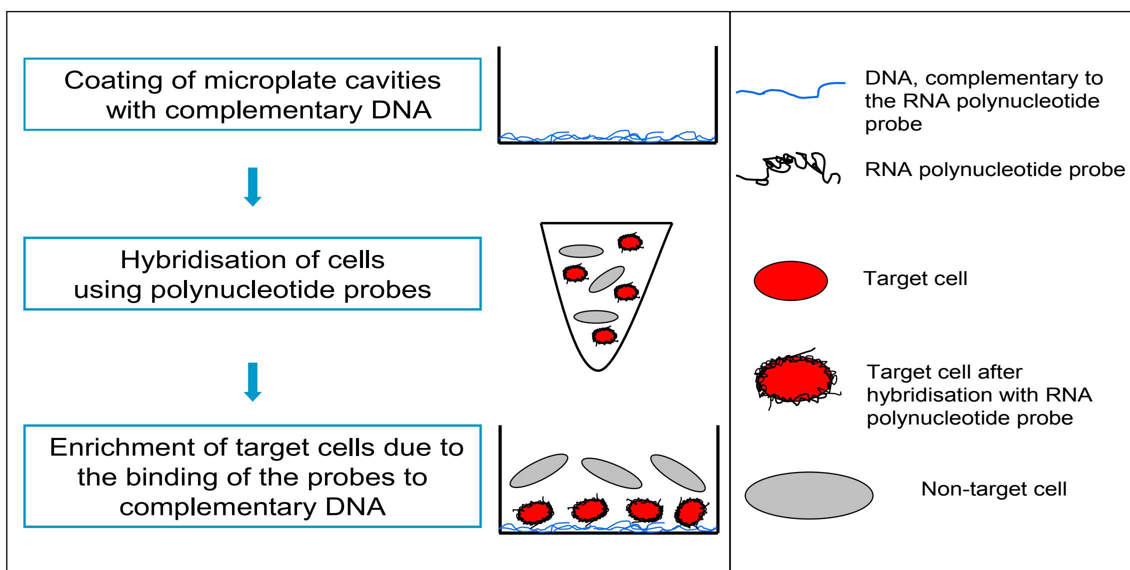
The annealing temperature is 50°C, other conditions are similar to a standard PCR protocol. After the amplification run agarose gel to control the result of the asymmetric PCR.

B.11. Hybridisation in coated microplates for the separation of bacteria from different biological material

This cell sorting method derived from MACS (magnetic activated cell sorter) is based on the separation of cells in solution due to the binding of magnetically labelled streptavidin microbeads to the target cells. Subsequently, the solution passes a column, which is located in the magnetic field of a MACS separator. The labelled material adheres to the column whereas the unlabelled fraction runs through. After removal of the column from the magnetic field, the labelled cells can be eluted (Stoffels et al., 1999).

This MACS-technique was adapted to coated microplates (Zwirglmaier, 2004). The hybridisation in coated microplates provides another opportunity to separate target cells from biological material. During the first step, a part of the polynucleotide probe hybridises to its target sequence within the cells. During the second step, the protruding part of the probe binds to the complementary nucleic acids on the surface of microplate cavities. After a washing step, only target cells should be enriched in the cavities. An important advantage of this technique is the two in one detection method, which allows the separation of target cells followed by further molecular investigation. The analysis of cells by PCR in the same well could be used for identification of e.g. genes coding antibiotic resistance or virulence factors. Figure B.2 (p. 57) shows the procedure of the enrichment technique.

Figure B.2 Schematic illustration of the immobilisation protocol



B.11.1. Coating of microplates with DNA

1-Ethyl-3-(3-Dimethylaminopropyl)-Carbodiimide (EDC) is a common agent to cross-link carboxyl- or amino groups via carbodiimide condensation e.g. in NucleoLink plates.

Material:

Microplates	NucleoLink (Nalge Nunc, Roskilde, Denmark)
PCR amplificate	(freshly prepared, see below)
PCR film	Peqlab (Erlangen, Germany)
PBS	see B.3. (p. 26)
PBS/$MgCl_2$	PBS containing $MgCl_2$ 0.1M
EDC solution	10 mM 1-Ethyl-3-(3-Dimethylaminopropyl) -Carbodiimide (EDC) in PBS/$MgCl_2$

Procedure

- amplify the respective PCR fragment, add 0.1 vol NaAc (5 M) and 2.5 vol $EtOH_{abs}$ and incubate 0.5 -1h at -80°C
- centrifuge 20 min at 14000 rpm, wash with 100 µl EtOH 70% and centrifuge again 20 min at 14000 rpm
- resuspend the pellet in 50-100 µl H_2O_{MQ}
- use 1 µg PCR product in 100 µl EDC-solution with PBS/$MgCl_2$ in each cavity and close the microplate with PCR film
- incubate for 10 min at 94°C (for optimal binding result use ssDNA complementary to the later used polynucleotide probe)
- incubate over night at 37°C
- discard supernatant and dry cavities 1-2h at 60°C, store coated plates at 4°C for several weeks

(- before continuing with the enrichment, wash cavities with 100 µl PBS to remove unbound DNA)

B.11.2. Hybridisation of bacteria in solution

B.11.2.1. Protocol for hybridisation with Gram-negative bacteria

- apply 10 µl (pure culture sample) or 50-100 µl (clinical sample) of PFA fixed cells in a 0.6 ml tube
- (for cells in blood samples: add 1 vol 50 mM Tris-HCl and incubate for 20 min at 37°C, centrifuge at 12000 rpm 5-10 min, and resuspend the pellet in 50-100 µl PBS)
- add 3 vol $EtOH_{abs}$ and incubate at 25°C for 3 min
- centrifuge at 12000 rpm 5-10 min
- resuspend cell pellet in 50-100 µl hybridisation buffer
- add 5-10 µl (5 µg) transcript probes
- denature polynucleotide probes at 80°C for 20-30 min and hybridise 5-12 h at 53°C

B.11.2.2. Protocol for hybridisation with Gram-positive bacteria

Table B.25 Pretreatments recommended for FISH with RNA oligo-oligonucleotide or RING-FISH probes in tubes

Bacteria	1st EtOH series	Enzymatic pretreatment at 37°C	Pretreatment at 200°C	2nd EtOH series
Enterococci	+	Lysozyme 20 min	1 min	+
Staphylococci	+	Lysostaphin 20-25 min	1 min	+
Streptococci	+	Mutanolysin 5 min	1 min	+

- apply 10 µl (pure culture sample) or 50-100 µl (clinical sample) of PFA fixed cells in a 0.6 ml tube
- (for cells in blood samples: add 1 vol 50 mM Tris-HCl and incubate for 20 min at 37°C, centrifuge at 12000 rpm 5-10 min, and resuspend the pellet in 50-100 µl PBS)
- use recommended pretreatment method (Table B.25, p. 59) dependent on the cell type
- centrifuge at 12000 rpm 5-10 min, wash with 50-100 µl PBS
- add 3 vol $EtOH_{abs}$ and incubate at 25°C for 3 min, centrifuge at 12000 rpm 5-10 min and resuspend cell pellet in 50-100 µl hybridisation buffer
- add 5-10 µl (5µg) transcript probes
- denature polynucleotide probes at 80°C for 20-30 min
- hybridise 1-1.5 h (oligo-oligonucleotide probes) or over night (RING-FISH probes) at 53°C

B.11.3. Immobilisation of bacteria in coated microplates

Material:

Microplates	NucleoLink (Nalge Nunc, Roskilde, Denmark)
PCR amplificate, purified	
PCR film	Peqlab (Erlangen, Germany)
PBS	see B.3. (p. 26)
MP buffer:	5 x SSC
(Microplate buffer)	0.02 % SDS
	2 % blocking reagent (Roche, Mannheim, Germany)
	0.1 % N-Laurylsarcosin
	X % formamide
Blocking reagent	10 % (w/v) blocking reagent (Roche, Mannheim, Germany) in maleic acid buffer (0.15 M NaCl, 0.1 M maleic acid, pH 7.5)

Procedure

- after the *in situ* hybridisation in solution centrifuge 5-10 min at 12000 rpm
- wash with 100 µl PBS and centrifuge again at 12000 rpm for 5-10 min
- resuspend the pellet in 50-100 µl MP buffer per sample of fixed cells
- wash microplate cavities with 100 µl PBS to remove unbound DNA
- add 50 µl of solution into each microplate cavity coated with DNA and into one uncoated microplate cavity (negative control)
- cover microplate cavities with adhesive PCR film
- incubate 1 h at 53°C (for rRNA targeted probes) or 37°C (for chromosomal DNA targeted probes)
- after enrichment hybridisation, pipette the solution carefully of the cavities and pool corresponding supernatants (needed for PCR)
- centrifuge at 12000 rpm for 10 min, resuspend in 5-10 µl PBS and use for PCR
- wash microplate cavities carefully with 100 µl PBS,
- immobilised target cells in cavities can be directly used for PCR

B.11.4. Detection of immobilised bacterial cells by PCR

- pool supernatants from all cavities and wash with PBS to remove non-enriched cells
- PCR detection in the microplate can be done
- perform PCR with immobilised cells in the cavities and 1 µl from each supernatant resuspension using required primers and conditions (Tables B. 26 and B.27, p. 61)
- run agarose gel electrophoresis

To enable amplification from whole cells, an initial denaturation step of 12 min is necessary. Other parameters of the PCR programme stay unmodified, only the elongation time depends on the length of the amplification fragment of interest.

Table B.26 Primers used for the amplification in microplate cavities resulting in an about 600 nt long amplicon of the 23S rRNA

Primer name	Sequence 5´→ 3´	GC [%]	T_m [°C]
939V	TTTGG(A/G)(A/G)GAGAACCAGCT	55	50
984R	AGTAGCCTGGCGAGCGAA	61	50

Table B.27 PCR programme used for the amplification of PCR fragments after immobilisation of cells in microplate cavities; x: T_D of primer; y: depending on fragment length, approximately 1 min per 1kb fragment length

Reaction	T	Time	Number of cycles
initial denaturation	94°C	12 min	1
denaturation	94°C	45 s	30
annealing	x °C	1 min	
elongation	72°C	y min	
final elongation	72°C	5 min	1

To control the specificity of probes and the enrichment results, amplicons showing correct fragment length were cloned and sequenced.

B.12. Cloning and Sequencing

B.12.1. Cloning with TOPO TA® Cloning Kit

To clone amplified PCR products, the TOPO TA® Cloning Kit (Invitrogen, Carlsbad, USA) was used. The pCR®II-TOPO® 4.0 kb long vector contains overhanging 3´deoxythymidine residues, which enable more efficient ligation of the PCR insert with the vector, due to the deoxyadenosine overhang of the PCR product added by Taq polymerase.

Material:

- pCR®II-TOPO® vector (Invitrogen, Carlsbad, USA)
- SOC medium (Invitrogen, Carlsbad, USA)
- One Shot® Mach1™ -T1® Competent Cells (Invitrogen, Carlsbad, USA)
- LB plates containing 50 µg/ml ampicillin (Sigma-Aldrich, Steinheim, Germany) and 40 µg/ml X-Gal (Sigma-Aldrich, Steinheim, Germany)

Cloning Reaction:

Salt solution	1 µl
pCR®II-TOPO® vector	1 µl
PCR product	1-4 µl
H_2O_{MQ}	ad 5 µl

- mix all components and incubate at 24°C for 5-10 min
- add 4 µl of the cloning reaction to One Shot® Mach1™ -T1® Competent Cells, mix gently and incubate at 4°C for 5-30 min
- heat-shock the cells for 30 sec at 42°C, put immediately at 4°C
- add 250 µl of SOC medium and incubate shaking at 37°C for 1h
- spread 50 and 80 µl of transformation on LB-Amp-X-Gal plate and incubate over night at 37°C

B.12.2. Identification of recombinant clones

The used vector contains in addition to *lacZ* gene, which encodes the beta-galactosidase, a multiple cloning site and genes conferring antibiotic resistances. The selection of potential clones consists of blue-white screening. When the fragment of interest was inserted into the vector, *lacZ* gene is interrupted and the β-galactosidase-activity eliminated. Due to the addition of X-gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside) to the LB_{amp}-plate, colonies containing the insert stay white, whereas blue colonies indicate that no insertion was carried out. The intact enzyme could hydrolyse X-gal, which is a chromogenic analogue to the usual substrate of the β-galactosidase.

- pick some white colonies of each LB_{amp}-plate, transfer them to LB_{amp}-medium and incubate them shacking over night at 37°C
- verify the insert by mini-plasmid preparation (QIAprep®Spin Miniprep Kit, Qiagen, Hilden, Deutschland) according to the manufacturer´s instruction
- to control the insert size of potential recombinant clones, a PCR reaction using M13V and M13R primers (Table B.28., p. 63) should be performed

Table B.28 Primers used for amplification of plasmid-DNA; V: forward primer, R: reverse primer

Primer name	Position in pCR®II-TOPO vector	Sequence 5´→ 3´	GC [%]	T_m [°C]
M13V	433	GTAAAACGACGGCCAG	56	50
M13R	205	CAGGAAACAGCTATGAC	47	50

B.12.3. Sequencing according to Sanger et al.,

The sequencing analysis of DNA fragments is known since 1975, the dideoxynucleotide sequencing (Sanger et al., 1977) combined with PCR amplification (Saiki et al., 1988) represents one possibility to sequence DNA. This technique relies on the application of 2',3'-dideoxynucleotide triphosphates (ddNTPs) possessing a hydrogen atom attached to the 3' carbon instead of a hydroxyl group commonly in deoxynucleotides. In contrast to a conventional PCR reaction, the sequencing mixture additionally contains labelled primers and ddNTPs. The incorporation of a ddNTP into an elongated sequence results in termination of the elongation process because the ddNTP cannot form a phosphodiester bond with the next dNTP.

For the sequencing PCR reaction Sequitherm Excel™ DNA Sequencing Kit-LC (66 cm, Epicentre, Madison, Wisconsin, USA) and primers labelled with IRDye 700/800 (LI-COR biosciences, Bad Homburg, Germany) were used. The detection of respective fragments obtained after the gel electrophoresis was performed in a LI-COR Global IR^2 DNA Sequencer (LI-COR Biosciences, Bad Homburg, Germany) and online detection via laser was used. To analyse the sequencing results, *e*-Seq DNA Sequencing and Analysis Software (LI-COR Biosciences, Bad Homburg, Germany) was applied.

Material:

Sequencing kit	Sequitherm Excel™ DNA Sequencing Kit-LC (66 cm, Epicentre, Madison, Wisconsin, USA)
Primers	5 µM IR labelled (MWG, Ebersberg, Germany; table B.28, p. 63)
Microtiter plate	polycarbonate, (Biozym, Hess. Oldendorf, Germany)
Microseals	Microseal™ (Biozym, Hess. Oldendorf, Germany)
Thermocycler	Type PTC-100™ (MJ Research Inc., Watertown, USA)

Reaction master mix for one reaction:

Sequitherm buffer	7.2 µl
Polymerase	1 µl
Primer (forward and reverse)	2 µl
DNA	250 ng
H_2O_{MQ}	ad 17 µl

- put 2 µl of the nucleotide mix (A, C, G, T containing ddNTPs) into each microplate cavity
- add 4 µl of the master mix to the nucleotides and mix
- cover microtiter plate with a seal and start programme shown in table B.29, p. 65

Table B.29 Programme for the sequencing reaction

Reaction	T [°C]	t [min]	Number of cycles
Initial denaturation	94	5	1
Denaturation	94	0.5	25
Annealing	50	0.5	
Elongation	70	1	
Final elongation	70	5	1

When the programme is terminated, add 3 µl of stop buffer into each cavity. Before loading the sequencing gel with the samples, a denaturation step of 5 min at 94°C should be performed or store the microtiter plate at -20°C.

Polyacrylamide gelelectrophoresis

Material:

TBE buffer 10 x	162 g Tris
(Tris-boric acid EDTA-buffer)	27.5 g boric acid
	9.3 g Na_2EDTA
	H_2O_{MQ}
TBE buffer 1x	
Urea, ultrapure	(Ultrapure, United States, Biochemicals, Cleveland, USA)

Silane stock solution	50 µl 3-Methacryloxypropyltrimethoxysilane 50 µl Acetate (100 %) 50 µl H_2O_{MQ} 9.62 ml $Ethanol_{abs}$
Silane solution	364 µl Silane stock solution 11 µl Acetate (10 %)
Long Ranger® Gel Solution	(Cambrex, Bio Science Rockland Inc., Rockland, ME, USA)
APS	10 % (w/v) ammoniumpersulfate
TEMED	N´-N´-N´-N´-tetramethylendiamine (Sigma Chemicals, St. Louis, USA)
Filter, sterile	0.2 µl pore diameter (Gelman Sciences, Support Acrodisk 25, Ann Arbor, USA)
Glass plates	66 cm (MWG, Ebersberg, Germany)
Spacer	0.25 mm (MWG, Ebersberg, Germany)
Comb	48 well shark tooth (MWG, Ebersberg, Germany)
Sequencing equipment	LI-COR Global IR^2 DNA Sequencer (LI-COR Biosciences, Bad Homburg, Germany)
Software	*e*-Seq DNA Sequencing and Analysis Software (LI-COR Biosciences, Bad Homburg, Germany)

Procedure for the preparation of the gel

- dissolve 25.2 g of urea in 32 ml H_2O_{MQ}
- add 7.2 ml 10 x TBE and 4.8 ml Long Ranger Gel Solution
- degas the mixture for 10 min in an ultra sonic bath
- add 400 µl of APS and 40 µl TEMED, sway the solution and filtrate sterilely
- prepare gel according to manufacturer´s instructions
- start prerun after positioning the gel in LI-COR Global IR^2 DNA Sequencer (Table B.30, p. 67)
- denature the sequencing reaction for 5 min at 94 °C and load the gel
- run the electrophoresis (Table B.30, p. 67) for 10 -12 h
- perform the analysis using software mentioned above

LI-COR parameters

Table B.30 LI-COR parameters for the gelelectrophoresis

Pre-run	Volt [V]	2000
	Ampere [mA]	25
	Watt [W]	45
	Time [min]	30
Run	Volt [V]	2000
	Ampere [mA]	25
	Watt [W]	45
	Time [h]	12
	Temperature [°C]	45
	Scan speed	2

C. Results

C.1. FISH on slides using oligonucleotide probes

The evaluation of taxon-specific oligonucleotide probes by FISH on slides was the precondition for the design of oligo-oligonucleotide probes. The procedure was carried out in three steps as follows: each oligonucleotide probe was tested in hybridisations with the target organism, the non-target organism and in a mixture of both. Oligo-oligonucleotide probes were developed of probes showing specific as well as the most intense whole cell fluorescence signals (bold oligonucleotide sequences in table C.1, p. 68). FISH experiments with these oligonucleotide probes are shown in figures C.1-C.12, pp. 69-72.

Table C.1 Bacteria, respective oligonucleotide probes and the target region within the 16S or 23S rRNA. Bold probe sequences were chosen for the design of oligo-oligonucleotide probes

Bacteria	Probe name / FA [%]	Probe sequence 5´→ 3´	Target 16/23S rRNA
A. baumannii	**Aba88/ 30** Aba79/30	**TCCGCCGCTAGGTCCGGT** TAGGTCCGGTAGCAAGCT	16S 16S
B. cepacia	Burcep/ 30	CTGTGCGCCGGTTCTCTT	16S
E. aerogenes	**Eae16S/ 0** Ent16S/15	**CGAGTAACGTCAATCGCC** CCCCCTCTTTGGTCTTGC	16S 16S
E. coli	**Eco271/ 10**	**CACACACTGATTCAGGCT**	23S
H. influenzae	Hin343/30	GTACCACAATATGGTTTTT	23S
K. oxytoca	Kox458/ 5	GAATAAGGTTATTAACCTC	16S
K. pneumoniae	**Kpn (1701)/ 30**	**CCTACACACCAGCGTGCC**	23S
P. aeruginosa	**PseaerB/ 10**	**TCTCGGCCTTGAAACCCC**	23S
P. fluorescens	Ps-Ag1/ 3	GATAACTCGTCATCAGCTC	23S
S. maltophilia	**Sma1158/ 5** Sma471/30	**AAGTTATGCACCCCAAG** GGTACCGTCATCCCAACC	23S 16S
E. faecium	**Efi58/ 10 •**	**TGACTCCTCTTCAGACTT**	23S
E. faecalis	**Efe286/0 •** Efe1473/ 11 •	**TCCTTCAACTATACTAAC** TAACTCTACTCAAGACTCAT	23S 16S
S. aureus	Sau(69)/10;20 * **Sau227/10;20 ***	GAAGCAAGCTTCTCGTCCG **AATGCAGCGCGGATCCAT**	16S 16S
S. epidermidis	**Sep16S/10 *** Sep1498/ 10 *	**GTCAGAGGAGCAAGCTCC** TCAGCCTTATGAGTGCCG	16S 23S
S. haemolyticus	Sha307/ 10 *	CTTCTTTGATTCAACTTTC	23S
S. agalactiae	**Sag210/ 10 •**	**CCATCTCACAGTGAAGCA**	16S
S. pyogenes	**Spy467/ 30 •**	**CCGTCACTTGGTGGATTT**	16S

•Pretreatment using lysozyme *Pretreatment using lysostaphin

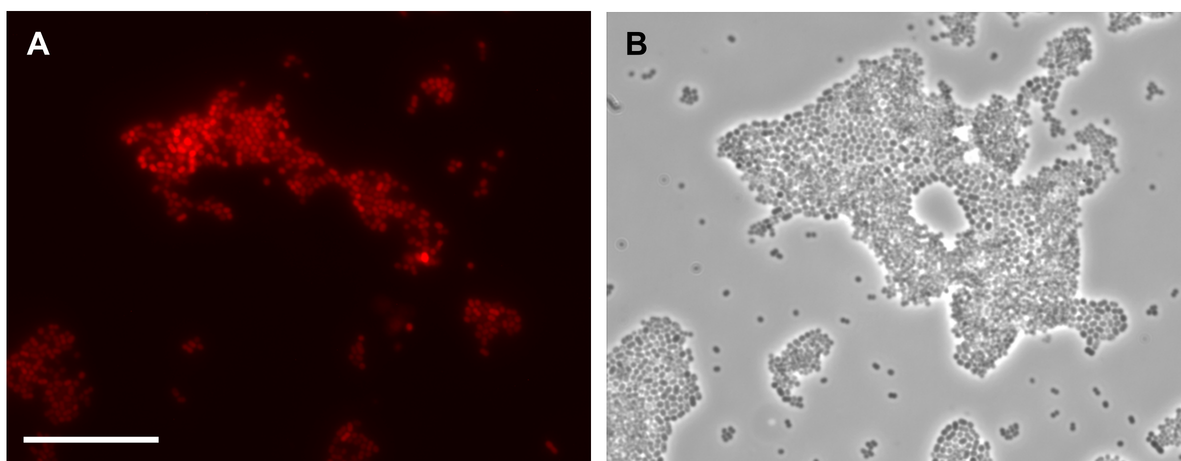


Figure C.1 A: Whole cell fluorescence signals after FISH (1.5 h; 30 % FA) using the Cy3 labelled oligonucleotide probe Aba88 targeting the 16S rRNA of *A. baumannii* **B:** Phase contrast image of *A. baumannii* and *Neisseria canis* (cocci: negative control); bar: 20 µm

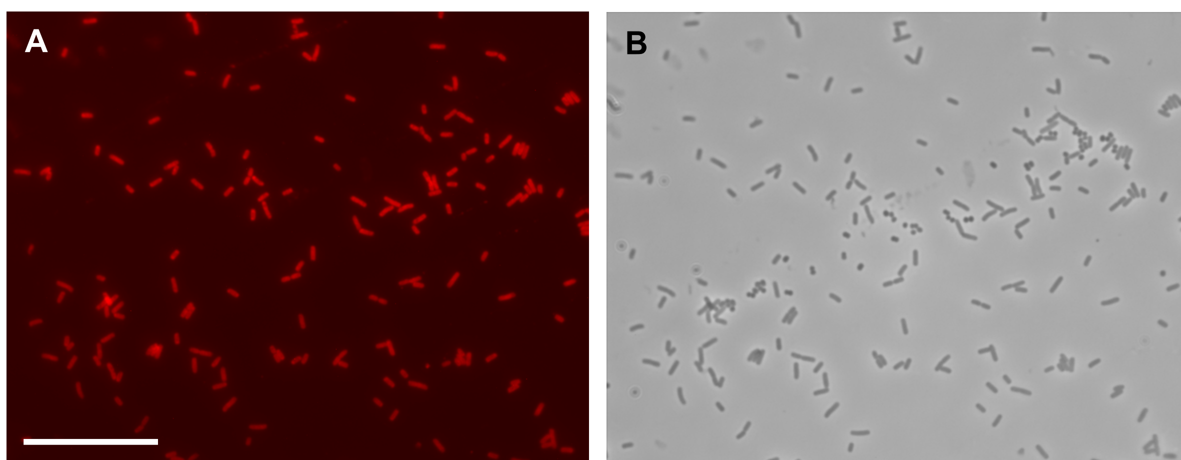


Figure C.2 A: Whole cell fluorescence signals after FISH (1.5 h; 0 % FA) using the Cy3 labelled oligonucleotide probe Eae16S targeting the 16S rRNA of *Enterobacter aerogenes.* **B:** Phase contrast image of *E. aerogenes* and *Neisseria canis* (cocci: negative control); bar: 20 µm

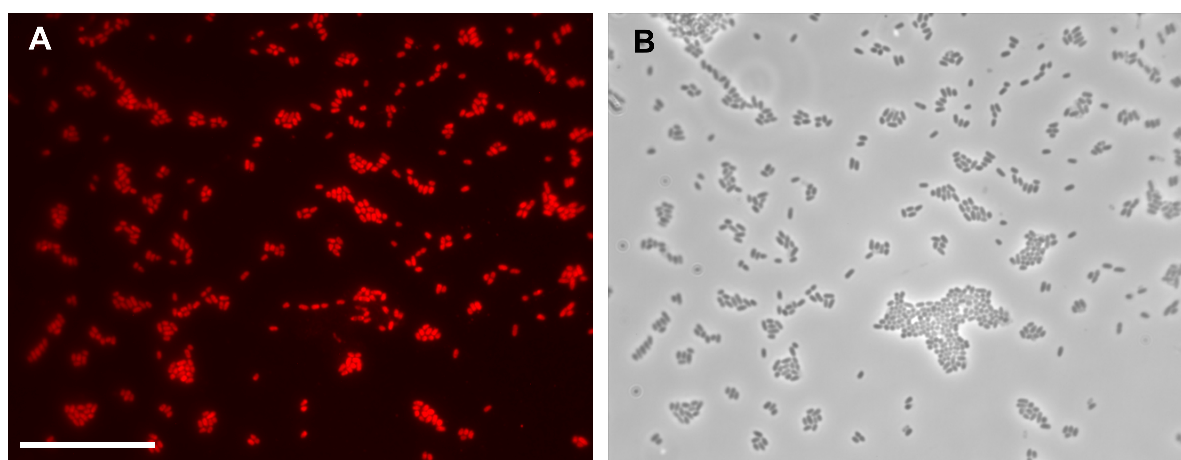


Figure C.3 A: Whole cell fluorescence signals after FISH (1.5 h; 10 % FA) using the Cy3 labelled oligonucleotide probe Eco271 targeting the 23S rRNA of *Escherichia coli.* **B:** Phase contrast image of *E. coli* and *Neisseria canis* (cocci: negative control); bar: 20 µm

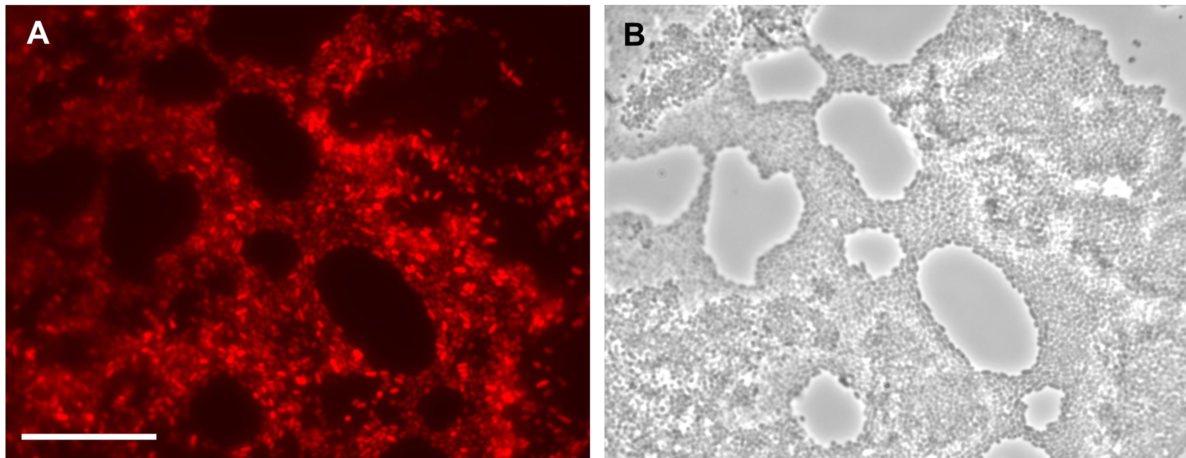


Figure C.4 A: Whole cell fluorescence signals after FISH (1.5 h; 30 % FA) using the Cy3 labelled oligonucleotide probe Kpn (1701) targeting the 23S rRNA of *Klebsiella pneumoniae.* **B:** Phase contrast image of *K. pneumoniae* and *Neisseria canis* (cocci: negative control); bar: 20 µm

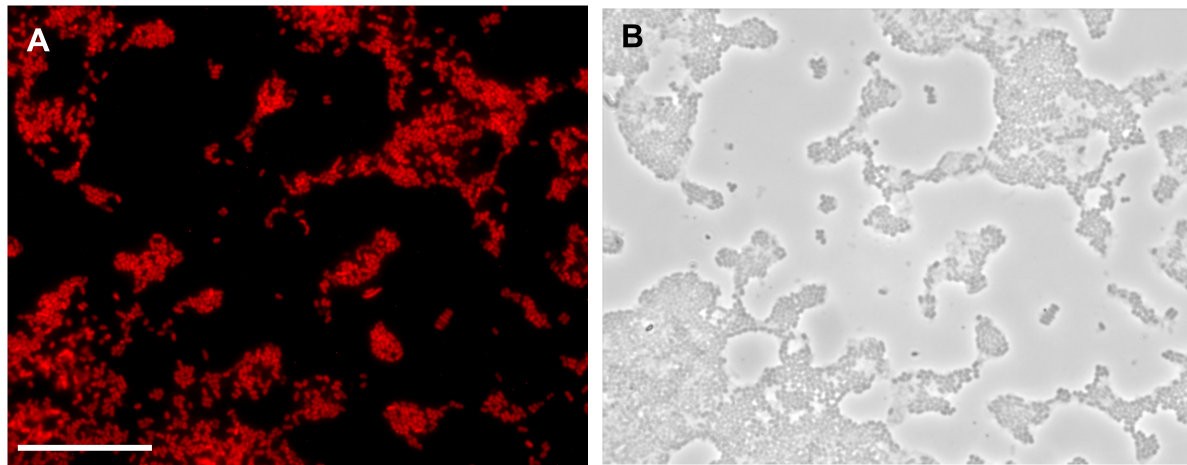


Figure C.5 A: Whole cell fluorescence signals after FISH (1.5 h; 10 % FA) using the Cy3 labelled oligonucleotide probe PseaerB targeting the 23S rRNA of *Pseudomonas aeruginosa.* **B:** Phase contrast image of *P. aeruginosa* and *Neisseria canis* (cocci: negative control); bar: 20 µm

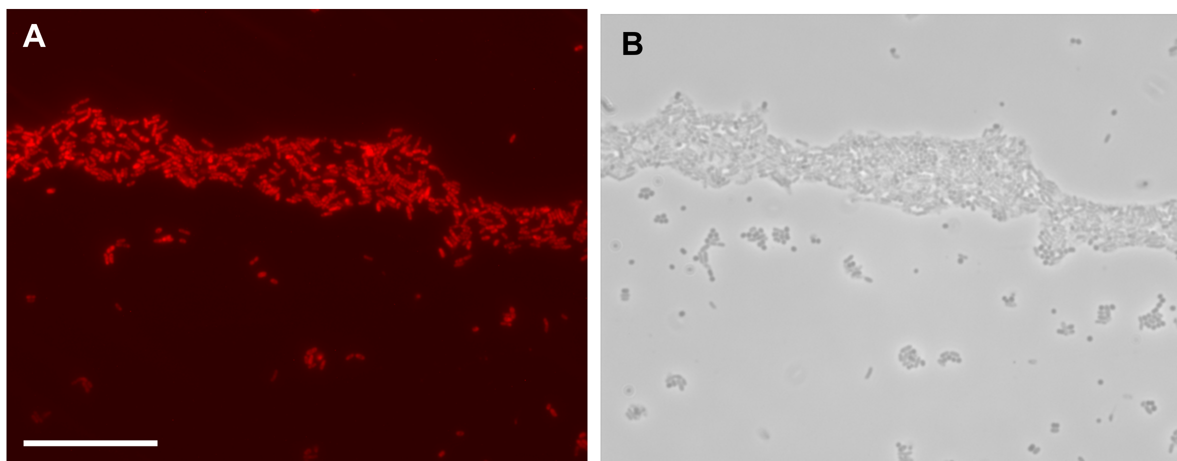


Figure C.6 A: Whole cell fluorescence signals after FISH (1.5 h; 5 % FA) using the Cy3 labelled oligonucleotide probe Sma1158 targeting the 23S rRNA of *Stenotrophomonas maltophilia.* **B:** Phase contrast image of *S. maltophilia* and *Neisseria canis* (cocci: negative control); bar: 20 µm

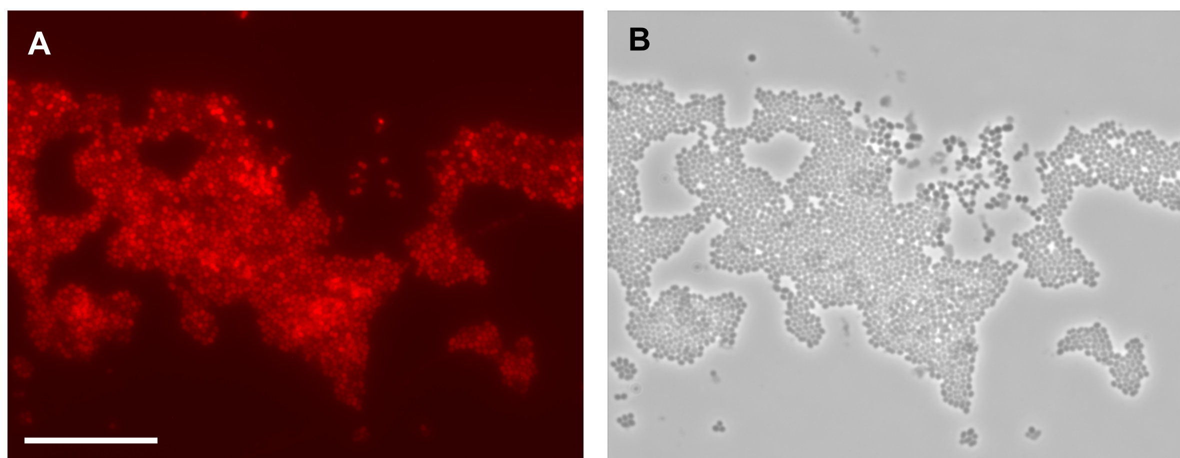


Figure C.7 A: Whole cell fluorescence signals after FISH (1.5 h; 10 % FA) using the Cy3 labelled oligonucleotide probe Efi58 targeting the 23S rRNA of *Enterococcus faecium.* **B:** Phase contrast image of *E. faecium* and *Streptococcus pyogenes* (dark cells under phase contrast: negative control); bar: 20 µm

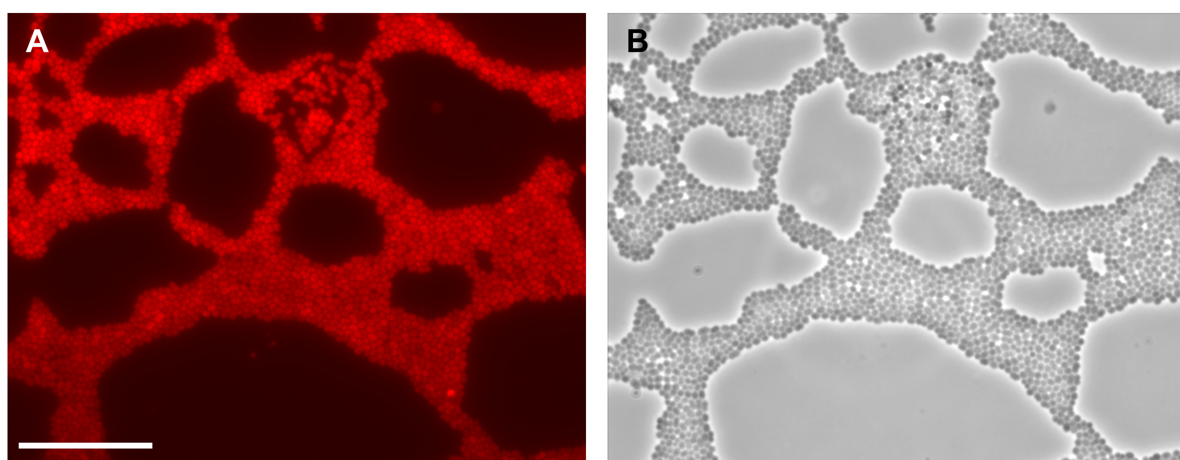


Figure C.8 A: Whole cell fluorescence signals after FISH (1.5 h; 0 % FA) using the Cy3 labelled oligonucleotide probe Efe286 targeting the 16S rRNA of *Enterococcus faecalis* **B:** Phase contrast image of *E. faecalis* and *Streptococcus pyogenes* (dark cells under phase contrast: negative control); bar: 20 µm

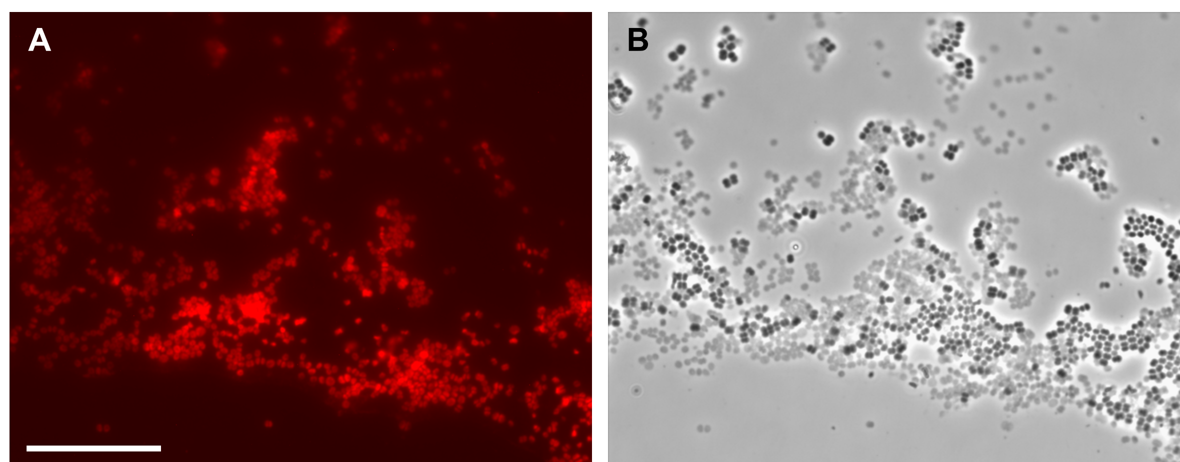


Figure C.9 A: Whole cell fluorescence signals after FISH (1.5 h; 10 % FA) using the Cy3 labelled oligonucleotide probe Sau227 targeting the 16S rRNA of *Staphylococcus aureus.* **B:** Phase contrast image of *S. aureus* and *Staphylococcus haemolyticus* (dark cells under phase contrast: negative control); bar: 20 µm

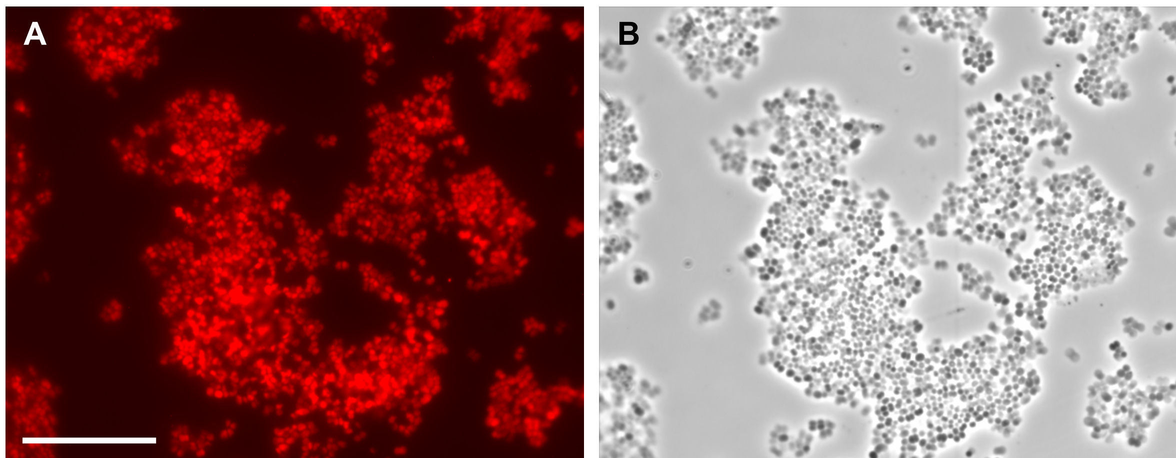


Figure C.10 A: Whole cell fluorescence signals after FISH (1.5 h; 10 % FA) using the Cy3 labelled oligonucleotide probe Sep16S targeting the 16S rRNA of *Staphylococcus epidermidis.* **B:** Phase contrast image of *S. epidermidis* and *Staphylococcus haemolyticus* (dark cells under phase contrast: negative control) bar: 20 µm

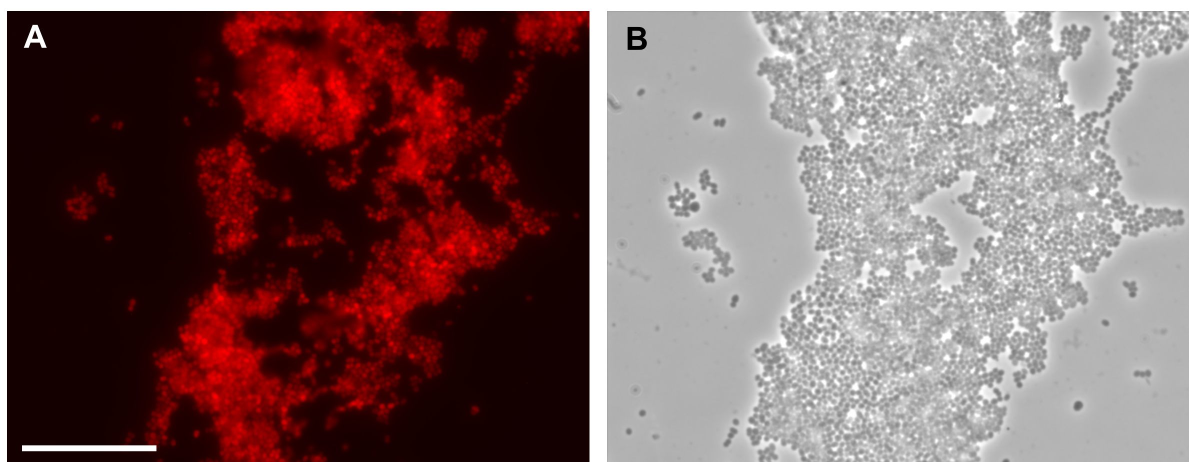


Figure C.11 A: Whole cell fluorescence signals after FISH (1.5 h; 10 % FA) using the Cy3 labelled oligonucleotide probe Sag210 targeting the 16S rRNA of *Streptococcus agalactiae.* **B:** Phase contrast image of *S. agalactiae* and *Enterococcus faecalis* (dark cells under phase contrast: negative control); bar: 20 µm

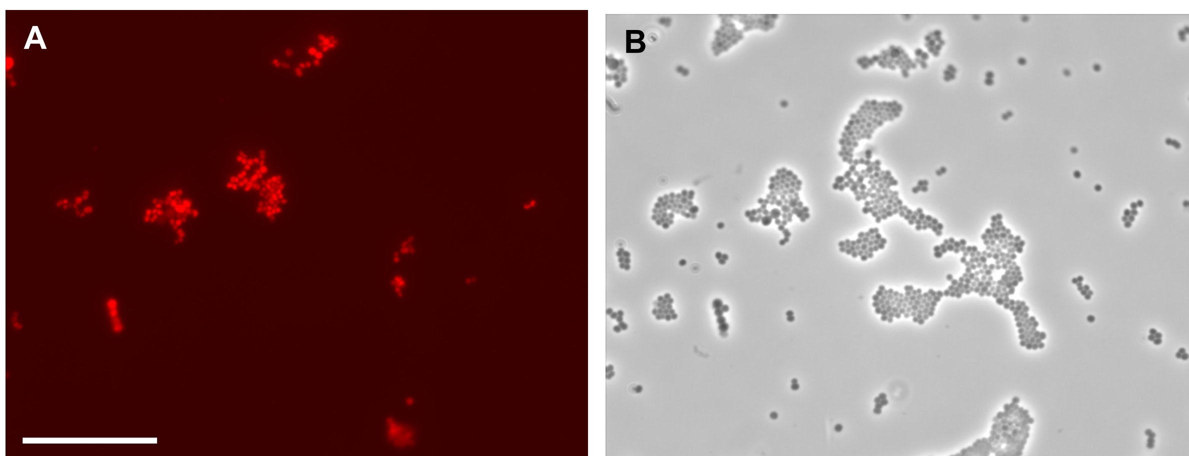
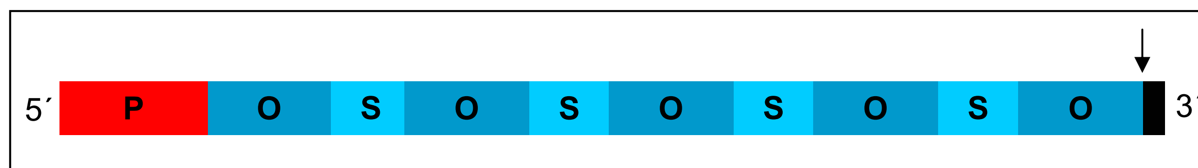


Figure C.12 A: Whole cell fluorescence signals after FISH (1.5 h; 30 % FA) using the Cy3 labelled oligonucleotide probe Spy467 targeting the 16S rRNA of *Streptococcus pyogenes* **B:** Phase contrast image of *S. pyogenes* and *Staphylococcus epidermidis* (cocci arranged in clusters, negative control); bar: 20 µm

C.2. Construction and application of RNA oligo-oligonucleotide probes for the identification of pathogens causing nosocomial infections

C.2.1. Design and generation of monospecific RNA oligo-oligonucleotide probes

Figure C.13 Schematic illustration of the monospecific oligo-oligonucleotide construct containing the promoter fragment (P), oligonucleotide sequences (O) and spacer fragments (S). The endonuclease site is indicated by the black arrow and box.



The main part of this study was focused on the development of polynucleotide probes composed of taxon-specific oligonucleotide probes and non-coding fragments, so-called spacers. Domain III targeted polynucleotide probes are rather specific concerning the identification of bacteria. Nevertheless, these polynucleotide probes did not allow a differentiation between enterococci and streptococci (Fichtl, 2005). The discrimination of bacteria by RING-FISH is also limited due to the probe length and possible sequence combinations derived from secondary structure formation. Therefore, another approach resulting in a more specific identification and separation of pathogens using polynucleotide probes is necessary. The concept for the design of oligo-oligonucleotide probes is based on the combination of features of oligonucleotide (specificity) and polynucleotide probes (formation of secondary structures). The oligonucleotide parts ensure the specificity and the insertion of spacers (GCGCG or AAAAA) ensures that the original oligonucleotide fragments stay apart. Additionally, the GC- or polyA-stretches avoid mismatch-hybridisation due to shifted oligonucleotide sequences. These novel polynucleotide probes should also be able to form secondary structures and a network within and around the cell envelope during hybridisation resulting in halo detection.

Several methods were refined and new approaches were developed to test and construct oligo-oligonucleotide probes. A single stranded construct comprising five taxon-specific oligonucleotides and four interjacent spacer regions was designed. Furthermore, a T3 promoter is located at the 5` terminus of the construct and a restriction endonuclease site at the 3´ terminus (Figure B.1, p. 50; Tables B.20 and

B.21, pp. 51 and 52). The single stranded fragment was denatured followed by the synthesis of a double stranded template via Klenow reaction (see B.10.2., p. 49). The direction of all oligonucleotide fragments within the whole construct corresponds to the 5´→3´ direction of all DNA oligonucleotide probes, which were used in FISH before (see C.1. and table C.1, p. 68). The double stranded product (generated by the Klenow enzyme) was polyadenylated, cloned into a vector and checked by PCR (see B.10.2., p. 49). Samples showing amplicons of the right size (about 380 nt) were double digested (see B.10.2., p. 49 and table B.23, p. 54). A restriction endonuclease (here: EcoRI) cut the template out of the vector. The second specific enzyme was separately chosen for each probe and possesses a restriction site within the designed construct at the 3´end (Figure B.1, p. 50 and figure C.13, p. 73). After the digestion, the purified fragment could be used for the synthesis of directly or indirectly labelled probes via *in vitro* transcription by incorporation of either fluorescein-12-UTP or biotin-16-UTP (see B.10.1., p. 47). In parallel, the cloned fragments (about 380 nt) were sequenced to assure the correct length (110 or 105 nt) and sequence of resultant oligo-oligonucleotide probes (see B.12., p. 62).

In this way, the synthesis of monospecific as well as multispecific oligo-oligo polynucleotide probes from a single stranded DNA-template was successful. The presented procedure for the design and generation of oligo-oligonucleotide probes was used for the development of mono- (Tables C.2 and C.3, pp. 75 and 76) and multispecific probes (Table C.6, p. 99), for the identification of six Gram-negative and six Gram-positive bacteria causing nosocomial infections. All probes are 110 nt long, with the exception of the probe specific for *Stenotrophomonas maltophilia.* This polynucleotide probe consists of 105 nt due to the 17 nt long oligonucleotide probe Sma1158 (Table C.1, p. 68) in contrast to other oligonucleotides of 18 nt. All listed RNA oligo-oligonucleotide probes (Tables C.2 and C.3, pp. 75 and 76) were tested and successfully applied in hybridisation and separation experiments from biological material of different origin.

Table C.2 Sequences of oligo-oligonucleotide probes targeting Gram-negative bacteria. Target bacteria, AF number, probe name, whole oligo-oligonucleotide probe sequence and target region: 16/23S rRNA. Oligonucleotide sequence: blue bold sequence; spacer fragment (GCGCG or AAAAA): black bold italics

Bacteria and AF number	Probe name	Oligo-oligonucleotide probe sequence 5´→ 3´ direction	16/23S rRNA
A. baumannii 438775	Aba88_GC	**UCCGCCGCUAGGUCCGGU*GCGCG*UCCGCCGCUAGGUCCGGU*GCGCG*UCCGCCGCUAGUCCGGU*GCGCG*UCCGCCGCUAGGUCCGGU**	16S
A. baumannii 438775	Aba88_AA	**UCCGCCGCUAGGUCCGGU*AAAAA*UCCGCCGCUAGGUCCGGU*AAAAA*UCCGCCGCUAGGUCCGGU*AAAAA*UCCGCCGCUAGUCCGGU**	16S
E. aerogenes 427068	Eae16S_GC	**CGAGUAACGUCAAUCGCC*GCGCG*CGAGUAACGUCAAUCGCC*GCGCG*CGAGUAACGUCAAUCGCC*GCGCG*CGAGUAACGUCAAUCGCC**	16S
E. coli 441612	Eco271_GC	**CACACACUGAUUCAGGCU*GCGCG*CACACACUGAUUCAGGCU*GCGCG*CACACACUGAUUCAGGCU*GCGCG*CACACACUGAUUCAGGCU**	23S
K. pneumoniae 429457	Kpn_GC	**CCUACACACCAGCGUGCC*GCGCG*CCUACACACCAGCGUGCC*GCGCG*CCUACACACCAGCGUGCC*GCGCG*CCUACACACCAGCGUGCC**	23S
P. aeruginosa 426470	PseaerB_GC	**UCUCGGCCUUGAAACCCC*GCGCG*UCUCGGCCUUGAAACCCC*GCGCG*UCUCGGCCUUGAAACCCC*GCGCG*UCUCGGCCUUGAAACCCC**	23S
S. maltophilia 484133	Sma1158_GC	**AAGUUAUGCACCCCAAG*GCGCG*AAGUUAUGCACCCCAAG*GCGCG*AAGUUAUGCACCCCAAG*GCGCG*AAGUUAUGCACCCCAAG**	23S

Table C.3 Sequences of oligo-oligonucleotide probes targeting Gram-positive bacteria. Target bacteria, AF number, probe name, whole oligo-oligonucleotide probe sequence and target region: 16/23S rRNA. Oligonucleotide sequence: blue bold sequence; spacer fragment (GCGCG): black bold italics

Bacteria and AF number	Probe name	Oligo-oligonucleotide probe sequence 5´→ 3´ direction	16/23S rRNA
E. faecium 458387	Efi58_GC	**UGACUCCUCUUCAGACUU*GCGCG*UGACUCCUCUUCAGACUU*GCGCG*UGACUCCUCUUCAGACUU*GCGCG*UGACUCCUCUUCAGACUU*GCGCG*UGACUCCUCUUCAGACUU**	23S
E. faecalis Staph/Strep 28	Efe286_GC	**UCCUUCAACUAUACUAAC*GCGCG*UCCUUCAACUAUACUAAC*GCGCG*UCCUUCAACUAUACUAAC*GCGCG*UCCUUCAACUAUACUAAC*GCGCG*UCCUUCAACUAUACUAAC**	16S
S. aureus 433088	Sau227_GC	**AAUGCAGCGCGGAUCCAU*GCGCG*AAUGCAGCGCGGAUCCAU*GCGCG*AAUGCAGCGCGGAUCCAU*GCGCG*AAUGCAGCGCGGAUCCAU*GCGCG*AAUGCAGCGCGGAUCCAU**	16S
S. epidermidis MRSE / S:23	Sep16S_GC	**GUCAGAGGAGCAAGCUCC*GCGCG*GUCAGAGGAGCAAGCUCC*GCGCG*GUCAGAGGAGCAAGCUCC*GCGCG*GUCAGAGGAGCAAGCUCC*GCGCG*GUCAGAGGAGCAAGCUCC**	16S
S. agalactiae 616311	Sag210_GC	**CCAUCUCACAGUGAAGCA*GCGCG*CCAUCUCACAGUGAAGCA*GCGCG*CCAUCUCACAGUGAAGCA*GCGCG*CCAUCUCACAGUGAAGCA*GCGCG*CCAUCUCACAGUGAAGCA**	16S
S. pyogenes 611333	Spy467_GC	**CCGUCACUUGGUGGAUUU*GCGCG*CCGUCACUUGGUGGAUUU*GCGCG*CCGUCACUUGGUGGAUUU*GCGCG*CCGUCACUUGGUGGAUUU*GCGCG*CCGUCACUUGGUGGAUUU**	16S

C.2.2. Development and optimisation of the FISH protocol for the detection of bacteria

Some general preconditions had to be evaluated and fulfilled before the new types of polynucleotide probes were tested for their applicability in hybridisations. The optimised parameters should lead to an optimal binding of the probe to its target.

C.2.2.1. Fixation of cells in different growth phases

A former study indicates (Schleifer, 1975) that the condition of the cell wall concerning permeability can vary during the growth process. The cell envelope is more permeable in the later exponential growth phase as long as the reproduction is not completely finished.

Therefore, hybridisations were accomplished and should demonstrate the correlation of bacterial cell wall condition with respect to the point of time of PFA fixation (see B.3., p. 26) and appearance of the signal after FISH. Hybridisations using the monospecific probe Aba88_GC targeting *Acinetobacter baumannii* fixed with PFA (4 %) at two different growing phase stages (OD_{600}: 0.8 and OD_{600}: 1.2) resulted in a different appearance of the signal under identical incubation conditions. Different signal intensities were observed after FISH under similar conditions (same temperature, formamide concentration, denaturation and incubation time). When *A. baumannii* cells were fixed at a later stage of exponential growth phase (OD_{600}: 0,6-0,8), the FISH showed intensive halo signals of almost all target cells. In contrast, hybridisations using bacteria fixed at the stationary growth phase (OD_{600}: >1) showed less intense signals. Moreover, not all target cells present on the microscopic slide showed a halo signal.

C.2.2.2. Pretreatments to ensure the accessibility of the probe to its target site

The cell wall of Gram-positive bacteria is characterised by a rigid multilayered peptidoglycan, polysaccharides and teichoic acids. These components form a network around the cell and can vary in structure and composition among bacterial groups. Peptidoglycan, also called murein, consists of alternating beta-1,4-linked residues of N-acetyl muramic acid and N-acetylglucosamine. To overcome that barrier different enzymes affecting the bacterial cell wall are available and were already used for FISH with oligonucleotide probes. Lysozyme and mutanolysin cleave the peptidoglycan by hydrolysing the beta-1,4 bond between N-acetyl muramic acid and N-acetylglucosamine. Especially mutanolysin is useful to permeabilise the cell wall of enterococci and streptococci. Lysostaphin, another enzyme, affects specifically staphylococci due to hydrolysis of the glycine-glycine bonds in the pentaglycine interpeptide linkages, which are typical for the peptidoglycan of staphylococci.

It is important to evaluate an adequate permeabilisation method to ensure the accessibility for the probe to its target region through the cell envelope. Concurrently a destruction of the bacterium arising from a too strong treatment should be avoided. Former studies (Sandjong, 2003; Fichtl, 2002) show some appropriate treatments for the cell walls of enterococci and staphylococci. In both studies the combination of an enzymatic and a heat treatment proved to be beneficial regarding an optimal permeabilisation and time reduction. In the present study the bacteriolytic enzymes were not only tested at different incubation times (from 5 to 30 min) but at different temperatures (0, 30, 37°C), too. The sole enzymatic treatment proved to be time consuming, e.g. 20 min lysostaphin at 37°C was necessary to permeabilise the cell wall of *Staphylococcus epidermidis*. In comparison to FISH using oligonucleotide probes (see B.9.1., p. 38) the incubation time was shorter and the permeabilisation consisted only of an enzymatic step. Lysozyme was effective at 0°C after 5 or 10 min (Table B.16, p. 40). The hybridisations using oligo-oligonucleotide probes showed that longer incubations (40 min) at higher temperature (37°C) are more efficient but time-consuming. In contrast to an incubation of 10 min at 37°C, long heating step at 200°C became manifest in lysed cells depending on the bacteria. While streptococci were lysed after 5 min at 200°C the cell wall of staphylococci was destroyed after longer incubation (up to 10 min) at this temperature. These results indicated that

neither an enzymatic treatment at 37°C (long duration) nor a sole heating step at 200°C (degradation of the cell) were applicable for the optimal accessibility of the cell wall. Finally, the combination of an enzymatic step followed by a short (1-7 min) heat pretreatment at 200°C proved to be the most efficient permeabilisation method for FISH on microscopic slides. An overview of recommanded pretretment methods is presented in table B.19 (p. 45) and the special pretreatments for respective bacteria are described in detail under C.2.2.6. (p. 83).

In Gram-negative bacteria, the cell wall is inter alia composed of few layers of peptidoglycan and relatively similar throughout most genera. The outer membrane protects the bacteria from several antibiotics or some detergents (Lengeler et al., 1999). Therefore, it is not necessary to pretreat the cell walls of Gram-negative bacteria prior to hybridisations. The fixation of Gram-negative cells with PFA (see B.3., p. 26) and the ethanol series (see B.9.1.1., p. 38) are sufficient for the permeabilisation of the cell envelope before hybridisation.

Table B.4 (p. 24) shows that Gram-negative and Gram-positive bacteria are often isolated from the same clinical sample. When the mixture contained bacteria with different cell walls (Gram-negative and -positive), the protocol concerning pretreatments was performed separately. One sample using the protocol for permeabilisation of Gram-positive bacteria was performed and the bacteria were pooled together afterwards (data not shown). Another approach concerning the permeabilisation of a sample containing Gram-positive and -negative bacteria, was to pretreat the Gram-negative bacteria, too. Figure C.23 B (p. 92) shows an artificial mixture of *Streptococcus agalactiae* and *P. aeruginosa* (Gram-negative bacteria) which stayed intact after the pretreatment. Additionally, the second attempt is timesaving.

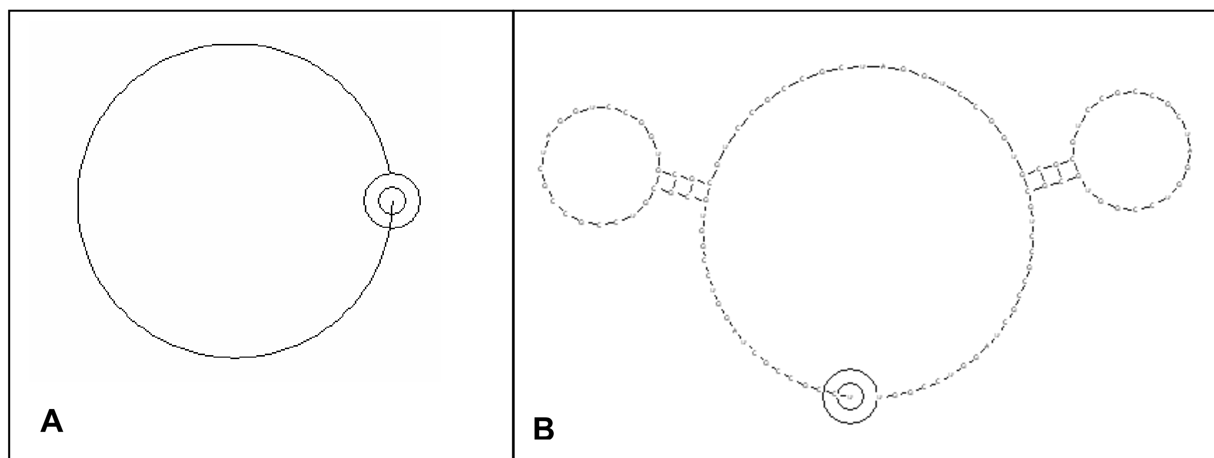
C.2.2.3. Selection of target and non-target organisms

For the evaluation of specific FISH conditions, each oligo-oligonucleotide probe first was tested in hybridisations using the target cell followed by hybridisations using the target and non-target cell. As shown in table B.4 (p. 24) and described in several studies (Pfaller et al., 1998; Johansen et al., 2006) some pathogens can cause particular diseases. Hence, they colonise special human tissues or fluids and could be isolated from their typical sites of infections (*Staphylococcus aureus* from blood or *Escherichia coli* from urine). Furthermore, in the most cases, one bacterial species triggers the illness. Seldom two or three bacterial species are responsible for the disease at the same time. Apart from *Neisseria canis,* which can be easily distinguished from most of the rod-shaped Gram-negative target cells due to the coccoid morphology, other bacteria were used as negative controls. The selection of additional non-target cells depended on the clinical sample and potentially coexistent bacteria during a real infection (Table B.4, p. 24). Table B.4 (p. 24) also shows that Gram-negative and -positive pathogens sometimes coexist in one clinical material e.g. *E. coli* and *Enterococcus* spp. were often isolated from the same urine sample.
Summa summarum, the selection of the positive and negative controls as well as the pretreatment method (see C.2.2.2., p. 78) corresponds as far as possible to the clinical situation and the proceedings in the diagnostic laboratories.

C.2.2.4. Sequencing of probe templates and in silico analysis of oligo-oligonucleotide probes

Before starting the *in vitro* transcription (see B.10., p. 47) for the synthesis of RNA oligo-oligonucleotide probes, the template sequences (Tables B.20 and B.21, pp. 51 and 52) were controlled by sequencing (see B.12., p. 62). The sequencing results showed that the templates have the correct length. Both, the defined sequence and size are preconditions for the generation of labelled probes (Tables C.2 and C.3, pp. 75 and 76). Additionally, *in silico* analyses (RNAdraw V1.1b2, Matzura et al., 1996) were performed to calculate potential secondary structures of all RNA oligo-oligonucleotide probes. As hypothesised in earlier studies (Zwirglmaier et al., 2003) these structures of labelled probes play an important role for the network formation, which is responsible for halo signals during hybridisations. After the binding of the polynucleotide probe to its target the protruding part of the probe interacts with other probe molecules and generate a network within and around the cell envelope. After hybridisation, this phenomenon can be observed as a halo under the microscope. Figure C.14 (p. 81) shows possible secondary structures of the *A. baumannii*-specific oligo-oligonucleotide probe Aba88_GC (Table C.2, p. 75) under certain conditions (denaturation at 80°C, hybridisation at 53°C, 30 % formamide in the buffer). For the calculation of secondary structures at different temperatures or (formamide concentrations), 1 % formamide was assumed to lower the melting temperature by 0.72°C (McConaughy et al., 1969). Probable secondary structures of some other generated oligo-oligonucleotide probes are shown in the appendix (part F, p. 170).

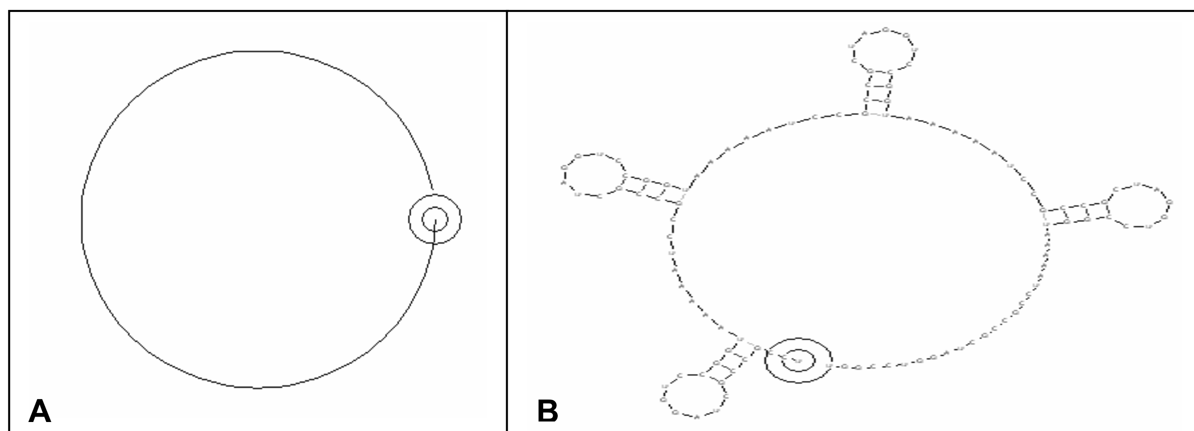
Figure C.14 Secondary structure model (RNAdraw V1.1b2, Matzura et al., 1996) of RNA oligo-oligonucleotide probe Aba88_GC (Table C.2, p. 75) with 30 % formamide in the buffer
A: under denaturation conditions at 80°C, **B:** under hybridisation conditions at 53°C



C.2.2.5. Generation of probes containing different spacer sequences

In addition to oligo-oligonucleotide probes containing the GC-spacer, a probe Aba88_AA targeting *A. baumannii* containing the AAAAA-stretch (Table C.2, p. 75) was tested for FISH on slides. The aim of these hybridisations was to determine whether different stretches (within the polynucleotide probe) have an influence on the appearance of the halo signal. The synthesis of the Aba88_AA probe was performed in the same way as for probes containing the GC-stretch (see C.2.1., p. 73). The *in silico* analysis of secondary structures (RNAdraw V1.1b2, Matzura et al., 1996) indicates that secondary structures are possible and could form a network. For the *in silico* analysis at different temperatures (or different formamide concentrations), 1 % formamide lowers the melting temperature by 0.72°C (McConaughy et al., 1969). Figure C.15 (p. 82) shows probable secondary structures of the probe Aba88_AA (Table C.2, p. 75) at 80°C (denaturation step) and 53°C (hybridisation step). Experiment conditions, which were evaluated with probes containing the GC-spacer (see C.2.2.6., p. 83; 30 min denaturation at 80°C, 0-40 % formamide in the poly-FISH buffer, 2-6 h hybridisation time) were applied for the probe Aba88_AA, too. The hybridisations at different incubation times resulted in weak whole cell fluorescence of some target cells. The typical halo signal of the target cell was not observed. Due to these results, further probes containing the stretch of five adenosines were not constructed. For other strains, monospecific polynucleotide probes, containing the GC-spacer, were developed and applied (Tables C. 2 and C.3, pp. 75 and 76).

Figure C.15 Secondary structure model (RNAdraw V1.1b2, Matzura et al., 1996) of RNA oligo-oligonucleotide probe Aba88_AA (Table C.2, p. 75) with 30 % formamide in the buffer
A: under denaturation conditions at 80°C, **B:** under hybridisation conditions at 53°C



C.2.2.6. Hybridisations on slides using monospecific oligo-oligonucleotide probes

After the evaluation of hybridisation preconditions (see C.2.2.1.-C.2.2.4., pp. 77-81), the optimisations of appropriate FISH protocols for each oligo-oligonucleotide probe were achieved. It was successful to develop (Table C.2, p. 75) and apply generated oligo-oligonucleotide probes for six Gram-negative bacterial species (*Acinetobacter baumannii*, *Enterobacter aerogenes*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia*). All these pathogens play an important role as causatives of nosocomial infections, especially sepsis, infections of the urinary as well as respiratory tract and of wounds.

In general, the hybridisation success is influenced by several factors, e.g. type of the buffer, formamide concentration, temperature, duration of the denaturation (if necessary) and incubation step as well as the introduction of washing steps. Inter alia, these parameters were modified or omitted during the development and optimisation of the protocol for FISH using oligo-oligonucleotide probes. Experiments using the buffer, which is usually applied for hybridisations using oligonucleotide probes (see B.9.1., p. 38), were performed for incubations with *A. baumannii*, *S. maltophilia* and *E. coli* using the respective oligo-oligonucleotide probes containing GC-spacers (Table C.2, p. 75). Different hybridisation times (0.5-3 h) at 46°C and 53°C and variable formamide concentrations (0-50 %) resulted in strong and specific whole cell fluorescence signals of all target cells. A subsequent washing step (see B.9.1. and table B.15, p. 38) of 15 min at 48°C or 55°C led to a specific fluorescence signal, which was distributed over the whole cell, too. Figures C.16 A-F (p. 84) show the results of the hybridisations using the oligo-FISH buffer (see B.9.1., p. 38) and the 16S rRNA targeted 110 nt long Aba88_GC oligo-oligonucleotide probe specific for *A. baumannii.* Figures C.16 A-F (p. 84) show the hybridisation results in the mixture of *A. baumannii* and *M. morganii* (A) or *N. canis* (F) without the washing step. In contrast, figure C.16 C (p. 84) demonstrates the result of the introduction of a subsequent washing step. The appearance of the signal (whole cell fluorescence) persists independently of the additional washing step after the hybridisation but the signal intensity was increased significantly after washing.

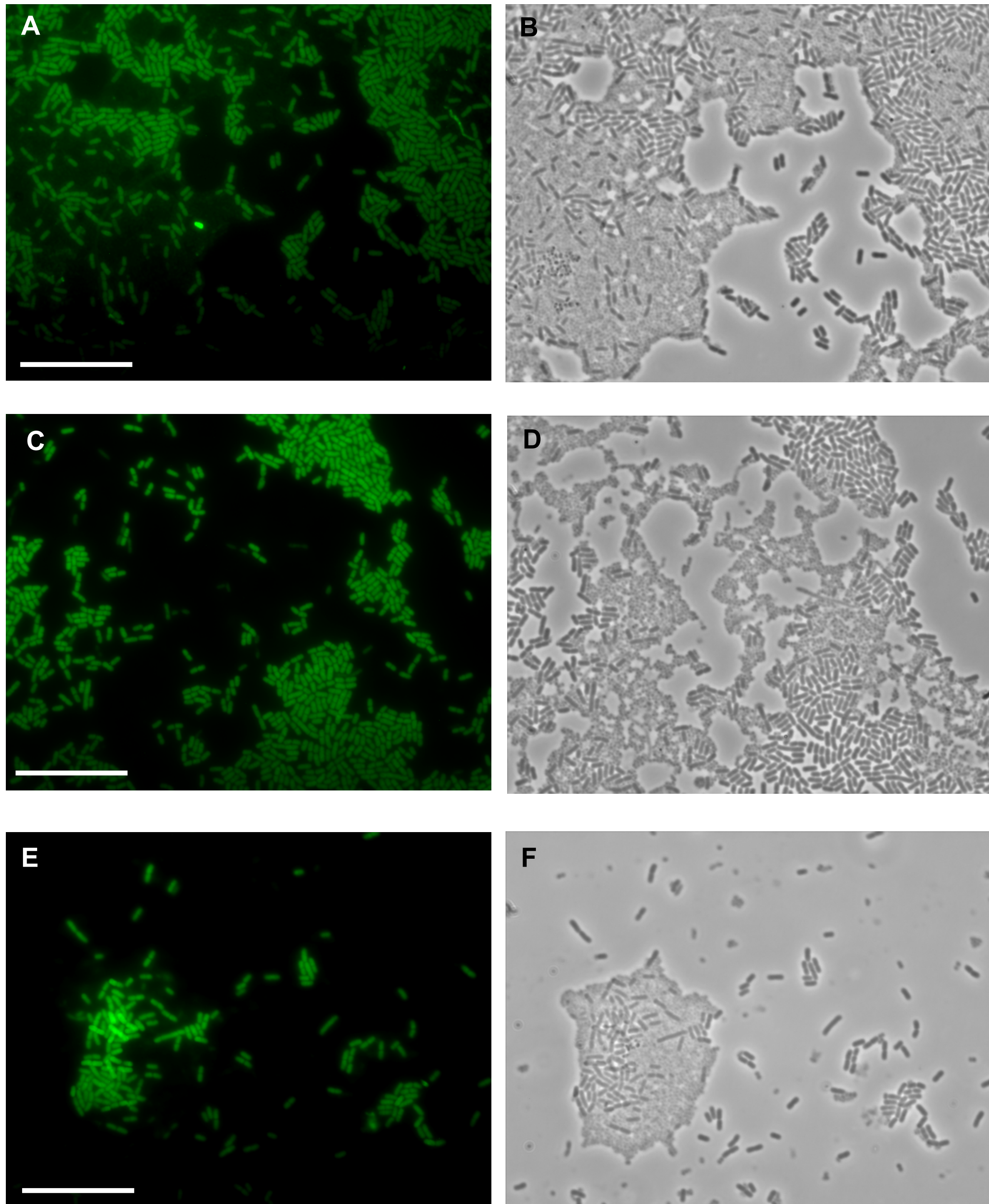


Figure C.16 Whole cell fluorescence after FISH using the oligo-oligonucleotide probe Aba88_GC targeting 16S rRNA of *Acinetobacter baumannii*. Results obtained after hybridisations at 30 % FA in the **oligo-FISH buffer** with *Acinetobacter baumannii* and *Morganella morganii* (smaller rod-shaped bacteria) (B+D) and *Neisseria canis* (cocci) (F) (negative controls). **A: without** subsequent washing step after 1.5 h, **C: with** subsequent washing step after 1.5h; **E: without** subsequent washing step after 2 h; **A, C, E:** epifluorescence images; **B, D, F:** phase contrast images; bar: 20 µm

The formation of a halo is the decisive criterion with regard to further application of oligo-oligonucleotide probes for immobilisations of cells in microplate cavities. Because of the results (whole cell fluorescence) obtained with the oligo-FISH buffer, the poly-FISH protocol (see B.9.2., p. 42), which is usually applied for hybridisations with RNA polynucleotide probes (Zwirglmaier, 2003) was tested. The procedure implies a changed buffer composition and a denaturation step at 80°C prior to hybridisation. After the oligo-oligonucleotide probe Aba88_GC targeting the 16S rRNA of *A. baumannii* was denatured (20 min at 80°C) and the hybridisation was performed between 1 to 3 h and at 0-15 % formamide in the poly-FISH buffer no or weak halo signals were observed. Consequently, further improvements of the protocol were necessary. Finally, the characteristic halo signals were observed after a prolonged denaturation step (30 min at 80°C) followed by a hybridisation of 4 h (20 and 30 % FA in the buffer) at 53°C. Figure C.17 (p. 85) illustrates the optimal result demonstrating strong and specific halo signals of the target bacteria. All target cells (*A. baumannii*) exhibited the ring-shaped signal. *N. canis* (negative control) did not show any fluorescence. The positive results point out that these conditions could be also adapted to hybridisations with other bacteria and probably enable the immobilisation of cells due to network formation.

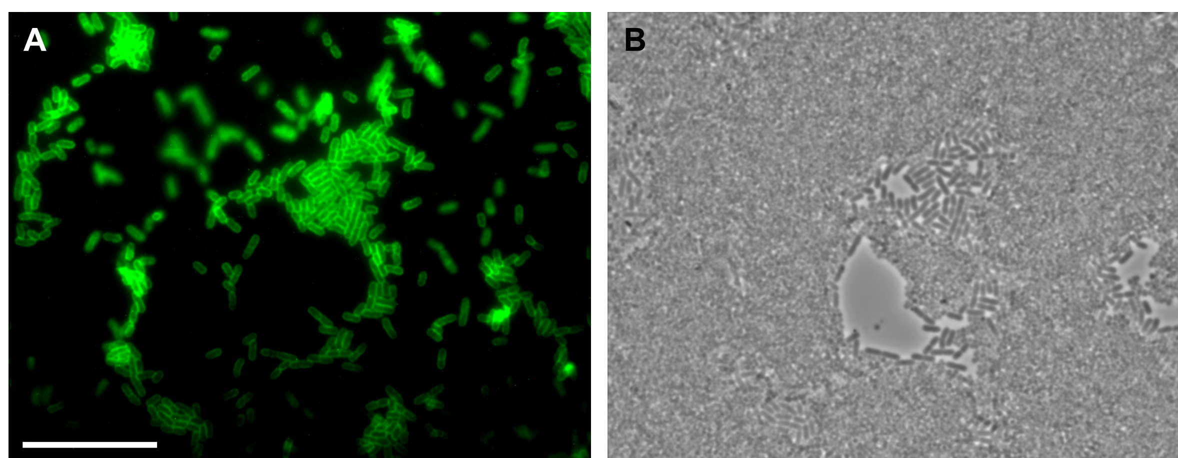


Figure C.17 Halo signals after FISH using the oligo-oligonucleotide probe Aba88_GC targeting 16S rRNA of ***Acinetobacter baumannii***. Results obtained after 3 h hybridisation at 30 % FA in the **poly-FISH buffer** with *Acinetobacter baumannii* and *Neisseria canis* (cocci, negative control). **A:** epifluorescence image; **B:** phase contrast image; bar: 20 µm

Other protocol modifications (reduction of the denaturation time, introduction of a washing step after the hybridisation) led to signal loss or did not show any effects. Elongation of the incubation time at 53°C sometimes caused false positive signals of non-target cells (*N. canis*, *P. aeruginosa, K. pneumoniae, E. coli, S. aureus, Enterococcus* spp.). The increased formamide concentration from 40 to 70 % changed the appearance of the halo signal. Either an unevenly distributed signal occurred in the periphery of the target cells (looking like a comet tail) or an expanded fluorescence signal (similar to fried egg, in which the egg yolk symbolises the target cell). These phenomena were probably caused by an accumulation of the remaining unbound probes, which could not be eliminated after the hybridisation. The described observations at higher formamide concentrations (> 40 %) correlated with hybridisation experiments performed with other target cells and specific oligo-oligonucleotide probes. These particular signals also appeared after incubations at increased formamide concentrations with *Enterobacter aerogenes*, *Klebsiella pneumoniae* and *Enterococcus faecium* as target organisms.

As mentioned before, due to the results obtained after hybridisations using the poly-FISH protocol, this proceeding (poly-FISH buffer, 30 min denaturation at 80°C, hybridisation at 53°C) was maintained for further experiments with oligo-oligonucleotide probes and different samples containing pathogens. For FISH series with five Gram-negative bacterial species (*E. aerogenes*, *E. coli*, *K. pneumoniae*, *P. aeruginosa* and *S. maltophilia*) a denaturation time of 30 min, hybridisation times between 2 to 6 h and formamide concentrations of 0 to 20 % were successfully used. Specific signals of the other target cells could be obtained after optimising the formamide concentrations and incubation times by using several negative controls. Figures C.18 and C.19 (p. 88) illustrate the ring-shaped fluorescence of *P. aeruginosa* and *S. maltophilia*. Figure C.18 (p. 88) shows the positive signals of *P. aeruginosa* after (5 h) incubation with the PseaerB_GC oligo-oligonucleotide probe. Since *P. aeruginosa* represents a frequent isolate from hospital-acquired diseases especially sepsis, infections of the respiratory or urinary tract as well as wound infections and sepsis, it was very important to identify this pathogen.

Figure C.19 (p. 88) shows further successful hybridisations using novel oligo-oligonucleotide probes. Figure C.19 B (p. 88) demonstrates specific binding of *S. maltophilia* targeted Sma1158_GC in a mixture of *S. maltophilia* and *M. morganii* (negative control). Both bacteria were incubated with the Sma1158_GC and only the target cell was detected after FISH. Other bacteria used as non-target organisms were *E. coli* and *P. aeruginosa.*

In addition to the characteristic ring-shaped signals in the periphery of the target organism, other fluorescence signals were also observed. The hybridisation using the Eae16S_GC probe targeting *E. aerogenes* and some hybridisations using the Sma1158_GC probe specific for *S. maltophilia* led to whole cell fluorescence of some target cells. However, figure C.20 A (p. 88) shows that the Eae16S_GC oligo-oligonucleotide probe is specific because the signal of the target cell can be observed (after 5 h, 0 % FA) but no signal of the negative control *Haemophilus influenzae* (Figure C.20 B, p. 88).
The other fluorescence signal that was noticed apart from halos was the so-called intermediate signal described by Fichtl (2005). The intermediate signals are characterised by soft whole cell fluorescence and a stronger ring-shaped signal in the periphery of the target cell. This phenomenon appeared after incubations (5 h, 10 % FA) with the Eco271_GC oligo-oligonucleotide probe specific for *E. coli* and is demonstrated in figure C.21 A (p. 89). Here, the successful and specific detection of one of the most important and frequent isolate (*E. coli*) from nosocomial infections is visualised. The negative control was *M. morganii*.

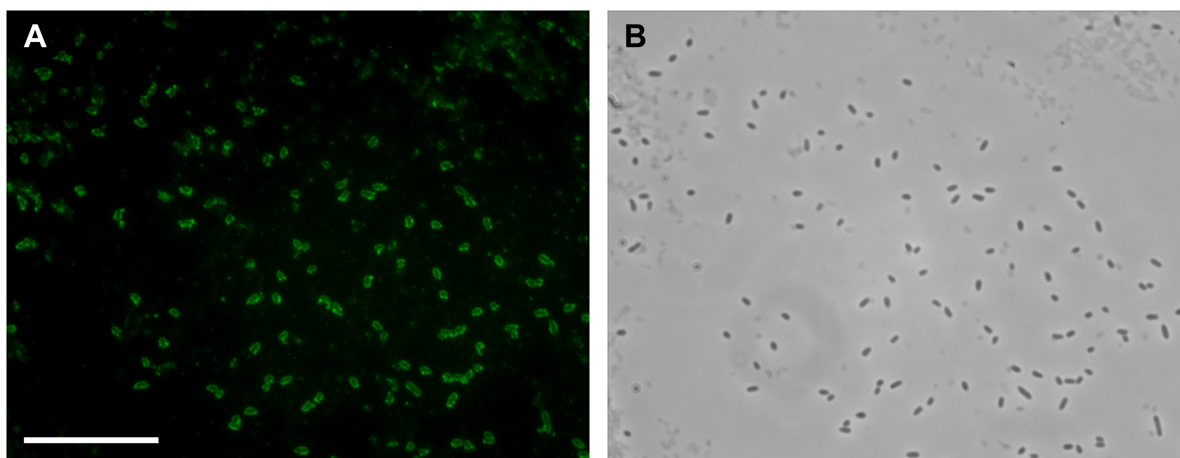


Figure C.18 Halo signals observed after FISH using the oligo-oligonucleotide probe PseaerB_GC targeting 23S rRNA of ***Pseudomonas aeruginosa***. Results obtained after 5 h hybridisation at 20% FA in the poly-FISH buffer with *P. aeruginosa* in pure culture;
A: epifluorescence image; **B:** phase contrast image, bar: 20 µm

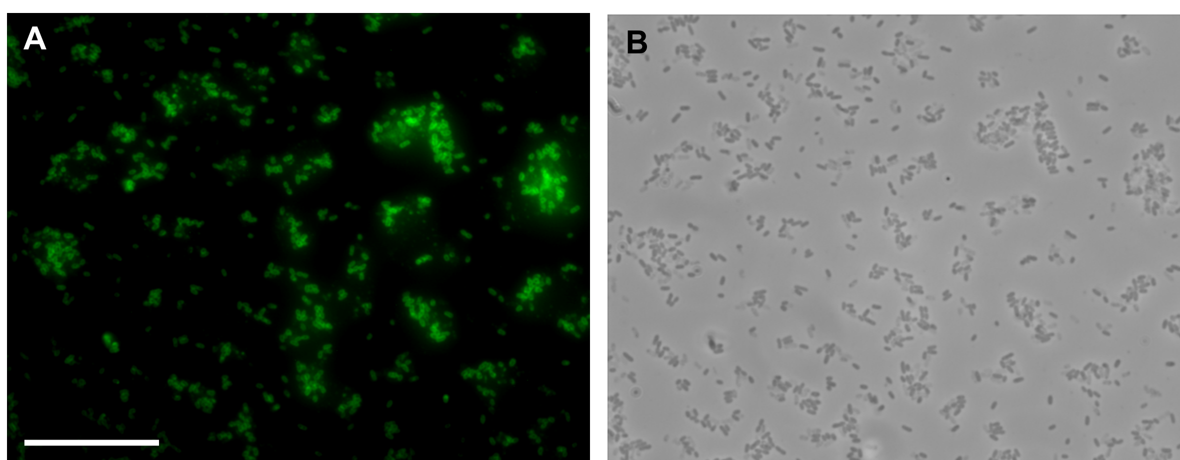


Figure C.19 Halo and whole cell fluorescence signals observed after FISH using the oligo-oligonucleotide probe Sma1158_GC targeting 23S rRNA of ***Stenotrophomonas maltophilia***. Results obtained after 2 h hybridisation at 20 % FA in the poly-FISH buffer with *S. maltophilia* and *M. morganii* (smaller and brighter rods under the phase contrast: negative control);
A: epifluorescence image; **B:** phase contrast image, bar: 20 µm

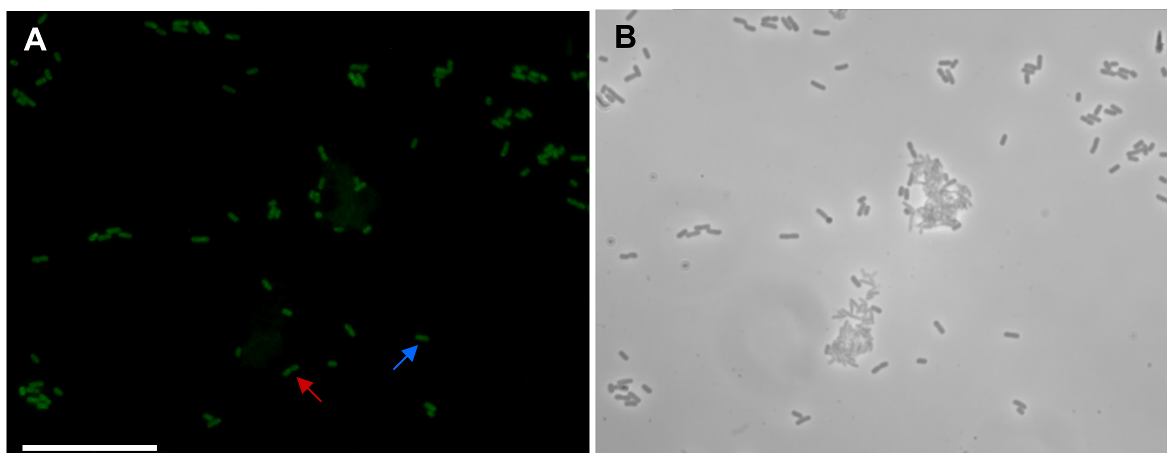


Figure C.20 Halo (red arrow) and whole cell fluorescence (blue arrow) signals observed after FISH using the oligo-oligonucleotide probe Eae16S_GC targeting 16S rRNA of ***Enterobacter aerogenes***. Results obtained after 5 h hybridisation at 0 % FA in the poly-FISH buffer with *E. aerogenes* and *Haemophilus influenzae* (thin rods under the phase contrast: negative control)
A: epifluorescence image; **B:** phase contrast image; bar: 20 µm

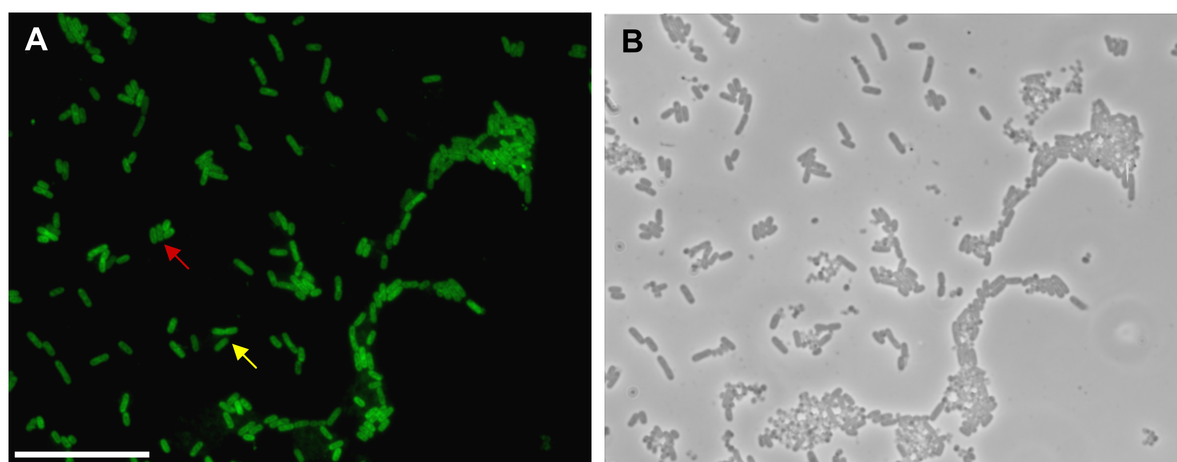


Figure C.21 Halo (red arrow) and intermediate signals (yellow arrow) observed after FISH using the oligo-oligonucleotide probe Eco271_GC targeting 23S rRNA of ***Escherichia coli***. Results obtained after 5 h hybridisation at 10 % FA in the poly-FISH buffer with *E. coli* and *Morganella morganii* (small rods under the phase contrast: negative control); **A:** epifluorescence image; **B:** phase contrast image; bar: 20 µm

Table C.4 (p. 89) summarises all results obtained after the application of taxon-specific oligo-oligonucleotide probes for the identification of six Gram-negative bacteria causing nosocomial infections.

Table C.4 Results obtained after FISH using oligo-oligonucleotide probes. Six Gram-negative bacteria causing nosocomial infections, AF number, oligo-oligonucleotide probe names, target region: 16/23S rRNA and FISH conditions which led to specific signals

Bacteria and AF number	**Probe name**	**16/23S rRNA**	**FISH conditions at 53°C t [h] / FA [%]**	**Detected signal**
A. baumannii 438775	Aba88_GC	16S	4 / 30	Halo
E. aerogenes 427068	Eae16S_GC	16S	5 / 0	Halo, WCF
E. coli 429457	Eco271_GC	23S	5 / 10	Halo +
K. pneumoniae 479983	Kpn_GC	23S	6 / 20	Halo
P. aeruginosa 426470	PseaerB_GC	23S	5-6 / 20	Halo (+)
S. maltophilia 484133	Sma1158_GC	23S	2-3 / 20	Halo, WCF

Halo+: intermediate signal; WCF: whole cell fluorescence

Further sequences of six monospecific oligo-oligonucleotide probes are listed in table C.3 (p. 76). They were synthesised and applied for the detection and separation of Gram-positive pathogens. These Gram-positive bacteria are *Enterococcus faecalis*, *Enterococcus faecium*, *Staphylococcus aureus*, *S. epidermidis*, *Streptococcus agalactiae* and *S. pyogenes*. All of them play an important role in the context of nosocomial infections like pneumonia, sepsis and infections of the urinary tract or wounds.
After the optimisation of an adequate permeabilisation method (see C.2.2.2., p. 78), the next challenge was the detection of specific signals using oligo-oligonucleotide probes. The evaluation of the protocol was carried out under the same conditions as for Gram-negative bacteria (as described above) between 1.5 to 6 h at 0-50 % formamide at 53°C. First, the target cells and the oligo-oligonucleotide probes were tested in pure cultures and afterwards in artificial mixtures containing non-target cells. The control organisms were chosen as presented under C.2.2.3 (p. 80). In addition to enterococci, staphylococci and streptococci, Gram-negative control organisms (*E. aerogenes, E. coli*, *P. aeruginosa*, *K. pneumoniae*) were tested. Similar to experiments using probes targeting Gram-negative bacteria, hybridisations at high formamide concentrations often resulted in uneven or expanded signals around the target cell. Mostly, prolonged incubation times led to unspecific signals and/or fluorescence distributed around the whole bacterium. The hybridisation conditions, which led to a successful identification of Gram-positive bacteria, are shown in table C.5 (p. 92).

For both *Enterococcus* species (*E. faecalis* and *E. faecium*), the same pretreatment method was effective (lysozyme for 10 min at 37°C and 5 min at 200°C, described under C.2.2.2., p. 78). The hybridisations using 16S rRNA targeted oligo-oligonucleotide probe Efe286_GC specific for *E. faecalis* was tested under different conditions. Specific halo and intermediate signals of the target cell were observed after 3 h and 30 % formamide. Specific halo and whole cell fluorescence signals of *E. faecium* cells were observed after 3 h at 10 % formamide using the Efi58_GC oligo-oligonucleotide probe. Ring-shaped signals obtained after incubation of *E. faecium* cells using the specific Efi58_GC probe are shown in figure C.22 (p. 91). Figure C.22 A (p. 91) visualises that all target cells show a strong fluorescence signal in the periphery of the bacterial cell.

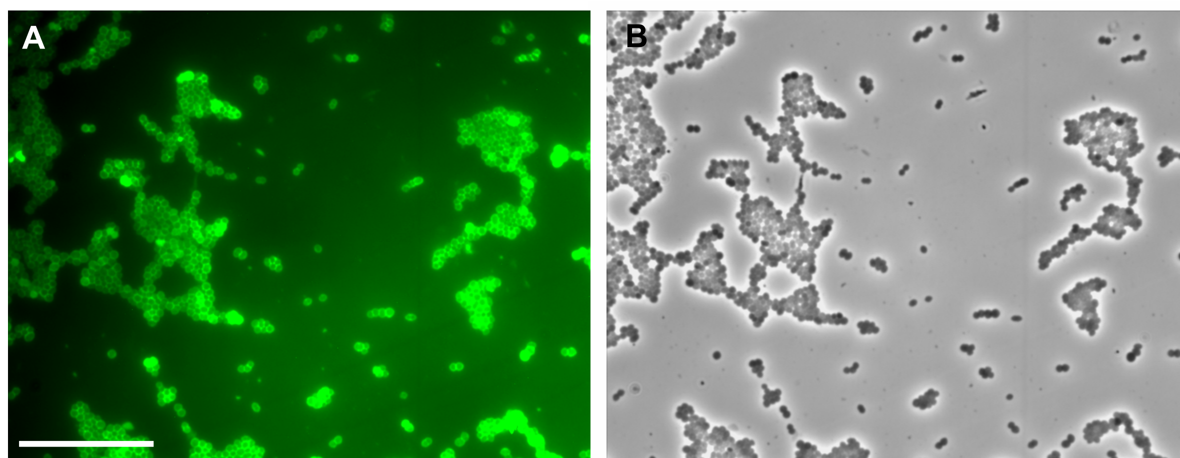


Figure C.22 Halo signals observed after FISH using the oligo-oligonucleotide probe Efi58_GC targeting 23S rRNA of ***Enterococcus faecium***. Results obtained after 3 h hybridisation at 10 % FA in the poly-FISH buffer with *E. faecium* cells in pure culture. **A:** epifluorescence image; **B:** phase contrast image; bar: 20 µm

For the Gram-positive and coagulase-positive *S. aureus* as well as for the coagulase-negative *S. epidermidis* a similar pretreatment protocol (10 min lysostaphin at 37°C followed by 7 min at 200°C) was used. The hybridisations (after 2 to 3 h at 10 % FA) resulted in halo and whole cell fluorescence signals. The exact hybridisation conditions for hybridisations with Gram-positive bacteria are listed in table C.5 (p. 92).

After the application of an adequate permeabilisation protocol (mutanolysin for 5 min at 37°C followed by 1 min at 200°C), FISH with *S. agalactiae* and *S. pyogenes* resulted in specific halo signals. Figure C.23 (p. 92) shows the halo signals obtained after hybridisations (3 h, 20 % formamide) with *S. agalactiae* using the Sag210_GC oligo-oligonucleotide probe in mixture with negative controls *P. aeruginosa* (Figure C.23 B, p. 92) and *S. epidermidis* (Figure C.23 D, p. 92). In both cases, FISH using the *S. agalactiae* targeted oligo-oligonucleotide probe Sag210_GC led to specific halos. Black arrows (Figures C.23 B and C.23 D, p. 92) indicate some of the non-target bacteria. Although *P. aeruginosa* (Gram-negative bacterium) was also pretreated, the cell integrity is still intact.

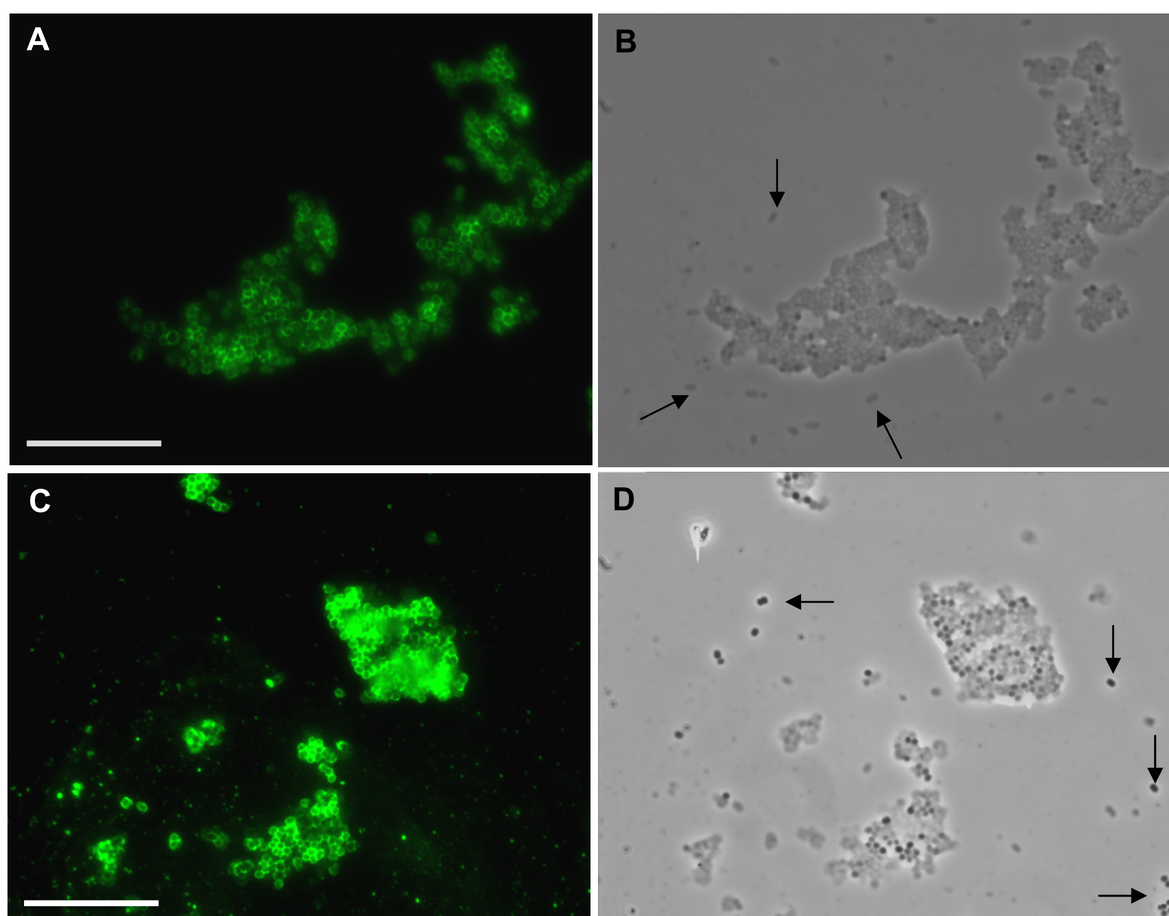


Figure C.23 Halo signals observed after FISH using the oligo-oligonucleotide probe Sag210_GC targeting 16S rRNA of ***Streptococcus agalactiae.*** Results obtained after 3 h hybridisation at 20 % FA in the poly-FISH buffer after the pretreatment (5 min mutanolysin and 1 min exposition at 200°C) of the target and non-target cells. Some of the non-target cells are indicated by black arrows) **A+C:** epifluorescence images of *S. agalactiae* after FISH; **B:** phase contrast image of ***S. agalactiae*** and ***P. aeruginosa*** (rods: negative control), **D:** phase contrast image of ***S. agalactiae*** and **S. *epidermidis*** (cocci arranged in clusters: negative control), bar: 20 µm

Table C.5 Results obtained after FISH using oligo-oligonucleotide probes. Six Gram-positive bacteria causing nosocomial infections, AF number, oligo-oligonucleotide probe names, target region: 16/23S rRNA and FISH conditions which led to specific signals

Bacteria and AF number	Probe name	Target rRNA	FISH conditions at 53°C t [h] / FA [%]	Detected signal
E. faecalis Staph/Strep 28	Efe286_GC	16S	3 / 30	Halo +
E. faecium 458387	Efi58_GC	23S	3 / 10	Halo
S. aureus 433088	Sau227_GC	16S	3 / 10	Halo +
S. epidermidis MRSE /:23	Sep16S_GC	16S	2-3 / 10	Halo, WCF
S. agalactiae 616311	Sag210_GC	16S	2-3 / 20	Halo
S. pyogenes 611311	Spy467_GC	16S	5 / 30	Halo

Halo+: intermediate signal; WCF: whole cell fluorescence

C.2.3. Adaptations for FISH on slides using monospecific oligo-oligonucleotide probes for the detection of bacteria from spiked clinical material

To test whether developed oligo-oligonucleotide probes are appropriate for the identification of pathogens from clinical samples, Gram-negative and -positive bacteria were available in spiked clinical samples, with the exception of *S. aureus* (Table B. 3, p. 23). Three chosen sterile clinical samples (EDTA-blood, plasma and urine) were spiked with target cells in defined dilutions ranging from 10^{-1} to 10^{-4}. Although these specimens only contained the target bacteria the composition and the consistency of this material was similar to real clinical samples. In addition, the dilutions 10^{-3} and 10^{-4} contained about 8 x 10^{3} bacterial cells corresponding to the real cell number of pathogens, e.g. during an infection of the urinary tract. Positive signals obtained in these samples could be considered as hints for a hybridisation success in real clinical material.

However, it was not always possible to detect all bacteria from spiked samples. Few target cells and eukaryotic cells or other sample characteristic compounds were sometimes observed under the microscope. In some cases, the autofluorescence of eukaryotic cells or cell fragments turned out to be problematic because of the strong signal of eukaryotic components present in the sample. The autofluorescence was so intense, in comparison to the naturally weaker halo signal, that it was hardly to see and not always possible to be visualised. Additionally, it was difficult to handle blood samples. The EDTA, which was added to blood samples, actually inhibited the agglutination of blood cells but the compact and tough mass of cells as well as other blood compounds accumulated in the cap's base. This complicated homogenous distribution of bacterial and other cells. Nevertheless, after the ethanol gradient (see B.9., p. 37), a red layer (from the blood sample) peeled off from the microscopic slide (or could be removed with tweezers). A transparent layer stayed on the slide containing mainly the bacterial target cells and fewer eukaryotic compounds or erythrocytes. Due to the ethanol effect, no additional pretreatment was necessary for FISH on slides using spiked clinical samples containing Gram-negative bacteria. Figure C.24 (p. 94) visualises an optimal hybridisation with *E. faecium* cells in a spiked urine-sample using the specific probe Efi58_GC. All target bacteria show a strong halo signal under evaluated FISH conditions (Table C.5, p. 92).

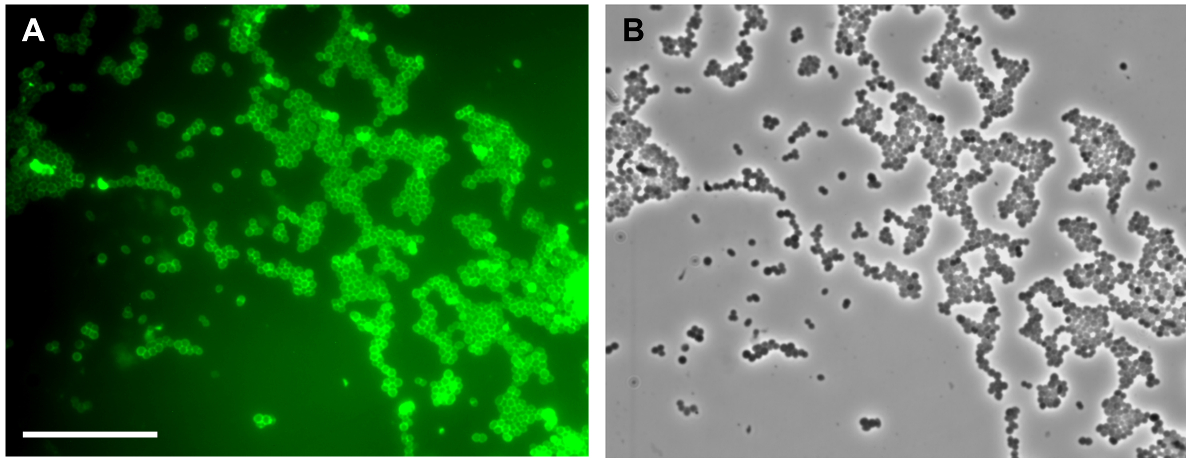


Figure C.24 Halo signals observed after FISH using the oligo-oligonucleotide probe Efi58_GC targeting 23S rRNA of ***Enterococcus faecium*.** Results obtained after 3 h hybridisation at 10 % FA in the poly-FISH buffer with the **spiked (dilution 10^{-1}) urine** sample.
A: epifluorescence image; **B:** phase contrast image; bar: 20 µm

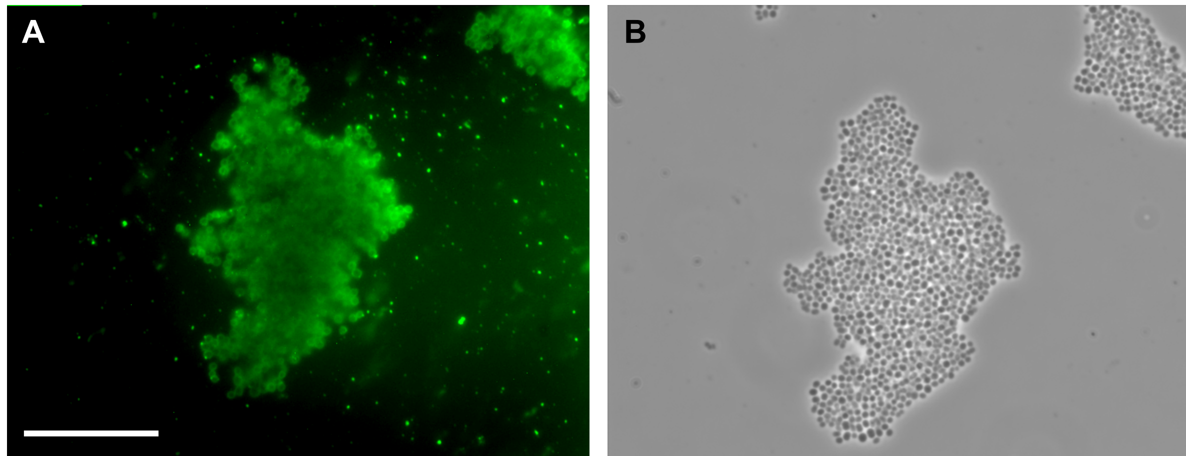


Figure C.25 Halo signals observed after FISH using the oligo-oligonucleotide probe Sag210_GC targeting 16S rRNA of ***Streptococcus agalactiae***. Results obtained after 3 h hybridisation at 20 % FA in the poly-FISH buffer with the **spiked (dilution 10^{0}) urine** sample. **A:** epifluorescence image; **B:** phase contrast image; bar: 20 µm

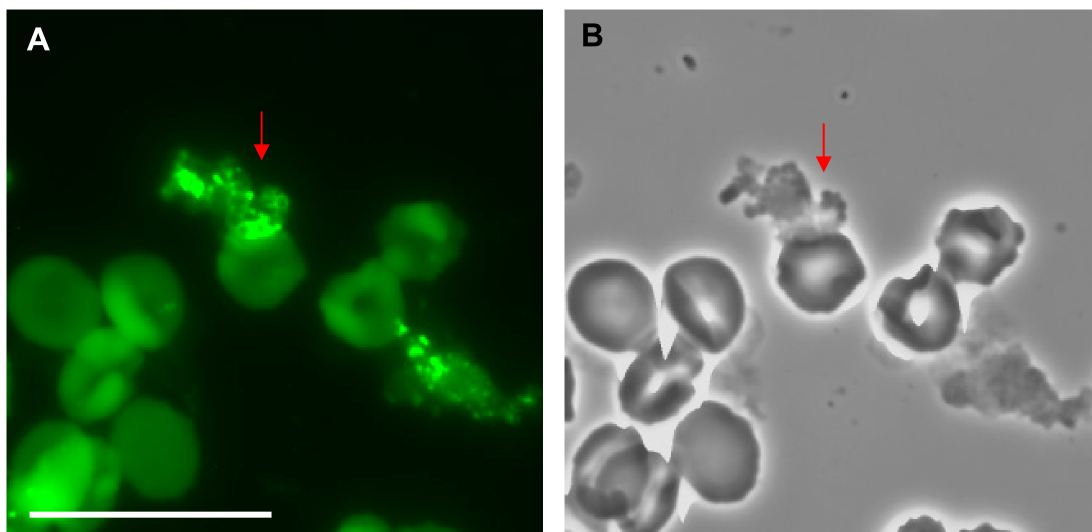


Figure C.26 Halo signals observed after FISH using the oligo-oligonucleotide probe Spy467_GC targeting 16S rRNA of ***Streptococcus pyogenes*** (small cells indicated by red arrows). Results obtained after 6 h hybridisation at 30 % FA in the poly-FISH buffer with the **spiked (dilution 10^{-3}) blood** sample. **A:** epifluorescence image; **B:** phase contrast image; big cells: eukaryotic cells and cell fragments; bar: 20 µm

In contrast, figures C.25 and C.26 (p. 94) show a less optimal but successful FISH result obtained after incubation of target bacteria (*S. agalactiae* and *S. pyogenes*) with the respective oligo-oligonucleotide probes in spiked samples. Figures C.25 A and B (p. 94) indicate that the Sag210_GC did not bind inside all target cells, so that some organisms cannot exhibit a halo signal. Figure C.26 (p. 94) illustrates the side effect of hybridisations using labelled probes in spiked samples containing typical components of the biological material. Due to strong autofluorescence of eukaryotic sample compounds (e.g. blood cells), the target bacteria can be hardly seen. The signals and the bacteria (*S. pyogenes*) are indicated by red arrows in figures C.26 A and B (p. 94). Furthermore, figure C.26 (p. 94) shows a hybridisation in a spiked sample where the bacterial cell number (dilution 10^{-3}) correlates with probable real bacterial count during an infection.
Altogether, the FISH assays in spiked clinical material using the oligo-oligonucleotide probes can be assessed as effective leading to the typical ring-shaped signals of target pathogens. Even if the success is influenced and hampered by eukaryotic factors or other components characteristic for the specimen, the ring-shaped signals were detected. These attempts pointed out that a FISH using oligo-oligonucleotide probes in real clinical samples could be possible (Figure C.26, p. 94). Moreover, the results show that enrichment experiments with these novel polynucleotide probes could be also performed in materials containing few target bacteria and interfering elements.

C.2.4. Adaptations for FISH on slides using monospecific oligo-oligonucleotide probes for the detection of bacteria from real clinical material

The previously achieved hybridisation results with clinically relevant pathogens and respective oligo-oligonucleotide probes in pure culture, artificial mixture and in spiked clinical samples were then applied and adapted to real clinical samples. One significant feature of available real clinical samples is the real number of pathogens, which individually depends on the infection. Furthermore, the condition of the bacteria during an infection could vary from that of bacteria, which are cultivated under laboratory conditions. It can be assumed that due to the immune response of the innate immune system especially the condition of the cell wall plays an important role (Janeway and Travers, 1997). With respect to permeability, the cell wall of

bacteria in real clinical samples should be more permeable as the cell wall of pure culture organisms cultivated in laboratory. Finally, other components of real samples (non-target bacteria or eukaryotic cells) which are present in the biological material should not be disregarded in the whole concept. The eukaryotic cells and non-target bacteria may influence the accessibility of the polynucleotide probe from buffer to its target organism as well as the binding of the probe to its target region within the cell. This factor could eventually have an effect on the hybridisation time. Based on this, the incubation duration possibly had to be increased. It is very important to evaluate adequate hybridisation parameters in pure culture as a precondition for starting with hybridisation and immobilisation experiments because the volume of spiked and real clinical samples is limited.

The most common representatives of bacterial pathogens causing nosocomial infections are *S. aureus*, coagulase negative staphylococci*, E. coli, P. aeruginosa* followed by *Enterococcus* spp. as well as *Enterobacter* spp.. In the present study, *E.coli*, *P. aeruginosa*, *E. faecalis*, *E. faecium*, *S. aureus*, *S. agalactiae* and *S. pyogenes* were available in real clinical material (Table B.4, p. 24). Different real clinical samples containing target bacteria, possible coexisting non-target bacteria and the information on the origin of the specimens are summarised in table B.4 (p. 24).

For all pathogens available in real clinical samples, oligo-oligonucleotide probes were developed and most of them were successfully tested in pure culture, artificial mixture and spiked samples. Like in spiked samples, apart from positive signals of target cells the autofluorescence of eukaryotic cell components was also detected. Examples of FISH results from clinical material are demonstrated in figures C.27 and C.28 (p. 97). Illustrated are efficient FISH results in real clinical samples using the novel types of polynucleotide probes targeting a Gram-negative (*P. aeruginosa*) and a Gram-positive (*S. pyogenes*) bacterium*.* Figure C.27 A (p. 97) shows halo signals, which were obtained after hybridisation of *P. aeruginosa* with the PseaerB_GC (Table C.2, p. 75) probes in a real urine specimen (number 16, table B.4, p. 24). Strong signals of target cells were detected. Figure C.28 (p. 97) shows an example for a further successful hybridisation of target cells (*S. pyogenes*) using the respective oligo-oligonucleotide probe (Spy467_GC, table C.3, p. 76) in a real clinical sample (number 13, wound smear, table B.4, p. 24). Strong halo signals (Figure C.28

A, p. 97) of all bacterial cells can be observed and weaker autofluorescence of eukaryotic cells.

The results obtained from real samples indicated the possibility to detect clinically relevant pathogens with novel oligo-oligonucleotide probes and to use them for the immobilisation of target cells in coated microplates.

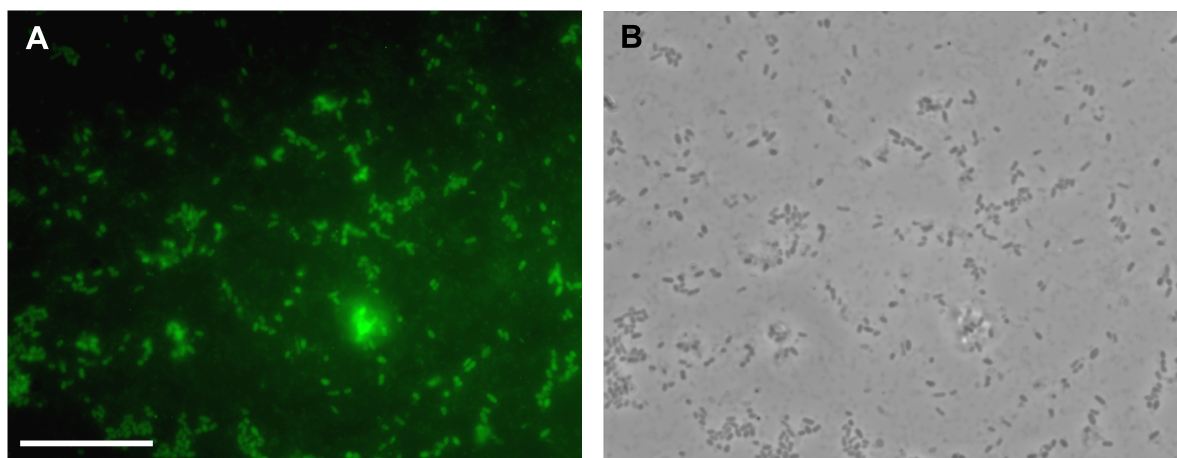


Figure C.27 Intermediate and whole cell fluorescence signals observed after FISH using the oligo-oligonucleotide probe PseaerB_GC targeting 23S rRNA of ***Pseudomonas aeruginosa***. Results obtained after 5 h hybridisation at 20 % FA in the poly-FISH buffer from a **real clinical urine** sample (number 16, table B.4, p. 24) containing the target organism. **A:** epifluorescence image; **B:** phase contrast image; bar: 20 µm

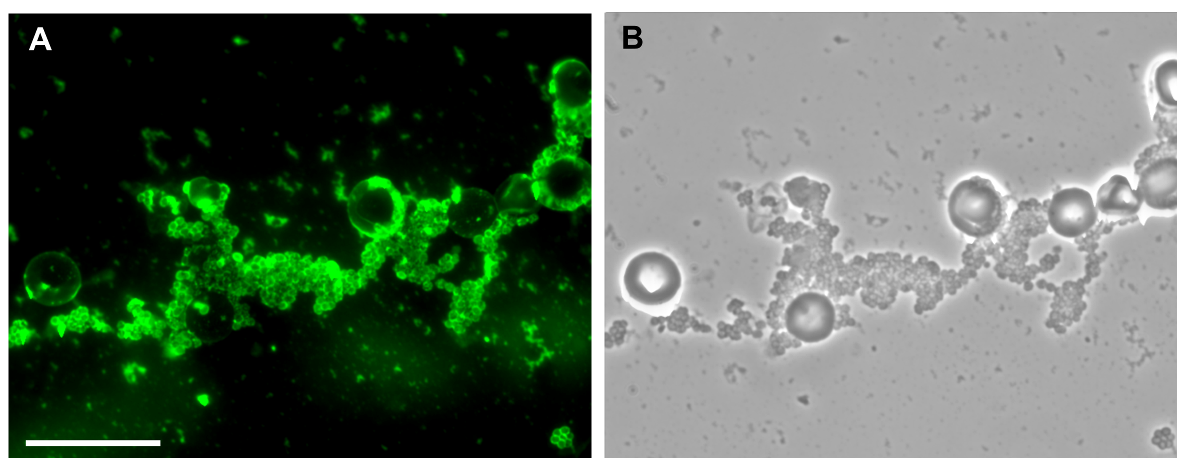
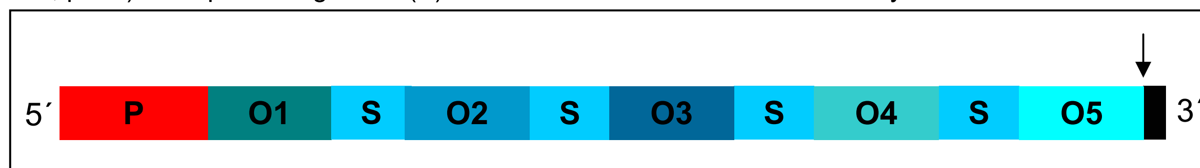


Figure C.28 Halo signals observed after FISH using the oligo-oligonucleotide probe Spy467_GC targeting 16S rRNA of ***Streptococcus pyogenes***. Results obtained after 4 h hybridisation at 30 % FA in the poly-FISH buffer from a **real clinical wound smear** sample (number 13, table B.4, p. 24) containing the target organism. **A:** epifluorescence image; **B:** phase contrast image (big cells under the phase contrast: eukaryotic components); bar: 20 µm

C.2.5. FISH on slides using multispecific oligo-oligonucleotide probes for the detection of bacteria from pure culture and artificial mixture

Figure C.29 Schematic illustration of the multispecific oligo-oligonucleotide construct for the first probe (MO1_GC) containing the promoter fragment (P), different oligonucleotide sequences (O1-O5, table C.6, p. 99) and spacer fragments (S). The endonuclease site is indicated by the black arrow and box.



Multispecific oligo-oligo-polynucleotide probes were designed in order to use only one single polynucleotide probe for the detection of pathogens of different taxa. They could be applied for the separation of typical pathogens from diverse clinical materials, e.g. staphylococci from skin or blood samples.

Five Gram-negative bacteria (*Acinetobacter baumannii*, *Stenotrophomonas maltophilia, Enterobacter aerogenes*, *Klebsiella pneumoniae*, *Escherichia coli*) were chosen as target cells for the development of the multispecific polynucleotide probe. The first 109 nt long oligo-oligonucleotide probe (MO1_GC, table C.6, p. 99) was designed according to the suggested model (see B.10.2., p. 49) of monospecific oligo-oligonucleotide probes and contained the short taxon-specific probes as illustrated in figure C.29 (p. 98). After hybridisation experiments in pure and mixed cultures using 0-40 % formamide in the poly-FISH buffer (see B.9.3., p. 44) halo signals or whole cell fluorescence of *A. baumannii* and *E. coli* at 30 % and 40 % were observed after 5 h, respectively. No signals of other target cells or negative controls (*Morganella morganii*, *Pseudomonas aeruginosa*) were observed.

Since the particular oligonucleotide fragments targeting *A. baumannii* or *E. coli* are located at both ends of the polynucleotide probe (MO1_GC, table C.6, p. 99), the assumption emerged that possibly the position of each oligonucleotide sequence within the probe influenced the hybridisation result. To test whether the arrangement of single oligonucleotide sequences within the probe plays a role, the composition of the fragments was changed by the generation of a second multispecific probe (MO2_GC, table C.6, p. 99). In this case, the probe targeting *E. aerogenes* was placed at the 5´end and the probe targeting *K. pneumoniae* at the 3´end of the template. The hybridisations were performed under the same conditions as for the first generated multispecific probe. Specific halo signals of *E. aerogenes* (30 %) and *A. baumannii* were observed after 5 h (20 and 30 % formamide). Other bacterial

species (*M. morganii*, *P. aeruginosa)* which were used as a negative control did not show any signals under these conditions. The results are summarised in table C.6 (p. 99).

Table C.6 Results obtained after FISH using multispecific oligo-oligonucleotide probes. Target bacteria, AF number, probe name, whole oligo-oligonucleotide probe sequence and hybridisation results (bold marked). Oligonucleotide sequence: coloured bold sequences; spacer fragment (GCGCG): black bold italics. The order of the microorganisms corresponds to the arrangement of oligonucleotide probes within the oligo-oligonucleotide probes.

Bacteria and AF number	Probe name	Whole sequence of the RNA probe 5´→ 3´direction	Halo/WCF signals obtained at 53°C after 5 h
A. baumannii 438775 *S. maltophilia* 484133 *E. aerogenes* 427068 *K. pneumoniae* 429457 ***E. coli*** 441612	MO1_GC	**UCCGCCGCUAGGTCCGG** **U*GCGCG*AAGUUAUGCAC** **CCCAAG*GCGCG*CGAGUA** **ACGUCAAUCGCC*GCGCG*** **CCUACACACCAGCGUGC** **C*GCGCG*CACACACUGAU** **UCAGGCU**	**→ 30 % FA** **→ 40 % FA**
E. aerogenes 427068 ***A. baumannii*** 438775 *E. coli* 441612 *S. maltophilia* 484133 *K. pneumoniae* 429457	MO2_GC	**CGAGUAACGUCAAUCGC** **C*GCGCG*UCCGCCGCUAG** **GUCCGGU*GCGCG*CACAC** **ACUGAUUCAGGCU*GCGC*** ***G*AAGUUAUGCACCCCAA** **G*GCGCG*CCUACACACCA** **GCGUGCC**	**→ 30 % FA** **→ 20, 30 % FA**

WCF: whole cell fluorescence

The detected signals (ring-shaped and whole cell fluorescence) observed after incubations using the first multispecific oligo-oligonucleotide probe (MO1_GC, table C.6, p. 99) are shown in figure C.30 (p. 100). The hybridisation using this multispecific probe was performed in pure culture with *E. coli* (40 % formamide in the buffer). The FISH experiments using the second probe (MO2_GC, table C.6, p. 99) were successful and the results are visualised in figure C.31 A-D. The multispecific oligo-oligonucleotide probe MO2_GC (Table C.6, p. 99) was applied in mixed culture of the positive and negative control. Only the target organisms *A. baumannii* and *E. aerogenes* showed intense halo signals. Although not all five target cells (*A. baumannii*, *E. aerogenes*, *E. coli*, *S. maltophilia*, *K. pneumoniae*) exhibited the halo signals after hybridisations with the multispecific polynucleotide probes MO1_GC and MO2_GC (Table C.6, p. 99), the achievements can be regarded as satisfactory. For the first time it was possible to generate synthetic polynucleotide probes containing different oligonucleotide sequences which led to positive signals.

Due to the specificity of these probes, they could be appropriate for e.g. the detection of pathogens from clinical material.

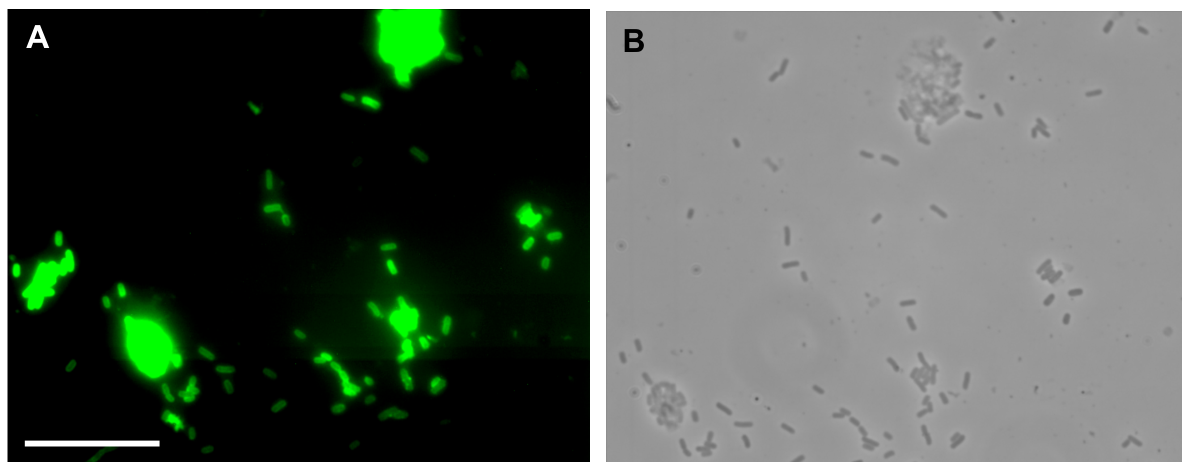


Figure C.30 Halo and whole cell fluorescence observed after FISH using the **first** multispecific oligo-oligonucleotide **probe MO1_GC** (Table C.6, p. 99). Results obtained after 5 h hybridisation at 40 % FA of ***Escherichia coli*** in pure culture. **A:** epifluorescence image; **B:** phase contrast image; bar: 20 µm

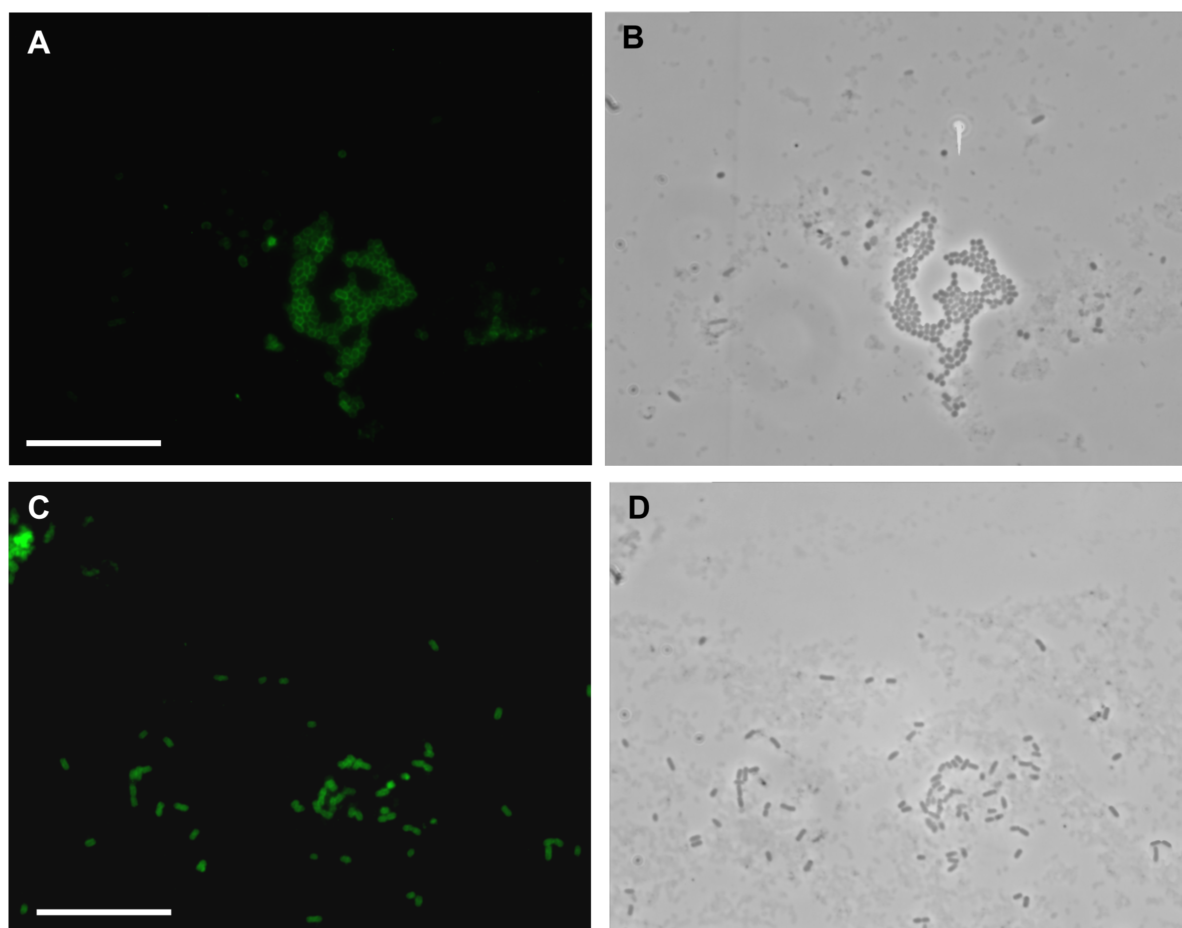


Figure C.31 Halo signals observed after FISH using the **second** multispecific oligo-oligonucleotide **probe MO2_GC** (Table C.6, p. 99). Results obtained after 5 h hybridisation with the poly-FISH buffer. **A:** ***Acinetobacter baumannii*** (20 % FA), **C:** ***Enterobacter aerogenes*** (30 % FA), epifluorescence images; **B+D:** target cells and *Pseudomonas aeruginosa* (larger rods under the phase contrast: negative control), phase contrast images; bar: 20 µm

C.3. Immobilisations of pathogens using monospecific RNA oligo-oligonucleotide probes

After the adaptation of FISH protocols with oligo-oligonucleotide probes on slides, the transfer to microplate cavities for the separation of target cells from material of different sources was accomplished. The procedure for the enrichment of whole cells is composed of three steps (Figure B.2, p. 57). First, the cavities were coated with DNA complementary to the respective polynucleotide probes. In the second step, a hybridisation in solution containing bacteria and polynucleotide probes took place in a 0.6 ml eppendorf tube under specific conditions, which were evaluated on slides before (see C.2.2.-C.2.4., pp. 77-95). Subsequently, the solution was transferred to the microplate cavities, coated with the complementary nucleic acids, followed by a second hybridisation (see B.11., p. 57). To demonstrate the immobilisation efficiency PCR was performed (see B.11.4., p. 61). For the confirmation of separated target cells in coated cavities or of the non-enriched (non-target) cells in the supernatant, a PCR using degenerated primers (Table B. 26, p. 61) was carried out.
Based on the combination of the cell sorting from biological material and the amplification with different primers, this identification method, offers many possibilities for the clinical diagnostics. Therefore, it is of great interest to evaluate an adequate protocol for the applicability of oligo-oligonucleotide probes for the enrichment of cells from different specimens.

C.3.1. Evaluation of the immobilisation procedure

C.3.1.1. Fixation of cells in different growth phases

An important precondition for successful immobilisations of bacteria is a stable halo formation in and around the cell envelope of the target organism obtained after hybridisations using polynucleotide probes. As mentioned under C.2.2.1. (p. 77) hybridisations on slides using cells fixed with PFA (see B.3., p. 26) at a stage of later exponential growth phase (OD_{600}: 0.6-0.8) led to strong ring-shaped signals in the periphery of target bacteria. Because of this, available cells (pure culture) fixed at the later exponential growth phase were applied for separation tests of several pathogens.

C.3.1.2. Pretreatments to ensure the accessibility of the probe to its target site

To enable the usage of compact blood samples, the specimens containing Gram-negative target cells (prior hybridisation) or Gram-positive target cells (prior respective pretreatments) were incubated with 2 volumes of 50mmol Tris-HCl for 20 min at 37°C. This procedure allowed the separation of cells from blood samples, which were rather viscous due to the compact mass of blood and other eukaryotic cells.

A pretreatment of fixed Gram-positive bacteria was necessary to obtain a better permeabilisation. The combination of an enzyme application at 37°C followed by 1 min incubation at 200°C turned out to be more effective than just a treatment with cell wall lytic enzymes. The challenge consisted in an optimal permeabilisation and the maintenance of the cell integrity. Several approaches were tested in parallel on slides to assure the intact condition of the cells. Pretreatments for *Enterococcus* spp. *E. faecalis* and *E. faecium* (lysozyme for 20 min at 37°C) as well as for *Streptococcus* spp. *S. agalactiae and S. pyogenes* (mutanolysin for 5 min at 37°C) followed by incubation for 1 min at 200°C in both cases, this procedure proved to be very effective. The treatment of *Staphylococcus* species demanded different enzymatic incubation times. For the pretreatment of *S. aureus* an incubation of 20 min with lysostaphin (at 37°C) were sufficient whereas for the permeabilisation of the cell wall of *S. epidermidis* a 5 min longer application was necessary. Table B.25 (p. 59) shows optimal pretreatments for enterococci, staphylococci and streptococci in more detail. The presented pretreatments were also applied for the immobilisation of whole cells from spiked or real clinical samples.

C.3.1.3. Modification of the standard protocol

In comparison to the procedure used in earlier studies (Zwirglmaier, 2003; Fichtl, 2005) several modifications were necessary for a successful immobilisation of cells in coated microplate cavities using monospecific oligo-oligonucleotide probes. Although in former protocols, an initial denaturation step is not especially recommended for the enrichment using nucleolink plates, the denaturation (10 min at 94°C) of nucleic acids prior to coating the cavities turned out to be very advantageous. Furthermore, some washing steps with PBS (prior or after pretreatments) leading to cell loss were omitted. To avoid a reduced microbial amount, the cells were washed only when necessary (after the hybridisation and immobilisation step, see B.11.2., p. 59 and B.11.3., p. 60). Moreover, to increase the cell amount, the centrifugation steps were prolonged to 5-10 min depending on sample material and the current step of the protocol (prior and after hybridisation, see B.11.2., p. 59). One problem often emerged after the first immobilisation assays: false negative results were achieved by PCR after the immobilisation. The enriched cells could not be detected by PCR because no amplicons from different cavities (containing enriched cells or supernatants) could be obtained. The procedure was modified several times and finally, the introduction of a Hot Start PCR enabled an effective amplification of rDNA indicating enriched cells.

C.3.1.4. Application of the time-reduced protocol

Another improvement of the whole method with regard to time consumption and applicability was the use of the time-reduced protocol, which was introduced by Fichtl (2005) for the immobilisation of cells using domain III targeted and RING-FISH polynucleotide probes. In this context, the time for cell fixation with PFA could be effectively reduced from originally 5-9 h to 0.5 h. The hybridisation time in tubes required 1-1.5 h instead of a minimum of 11 h. Only the enrichment duration was not changed. Altogether, a successful immobilisation experiment took 2.5 to 4 h. The following overview (Figure C.32, p. 104) summarises the modified protocols.

Figure C.32 Overview of protocols for the immobilisation of target cells using different types of polynucleotide probes with regard to time consumption derived from Fichtl (2005) and modified for hybridisations using oligo-oligonucleotide probes.

Procedure / time	old protocol (for DIII polynucleotide probes)	new protocol (for DIII polynucleotide probes; K. Fichtl)	new protocol (for oligo-oligonucleotide and RING-FISH probes)
fixation of cells	5 - 9 h	0.5 h	0.5 h
hybridisation in solution	5 - 12 h	0.33 - 1 h	1 - 1.5 h
enrichment hybridisation	1 - 2 h	1 - 2 h	1 - 2 h
summa summarum	11 - 23 h	1.5 - 3.5 h	2.5 - 4 h

C.3.1.5. Immobilisations using monospecific oligo-oligonucleotide probes

Compared to hybridisations on slides, here the first immobilisation experiments were also performed in pure culture to evaluate the optimal incubation conditions, adequate pretreatment methods as well as required protocol modifications. Corresponding to former studies (Fichtl, 2005) the hybridisation time in solution and in the following second hybridisation in microplate cavities took usually about 1 h.

All existing RNA oligo-oligonucleotide probes (Tables C.2 and C.3, pp. 75 and 76) were successfully tested in immobilisation experiments in pure culture. The formamide concentration corresponded to the evaluated buffer composition for FISH on slides (Tables C.4 and C.5, pp. 89 and 92). The immobilisation efficiency after the enrichment was controlled by amplification of an about 600 nt long part of the 23S rDNA using degenerated primers (Table B.26, p. 61). The PCR was done in coated and uncoated cavities as well as in cavities containing supernatants. After an optimal separation process, a positive PCR signal should be detected in all coated wells and in the cavity containing the supernatant of the uncoated well due to the presence of

cells in these fractions. In contrast, the uncoated well and the supernatant of coated cavities should not contain cells, so that no PCR amplicons should be observed. Since in pure culture and spiked clinical material only the target organisms were present, the PCR products arising from these samples were not always cloned and sequenced. Usually, for the identification of the positive and negative control bacteria from real clinical samples, the PCR fragments were cloned and sequenced.
Figure C.33 (p. 105) illustrates the PCR analysis of coated and uncoated cavities as well as of the supernatants of these cavities obtained after the immobilisation of the Gram-negative *A. baumannii* from pure culture using the oligo-oligonucleotide probe Aba88_GC. In coated cavities, amplicons could be generated during the PCR but the second and the third coated cavity exhibit a very weak band. Probably, the amount of *A. baumannii* cells in these cavities was reduced and led finally to this effect. The uncoated well (negative control) and the cavity with the supernatants of the three coated cavities (S+) do not show a band. A strong PCR signal was detected in the cavity containing the supernatant of uncoated cavity (S-). This result indicates that the bacterial cells present in the uncoated cavity (negative control) were not separated (Figure C.33, p. 105).

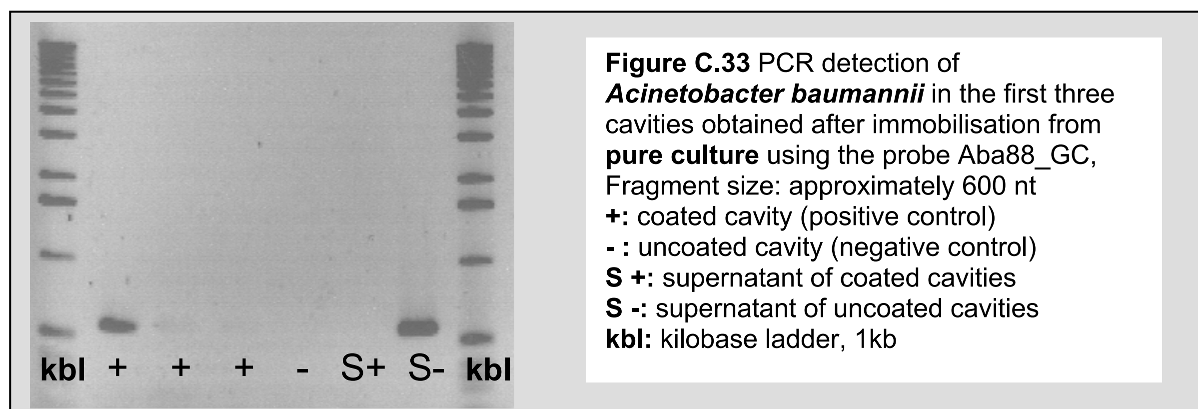


Figure C.33 PCR detection of ***Acinetobacter baumannii*** in the first three cavities obtained after immobilisation from **pure culture** using the probe Aba88_GC, Fragment size: approximately 600 nt
+: coated cavity (positive control)
- : uncoated cavity (negative control)
S +: supernatant of coated cavities
S -: supernatant of uncoated cavities
kbl: kilobase ladder, 1kb

On the one hand, it was often observed that not in every coated cavity, a separation of cells was possible. On the other hand, the supernatant of coated cavities contained some non-enriched target cells. This result is presented in figure C.34 (p. 106). Figure C.34 (p. 106) shows a less optimal separation of the Gram-positive *Streptococcus pyogenes* from pure culture. From all coated wells, only in the first one a positive PCR signal was detected. Additionally, the strong band of the supernatant of coated wells (S+) indicated an inefficient immobilisation.

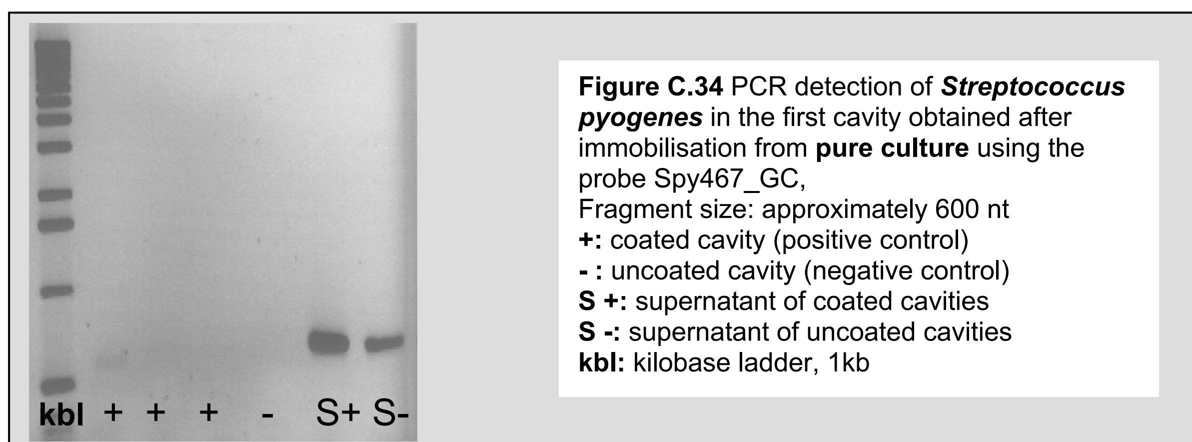


Figure C.34 PCR detection of ***Streptococcus pyogenes*** in the first cavity obtained after immobilisation from **pure culture** using the probe Spy467_GC,
Fragment size: approximately 600 nt
+: coated cavity (positive control)
- : uncoated cavity (negative control)
S +: supernatant of coated cavities
S -: supernatant of uncoated cavities
kbl: kilobase ladder, 1kb

C.3.2. Immobilisation results obtained from spiked clinical material

After the oligo-oligo-polynucleotide probes were tested and the pretreatment attempt was evaluated in pure cultures, the next step was the application of these probes for the separation of target cells from spiked clinical material (Table B. 3, p. 23). The experiments with different specimens and the particular probes were performed according to the real situation in a hospital. Not all used pathogens colonise and infect all human bodily parts (organs, ichors), e.g. *A. baumannii* or *S. aureus* were rather isolated from blood and *E. faecalis* and *E. coli* rather from urine (see C.2.2.3., p. 80 and table B.4, p. 24).
An important criterion of spiked material is that (apart from *S. aureus*) all three clinical samples (EDTA-blood, plasma and urine) were spiked with the target bacteria in the dilutions 10^{-1} to 10^{-4}. The bacterial count in dilutions 10^{-3} and 10^{-4} is about $8x10^{3}$ bacteria corresponding to the real cell number in samples from infected patients. Immobilisation experiments were performed with spiked material when the respective bacteria were not available in a real clinical sample. For special clinical material or samples containing Gram-positive bacteria, several pretreatments were applied as described under C.3.1.2. (p. 102). Table C.7 (p. 108) summarises the results achieved with RNA oligo-oligonucleotide probes after the separation of cells from different spiked samples. Figures C.35-C.37 (p. 107) illustrate the success and effectiveness of this immobilisation technique, which was performed with these novel types of polynucleotide probes for the first time. Figure C.35 (p. 107) shows the immobilisation of *A. baumannii* from a spiked plasma sample. The dilution 10^{-4} is similar to the real cell number of pathogens during an infection. Figure C.36 (p. 107) illustrates a successful separation of *Escherichia coli* cells from spiked urine samples

(undiluted (10^0) and dilution 10^{-1}). Figure C.37 (p. 107) shows the immobilisation of *Klebsiella pneumoniae* from spiked blood sample (dilution 10^{-4}).

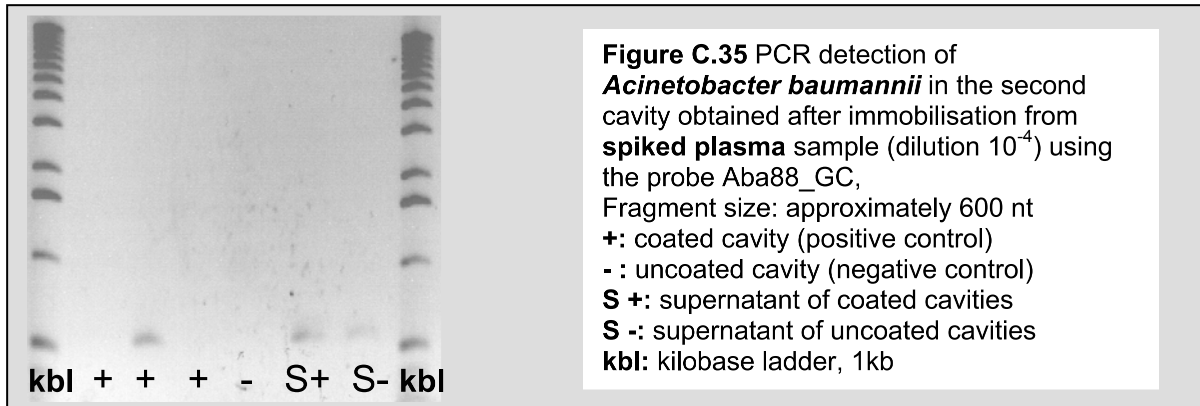


Figure C.35 PCR detection of ***Acinetobacter baumannii*** in the second cavity obtained after immobilisation from **spiked plasma** sample (dilution 10^{-4}) using the probe Aba88_GC,
Fragment size: approximately 600 nt
+: coated cavity (positive control)
- : uncoated cavity (negative control)
S +: supernatant of coated cavities
S -: supernatant of uncoated cavities
kbl: kilobase ladder, 1kb

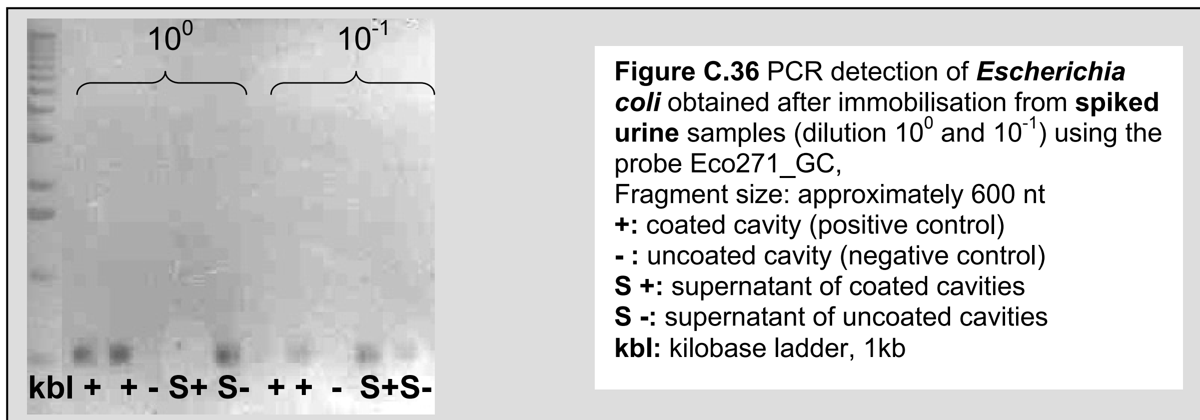


Figure C.36 PCR detection of ***Escherichia coli*** obtained after immobilisation from **spiked urine** samples (dilution 10^0 and 10^{-1}) using the probe Eco271_GC,
Fragment size: approximately 600 nt
+: coated cavity (positive control)
- : uncoated cavity (negative control)
S +: supernatant of coated cavities
S -: supernatant of uncoated cavities
kbl: kilobase ladder, 1kb

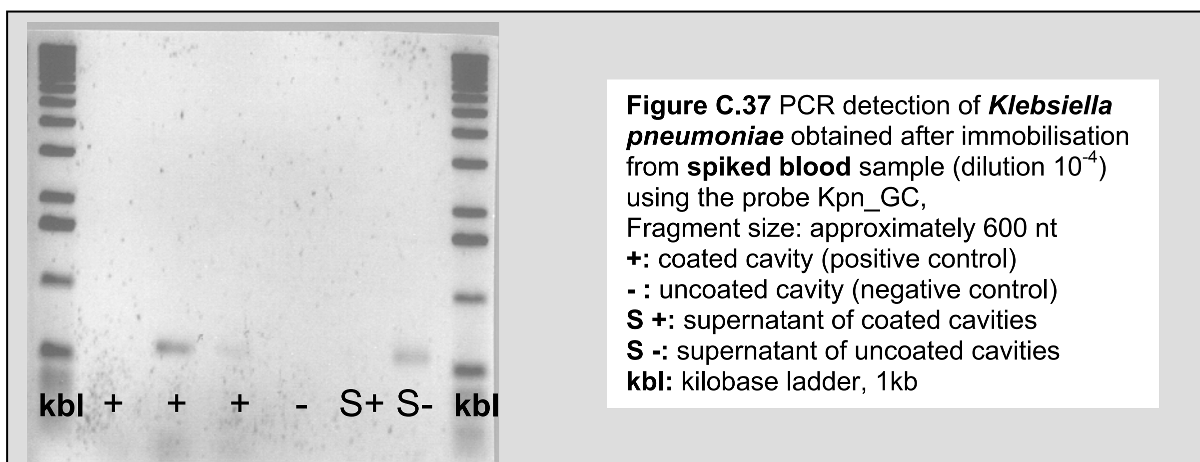


Figure C.37 PCR detection of ***Klebsiella pneumoniae*** obtained after immobilisation from **spiked blood** sample (dilution 10^{-4}) using the probe Kpn_GC,
Fragment size: approximately 600 nt
+: coated cavity (positive control)
- : uncoated cavity (negative control)
S +: supernatant of coated cavities
S -: supernatant of uncoated cavities
kbl: kilobase ladder, 1kb

Table C.7 (p. 108) summarises all enrichment experiments obtained from pure culture and spiked EDTA-blood, urine and plasma samples. Some bacteria (dilutions marked in red) could be separated from specimens, which correlate with the real amount of pathogens in a real clinical material arising from an infection.

Table C.7 Immobilisation results of bacteria present in pure culture and spiked clinical samples using oligo-oligonucleotide probes targeting respective pathogens; dilutions corresponding to real cell amount in the respective specimens are 10^{-3} and 10^{-4} (marked in red)

Pure culture/ Spiked clinical samples Bacteria	AF number	Pure culture	EDTA - Blood	Urine	Plasma
A. baumannii	438775	+	10^{-4}	n.t.	10^{-4}
E. aerogenes	427068	+	10^{-4}	n.t.	-
E. coli	441612	+	-	10^{0} - 10^{-4}	10^{0} - 10^{-2}
K. pneumoniae	429457	+	10^{-4}	10^{-4}	10^{-4}
P. aeruginosa	426470	+	n.t.	n.t.	n.t.
S. maltophilia	484133	+	n.t.	n.t.	n.t.
E. faecium	458387	+	n.t.	n.r.	n.r.
E. faecalis	Staph/Strep28	+	n.r.	n.t.	n.t.
S. aureus	678848	+	n.a.	n.a.	n.a.
S. epidermidis	MRSE/S:23	+	10^{-3}	n.t.	n.t.
S. agalactiae	616311	+	10^{-4}	10^{0}	n.t.
S. pyogenes	611333	+	n.r.	n.t.	n.t.

n.t.: not tested, n.r.: no results, n.a.: not available, - : not successful

C.3.3. Immobilisation results obtained from real clinical material

A successful immobilisation of whole cells from real clinical samples will show whether the enrichment technique can also be applied in the clinical diagnostics for a faster identification of pathogens. Different real specimens were isolated and fixed. Real clinical samples used in this study are listed in table B.4 (p. 24). Table B.4 (p. 24) shows that several pathogens (*E. coli, P. aeruginosa*, *S. aureus*) were often found in different samples and are mostly the causative species of the infection. Seldom two coexisting pathogens (*E. coli* and *E. faecium*) were present in one sample. The non-target bacteria were used as a naturally occurring negative control. Important factors of real clinical material with regard to the applicability were the real cell number of bacteria, the composition of this material as well as the viscosity and volume. All parameters varied from sample to sample and could have a positive or negative impact on the accessibility of the probe to its target inside the cell.

The real samples were isolated during an infection of the patient. It should be noticed that the pathogens could be recognised by the innate immune system of the host, which immediately confers protection against the foreign organism. Therefore, the human immune system plays an important role and should not be disregarded with respect to a possibly increased permeabilisation of the cell envelope. Significant factors of the innate immune system, which affect the cell wall, are especially lysozyme (often present in many human and non-human secretions) and the complement system (Janeway and Travers, 1997). Both components (lysozyme and the complement system) can increase the permeabilisation of the cell envelope and facilitate the access of the polynucleotide probe to its target region. Microscopic control confirmed that the bacteria were not destroyed and that the cell integrity remained intact.

The enrichment experiments were performed after successful application of oligo-oligonucleotide probes in hybridisations on slides with real samples and specific signals were observed (see C.2.4., p. 95). Here too, the optimised pretreatment protocol concerning the permeabilisation of the Gram-positive cell wall or other recommended treatments prior to hybridisations was adopted from pure culture or spiked specimens (see C.3.1.2., p. 102 and table B.25, p. 59). Furthermore, the incubation times were prolonged from 1 h to 1.5 or 2 h and the sample volume was increased to 50-100 µl. Finally, the efficient immobilisations showed that it is possible to separate whole cells from real clinical material, too. The effectiveness varies from sample to sample arising from relatively low amount of the target cells and the composition of the sample. Figures C.38 (p. 110) and C.39-C.40 (p. 111) illustrate the PCR results obtained after immobilisation approaches with oligo-oligonucleotide probes from real clinical samples. The identification of enriched or non-enriched cells was controlled by cloning and sequencing of all PCR fragments (see C.3.4., p. 112). However, in some cases the presence of amplicons of the target bacterium in supernatants (of coated wells) indicated that not all target cells could be immobilised.

Figure C.38 (p. 110) shows an amplification, which demonstrates a significant separation efficiency of the *Escherichia coli*-targeted oligo-oligonucleotide probe Eco271_GC during the enrichment from real clinical urine sample (number 15, table B.4, p. 24). In most of the coated cavities, the cells could be enriched. The unspecific PCR signals (bands larger than 600 nt) from the supernatants could derive of *Enterococcus faecalis* cells, also present in this real clinical sample. This assumption

was controlled by sequencing (see B.12., p. 62). To identify the enriched and non-enriched bacteria, the partial sequences of 23S rDNA were compared to DNA sequence similarities by BLAST (Basic Local Alignment Search Tool) followed by analysis of the sequences in a 23S rRNA ARB sequence software package. The presence of the non-target organism *E. faecalis* in the supernatants (of coated and uncoated wells) was confirmed.

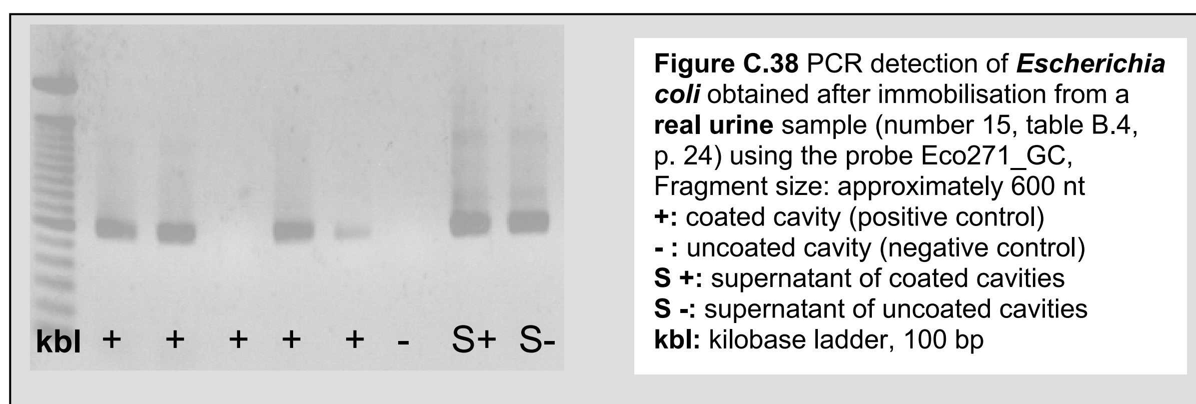


Figure C.38 PCR detection of ***Escherichia coli*** obtained after immobilisation from a **real urine** sample (number 15, table B.4, p. 24) using the probe Eco271_GC, Fragment size: approximately 600 nt
+: coated cavity (positive control)
- : uncoated cavity (negative control)
S +: supernatant of coated cavities
S -: supernatant of uncoated cavities
kbl: kilobase ladder, 100 bp

The genotyping of enriched *E.coli* cells (from a parallel approach) from real urine sample (number 15, table B.4, p. 24) for the detection of current SNPs (single nucleotide polymorphisms) emerging in antibiotic resistance genes should be determined by the project partners on DNA microarrays (Leinberger and Barl, Institute for Technical Biochemistry, Stuttgart). The microarrays were developed for the analysis of known SNPs occurring in antibiotic resistance genes (Grimm et al., 2004). In general, the procedure consists of three steps (DNA-extraction from a sample, amplification of target genes via PCR and hybridisation). During the PCR, the amplicons can be fluorescently labelled and subsequently applied for a hybridisation using DNA probes on a DNA microarray. Afterwards the fluorescence signals can be analysed.

The genotypic screening was performed using GyrA primers (detection of a fluorochinolone resistance) and TEM, SHV and CTX-M primers (detection of different beta-lactamases) in multiplex PCR (Grimm et al., 2004; Woodford et al., 2006). The PCR using GyrA primers led to amplification of a fragment (350-360nt long). After sequencing, the obtained sequence was compared to a reference sequence of *gyrA* gene of *E.coli* K12. The analysis of *gyrA* gene sequence of *E.coli* (real urine sample, number 15, table B.4, p. 24) showed three point mutations. The PCR using TEM, SHV and CTX-M primers did not show any signals. All results reflect the phenotypic tests (disc diffusion test) of the *E. coli* (isolate number 15, table B.4, p. 24) performed

by microbiologists at Robert-Bosch-hospital in Stuttgart. A ciprofloxacin and levofloxacin (fluorochinolone) resistance could be shown and no resistances against TEM-, SHV- and CTX-M-beta-lactamases. These approaches demonstrate a possible combination of the immobilisation technique (about 3 h) and subsequent genotyping of resistance genes (about 5 h) with regard to clinical importance. Indeed, the results of the genotypic analyses reflect the findings obtained using conventional analyses of pathogens but the conventional testing need at least 2 days. Furthermore, this procedure shows that the whole and intact cells could be also used for other screenings using molecular methods.

The probe PseaerB_GC targeting *Pseudomonas aeruginosa* is appropriate for a successful separation of target cells from real urine sample number 16 (Table B.4, p. 24) indicated by PCR signals, which were detected in all coated cavities. This result is shown in figure C.39 (p. 111).

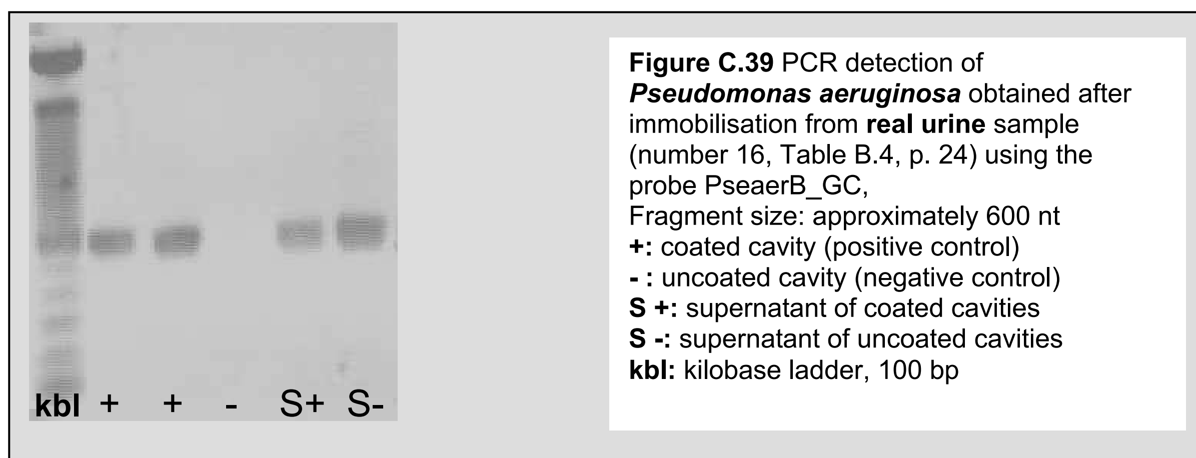


Figure C.39 PCR detection of ***Pseudomonas aeruginosa*** obtained after immobilisation from **real urine** sample (number 16, Table B.4, p. 24) using the probe PseaerB_GC,
Fragment size: approximately 600 nt
+: coated cavity (positive control)
- : uncoated cavity (negative control)
S +: supernatant of coated cavities
S -: supernatant of uncoated cavities
kbl: kilobase ladder, 100 bp

The pathogen *Staphylococcus aureus* was immobilised in two of three coated cavities from a real swab sample number 11 (Table B.4, p. 24) in enrichments using the oligo-oligonucleotide probe Sau227_GC. Figure C.40 (p. 111) shows this result.

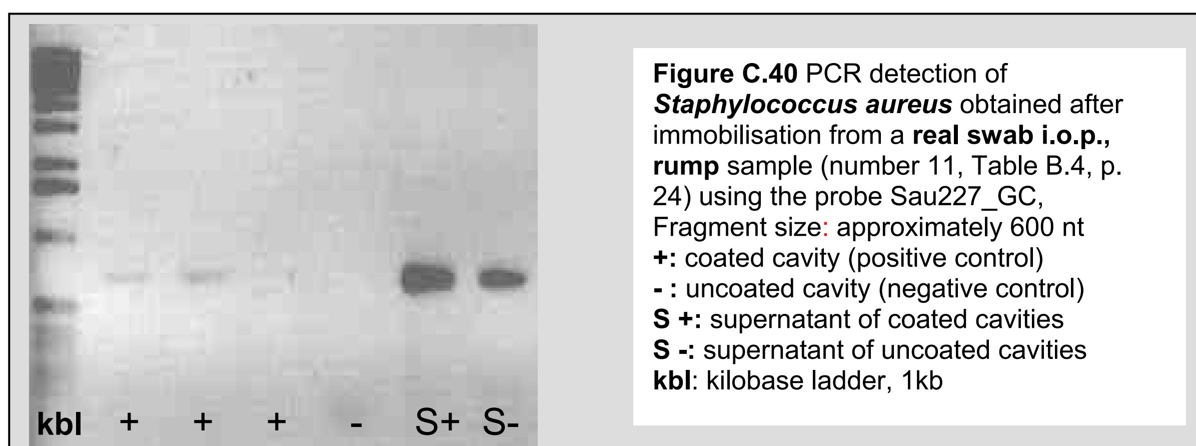


Figure C.40 PCR detection of ***Staphylococcus aureus*** obtained after immobilisation from a **real swab i.o.p., rump** sample (number 11, Table B.4, p. 24) using the probe Sau227_GC,
Fragment size: approximately 600 nt
+: coated cavity (positive control)
- : uncoated cavity (negative control)
S +: supernatant of coated cavities
S -: supernatant of uncoated cavities
kbl: kilobase ladder, 1kb

C.3.4. Cloning and sequencing of PCR products from immobilisations of real clinical samples

Amplicons (about 600 nt long, table B.26, p. 61) obtained after efficient immobilisation experiments from real clinical samples containing the target cell and coexistent pathogens were cloned and sequenced as described under B.12 (p. 62). The identification of sequences of enriched and non-enriched cells was performed using BLAST (Basic Local Alignment Search Tool) and the ARB software package. Table C.8 (p. 112) summarises some sequencing results. The sequenced clones belonged to the group of the target bacteria, which were assumed as the immobilised organism from the respective sample. In the supernatant of the real clinical material wound smear (number 10, table B.4, p. 24) the fragment of the 23S rRNA of *K. pneumoniae* was identified instead of the sequence of *K. terrigena*. The sequence of 23S rDNA of *K. terrigena* is not available and only physiological tests allow the differentiation of these *Klebsiella* species (Westbrook et al., 2000).

Table C.8 Results of sequence annotations of cloned and sequenced amplicons obtained from efficient immobilisations of target bacteria from real clinical specimens (number 10, 15, 12 and 11, table B.4, p. 24)

Target bacteria and other organisms present in real clinical samples	**Name of clones**	**Result of sequence annotation**	
		ARB	**BLAST similarity**
E. coli **+** *K. terrigena* Wound smear nr 10 **coated cavities**	10.2.a	*E. coli*	99 % *E. coli* 23S rRNA to CP000247
	10.2.b	*E. coli*	97 % *E. coli* 23S rRNA to CP000247
E. coli **+** *K. terrigena* Wound smear nr 10 **supernatant of coated cavity**	10.4.b	*K. pneumoniae*	94 % *K. pneumoniae* 23S rRNA to X87284
E. coli **+** *E. faecalis* Urine nr 15 **coated cavities**	15.2.a	*E. coli*	98 % *E. coli* 23S rRNA to CP000243
	15.2.c	*E. coli*	97 % *E. coli* 23S rRNA to CP000243
E. coli **+** *C. albicans* Urethra smear nr 12 **coated cavity**	12.2.a	*E. coli*	99 % *E. coli* 23S rRNA to AE014075
S. aureus **+** other Gram-positive bacteria Swab i.o.p. nr 11 **coated cavities**	11.1.a	*S. aureus*	99 % *S. aureus* 23S rRNA to CP000046
	11.1.e	*S. aureus*	99 % *S. aureus* 23S rRNA to CP000253

C.4. FISH on slides using DNA oligo-oligonucleotide probes

Domain III targeted DNA polynucleotide probes were successfully applied in hybridisations on slides for the detection of several *Acinetobacter* species and led to halo signals of the positive controls (Zimmermann et al., 2002). DNA oligo-oligonucleotide probes were also developed and tested because the synthesis of DNA polynucleotide probes is easier and less time-consuming in comparison to the generation of RNA transcript probes.

In the present study, monospecific DNA oligo-oligonucleotide probes were designed and compared with RNA oligo-oligonucleotide probes in FISH. Several hybridisation experiments were performed using DNA oligo-oligonucleotide probes with the same length as RNA probes and containing a spacer (repetition of five As or a GCGCG-stretch). The templates did not contain the T3 promoter sequence at the 5´end and were produced via asymmetric PCR (see B.10.3., p. 56). This modification of the standard PCR allowed the synthesis of a single stranded fragment out of a double stranded template depending on the usage of a sense or antisense primer. The labelling of the probe was performed by incorporation of fluorescein-12-dUTP during the amplification. For *A. baumannii* the sense stranded DNA oligo-oligonucleotide probe was generated and tested. As these probes are deoxynucleotides, the oligo-FISH buffer was used in the experiments at 46°C (see B.9.1., p. 38). The hybridisations using monospecific DNA oligo-oligonucleotide probes including either the polyA- or the GC-spacer resulted in whole cell fluorescence of the target cells (*A. baumannii*) after different hybridisation times (1-4 h, at 10-30 % formamide). However, a reproducible halo formation is a precondition for the applicability of these polynucleotide probes for the separations of whole cells in microplate cavities.

The results obtained with monospecific DNA oligo-oligonucleotide probe targeting the Gram-negative *A. baumannii* suggested that they are probably not appropriate for the detection and immobilisation of bacteria.

C.5. FISH on slides using RING-FISH probes for the detection of antibiotic resistance genes harboured by pathogens

Another part of the study was focused on the generation of RNA polynucleotide probes targeting drug resistance genes of pathogens causing nosocomial infections. The resistance genes chosen for the detection on slides and immobilisation in microplate cavities belong to three different functional groups of antibiotics. *Enterobacteriaceae* often possess chromosomally or plasmid encoded genes for beta-lactamases that bind and inhibit enzymes essential for the cross linkage of the peptidoglycan during cell wall synthesis. Hence, they affect only dividing/growing bacteria. Aminoglycosides belong to the group of broad-spectrum antibiotics. They interfere with the 30S ribosomal subunit by impeding the translocation of the peptidyl-tRNA from the A-site to the P-site during the initiation of the translation process and induce misreading of mRNA resulting in synthesis of abnormal proteins. Vancomycin disturbs the cell wall synthesis by interacting with the terminal D-alanyl-D-alanine moieties of the peptide subunit and prevents the cross linkage of the peptidoglycan (Madigan et al., 2000).

C.5.1. Optimisation of the RING-FISH protocol for FISH on slides

C.5.1.1. Sequencing of the templates and in silico analysis of RING-FISH probes

The sequences of all RING-FISH probes was determined prior to FISH experiments by sequencing. All developed polynucleotide probes were tested as a sense and an antisense probe to show that the specific signals derive from a stable interaction with the DNA and not from an mRNA-probe hybrid (Tables B.5-B.13, pp. 31-34). Additionally, for *Klebsiella oxytoca*, *K. pneumoniae* and *Pseudomonas fluorescens* polynucleotide probes of different lengths targeting one resistance gene were designed (Tables B.9-B.11, pp. 32 and 33). Applied in FISH, the formation of secondary structures resulting in a halo signal eventually depending on the probe length was evaluated. The potential to form secondary structures was analysed *in silico* (RNAdraw V1.1b2, Matzura et al., 1996). It was suggested (Zwirglmaier et al., 2003) that the feature of RNA polynucleotide probes to form a network within and

around the cell envelope is decisive for the appearance of halo signals of target bacteria. The sequences and secondary structures are shown in the appendix (part F, pp. 177-180).

C.5.1.2. Evaluation of the pretreatment protocol

The permeabilisation of the cell wall of Gram-positive bacteria posed a challenge for the evaluation of an appropriate hybridisation protocol. The combination of an enzymatic treatment followed by a heating step at 200°C was the most effective procedure. These pretreatment methods were also used for oligo-oligonucleotide probes. Table B.18 (p. 43) shows optimal pretreatments for the detection of bacteria by RING-FISH on slides.

C.5.1.3. Calculation of the GC content of polynucleotide probes and targets

In current study (Pavlekovic, unpublished) it is assumed that high GC content (> 60 %) eventually plays a role in the formation of halo signals. Due to strong intermolecular bonds between nucleotides (which should be dissolved during FISH), high formamide values are necessary for the linearisation of nucleic acids to enable a target-probe hybrid. The hypothesis is based on the suggestion that a high GC content of the target region recommends high formamide concentration in the buffer or increased temperatures. On the one hand, high formamide concentration (or high temperature) is necessary for the accessibility of the target region for its probe. On the other hand, high formamide concentration (or high temperature) might result in a complete denaturation, which would make the formation of a target-probe hybrid impossible. To evaluate this suggestion, the GC contents of all sequences of resistance genes and target regions within these genes were determined (Table C.9, p. 120). The lowest GC content of 31 mol % was determined for *mecA* gene of *S. aureus* and the highest of 66 mol % for the polynucleotide probe targeting a region of beta-lactamase$_{SHV-1}$ gene of *Klebsiella pneumoniae*. The GC contents of almost all sequences of resistance genes or respective probes range from 50 to 60 mol %. The respective results are described under C.5.1.4 (p. 116).

C.5.1.4. Application of the developed RING-RISH protocol

In general, the RING-FISH probes were tested at hybridisation times varying from 18 to 26 h at 53°C and formamide concentrations ranging from 0-50 % according to the protocol described under B.9.2 (p. 42). Depending on the RNA polynucleotide probe and target region, successful hybridisations allowed a detection of resistance genes at 0 and 10 % formamide in the buffer after 17 to 24 h. Some Gram-negative bacteria (e.g. *N. canis*, *M. morganii*, *E. coli*, *P. aeruginosa*) and Gram-positive bacteria (enterococci, staphylococci, streptococci) were selected as negative controls based on their coexistence during the infection. Mostly specific halo signals and intermediate fluorescence signals of target cells were observed. Hybridisations using RING-FISH probes at higher formamide concentrations (> 40 %) resulted in unusual appearance of the signal (probable accumulation of unbound probes around the bacterial cell) or signal loss (see also C.2.2., p. 77). The determination of the appropriate incubation time posed another challenge. On the one hand, the hybridisation time should be long enough to ensure a stable binding of the target and the polynucleotide probe. On the other hand, too long incubation time could possibly lead to whole cell fluorescence of the target cell or to false positive signals of the negative controls.

New RING-FISH polynucleotide probes targeting a chromosomally encoded gene for beta-lactamase$_{OXY-2-5}$ of *Klebsiella oxytoca* (Table B.9, p. 120) were designed. This gene confers resistance inter alia to ceftazidime (a cephalosporin antibiotic) by hydrolysing the drug (Mammeri et al., 2003). Two polynucleotide probes (336 and 587 nt long; (Table B.9, p. 120 and appendix, p. 173, part F) were generated from the 1097 long reference sequence (NCBI, AY303806) as sense and antisense probes. Halo signals were observed (0 % formamide) after 22 h using the shorter probes (336 nt). 24 h were required for successful hybridisations using the longer probes (587 nt).

The chromosomal beta-lactamase$_{SHV-1}$ gene of *K. pneumoniae* confers among others resistance to piperacillin (Rice et al., 2000). For its identification, 365 and 536 nt long polynucleotide probes were designed (Table B.10, p. 33 and appendix, p. 174, part F). Hybridisations using inter alia *P. aeruginosa*, *M. morganii* and *S. aureus* as

negative controls resulted in specific halo signals after 23 h (365 nt long probe) and 24 h (536 nt long probe) at 0 % formamide. Figure C.41 (p. 117) shows positive signals of *K. pneumoniae,* which appeared after the hybridisation using the 536 nt long probe under described conditions. All cells exhibit the halo signal. Higher formamide concentrations in the buffer (due to GC content of 66 mol % of the 536 nt long probe) led to signal loss or accumulation of the probe around the cell. The RING-FISH polynucleotide probes targeting beta-lactamase genes of *K. oxytoca* and *K. pneumoniae* were not tested against each other due to sequence similarities.

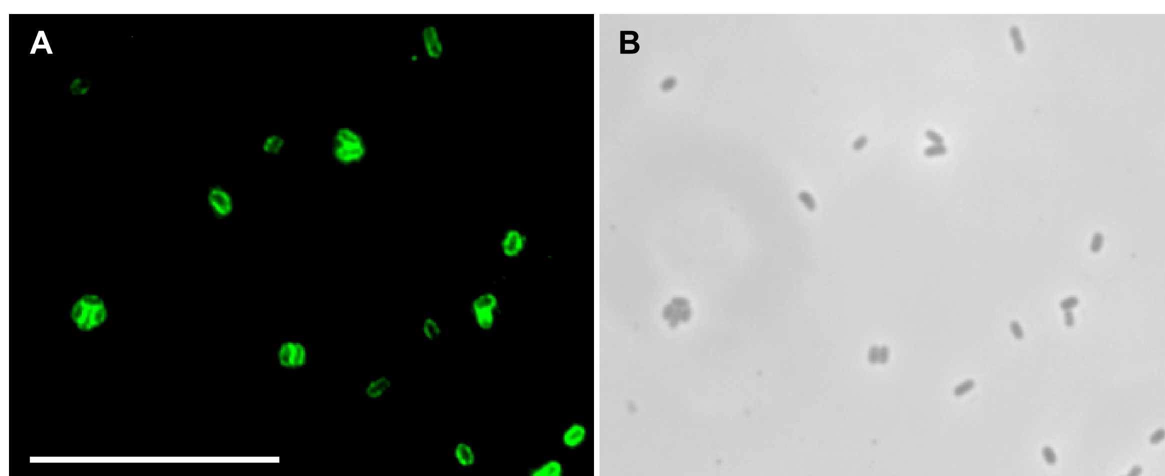


Figure C.41 Halo signals observed after FISH using the fluos-12-UTP labelled and 536 nt long RING-FISH probe KpBlaSHV targeting $bla_{SHV\text{-}1}$ gene of ***Klebsiella pneumoniae*** after **24 h** (0 % FA) **A:** *K. pneumoniae,* epifluorescence image, **B:** phase contrast image of the pure culture; bar: 20 µm

Bacterial resistance to aminoglycosides is mostly based on enzymatic modification by aminoglycoside 6´-N-acetyltransferases encoded by several *aac(6´)*-genes on chromosomal- or plasmid-borne transposable elements (Lambert et al., 1994). The *aac(6´)*-gene of *Pseudomonas fluorescens* also mediates resistance to gentamicin. Three differently long (188, 282 and 409 nt) RNA polynucleotide probes were generated that target a defined fragment of a maximum of 409 nt (Table B. 11, p. 33 and appendix, p. 176, part F). The intent was to evaluate if the probe length is essential for the formation of secondary structures and halo signals. The positive ring shaped signals and whole cell fluorescence of some target bacteria were observed after 18 h at 0 % formamide using the 188 nt long probe. Hybridisations using the 282 nt and 409 nt long probes showed halos after 18 and 22 h, respectively. Figure C. 42 (p. 118) demonstrates the hybridisation using the 282 nt long probe in a mixture of *P. fluorescens* and *N. canis* after 18 h. Only the target bacteria show halo signals.

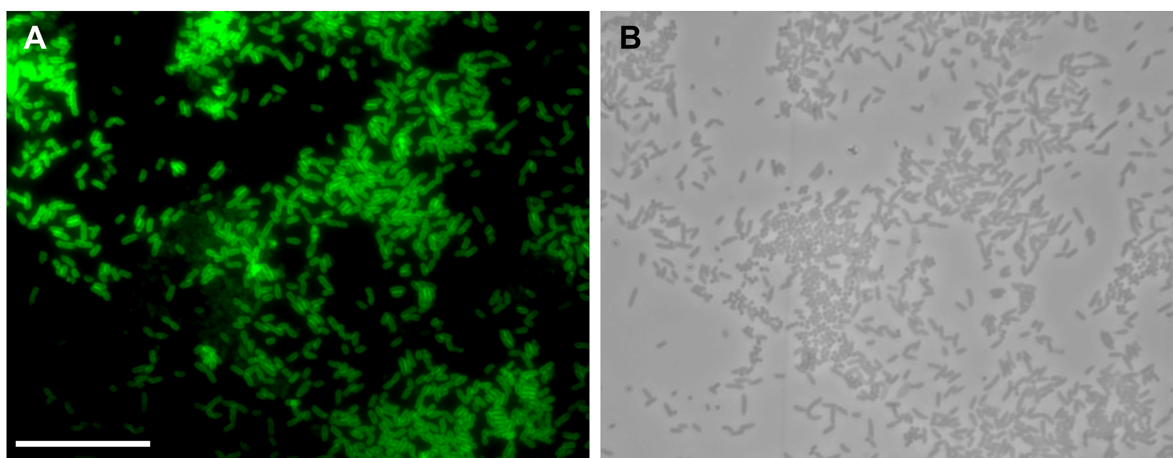


Figure C.42 Halo signals observed after FISH using the fluos-12-UTP labelled RING-FISH probe Pflaac282 (282 nt long) targeting *aac(6´)* gene of ***Pseudomonas fluorescens*** after **18 h** (0 % FA). **A:** *P. fluorescens,* epifluorescence image **B:** *P. fluorescens* and *N. canis* (cocci: negative control*)*, phase contrast image; bar: 20 µm;

Both *Enterococcus* spp. used in the present study, *E. faecalis* and *E. faecium*, harbour a gene, which is responsible for vancomycin resistance. The most common resistance phenotypes are VanA and VanB. The VanA phenotype confers resistance to vancomycin and teicoplanin. The VanB phenotype is characterised by resistance to vancomycin and susceptibility to teicoplanin (Torres et al., 2006). In the present study, *vanB* gene of enterococci was targeted by RING-FISH probes. Due to complete sequence identity of this gene in both *Enterococcus* isolates, one primer pair was designed for the generation of a 409 nt long polynucleotide probe (Table B.12, p. 34 and appendix, p. 172, part F). A FISH experiment using specific oligonucleotide probes (probes: Efi58 and Efe286, table C.1, p. 68) confirmed that these entorococci belong to two different species. The optimal pretreatment (Table B.18, p. 43) and the hybridisation experiments resulted in positive signals (18 h at 0 and 10 % formamide). Figure C.43 (p. 119) shows the successful RING-FISH in a mixture of *E. faecium* and *S. aureus* (non-target organism). The hybridisation conditions led to intermediate signals of the target bacteria.

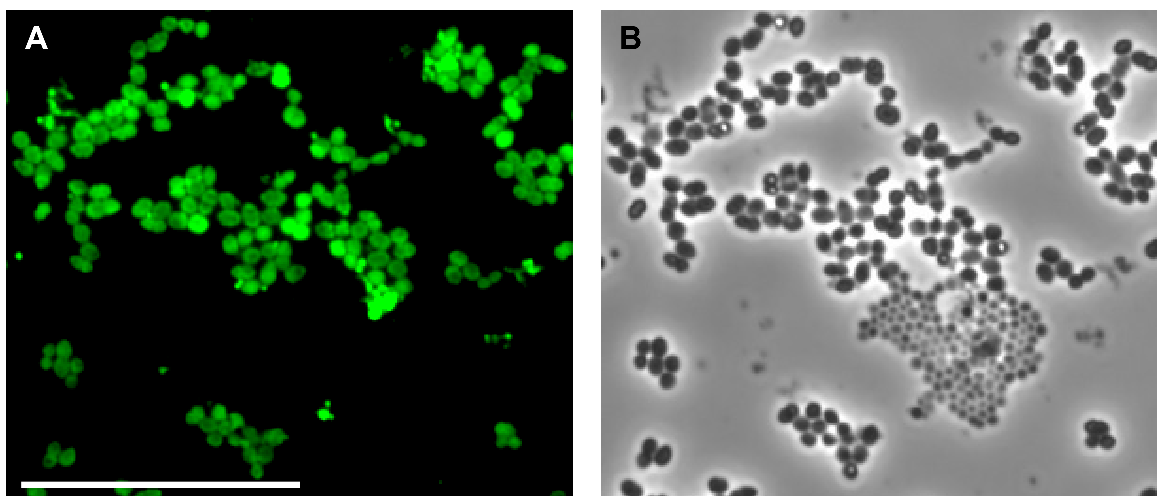


Figure C.43 Intermediate signals observed after FISH using the fluos-12-UTP labelled and 409 nt long RING-FISH probe EfVanB targeting *vanB* gene of ***Enterococcus faecium*** after **18 h** (0 % FA). **A:** *E. faecium,* epifluorescence image **B:** *E. faecium* and *S. aureus* (small cocci under the phase contrast: negative control), phase contrast image; bar: 20 µm

A dreaded human infection is the colonisation with a methicillin-resistant *Staphylococcus aureus* (MRSA). MRSA possesses antimicrobial drug resistance to all penicillins, including methicillin which is beta-lactamase stable. In the past, MRSA traditionally has emerged as a hospital-acquired strain (HA-MRSA), nowadays increasingly community-acquired MRSA (CA-MRSA or cMRSA) have been isolated (Bressler et al., 2005). Methicillin ineffectivity is attained by production of the penicillin binding protein 2a (PBP2a) encoded by the *mecA* gene. For the detection of a methicillin-resistant *Staphylococcus aureus* a 500 nt long RING-FISH probe (Table B.13, p. 34 and appendix, p. 175, part F) targeting *mecA* gene was generated and tested in several hybridisations. An appropriate pretreatment procedure (Table B. 18, p. 43) and hybridisation performed at 0 % led to intermediate and whole cell fluorescence signals after 22 h. Enterococci and streptococci were used as negative controls. The Gram-negative non-target bacteria were *E. coli, M. morganii* and *P. aeruginosa*. A temperature reduction to 50°C (due to the low GC content of 31 mol % of the target gene) to control whether the typical halo signals will appear in contrast to hitherto occurring intermediate and whole cell fluorescence, did not led to halo signals. Further temperature reductions were not tested because of strong secondary structures *in silico* (data not shown).

Table C.9 (p. 120) shows all results obtained using RNA polynucleotide probes targeting the selected drug resistance genes.

Table C.9 Results of hybridisations using RING-FISH probes. Bacteria, AF number, target gene, length and GC-content of the whole gene and respective probe and the FISH conditions, which led to positive signals at 53°C

Bacteria and AF number	**Target gene**	**Length of target gene [nt] / GC content [%]**	**Length of probe [nt] / GC content [%]**	**FISH conditions FA [%] / t [h]**
E. aerogenes 427068	*ampC*	1291 / 58	1078 / 60	0, 10 / 17, 18
K. oxytoca 479983	$bla_{OXY-2-5}$	1097 / 55	336 / 50 587 / 58	0 / 22 0 / 24
K. pneumoniae 429457	bla_{SHV-1}	1342 / 59	365 / 58 536 / 66	0 / 23 0 / 24
P. fluorescens *473825*	*aac(6´)*	1707 / 58	188 / 59 282 / 57 409 / 55	0 / 18 0 / 22
E. faecalis Staph/Strep 28 *E. faecium* 458387	*vanB*	502 / 50	409 / 50	0, 10 / 18
S. aureus 678848	*mecA*	2007 / 31	500 / 31	0 / 22

C.5.2. Adaptation of the RING-FISH protocol for the detection of inducible *ampC* gene of *Enterobacter aerogenes*

The protocol for the already existing polynucleotide probe (Table B.5, p. 31) targeting the inducible AmpC beta-lactamase of *Enterobacter aerogenes* (Ludyga, 2004) was improved. Jacobs (1997) and Wiegand (2003) described the expression of inducible *ampC* genes often found in *Enterobacteriaceae*. The expression is associated with a complicated regulation mechanism (Wiegand, 2003) resulting in the cleavage of the beta-lactam bond. Growth in the absence of beta-lactam antibiotics leads to a stable ratio of murein synthesis and degradation products inside the cell. The AmpR regulator is repressed by UDP-MurNAc-pentapeptide so that *ampC* gene cannot be expressed. After entering the periplasm, the beta-lactams bind to their targets, the penicillin binding proteins (PBPs). PBPs are membrane bound enzymes involved in peptidoglycan synthesis. The inhibition of PBPs leads to the accumulation of the murein intermediate N-acetylglucosamyl-anhMurNAc-tripeptide, which is transported into the cytoplasm by the AmpG permease. These molecules are cleaved by the beta-N-acetylglucosaminidase (Gmase) into anh-monosaccharide-peptides and then hydrolysed by AmpD into the peptide and a saccharide residue. The peptides are used in the murein biosynthetic pathway, but due to the overexpressed level of muropeptides, AmpD is saturated and anhMurNac-tripeptides accumulate inside the cell. Finally, the high concentration of the tripeptide is sufficient to replace the UDP-MurNAc-pentapeptide from the transcriptional factor AmpR that triggers *ampC* gene expression.

After the pretreatment of *E. aerogenes* cells with ampicillin (35 µg/µl) during cultivation (see B.2, p. 25) the hybridisation time using RING-FISH probes could be reduced from 25 to 17 or 18 h (Table C.9, p. 120). Figures C.44 to C.46 (pp. 123 and 124) show the detection of whole cell fluorescence and halo signals obtained with the sense as well as with the antisense probes. The signals occurred after hybridisations of ampicillin affected *E. aerogenes* cells using the *ampC* beta-lactamase targeted 1078 nt long RNA polynucleotide probe. FISH results obtained after incubations with sense as well as antisense probes confirmed the probe-target interaction indicating that the halos derive not from an mRNA-probe binding.

Figure C.44 (p. 123) shows the results of experiments performed with ampicillin affected *E. aerogenes* cells under conditions (25 h, 10 % FA) evaluated for hybridisations with untreated cells in a former study (Ludyga, 2004). Figure C.44 (p. 123) shows whole cell fluorescence of all targets cells after the incubation using the 1078 nt long RING-FISH probe targeting *ampC* beta-lactamase gene of *E. aerogenes*. Moreover, figure C.44 B (p. 123) demonstrates the intact condition of the bacteria exposed to an antimicrobial substance (ampicillin) during cultivation. Figure C.45 (p. 123) visualises the effect of reduced hybridisation time (from 25 to 18 h) using ampicillin-treated *E. aerogenes* cells. The experiments were carried out with the sense and antisense polynucleotide probes. After both incubation attempts strong ring-shaped signals of all target cells were observed.

To test the conditions (17, 18 h and 0, 10 % FA) for specific detection of *ampC* gene of *E. aerogenes,* the respective polynucleotide probes were applied in mixtures containing Gram-negative and -positive control organisms. The non-target bacteria were inter alia *N. canis*, *M. morganii* and *P. aeruginosa*. Some potential negative controls (*A. baumannii*, *E. coli* or *K. oxytoca*) also harbour *ampC* beta-lactamase genes with significant sequence similarities (Figures D.2, p. 149; D.3, p. 150; D.4, p. 165) and could not be used as non-target organisms in control experiments. The hybridisations under described conditions (17, 18 h and 0, 10 % FA) led to the appearance of specific halo signals of all *E. aerogenes* cells (Figure C.46, p. 124). After incubation applying the RING-FISH probe, a subsequent FISH experiment (see B.9.1., p. 38) using the specific oligonucleotide probe Ent16S targeting 16S rRNA of *E. aerogenes* was performed. This hybridisation resulted in whole cell fluorescence of the rod-shaped bacteria (Figure C.46 D, p. 124). The overlay of the images obtained after RING-FISH and FISH using oligonucleotide probes (Figure C.46 E, p. 124) shows that *E. aerogenes* cells were also detected with the 1078 nt long RING-FISH probe. Due to these facts, the RNA polynucleotide probe targeting *ampC* gene of the positive control could be appropriate for the identification of antibiotic resistance genes harboured by the nosocomial pathogen *E. aerogenes.*

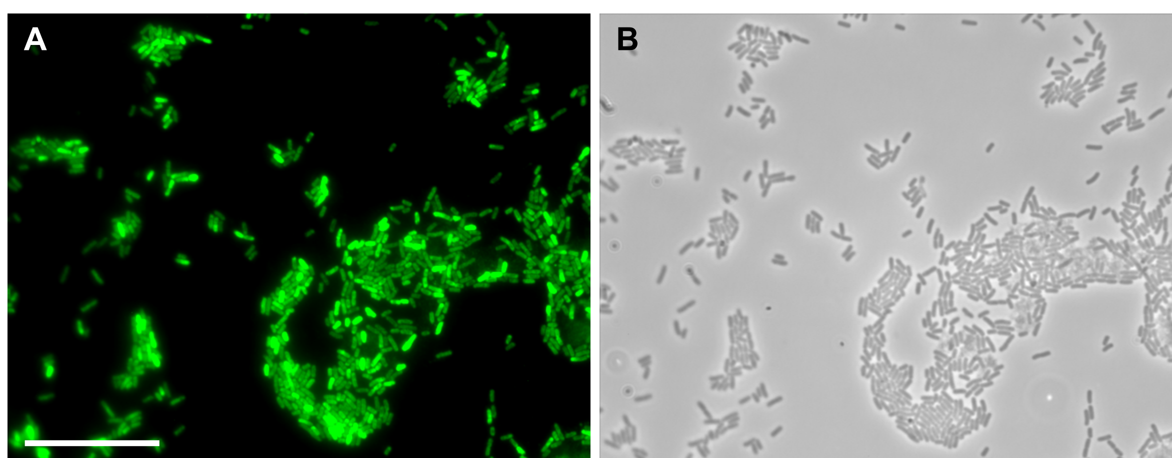


Figure C.44 Whole cell fluorescence observed after FISH (**25 h**, 10 % FA) using the antisense fluos-12-UTP labelled and 1078 nt long RING-FISH probe EaAmpC targeting *ampC* gene of ***Enterobacter aerogenes***. **A:** epifluorescence image; **B:** phase contrast image; bar: 20 µm

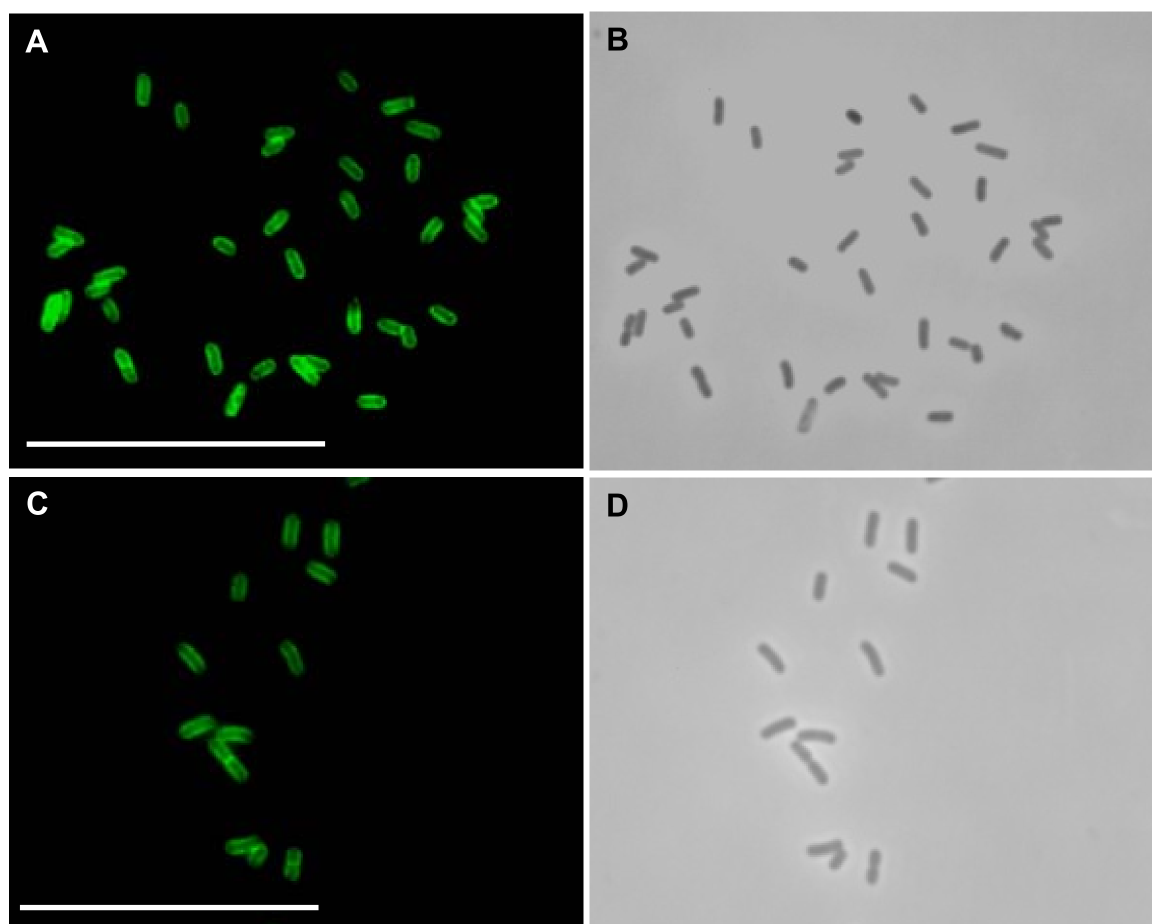


Fig.C.45 Halo signals observed after FISH using the fluos-12-UTP labelled and 1078 nt long RING-FISH probe EaAmpC targeting the *ampC* gene of ***Enterobacter aerogenes***. Halo signals obtained **after 18 h** at 10 % FA in the buffer. **A:** halos observed after FISH using the **sense probe**, **C:** halos observed after FISH using the **antisense probe**; epifluorescence images; **B+D:** phase contrast images; bar: 20 µm

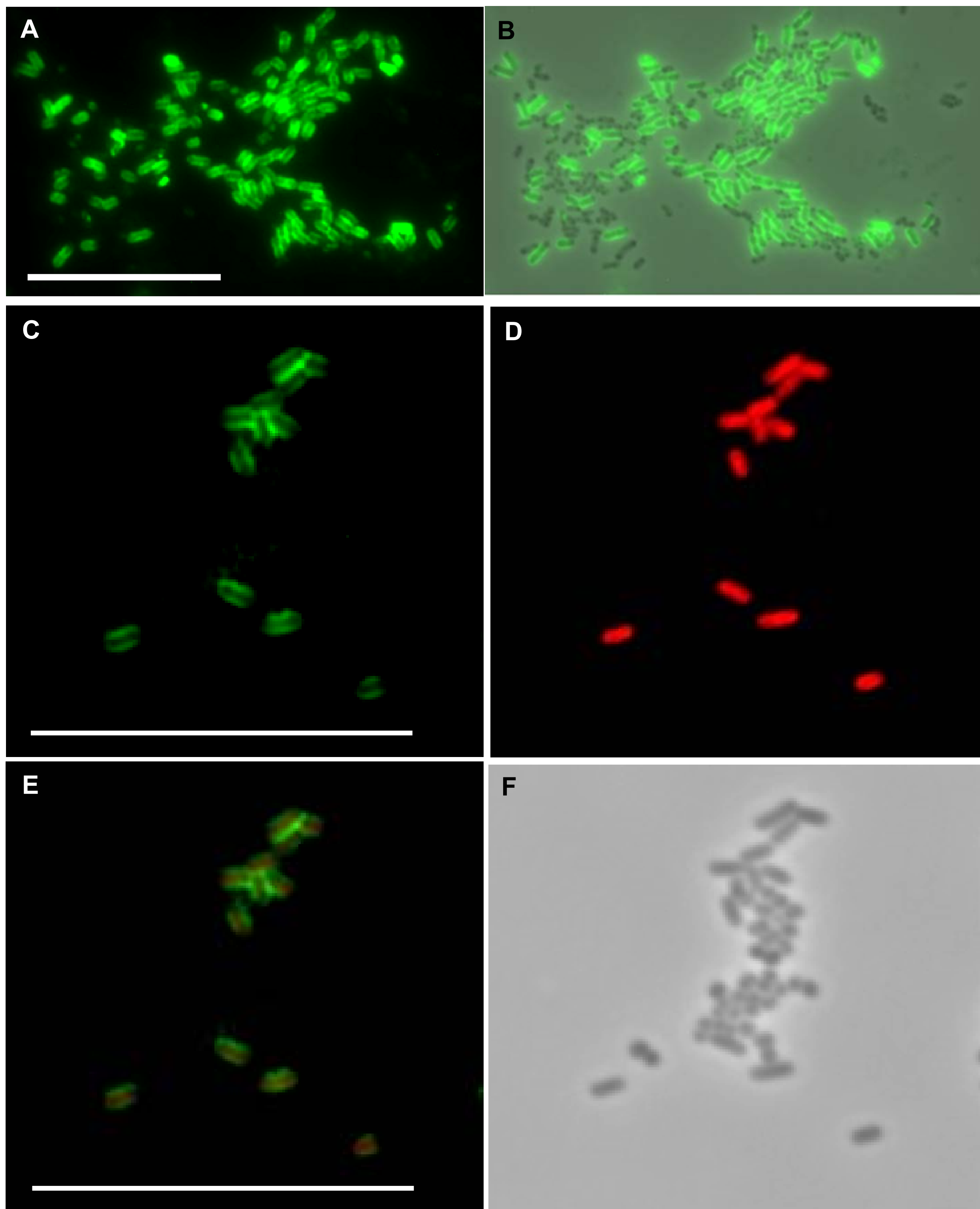


Figure C.46 A, C, E: Halo signals observed after FISH (10 % formamide) using the fluos-12-UTP labelled and 1078 nt long RING-FISH probe EaAmpC targeting the *ampC* gene of ***Enterobacter aerogenes*** **after 18 h A:** *E. aerogenes* exhibiting halos **B:** *E. aerogenes* exhibiting halos and *M. morganii* (smaller rods under the phase contrast: negative control); overlay of the epifluorescence and phase contrast images **C:** *E. aerogenes* exhibiting halos **D:** Whole cell fluorescence signals of *E. aerogenes* after FISH using the Cy3 labelled oligonucleotide probe Ent16S **E:** overlay of images C and D **F:** phase contrast image of *E. aerogenes* and *N.canis* (cocci: negative control); bar: 20 µm

C.5.3. Adaptation of the RING-FISH protocol for spiked clinical material

FISH results obtained in pure and mixed cultures (see C.5.1, p. 114) were applied to spiked clinical material containing the target organism in a defined dilution. The cell amount in a 10^{-3} and 10^{-4} spiked sample corresponded to the cell number of about $8x10^3$ in a real clinical specimen, e.g. in an infected urine sample. These spiked samples offered the possibility to evaluate the accessibility of the polynucleotide probe to its target cell in the presence of eukaryotic cells, cell compounds or other elements typical for clinical samples. Spiked clinical material was available for all bacteria listed in table B.3 (p. 23) with the exception of *Staphylococcus aureus*. Hybridisations using RING-FISH probes in spiked samples containing the target bacteria also showed halo signals but sometimes the FISH led to intermediate signals or whole cell fluorescence of the target bacteria. Moreover, the autofluorescence of eukaryotic cells or cell compounds impeded the visualisation of the hybridisation success. Due to the reduced cell number (compared to pure culture) the already few signals were sometimes superposed by background fluorescence.
Figures C.47 and C.48 (p. 126) show examples of FISH in spiked clinical samples containing a Gram-negative (Figure C.47, p. 126) and a Gram-positive bacterium (Figure C.48, p. 126). Figure C.47 (p. 126) demonstrates an effective detection of *Klebsiella oxytoca* in a spiked urine sample (dilution 10^{-1}) by RING-FISH using the 336 nt long probe targeting $bla_{OXY-2-5}$ gene. Furthermore, figure C.47 B (p. 126) shows the conditions of clinical samples containing eukaryotic components. An efficient hybridisation in a spiked blood (10^{-3}) sample (containing *E. faecium*) using the 409 nt long *vanB* gene targeted polynucleotide probe is shown in figure C.48 (p. 126). Although many blood cells and other eukaryotic compounds hampered the visualisation of positive ring-shaped fluorescence in the periphery of the target cells, clear halo signals can be observed. In addition, figure C. 48 (p. 126) indicates that hybridisations using RING-FISH probes in spiked samples containing clinically realistic bacterial counts are successful. For this reason, these results suggest the possibility to apply RING-FISH probes for the identification of pathogens in real clinical material.

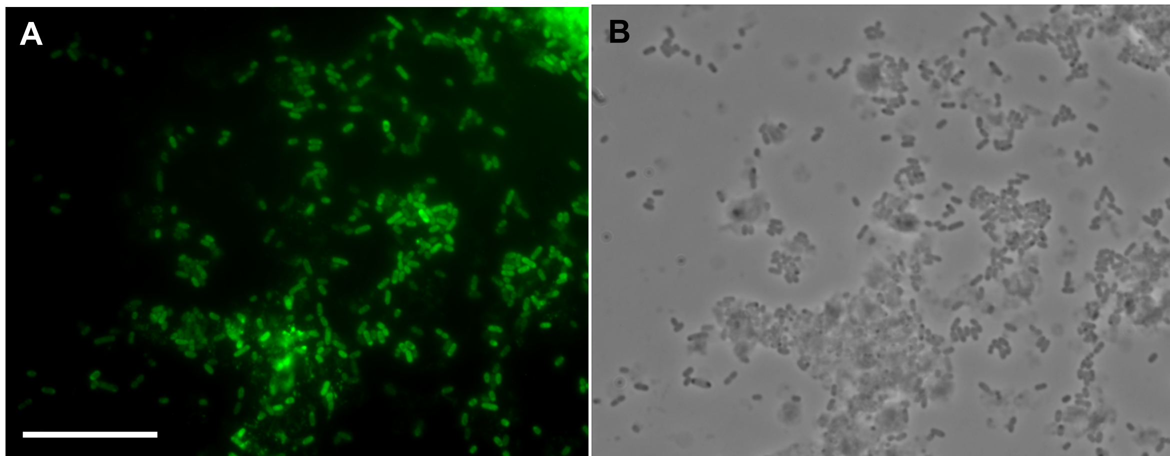


Figure C.47 Halo and intermediate signals observed after FISH (22 h, 0 % FA) using the fluos-12-UTP labelled and 336 nt long RING-FISH probe KoBlaOXY targeting $bla_{OXY\text{-}2\text{-}5}$ gene of ***Klebsiella oxytoca*** in the **spiked urine** sample (dilution 10^{-1}). **A:** epifluorescence image **B:** phase contrast image showing target bacteria and eukaryotic compounds; bar: 20 µm

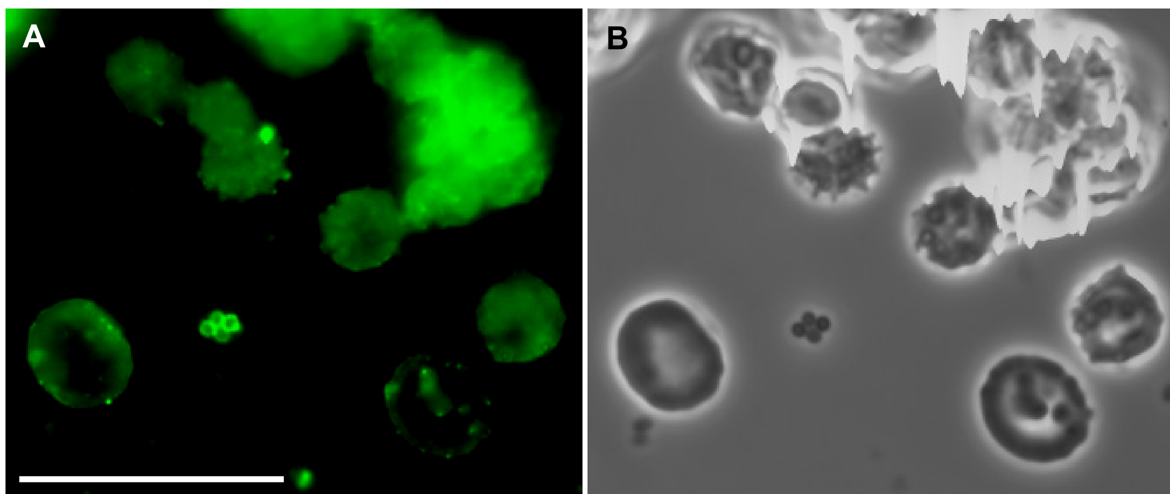


Figure C.48 Halo signals observed after FISH (20 h, 10 % FA) using the fluos-12-UTP labelled and 409 nt long RING-FISH probe EfVanB targeting *vanB* gene of ***Enterococcus faecium*** in the **spiked blood** sample (dilution 10^{-3}). **A:** epifluorescence image **B:** target bacteria and eukaryotic cell components, phase contrast image; bar: 20 µm

C.5.4. Adaptation of the RING-FISH protocol for real clinical material

To test whether RING-FISH probes are applicable for the detection of antibiotic resistance genes in real clinical material, the optimised protocol (see C.5.1., p. 114) was used for these samples.

Real clinical samples containing resistant organisms, for which developed RING-FISH probes could be used, were only available for *S. aureus* and *E. coli*. Figure C.49 (p. 127) shows the hybridisation using the RING-FISH polynucleotide probe for the detection of *ampC* gene (Table B.7, p. 31) of ampicillin-resistant *E. coli* and *K. terrigena* in a real clinical wound smear (sample number 10, table B.4, p. 24). Figure C.49 A (p. 127) demonstrates intermediate fluorescence signals of the target bacteria in the real clinical sample (number 10, table B.4, p. 24). Figure C.49 B (p. 127) shows that only few bacteria and eukaryotic compounds were present in this sample.

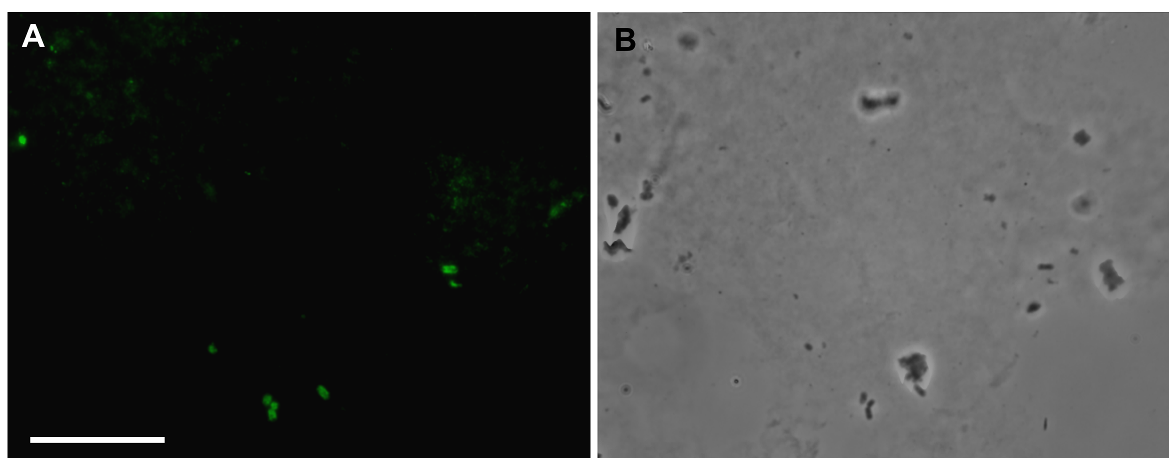


Figure C.49 Intermediate signals obtained after FISH (23 h, 0 % FA) using the fluos-12-UTP labelled and 495 nt long RING-FISH probe EcAmpC targeting *ampC* gene of ***Escherichia coli*** in the **real clinical wound smear** sample (number 10, table B.4, p. 24) containing *K. terrigena*
A: epifluorescence image **B:** phase contrast image; bar: 20 µm

C.6. Immobilisations of pathogens using RING-FISH probes

C.6.1 Optimisation of the immobilisation procedure

The evaluation of the FISH procedure on slides using resistance genes targeted polynucleotide probes was the precondition for the enrichment of target cells in coated cavities from samples of different origin. The protocol (see B.11., p. 57) comprised the same steps (coating of cavities, hybridisation in solution and incubation in the microplate) as for the immobilisation procedure with oligo-oligonucleotide probes.

Here, the modifications of the procedure (see C.3.1., p. 101) were also necessary for the improvement of the separation effectiveness. The modifications also included the denaturation of nucleic acids prior to the coating of cavities, the treatment of blood samples with Tris-HCl as well as the reduction of washing steps with PBS. Furthermore, the prolonged centrifugation step and the application of a Hot Start PCR after the immobilisation of cell were also necessary (see C.3.1., p. 101). The time-reduced assay, introduced by Fichtl (2005) for the immobilisation of cells using domain III targeted probes could be partially applied for separations using RING-FISH polynucleotide probes. It was also possible to reduce the duration of fixation with PFA to 0.5 h but the first hybridisation in solution could not be reduced to 1-1.5 h. The first hybridisation in solution using RING-FISH probes required a minimum of 5 h and a maximum of 12 h. Only the enrichment time was unchanged. The permeabilisation of the cell wall of Gram-positive pathogens was performed as in immobilisations using the oligo-oligonucleotide probes (Table B.25, p. 59) prior to the second ethanol series. In summary, for the immobilisation experiments applying RING-FISH probes for the detection of antibiotic resistance genes of pathogens 6 to 14.5 h were necessary.

The following new RING-FISH probes targeting resistance genes were generated for *Klebsiella oxytoca* (*bla*$_{OXY-2-5}$ gene conferring inter alia a ceftazidime resistance), *Klebsiella pneumoniae* (piperacillin resistance encoded by *bla*$_{SHV-1}$ gene) and *Pseudomonas fluorescens* (*aac(6´)* gene mediating gentamicin resistance). Furthermore, RING-FISH polynucleotide probes were generated for *Enterococcus faecium* and *Enterococcus faecalis* (*vanB* gene coding for vancomycin resistance) as well as for *Staphylococcus aureus* (methicillin resistance due to presence of *mecA*

gene). In a former study (Ludyga, 2004) developed polynucleotide probe targeting *ampC* beta-lactamase genes (among others conferring resistance to ampicillin) of *Acinetobacter baumannii*, *Enterobacter aerogenes*, *Escherichia coli* and *Klebsiella oxytoca* were also used for immobilisation experiments.

First, the experiments were carried out in pure culture to ascertain whether the enrichment is possible under the chosen conditions. Figure C.50 (p. 129) shows an optimal immobilisation of *Staphylococcus aureus* from pure culture using RING-FISH probes targeting *mecA* gene. All coated cavities and the supernatant of non-coated wells showed positive PCR signals. The uncoated well and the supernatant of coated cavities did not show any amplicons indicating that the target cells could be enriched. Table C.10 (p. 130) shows the enrichments of antibiotic resistant pathogens from pure culture and spiked clinical material in detail.

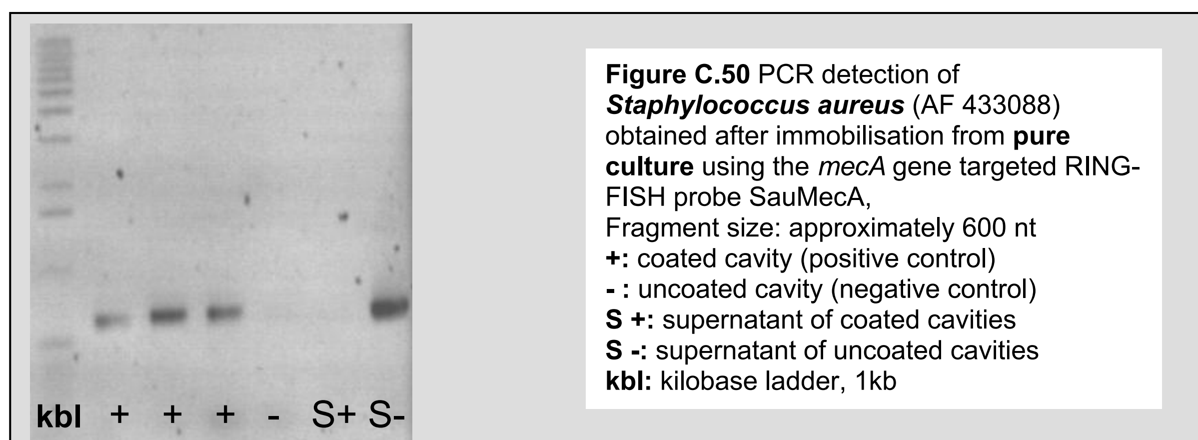


Figure C.50 PCR detection of ***Staphylococcus aureus*** (AF 433088) obtained after immobilisation from **pure culture** using the *mecA* gene targeted RING-FISH probe SauMecA,
Fragment size: approximately 600 nt
+: coated cavity (positive control)
- : uncoated cavity (negative control)
S +: supernatant of coated cavities
S -: supernatant of uncoated cavities
kbl: kilobase ladder, 1kb

C.6.2. Immobilisation results obtained from spiked clinical material

The features and the importance of tests with spiked clinical material are mentioned under C.2.2. (p. 77). Despite the fact that not in all coated cavities PCR signals were detected, bands in one or more coated wells demonstrated that separations of bacteria from spiked samples using RING-FISH probes were possible. *P. fluorescens* and *E. coli* were enriched from a spiked blood sample containing the target bacteria in the dilution 10^{-3}, corresponding to an authentic bacterial number in samples isolated from infected patients. This result suggests a possible immobilisation of target bacteria from samples containing pathogens in real bacterial numbers occurring during an infection. *P. fluorescens, A. baumannii* and *E. aerogenes* could be immobilised from undiluted spiked urine. Separations of *A. baumannii*, *E. coli*,

K. oxytoca and *K. pneumoniae* from other clinically relevant spiked samples were not successful. All results obtained after immobilisations from pure and spiked clinical samples are summarised in table C.10 (p. 130).

Additionally, figure C.51 (p. 130) shows the efficient enrichment of *E. faecium* from a spiked blood sample (dilution 10^{-4}) using *vanB* gene targeted RING-FISH probe. PCR bands were observed after amplifications in all coated cavities obtained after the enrichment. In the cavity, which contained the supernatant of coated wells (S+) a very weak PCR signal was perceived. This result indicates that a large part of *E. faecium* cells was immobilised. Moreover, the efficient enrichment of target cells in the dilution 10^{-4} (from a blood sample) suggests successful immobilisations of bacteria from real clinical samples.

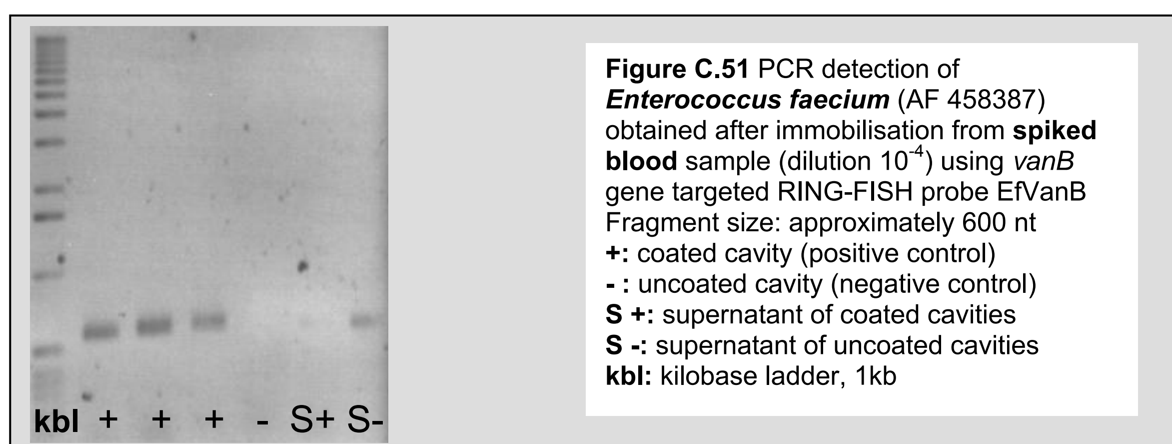


Figure C.51 PCR detection of ***Enterococcus faecium*** (AF 458387) obtained after immobilisation from **spiked blood** sample (dilution 10^{-4}) using *vanB* gene targeted RING-FISH probe EfVanB
Fragment size: approximately 600 nt
+: coated cavity (positive control)
- : uncoated cavity (negative control)
S +: supernatant of coated cavities
S -: supernatant of uncoated cavities
kbl: kilobase ladder, 1kb

Table C.10 Immobilisation results of bacteria present in pure culture and spiked clinical samples using RING-FISH polynucleotide probes targeting resistance genes; dilutions, which correspond to real cell amount in the respective specimens are 10^{-3} and 10^{-4} (marked in red)

Pure culture/ Spiked clinical sample Bacteria	AF number	Pure culture	EDTA - Blood	Urine	Plasma
A. baumannii	438775	+	n.t.	10^{0}	-
E. aerogenes	427068	+	n.t.	10^{0}	n.t.
E. coli	441612	+	10^{-3}	-	-
K. oxytoca	479983	+	n.t.	-	-
K. pneumoniae	429457	+	n.t.	-	-
P. fluorescens	473825	+	10^{-3}	10^{0}	10^{0}
E. faecalis	Staph/Strep 28	+	10^{-4}	n.t.	n.t.
E. faecium	458387	+	10^{-4}	n.r.	10^{0}
S. aureus	433088	+	n.a.	n.a.	n.a.

n.t.: not tested, n.r.: no results, n.a.: not available, - : not successful

C.6.3. Immobilisation results obtained from real clinical material

Available real clinical samples containing pathogens were used for the demonstration whether RING-FISH polynucleotide probes are appropriate for the immobilisation of bacteria from these samples. The properties and differences of real specimens in comparison to pure culture and spiked material are described under C.2.4. (p. 95). *Staphylococcus aureus* and *Escherichia coli* coding antibiotic resistance genes are relevant target organisms in real clinical samples. RING-FISH polynucleotide probes were generated for the *ampC* gene and the *mecA* gene. A real clinical specimen containing *S. aureus*, which harbours *mecA* gene (swab number 8, table B.4, p. 24), was available. Additionally, *E. coli* possessing *ampC* gene was fixed in vaginal smear, urine and wound smear (numbers 2, 10, 15, respectively, table B.4, p. 24). The urine contained apart from the target cells a naturally occurring negative control *E. faecalis*. The immobilisations resulted in successful enrichments in some coated cavities. These results indicated that this technique is also applicable for the separation of pathogens from real samples using RING-FISH probes.

Figure C.52 (p. 131) shows the immobilisation of methicillin-resistant *S. aureus* from a real rump swab sample (number 8, table B.4, p. 24) applying the respective RING-FISH polynucleotide probe. Because an amplicon in the cavity containing supernatants of coated wells (S+) was observed it could be assumed that not all target cells were enriched.

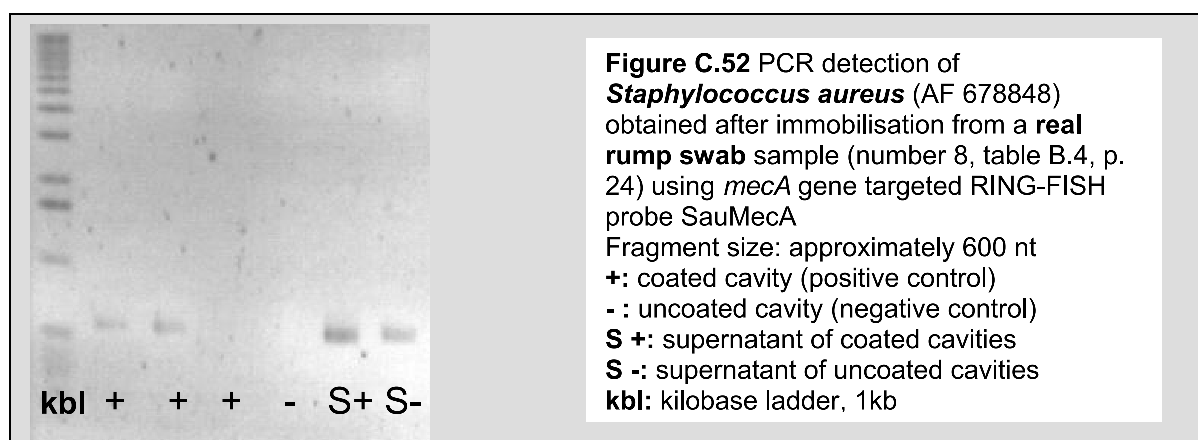


Figure C.52 PCR detection of ***Staphylococcus aureus*** (AF 678848) obtained after immobilisation from a **real rump swab** sample (number 8, table B.4, p. 24) using *mecA* gene targeted RING-FISH probe SauMecA
Fragment size: approximately 600 nt
+: coated cavity (positive control)
- : uncoated cavity (negative control)
S +: supernatant of coated cavities
S -: supernatant of uncoated cavities
kbl: kilobase ladder, 1kb

The sequencing analysis of the amplicons from coated cavities and supernatants generated after the separation of *E. coli* from the sample number 10 (wound smear, suture, table B.4, p. 24) showed that the coexistent ampicillin-resistant *K. terrigena* was also immobilised. This immobilisation demonstrated that RING-FISH probes could be used for the enrichment of bacteria belonging to different taxa due to the high similarity of *ampC* gene sequences among *Enterobacteriaceae.* The particular sequences differ from each other only in single point mutations (Wiegand, 2003).

Table C.11 (p. 132) summarises the results obtained from immobilisations of resistant bacteria from real clinical material with RING-FISH probes.

Table C.11 Immobilisation results achieved from real clinical samples using RING-FISH polynucleotide probes. Real clinical samples, number: sample number of real clinical specimens (Table B.4, p.24), AF number, target gene, enriched cells and non-target cells, which were not enriched.

Real clinical sample	Number	AF number	Target gene	Enriched target cell	Non-target cell, present in the sample
Vaginal smear	2	478499	*ampC*	*E. coli*	-
Wound smear	10	682242	*ampC*	*E. coli,* *K. terrigena*	-
Urine	15	405639	*ampC*	*E. coli*	*E. faecalis*
Swab i. o.p., rump	8	678848	*mecA*	*S. aureus*	-

D. Discussion

D.1. Application of RNA oligo-oligonucleotide probes

Nowadays, it is possible to identify bacteria without cultivation by fluorescence *in situ* hybridisation (FISH) using short (16-25 nt) synthetic DNA oligonucleotide probes labelled with a fluorescent dye. In recent years, further methodological improvements and the combination of FISH with other techniques allowed the detection of bacteria from natural habitats such as environmental or clinical samples (Amann et al., 2000; Hogardt et al., 2000). Basically, rRNAs are ideal as target molecules because they are present in all living organisms in high copy numbers and they contain variable as well as conserved sequences (Woese, 1987; Amann et al., 1995; Ludwig et al., 1994). The phylogenetic classification of prokaryotes is based on the comparative sequence analysis of rRNA molecules. Apart from successful application, the conventional FISH also reveals limitations. A drawback is the low signal intensity that is inter alia associated with inadequate cell permeability reducing the accessibility of the probe to its target. Another reason is the low ribosome content of slowly growing or metabolically inactive cells especially from environmental samples. To improve the signal intensity, in the last two decades among others single stranded multilabelled RNA polynucleotide probes (several hundred nt long) targeting 23S rRNA were developed and successfully applied in hybridisations (Trebesius et al., 1994; Zwirglmaier, 2003). The domain III of 23S rRNA is the fragment that is the most variable within the 23S rRNA (Höpfl et al., 1989) allowing taxon-specific differentiation. The signal amplification relies on both the multi-labelling and the formation of secondary structures, which is visible as the so-called halo, a strong fluorescence signal in the periphery of the cell. Trebesius et al. (1994) and Stoffels et al. (1999) described the phenomenon of the halo signal. The theory was that only a part of the polynucleotide probe binds to its target region while the remaining fragment protrudes out of the cell and encompasses the cell envelope with unbound probes. Zwirglmaier et al. (2003) refined this theory and postulated the “network hypothesis”. The hypothesis suggests that the polynucleotide probe partially hybridises to its target and the protruding fragment interacts with other (unbound) probe molecules resulting in a network in the area of the cell envelope. The hybridisations using RNA polynucleotide probes lead to a ring-shaped signal within

and around the cell periphery and enable a subsequent separation of target cells from different samples. Thereby, the protruding probe fragment is a prerequisite for cell sorting by application of the MACS technique (Magnetic Activated Cell Sorting, Stoffels et al., 1999) using biotin-labelled probes, which hybridise with streptavidin-coated magnetic beads. Zwirglmaier et al. (2004) used this principle for immobilisations of cells in microplates coated with probe-complementary nucleic acids.

Although, it is assumed that DNA-RNA hybrids are less stable in comparison to RNA-RNA hybrids, single stranded DNA polynucleotide probes targeting 23S rRNA were developed and used for the detection of several *Acinetobacter* spp. even from environmental samples (Zimmermann et al., 2001). The advantages of DNA polynucleotide probes consist in increased stability and faster synthesis as well as convenient labelling via PCR. However, the hybridisations resulted not always in halo signals, which are an essential precondition for the enrichment of bacteria from different habitats.

Due to the stable network formation leading to halos in hybridisations with RNA polynucleotide probes, the targeted spectrum was extended to regions localised outside of ribosomes. Genetic elements mediated by plasmids or chromosomally encoded could be these target regions in bacterial cells. In previous studies, successful hybridisations using single stranded RNA polynucleotide probes (ranging in length between 100 and several hundred nt) were described, which led to halos and allowed the detection of even single genes by RING-FISH (Zwirglmaier et al., 2004; Fichtl, 2005). However, the length of the polynucleotide probes (450-1000 nt) has a negative effect on the specificity (Ludyga, 2004). A higher specificity could be achieved by the application of about 230 nt long 23S rRNA domain III targeted polynucleotide probes but it was not sufficient for the discrimination of streptococci from enterococci (Fichtl, 2005).

The major task of the present study was the design and application of probes, which combine the advantages of oligonucleotide (specificity) and polynucleotide probes (network formation). The novel so-called oligo-oligonucleotide probes are composed of an alternating sequence of repetitive oligonucleotide fragments and spacer elements. The insertion of spacer fragments should avoid mismatch binding due to shifted oligonucleotide sequences. The development and application of oligo-oligonucleotide probes should be appropriate for a specific detection on slides (see

B.9.3., p. 44) and finally for the separation of target cells in coated microplate cavities (see B.11., p. 57).

In a former study (Fichtl, 2005), different approaches with synthetic oligo-oligonucleotide probes were tested. Probes containing only the specific repetitive elements (without spacer regions) were constructed but the typical halo signals could not be detected. Either no signals or whole cell fluorescence were observed after hybridisations. The relatively short sequence containing several repetitions complicated the generation of a double stranded template. In the present study, an effective protocol could be established which allowed the generation and application of oligo-oligonucleotide probes targeting 16S or 23S rRNA of bacteria.

D.1.1. Design and generation of oligo-oligonucleotide probes containing different spacer sequences

The exact procedure for the design and development of oligo-oligonucleotide probes is described in detail under C.2.1. (p. 73). A precondition for the construction of the oligo-oligonucleotide construct was the selection of reliable taxon-specific oligonucleotide probes.

The evaluated procedure (see C.2.1., p. 73) comprised a combination of several techniques (inter alia: Klenow reaction, cloning, digestion, *in vitro* transcription). It was successfully used for the synthesis of novel polynucleotide probes (Tables C.2, p. 75; C.3, p. 76 and C.6, p. 99). In comparison to earlier attempts, the important improvements of the new protocol were the use of the Klenow reaction and the double digestion. The Klenow reaction allowed the generation of high amounts of the double stranded template from a synthetic single stranded construct. The double stranded template was necessary for the generation of a RNA polynucleotide probe via *in vitro* transcription (see B.10.1., p. 47). The double digestion, using two different enzymes simultaneously, ensured a defined probe length and sequence (see C.2.1., p. 73). Although the single stranded construct is not low-priced, ultimately this method is more cost-effective than ordering templates and additionally directly and indirectly labelled RNA polynucleotide probes for further applications. A limitation of this concept is the maximum length (140 nt) of synthetically produced constructs resulting in a final probe length of about 110 nt. Probably, the probe length also plays a role with regard to halo formation (Zwirglmaier et al., 2003). In this study, the length

of 23S rRNA domain III targeted probes varied between 50 and 1188 nt and hybridisations led to ring-shaped signals. In another attempt (Fichtl, 2005) the hybridisations with about 70 nt long synthetic transcript probes resulted in intermediate and whole cell fluorescence signals.
However, in comparison to the generation of transcript probes from double stranded PCR products, the newly developed method for the generation of oligo-oligonucleotide probes is more time-consuming. On the other hand, the generation of the novel polynucleotide probes is significantly less expensive. Moreover, this method provides an opportunity to design monospecific and multispecific oligo-oligonucleotide probes as well as further polynucleotide probes not necessarily restricted to rRNA or rDNA.

D.1.2. Application of monospecific oligo-oligonucleotide probes in hybridisations on slides and in immobilisation experiments

Twelve monospecific oligo-oligonucleotide probes were constructed from taxon-specific oligonucleotide probes (see C.2.1., p. 73). These probes were applied for the identification and separation of pathogens causing nosocomial infections. Six of them target Gram-negative and further six Gram-positive bacteria, respectively. Optimal FISH conditions on slides led to a halo signal of the target cell and were essential for a successful separation. The obtained FISH parameters on slides (pretreatment, formamide concentration and hybridisation time) were easily adapted to immobilisation experiments in microplate cavities.
In the present study, successful hybridisations on slides demonstrated the possibility to detect specifically pathogens in different clinical material (see C.2.3., p. 93 and C.2.4., p. 95) independent of cell amount and non-bacterial components. Furthermore, the effective immobilisations of clinically relevant bacteria from specimens of different origins (see C.3.2., p. 106 and C.3.3., p.108) showed the applicability of this technique for the separation of target cells at a defined taxonomic level. The advantage of this method is that immobilised bacteria present in cavities and non-immobilised bacteria in supernatants can be used for further studies by applying microbiological, serological or molecular techniques. Based on this, oligo-oligonucleotide probes can be used for diagnostic investigations of infectious agents.

D.1.2.1. Selection of positive and negative controls and pretreatments

For the design of oligo-oligonucleotide probes, bacteria and oligonucleotide fragments were chosen which showed very strong whole cell fluorescence signals after hybridisations with appropriate oligonucleotide probes. It was assumed that the interaction of each oligonucleotide fragment within an oligo-oligonucleotide probe with the target would also be strong and result in intense signals. Furthermore, the selection of positive controls was based on the spectrum of clinical relevance and frequency of some pathogens occurring as causatives of nosocomial infections. The bacterial candidates as target organisms were inter alia *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and enterococci.
Neisseria canis cells were often used as non-target cells. In contrast to the mostly rod-shaped Gram-negative target bacteria, cells of *N. canis* are coccoid. Additional non-target cells were chosen dependent on the probable coexistence during an infection and were tested against each other.

Since Gram-negative and -positive pathogens (e.g. *E. coli* and enterococci) often coexist in a real clinical sample, problems appeared how to proceed with specimens containing bacteria with differently composed cell walls. Samples containing Gram-positive or Gram-negative bacteria were separately pretreated and afterwards they were pooled together prior to the hybridisation. Another attempt for the pretreatment of such samples consisted in the simultaneous treatment of Gram-positive and -negative bacteria in one sample. This approach implicated a pretreatment of Gram-negative bacteria, too. Enzymatic treatments of Gram-negative bacteria were described in former studies (e.g. Pernthaler et al., 2002). In the present study, hybridisations led to specific signals of the positive control and showed that the cell wall of the Gram-negative non-target cell remained intact (Figure C.23 A and B, p. 92). An advantage of this simultaneous pretreatment is that the second attempt is less time-consuming.

D.1.2.2. Detection of bacteria in pure culture, spiked and real clinical material on slides

Pure culture, artificial mixtures, spiked and real clinical samples were hybridised with fluorescently labelled oligo-oligonucleotide probes. The pure cultures contained only the target organisms in an unrealistic high number and the cells were suspended in a synthetic buffer, which does also not correspond to real conditions. At the beginning of the experiments, it was important to optimise hybridisation parameters like formamide concentration, denaturation or incubation time and adequate pretreatment methods. After testing of oligo-oligonucleotide probes in hybridisations in pure culture, which resulted in the detection of halo signals, stringent conditions were evaluated in artificial mixed cultures, spiked and real clinical samples. The ring-shaped fluorescence occurring in the periphery of the target cell after FISH on slides was a prerequisite for immobilisations for which the protruding fragment of the probe binds to complementary nucleic acids on the cavity. Until now, separations were carried out with polynucleotide probes of a minimum length of about 230 base pairs (targeting the domain III of the 23S rRNA; Fichtl, 2005) or longer (RING-FISH probes; Ludyga, 2004).

Hence, the question arose whether 110 or 105 nt long oligo-oligonucleotide probes possess the same ability to form a network like other already tested short polynucleotide probes (Zwirglmaier et al., 2003). Several hybridisation experiments led to the typical signals under evaluated conditions but whole cell fluorescence or so-called intermediate signals also occurred. The intermediate signals were characterised by weak whole cell fluorescence and a strong signal in the periphery of the cell (e.g. figure C.21, p. 89). The appearance of the intermediate signal may be explained by a usual network of bound and unbound probes in the periphery of the bacterium as expected and a weak network formation inside the cell.

The spiked samples also contained only the target organisms but they are suspended in blood, plasma and urine in the dilutions 10^{-3} and 10^{-4}, which correspond to the real cell number of about $8x10^3$ cells, usually present in a clinical material from infected patients. The choice of spiked samples depended on the frequency of a pathogen existing in a typical infected material. It is more probable that *Staphylococcus aureus* or *Acinetobacter baumannii* colonise blood as urine. *E. coli* is more often isolated from urine than from respiratory tract. The selection of

non-target organisms also was focused on the real situation during an infection (see D.1.2.1., p. 137).
Mostly, the same hybridisation times (Tables C.4, p. 89 and C.5, p. 92) were applied for FISH in spiked clinical material as in pure or mixed cultures. In general, the target cells could be successfully detected but sometimes the fluorescence appearance varied in the signal from ring-shaped over intermediate signals to whole cell fluorescence. As documented in tables C.4 (p. 89) and C.5 (p. 92) the formamide concentrations were well-defined but the incubation time depended on the clinical material. It could be stated that hybridisations in spiked clinical samples using oligo-oligonucleotide probes often needed an additional hour. The reason for different fluorescence signals or different hybridisation times is associated with the specimen itself. Evidently, in a clear material such as urine, the accessibility of the polynucleotide probe to its target cell is better than in a more viscous sample such as blood. Thus, in a transparent and liquid sample, halo signals could be obtained after shorter hybridisation time. In contrast, FISH in a viscous inhomogeneous material (containing several cell components of different size) required longer exposure times due to hindering compounds, which hamper the polynucleotide probe to reach the target organism and region. Additionally, many eukaryotic compounds showed autofluorescence and impeded the visualisation. To confirm that these signals did not derive from unspecific interaction of non-bacterial components with the polynucleotide probes, clinical samples were also observed microscopically without a probe. The eukaryotic components showed the same signal intensities even under different filters. Many kits from diagnostic screenings are known to remove bacteria from inter alia blood samples resulting in the destruction of pathogens but protocols for nondestructive separation of both fractions are not described. Indeed, FACS (Fluorescence Activated Cell Sorting) is usually used for the separation of different cells, but this method is too expensive and time-consuming. Otherwise, samples lacking naturally coexisting components will not reflect the authentic situation of this kind of material and probably query the functionality of the presented FISH protocol. Nevertheless, the hybridisations in spiked material were efficient as illustrated in figure C.26 (p. 94) showing *S. pyogenes* in a spiked blood sample (dilution 10^{-3}).
Finally, oligo-oligonucleotide probes were applied for hybridisations in real clinical samples isolated from infected patients. Different material was available containing only the target organism or seldom two. Usually, the second pathogen was a

coexistent bacterium and could be used as negative control. Astonishingly, the experiments with real clinical material often resulted in halo signals after shorter hybridisation times (minus one hour in comparison to results in tables C.4, p. 89 and C.5, p. 92). For sure, the composition of the material also affected the hybridisation as in spiked clinical samples. Another possible explanation for this phenomenon could be the influence of the innate immune system of the host. Here, the human immune system, which could affect the pathogen, plays an important role (Janeway and Travers, 1997). Especially, the factors (lysozyme and components of the complement system) which have an effect on the cell wall of bacteria may have an impact on a better accessibility of the probe to its target.

In general, it can be stated that reproducible results could be observed but the results could vary concerning the hybridisation time or observed signals from pure culture to spiked and real specimens. This means that the specificity of designed oligo-oligonucleotide probes could be verified but a defined protocol does not exist for the respective probe in different samples. In other words, it could happen that the hybridisation under the same conditions (formamide concentration, denaturation and incubation time, temperature) could result in different signal intensities or signal nature ranging from halo signals to whole cell fluorescence depending on the sample (pure culture, spiked or real). Obviously, FISH in a less complex sample containing the target cell and few hampering components will more rapidly result in halo signals than a FISH in a viscous material composed of the target cells and many interfering eukaryotic substances.
As the oligo-oligonucleotide probe based method is a promising concept for the specific identification of bacteria, the incubations times under evaluated conditions (reference values from pure culture) can be adjusted to the composition and consistency of respective clinical material. Usually, urine samples are clear, liquid and easy to handle. In contrast, blood, wound smear as well as tracheal swab are viscous or compact samples, which innately comprise many eukaryotic components indicating a hampered accessibility of the probe to its target nucleic acid. Dilution series (with a buffer) of the clinical material are possible but should also be adapted to the bacterial cell amount of the specimen. Empirical values are known which indicate the pathogen number of infected samples.

Figure D.1 (p. 141) shows halo signals detected after hybridisations using *S. pyogenes* cells and the oligo-oligonucleotide probe Spy467_GC in pure culture (Figure D.1 A, p. 141), spiked clinical sample (dilution 10^{-3}, figure D.1 B, p. 141) and in real clinical sample (Figure D.1 C, p. 141) after different incubation times.

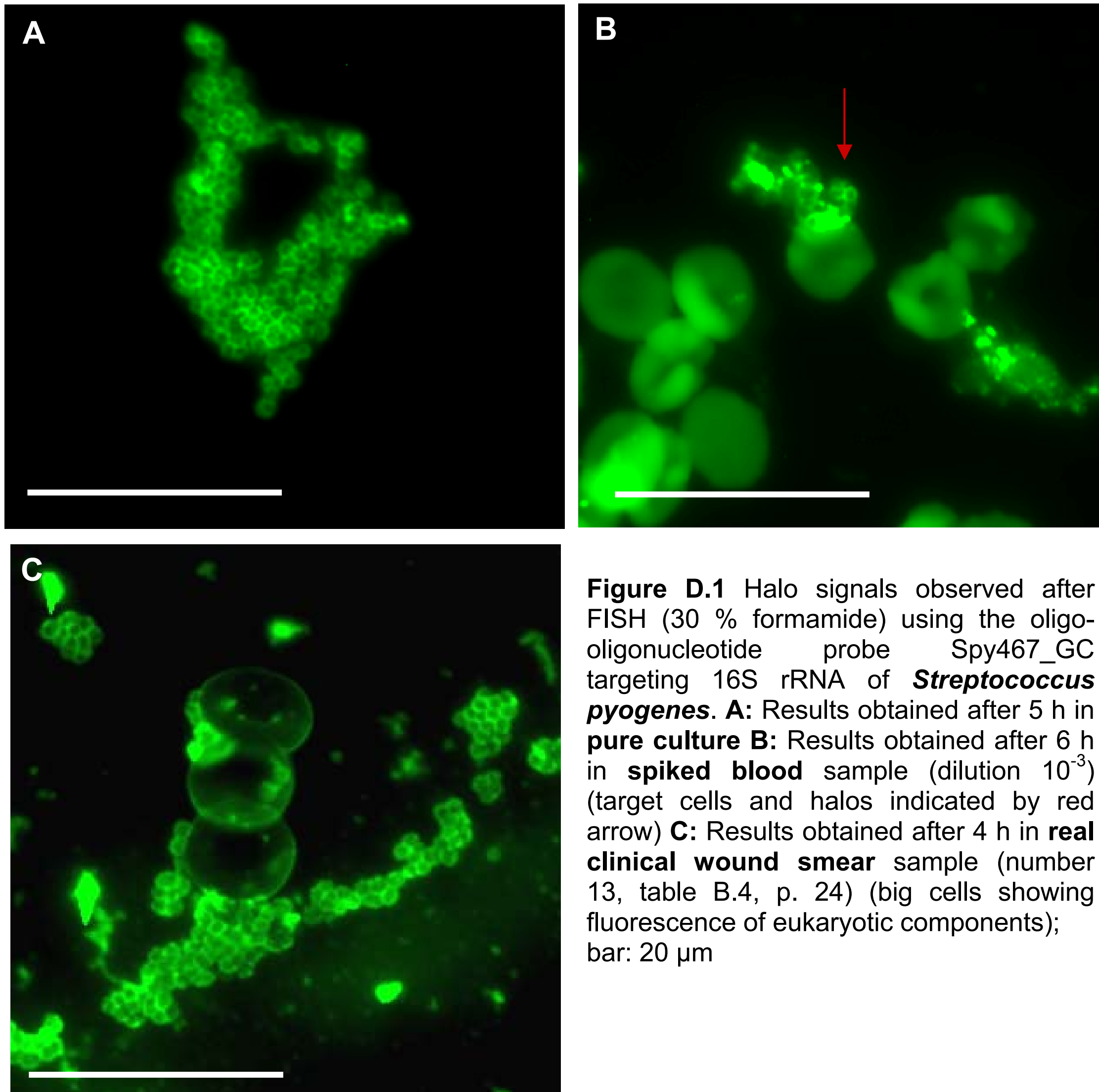


Figure D.1 Halo signals observed after FISH (30 % formamide) using the oligo-oligonucleotide probe Spy467_GC targeting 16S rRNA of ***Streptococcus pyogenes***. **A:** Results obtained after 5 h in **pure culture B:** Results obtained after 6 h in **spiked blood** sample (dilution 10^{-3}) (target cells and halos indicated by red arrow) **C:** Results obtained after 4 h in **real clinical wound smear** sample (number 13, table B.4, p. 24) (big cells showing fluorescence of eukaryotic components); bar: 20 µm

D.1.2.3. Separation of bacteria in pure culture, spiked and real clinical material

Analogue to FISH on slides the applicability of oligo-oligonucleotide probes for the immobilisation of cells was at first tested in pure culture then in spiked samples and finally in real clinical material. Positive results from pure culture indicated that the enrichment in general is possible using this probe. At the same time, they are a precondition for further investigations. Moreover, the time-reduced protocol (Fichtl, 2005) could be applied in the most cases (see C.3.1.4., p. 103). The main modification was the reduction of the time needed for PFA fixation. Incubation with PFA for 30 minutes is sufficient and was practised with pure cultures listed in tables B.1 (p. 22) and B.2 (p. 23). Only the first hybridisation (in spiked and real specimens) in tubes still required 1 to 1.5 hours (instead of about 20 min to 1 h). A prolonged incubation time may be necessary due to the hampered accessibility of the probe to its target nucleic acid. Some refining of the conventional procedure (see C.3.1., p. 101) led to successful results. For example, the denaturation of nucleic acids prior to the coating of cavities with DNA increased the enrichment efficiency. The reason is probably a stronger interaction of the probe and the nucleic acid. Originally, the incubation at 94°C was not applied for immobilisations using nucleolink microplates. Additionally, some redundant washing steps were abolished and contributed to a time reduction of the whole protocol, too. The washing steps often led to cell loss and elongated the procedure. Moreover, the treatment of blood samples with 50 mmol Tris-HCl allowed an improved separation of bacteria from these samples. This agent caused lysis of blood cells leading to a better accessibility of the probe to its target cell. Another detail (application of a Hot Start PCR subsequent to the enrichment) eliminated false negative results of some immobilisations. Occasionally, few PCR fragments were detected after the amplification suggesting either cell loss or ineffective PCR. Cell loss was excluded because of reduced washing steps and longer centrifugations (from 1 to 5 min). The effect of the Hot Start PCR may be that components inhibiting the polymerase like PFA or other enzymes were inactivated during 10 min denaturation at 94°C and the addition of the polymerase afterwards resulted in an increased amplification rate. Moreover, PCR signals were not observed in every coated cavity after cell sorting (Figures C.33 - C.35, pp. 105-107). A possible explanation could be that defective material caused wash out of both the enriched cells and nucleic acids by following washing steps.

After the separation method was optimised (see C.3.1., p. 101) and the applicability of oligo-oligonucleotide probes was confirmed in tests with pure samples, the immobilisations of bacteria from spiked clinical specimens were approached. Not all enrichment tests led to positive results, separations of *E. coli* from blood or *Enterobacter aerogenes* from plasma samples failed. Cell loss after some needed washing steps may be the reason for negative PCR result in some wells after the immobilisation. However, the effectiveness of successful pathogen separation by these novel polynucleotide probes was confirmed in other attempts. Data shown in table C.7 (p. 108) demonstrate the accomplishments obtained from pure culture and spiked clinical material. The achievements from spiked specimens in dilutions 10^{-3} and 10^{-4} suggested an efficient cell sorting of bacteria from authentic clinical material.

The enrichments from real clinical samples were very successful and all bacteria listed in table B.4 (p. 24) could be separated. In comparison to the detection on slides, the accessibility of the polynucleotide probe to its target bacterium and region was generally better in solution. For the identification of enriched and non-enriched bacteria, PCR-products generated after cell sorting from real clinical samples were cloned and sequenced (Table C.8, p. 112). The sequencing analyses affirmed the specificity of these novel polynucleotide probes but at the same time investigation of supernatants from coated cavities indicated that sometimes not all target bacteria could be enriched. A second immobilisation (data not shown) of target cells increased the efficiency of this method. For this purpose, the supernatant containing non-enriched target cells was transferred to fresh coated wells and incubated again.
Furthermore, within the PathoGenoMik-project, project partners from ITB (Institute for Technical Biochemistry at the University of Stuttgart) performed genotypic screenings of enriched *E. coli* cells from real urine for the detection of antibiotic resistance genes (see C.3.3., p. 108). The analyses showed that this pathogen harboured antibiotic resistance genes conferring resistance to fluorochinolones. In fact, this result reflects the phenotypic investigation (disc diffusion test) after 2-day cultivation of pathogens but the combined technique (immobilisation in microplates and subsequent genotyping) needs overall 8 h. This result confirmed the applicability of the separation method in combination with other diagnostic screenings.

In summary, the conditions for the specific and efficient oligo-oligonucleotide probe based immobilisation of pathogens causing nosocomial infections were largely optimised and successfully applied in samples of different sources. Especially, enrichments of bacteria from real clinical samples containing reduced bacterial counts demonstrated their applicability in samples isolated from infected patients. This technique provides an opportunity in clinical laboratories with regard to rapid and specific separations. Despite the enriched and non-enriched bacteria could not be cultivated, they are applicable for further research of the whole genome or organism by common PCR-methods, immunoassays, FACS, *in situ* hybridisations or microarrays.

D.1.3. Real clinical samples and the role of the human immune system

Real clinical samples are characterised by some typical parameters namely low amount of target cells, eventually hindering components, naturally emerging negative controls and consistency.
By the adjustment of an adequate protocol apart from these factors, the influence of the innate human immune system eventually leading to an increased accessibility of the probe to its target region over the cell envelope should be considered. Interestingly, particular components involved in the immune response like lysozyme and the complement system affect the bacterial cell envelope (Janeway and Travers, 1997). Due to the intact cell morphology, which was microscopically checked, it could be assumed that the fixation of bacteria took place at the beginning or during the infection when the pathogens were not completely destroyed. These factors (lysozyme and the complement system) of the host obviously contributed to an increased permeabilisation of the cell wall, too. Lysozyme is part of the innate immune system and is found in many secretions (sweat, tears, cytoplasmic granules of polymorphonuclear neutrophils, breast milk, saliva, nasogastric and bronchial secretions). It cleaves the peptidoglycan of both Gram-negative and Gram-positive bacteria by hydrolysing the beta-1,4 bond between N-acetyl muramic acid and N-acetylglucosamine and leads to a more permeable cell wall of pathogens. The next factor, which surely affected the permeabilisation of bacteria is the complement system. The complement system is a complex regulation mechanism composed of various complement proteins and receptors found in blood and other fluids or tissues.

Based on the activator components three pathways are known which finally result in porin formation inside the cell envelope and afterwards in cell lysis (Janeway and Travers, 1997).

Furthermore, it is well known that the bacterial growth in the host may be quite different from that under laboratory conditions. The growing bacteria may differ in their cell wall structure and this may result in a better accessibility of the target nucleic acid. A drug medication of patients especially with beta-lactamase antibiotics affecting the cell wall could not be excluded and will also influence the accessibility of the polynucleotide probe to its target inside the bacterial cell. Hence, it is important to consider all factors concerning bacterial survival strategies occurring during evolution. In the same manner it is important to consider mammalian or human adaptation and defending mechanisms to their environment containing microbial pathogens. Evidently, the condition of the pathogens and of their cell walls cultivated under laboratory conditions differ from the condition of bacteria isolated from infectious sources and should be considered in the development of an adequate protocol.

Probably, the described factors (components of the human immune system and possible drug medication) increased the accessibility of the polynucleotide probe through the cell envelope to its target site reducing the incubation time in hybridisations in real clinical samples. On the one hand, the permeabilisation of the bacterial cell wall and membrane was improved due to the immune response or medication. On the other hand, the incubation time could not be significantly reduced (only minus an hour in comparison to incubation times in tables C.4, p. 89 and C.5, p.92) for all clinical samples. The reasons for this effect were the presence of eukaryotic components and sample consistency (varying from sample to sample) impeding the accessibility of the probe to its target within the cell.

It can be stressed that the FISH success in biological materials depends on hybridisation conditions (formamide concentration, incubation time, pretreatment) as well as on factors of the individual sample, such as the property of the sample and the condition of the envelope of the target cell.

D.1.4. Growth stage of bacteria and appearance of the signal

As described under C.2.2.1. (p. 77) many bacteria showed different signal intensities after FISH under the same conditions, which could be related to the growth stage of the bacteria. This phenomenon is obvious and depends on the amount of target regions and the condition of the cell wall of bacteria. The hybridisations using *Acinetobacter baumannii* cells fixed at the late exponential growth phase (OD_{600}: 0,6-0,8) resulted more frequently in intense halo signals than cells fixed at the stationary phase (OD_{600}: >1).
The observations could be explained by the increased number of targets, which consists usually of 10^5 or more ribosomes during the exponential phase and offer therefore more binding sites for a strong halo due to a stable network formation. In contrast, the amount of ribosomes during the stationary phase is approximately 10^3 in *E. coli* (DeLong et al., 1989). The lower amount of target regions led to weaker fluorescence signals. Another reason for the augmented signal intensity after FISH using bacteria, which were fixed at OD_{600}: 0,6-0,8 may be the more permeable cell wall. Both facts, the high ribosome amount and a better permeabilisation contribute to the more intense halo signals.
Therefore, pathogens present as living pure culture (Tables B.1 and B.2, p.22) were cultivated and afterwards fixed at different growth stages and then used for hybridisations on slides as well as in separation approaches. These attempts also contributed to the application of the time-reduced protocol.

D.1.5. 23S rRNA targeted polynucleotide probes versus oligo-oligonucleotide probes

The novel oligo-oligonucleotide probes allowed the differentiation of streptococci and enterococci in artificial mixed cultures by FISH. The specific signals derived from specific oligonucleotides present within the polynucleotide probes.
In previous studies, it was not possible to separate streptococci from enterococci by using polynucleotide probes due to sequence similarities of the domain III of the 23S rRNA. To discriminate bacteria by FISH at 53°C and 80 % formamide in the hybridisation buffer, a dissimilarity of 24-30 % of the domain III of the 23S rRNA is necessary (Fichtl, 2005).

D.1.6. Application of multispecific oligo-oligonucleotide probes

As the hybridisations using monospecific oligo-oligonucleotide probes were specific, it was tried to develop a polynucleotide probe containing different taxon-specific oligonucleotides, which were interrupted by the same (GCGCG) spacer fragments. A multispecific oligo-oligonucleotide probe targeting simultaneously different bacteria was designed. The procedure for the generation of this multispecific oligo-oligonucleotide probe corresponded to the synthesis of monospecific probes described under B.10.2., p. 49; Tables B.22, p. 52 and C.6, p. 99). The multispecific construct probe targeted five Gram-negative pathogens causing nosocomial infections. The multispecifc polynucleotide probe was composed of oligonucleotides tested by FISH with oligonucleotide probes (Table C.1, p. 68) as well as in hybridisations with monospecific oligo-oligonucleotide probes (Table C.4, p. 89) on slides. The already tested monospecific probes resulted at different formamide concentrations (0-30 %) in specific detection of pathogens and the different incubation times (2-6 h) posed a challenge with respect to the evaluation of an optimal FISH protocol (see C.2.5., p. 98).
First experiments using the multispecific polynucleotide probe MO1_GC showed positive results for *Acinetobacter baumannii* and *Escherichia coli* at 30 and 40 % formamide after 5 h, respectively (Table C.6, p. 99). The specific halo signals of species were detected when the corresponding oligonucleotide sequences were positioned at the termini (5´ and 3´) of the whole probe. Based on this observation, it is quite possible that the arrangement of the internal oligonucleotide probes influenced the result of hybridisations, too. It was postulated that the fragments located at the termini could access their target more effectively in comparison to internal regions of the polynucleotide probe. Based on this assumption, a second multispecific oligo-oligonucleotide probe was designed and examined (MO2_GC, table C.6, p. 99). This probe contained the oligonucleotide fragment targeting *Enterobacter aerogenes* and *Klebsiella pneumoniae* at the respective end. Specific and characteristic signals of *Enterobacter aerogenes* at 30 % and of *Acinetobacter baumannii* appeared at 20 and 30 % after 5 h.
The hybridisations performed with the first and the second multispecific oligo-oligonucleotide probe indicated that on the one hand, the arrangement of the oligonucleotide fragments could play a role concerning the hybridisation result. On

the other hand, the FISH carried out with the second multispecific probe (Table C.6, p. 99) showed that not only the position of the oligonucleotide fragment within the polynucleotide probe influenced the results. The probe, which was localised on the 3´ terminus and targeted *K. pneumoniae* did not show any signals after FISH under different conditions. It was suggested that several fragments possess a stronger affinity to their complementary counterpart.
Probably, both factors the arrangement of the probes and their ability to interact strongly and stable with the complementary region found in the bacterial organism are decisive with regard to the hybridisation result. All results obtained with the 109 nucleotides long multispecific polynucleotide probes are illustrated in figures C.30 and C.31 (p. 100).

D.1.7. Application of DNA oligo-oligonucleotide probes

Due to an easier and less time-consuming synthesis of 23S rRNA domain III targeted DNA polynucleotide probes in comparison to the generation of RNA polynucleotide probes (Zimmermann et al., 2002), monospecific DNA oligo-oligonucleotide probes were synthesised, too. These DNA oligo-oligonucleotide probes were examined for their applicability to detect bacteria by FISH. To identify *Acinetobacter baumannii* hybridisations on slides using DNA monospecific polynucleotide probes containing five adenosines or GCGCG as spacer were tested (see C.4., p.113). After several hybridisations, only whole cell fluorescence was observed. The use of probes with different spacer fragments did not change the appearance of the signal. It can be assumed that the DNA oligo-oligonucleotide probes did not form secondary structures, which generated a solid network in the area of the bacterial cell wall.
Consequently, no further DNA oligo-oligonucleotide probes targeting other pathogens were developed and applied in FISH due to the absence of the halo, which is a prerequisite for successful immobilisation experiments. These experiments rather demonstrated that these DNA oligo-oligo-polynucleotide probes are not appropriate for the separation of bacteria from different samples.

D.1.8. Future perspectives

The evaluated protocol for the development of novel types of polynucleotide probes as well as successful hybridisations demonstrated their possible application for different samples.

Nowadays, many fluorescent dyes (Cy3, Cy5, DsRed or YFP) are known which could be incorporated into further oligo-oligonucleotide probes during their generation or afterwards. This method will allow a hybridisation with several probes, which target simultaneously different organisms in one sample.

Another concept could be the design of monospecific oligo-oligonucleotide probes not only restricted to the 16S or 23S rRNA/rDNA regions. As additional targets other relevant fragments of the genome of chromosomally or plasmid mediated genetic elements are possible. A possible use is the development of a multispecific oligo-oligonucleotide probe targeting several antibiotic resistance genes. For this, the whole sequences of the same antibiotic resistance gene (of several pathogens) should be aligned. Regions containing conserved fragments could be selected for the generation of oligo-oligonucleotide probes. The alignment shows a part of *ampC* beta-lactamase gene sequences of some *Enterobacteriaceae* and other bacteria which could be used for this project (Figure D.2, p. 149).

Figure D.2 A part of the alignment (ARB software) of *ampC* gene sequences of several Gram-negative bacteria; the red area highlights the sequences of 20 nucleotides long oligonucleotide region appropriate for the generation of a **monospecific** oligo-oligonucleotide probe

```
1851       1861       1871       1881       1891      1900
|          |          |          |          |         |
GACCCGCTAC TATAAAACCG CCGCGATTAA CCAGGGGCTG GGCTGGGAAA   971   EscCo516
GACCCGCTAC TATAAAACCG CCGCGATTAA CCAGGGGCTG GGCTGGGAAA   972   KleOxyto
GACCCGCTAC TATAAAACCG CCGCGATTAA CCAGGGGCTG GGCTGGGAAA   976   KlePneu3
GACCCGCTAC TATAAAACCG CCGCGATTAA CCAGGGGCTG GGCTGGGAAA  1860   SalEnter
GACCCGCTAC TATAAAACCG CCGCGATTAA TCAGGGTCTG GGCTGGGAAA  1419   MorMorga
GTCGCGCTAT TGGCGGGCTG GGGAGATGTA TCAGGGGCTC GGCTGGGAGA  1109   EntAerog
GTCACGTTAC TGGCGTATTG GTGAGATGTA CCAGGGATTA GGCTGGGAGA  1005   CitFreun
GTCTCGCTAC TGGCGTATTG GCGATATGTA CCAGGGATTA GGCTGGGAGA   877   ProMirab
ATCTCGCTAC TGGCAAACCG GCGATATGTA TCAGGGTCTG GGCTGGGAAA   865    EscColi
TCAAGGTCGC TATCAAGTAA ATACCATGTA TCAAGCGCTT GGTTGGGAAG   889   AciBauma
TCAAGGTCGC TATCAAGTAA ATACCATGTA TCAGGCACTC GGTTGGGAAG   889   AciBaum2
GTCGCGCTAC TGGCGGGCTG GGGAGATGTA TCAGGGGCTC GGCTGGGAGA   932   EntAero2
ATCTCGCTAC TGGCAAACCG GCGATATGTA TCAGGGTCTG GGCTGGGAAA   865   EscColi|
AAAGGCAATA CCACCGGCGG GCAAAGCATT CGCGCGGGCC TGCCTGAAAG   790   KleOxyt2
TGCAGGCCGC GGGCGAACTC CCGCCGT-CA CCAACGCATC GGGTGATGGT   802   EntCloac
TGCCGGTCTG CTTCGAGGCG ACGCCTAACG GGTAATACTG CTGTTTGCCG  1046   SerMarce
CCATCGCGGT TACTACAAGG TCGGCGACAT GACCCAGGGC CTGGGCTGGG   895   PseAe253
```

The evaluation of different multispecific oligo-oligonucleotide probes is necessary to design the optimal arrangement of oligonucleotide fragments. Other parameters should be also considered. It would be very helpful to construct one polynucleotide probe, which could be used for the simultaneous identification of several pathogens in biological material. The targets can be also expanded to regions localised outside of ribosomes encoding genes for drug resistances or other clinically relevant factors. The multispecific polynucleotide probes targeting several pathogens are less cost-intensive and time-consuming than monospecific oligo-oligonucleotide probes. Figure D.3 (p. 150) shows possible regions of *ampC* beta-lactamase gene of several *Enterobacteriaceae* and other bacteria, which could be selected for the generation of a multispecific polynucleotide probe.

Figure D.3 A part of the alignment (ARB software) of *ampC* gene sequences of several Gram-negative bacteria; the red area highlights the sequences of 15 and 16 nucleotides long oligonucleotide regions appropriate for for the generation of a **multispecific** oligo-oligonucleotide probe

```
1251       1261       1271       1281       1291       1300
|          |          |          |          |          |
CTGTAAGTAA AACTTTCACA GGTGTGCTGG GTGCGGTTTC TGTGGCGAAA    394  EscCo516
CTGTAAGTAA AACTTTCACA GGTGTGCTGG GTGCGGTTTC TGTGGCGAAA    395  KleOxyto
CTGTAAGTAA AACTTTCACA GGTGTGCTGG GTGCGGTTTC TGTGGCGAAA    399  KlePneu3
CTGTAAGTAA AACTTTCACA GGTGTGCTGG GTGCGGTTTC TGTGGCGAAA   1283  SalEnter
CTGTAAGTAA AACTTTCACA GGTGTGCTGG GTGCGGTTTC CGTGGCGAAA    842  MorMorga
CTGTTAGTAA GACTTTCACC GGCGTGCTGG GCGGCGATGC GGTGGCGCGC    532  EntAerog
CGGTCAGTAA AACCTTCAAT GGTGTGCTGG GTGGCGACGC TATCGCTCGC    428  CitFreun
CGGTTAGTAA GACGTTTAAC GGCGTGTTGG GCGGCGATGC TATCGCCCGC    300  ProMirab
CGGTCAGCAA AACATTTACG GGCGTGCTTG GTGGCGACGC TATTGCTCGA    288  EscColi
CTGTCAGTAA ATTATTTACT GCGACAGCAG GTGGATATGC AAAAAATAAA    312  AciBauma
CTGTCAGTAA ATTATTTACT GCGACAGCAG GTGGATATGC AAAAAATAAA    312  AciBaum2
CTGTTAGTAA GACTTTCACC GGCGTGCTGG GCGGCGATGC GGTGGCGCGC    355  EntAero2
CGGTCAGCAA AACATTTACG GGCGTGCTTG GTGGCGACGC TATTGCTCGA    288  EscColi|
CTTATATCGC GGCGACGAGC GTTTTGCCAT GTGCAGCACC AGTAAAGTGA    330  KleOxyt2
CACCCATTCA GCCATCAGTC AGCACGTTAA GACGC----T GGAACAACAT    293  EntCloac
CAGCGCGTCG CGCCATGGCT GCGCCACCTT CACCTGCTGC AGGTTGGCGT    447  SerMarce
GCTCGGTGAG CAAGACCTTC ACCGCCACCC TCGCCGGCTA TGCCCTGGCC    315  PseAe253
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D.2. Application of RING-FISH probes

The specific detection with short labelled oligonucleotide probes is very helpful for characterisation of living, non cultivable or slowly growing bacteria in natural habitats. In recent years, RNA polynucleotide probes targeting the 23S rRNA domain III were increasingly applied for the identification and separation of bacteria (Trebesius et al., 1994). The characteristic fluorescence signal obtained after hybridisations with labelled RNA polynucleotide probes is the so-called halo. Some explanations for the halo formation were postulated (Trebesius et al., 1994; Stoffels et al., 1999). Finally, Zwirglmaier et al. (2003) suggested the “network hypothesis”. It was assumed that the polynucleotide probe binds partially to the target while the remaining part of the probe interacts with other (unbound) probes forming a network within and around the cell envelope. The protruding fragment of the probe could be also used as an anchor for complementary nucleic acids localised on the surface of microplate wells. A protocol for the detection of bacteria on slides and in microplates was developed and successfully applied in former studies (Zwirglmaier et al., 2003; Fichtl, 2005). The most important benefit of these methods is the possibility to separate bacterial cells that can be used for further analyses. The fixed bacteria are no longer cultivable but still usable for molecular studies.

In former studies, DNA was targeted (chromosomal painting) to identify whole eukaryotic chromosomes or chromosomal regions using labelled DNA probes in FISH (Lichter et al., 1988; Lawerence et al., 1990; Speicher et al., 1996). Based on DNA:DNA reassociation, bacterial chromosomal painting (BCP) was developed to identify bacteria (Lanoil and Giovannoni, 1997). The hybridisations (after 48 h) applying 100-150 nt long DNA probes allowed a differentiation of distantly related proteobacteria but they did not result in halo signals.

Meanwhile, the detection and enrichment method using RNA polynucleotide probes was extended to FISH targeting individual genes (RING-FISH) (Zwirglmaier, 2004). This opportunity expanded the detection spectrum for single genes encoding antibiotic resistances or other clinically relevant factors. The RING-FISH RNA polynucleotide probes are especially useful when the immobilisation is focused on characteristic pathogenic properties of these bacteria. Another study (Kenzaka et al., 2005) was also focused on the detection of single bacterial genes by cycling primed *in situ* amplification-fluorescence *in situ* hybridisation (CPRINS-FISH). This method

uses one primer, which hybridises to the target and results in a linear amplification of the target DNA. The product length can be several kb. Due to more than one polymerisation cycle, this technique is called cycling primed *in situ* amplification (CPRINS). Multiple labelled fluorescent probes were subsequently applied in FISH, but the hybridisation did not allow the detection of halo signals. On the one hand, this method is impaired by the balance of a sufficient permeabilisation of the cell envelope. On the other hand, the efflux of amplification products should be avoided. Nevertheless, the RING-FISH technique leads to the appearance of halos, the prerequisite for successful immobilisations. Based on this, RNA polynucleotide probes were used in the present study for the detection of single genes by FISH.

D.2.1. To be resistant or not to be resistant - genetic regulation mechanisms responsible for antibiotic resistance patterns

After Alexander Fleming discovered penicillin a member of beta-lactams in 1928, new possibilities concerning the combat of bacterial infections were established and realised by its production since 1940. Nowadays, many other antimicrobial substances with different mechanisms are known which attack dividing and non-dividing cells. In 1944, a penicillin-resistant *Staphylococcus aureus* emerged and faster than expected various resistances against common antibiotics with diverse modes of action evolved (D´Costa et al., 2006).

The conventional methods (disc diffusion test, determination of the minimum inhibitory concentration) only detect the drug insusceptibility of some antimicrobial agents at a defined antibiotic concentration.

Bacterial resistance to antibiotics is intrinsic and inherited. This is achieved by mutation or horizontal gene transfer via transformation, transduction or conjugation. The bacteria can acquire genes coding for antibiotic resistance and develop strategies to mobilise and to spread them. The respective genetic elements are usually located on a bacterial chromosome or plasmid (Sheldon, 2005). The process of transformation implicates the transfer of naked DNA (e.g. a plasmid) from the surrounding. Transduction is defined by gene transport due to infection of the bacterial host by bacteriophages or viruses. After cell lysis, some bacterial DNA could be incorporated in the viral genome during assembling of the virus particle. A direct cell-to-cell contact is necessary for the transfer of DNA via conjugation.

Apart from the ability to switch DNA sequences from one cell to another, various recombination mechanisms have evolved leading to the mobilisation of elements within the genome of a cell. The classical or homologous recombination is RecA-dependent, which enhances a recombination of highly homologue recombining DNA regions and is possible during transformation or transduction. This is typical for the development of the penicillin-binding protein gene mosaics in penicillin-resistant *Streptococcus pneumoniae* and *Neisseria gonorrhoea* (Bennett, 1999). In contrast, the transposition or non-homologous recombination requires separate transposable elements without sequence homology between recombining sites. Bacterial transposons enable the insertion of genes from chromosomal DNA to plasmid DNA and vice versa. They are often responsible for the spread of multi-drug resistances (Poirel et al., 2006). Transposons mediate their own transposition and harbour genes for proteins, which are involved in this process.
Integrons are also genetic mobile fragments located on plasmids, chromosomes and transposons, which capture genes located on short (500-1000 bp) gene cassettes and are involved in site-specific recombination. Integrons contain an integrase gene (*int*), a site necessary for the integration of DNA (*attI*) and a promoter. Normally, gene cassettes possess a single gene seldom two and a 59 base element (*attC*) essential for the insertion of the DNA at *attI* on the integron. As a promoter is not located on the cassettes, the genes are expressed from a promoter, which is present on the integron (Bennett, 1999). Based on different integrase genes the integrons are classified in class I, II and III integrons. These genetic mobile elements are often responsible for the expression of unrelated antibiotic resistance genes (Hall and Collis, 1995; Rodriguez-Martinez et al., 2006).

Although many bacteria encode antibiotic resistance genes, several factors (genetic organisation, promoter modifications, genetic background or gene copy number) play a role with regard to their expression (Reisbig et al., 2003). These facts should be also considered in clinical settings where resistant bacteria are phenotypically screened without genotypic investigations (Enne et al., 2006).

A majority of *Enterobacteriaceae* encode the chromosomally mediated *ampC* gene conferring resistance to inducible and non-inducible beta-lactamases but the low amount of beta-lactamases is not sufficient for clinically relevant resistance. In some

strains, the expression is inducible by beta-lactam antibiotics but this is also not always sufficient to trigger clinical resistance. However, overproduction of beta-lactamases can be easily achieved by mutations leading in significant resistance to antimicrobial drugs (Wiegand, 2003; Resibig et al., 2003). Inducible beta-lactamases (e.g. of *Enterobacter aerogenes*) require apart from the respective resistance gene, AmpG (permease), AmpD (amidase), AmpR (a DNA binding regulation protein) as well as an inducing substance. The cell wall recycling pathway is described in detail under C.5.2. (p. 121) Mutations e.g. on AmpD or the *ampC* promoter result in high-level expression of the chromosomal *ampC* gene. However, regulation mechanisms known from chromosomally harboured genes do not correspond to the regulation system of plasmid mediated resistance genes. Most of the plasmid-mediated *ampC* genes are not associated with *ampR* gene on the plasmid. Here the gene expression correlates with high copy-numbers of plasmids.
Additionally, recent studies indicate that the expression of drug resistances evolved by integrons. It has been shown that the position of the promoter sequence is important for the expression. The closer a cassette is located to the integron promoter the higher the level of expression and vice versa (Fluit and Schmitz, 1999).

Finally, gene silencing in *E. coli* was postulated for the emergence of unexpressed intact drug resistance genes independent on effective promoters or integrons. The expression of silent intact resistance genes is reversible (Enne et al., 2006). It can be switched on or off and is related to the changes from one host or habitat to a new one. Maybe this is responsible for initial drug susceptibility of strains, which were screened phenotypically at low concentrations of few antibiotics followed by therapy failure at different concentrations of antimicrobial substances (Enne et al., 2006).

Based on these suggestions it is not only decisive to detect the concentration dependent drug resistance to some antimicrobial agents but to investigate the genetic arsenal for possible antibiotic resistances in hospital diagnostics, too. The clinically relevant drug resistance often emerges under special conditions influenced by the surroundings or other coexistent pathogens. RING-FISH polynucleotide probes targeting individual genes could provide a tool to verify the presence of encoded but inactive drug resistance genes or genetic elements independent of their expression.

D.2.2. Selection of target organisms and target regions

The necessity of techniques, which directly determine the presence of drug resistance genes, has been mentioned under D.2.1. (p. 152). Therefore, RNA polynucleotide probes targeting antibiotic resistance genes were generated via *in vitro* transcription (see B.10.1., p. 47) and applied in hybridisations.
The selection of positive controls was focused on the real clinical situation where several pathogens coexisted with special bacteria possessing typical antibiotic resistances. In the last decades, some new tendencies of resistance patterns were observed, e.g. extended-spectrum beta-lactamases (ESBLs) among *Enterobacteriaceae*, MRSA (methicillin-resistant *Staphylococcus aureus*) or VRE (vancomycin-resistant enterococci) (D´Costa et al., 2006).

In general, many different resistance phenotypes are known of bacteria causing nosocomial infections. Nevertheless, it was not possible to generate RNA polynucleotide probes targeting all drug resistance genes of the studied pathogens because many antibiotic resistance gene sequences are not known. The completely amplified sequence or even a part of this sequence was necessary for the synthesis of labelled RNA polynucleotide probes via *in vitro* transcription (see B.10.1., p. 47). Thus, the generation of several polynucleotide probes was limited to the availability of their reference DNA sequences.

D.2.3. Application of RING-FISH probes on slides and in immobilisation experiments

For the detection and immobilisation of bacteria causing nosocomial infections polynucleotide probes from a previous study (Ludyga, 2004) were applied and new RING-FISH polynucleotide probes were designed and developed (see C.5.1., p. 114).

D.2.3.1. Modified procedure for the detection of inducible ampC gene of Enterobacter aerogenes

In a former study (Ludyga, 2004), a RING-FISH probe (1078 nucleotides long, table B.6, p. 31) targeting *ampC* gene of *Enterobacter aerogenes* was developed and halo signals were obtained after 25 hours. Wiegand (2003) described the effect of beta-lactams on *Enterobacteriaceae* harbouring a chromosomal and inducible *ampC* gene. The addition of an inducer resulted in an increased permeability of the intact cell envelope of *Enterobacteriaceae* and in the dissociation of the repressor AmpR from the target region. Evidently, the permeability of the cell envelope and the free target region could lead to a faster binding of the polynucleotide probe to its target region. Since the chromosomally encoded AmpC beta-lactamase of *E. aerogenes* is an inducible enzyme, ampicillin was added to the medium during cultivation (see B.2., p. 25). FISH experiments using the 1078 long RING-FISH probe resulted in shorter incubation times (17 to 18 h instead of 25 h) and specific halo signals of the target bacterium (Figure C.46, p. 124). Additionally, sense and antisense probes were applied in hybridisations to verify that the positive signals derive from the target-probe hybrid and not from an mRNA-probe interaction (see C.5.2., p. 121).
The condition of the cell envelope of *E. aerogenes* cells (containing inducible beta-lactamases) which colonise a patient was simulated by the addition of ampicillin *in vitro*. *In vivo*, the immune response of the host (see D.1.3., p. 144) contributed to a more permeable cell envelope. Moreover, it was assumed that a possible treatment of the patient with beta-lactams (acting as inducers) could affect the cell wall of this bacterium. Both factors could lead to the formation of halo signals after a shorter incubation time and the condition of the cell envelope of treated cells rather

corresponded to the conditions of pathogens than to those of untreated *E. aerogenes* cells cultivated under laboratory conditions.

D.2.3.2. Detection of bacteria in pure culture, spiked and real clinical material on slides

Similar to FISH using oligo-oligonucleotide probes the hybridisations using RING-FISH polynucleotide probes started in pure cultures. Parameters such as denaturation or incubation time, formamide concentrations or necessary pretreatments of the cell envelope were studied in detail. The problems concerning RING-FISH application are the low copy number of the target and the unknown conformation of the target DNA molecule (supercoiled or open circular). In contrast to rRNA targeted probes, the gene of interest exists in some copies or only in one. The correctness of all templates was determined by sequencing. New RING-FISH probes (Tables B.9-B.13, pp. 32-34) were designed and comparative sequence analyses are listed in the appendix (part F, pp. 172-176). Furthermore, potential secondary structures were calculated (Matzura et al., 1996) and possible configurations are shown in the appendix (part F, pp. 177-180). Finally, for all tested pathogens characteristic signals in the periphery of the cell (the typical halo) as well as intermediate signals or seldom whole cell fluorescence were observed. The exact conditions are listed in table C.9. (p. 120).

Almost all hybridisations using RNA polynucleotide probes resulted in specific halo signals at 0 % or 10 % formamide. Sometimes negative controls (*Neisseria canis* or *Haemophilus influenzae*) showed positive signals, too. In hybridisations applying the 23S rRNA domain III targeted probes, the stringent conditions were assured by a high (80 %) formamide concentration (Fichtl, 2005). Formamide concentrations > 10 % resulted in unspecific signals of non-target cells. Formamide concentrations > 40 % resulted in accumulation of the probe around the cell (looking like a comet) or in signal loss. The unspecific fluorescence signals could arise from the probe length, which possesses possible binding sites for several genome fragments of other bacteria. The interaction with other sequences could be augmented by the formation of secondary structures leading to new sequence combinations. Another possibility, which should be considered, is that after a long hybridisation time (longer than 20

hours), RNases occurring in the environment could damage the sensitive RNA polynucleotide probes. Hence, the smaller fragments could hybridise to several regions resulting in unspecific signals. As a precaution, RNase inhibitors were added to the buffer during and after the *in vitro* transcription. To ascertain that the polynucleotide probes remained intact after FISH, the unbound fraction was analysed by gel electrophoresis. On the one hand, the investigation demonstrated that the polynucleotide probes were complete even after extended incubation. On the other hand, it cannot be excluded that the probes, which bind to their target inside the cell were damaged. Even though the signals and the specificity were not perfect, the evaluated conditions (Table C.9, p. 120 and C.5.1.4., p. 116) were considered as stringent because other clinically more important non-target bacteria (*M. morganii*, *P. aeruginosa, E. coli*, *S. aureus*, *S. epidermidis*) exhibited no positive signals. In several cases, a subsequent hybridisation using taxon-specific oligonucleotide probes (oligo-FISH) was performed to test whether cells showing the halo could be detected by these short probes. In most cases, the attempts were successful (Figure C.46 D, p. 124). In general, the combined hybridisation with polynucleotide probes prior to oligo-FISH or vice versa is difficult due to different formamide concentrations and incubation temperatures. When RING-FISH (see B.9.2., p. 42) was performed before oligo-FISH (see B.9.1., p. 38), the network around and within the cell envelope could hamper the accessibility of the oligonucleotide probe to its target inside the cell. In contrast, when oligo-FISH is performed before RING-FISH, the denaturation step (80°C) followed by a higher hybridisation temperature (53°C) could destabilise the oligonucleotide probe-target hybrid and may lead to signal loss.

The evaluated protocols in pure cultures were tested in spiked clinical samples. The aim of these tests was to investigate whether the RING-FISH probes were applicable in clinical specimens, too. For *Pseudomonas fluorescens*, the *Enterobacteriaceae* and both *Enterococcus* spp. spiked specimens were available (Table B.3, p. 23). Mostly, the target cells showed halos. However, in some cases intermediate or fluorescence signals distributed over the whole cell were observed. Background fluorescence of non-bacterial origin also occurred and hampered the microscopic visualisation. The autofluorescence was confirmed by hybridisations without any probes resulting in the same appearance of the signal.

Hybridisations using RING-FISH probes in real clinical material demonstrated that these probes are usable for the laboratory routine in hospitals. For methicillin-resistant *Staphylococcus aureus,* one real sample was available (rump swab, number 8, table B.4, p. 24). Ampicillin-resistant *E. coli* was available in three real clinical samples (vaginal smear, wound smear and urine; number 2, 10 and 15 respectively; table B.4, p. 24). The signals occurring after hybridisations using RING-FISH probes differed sometimes from the typical ring-shaped fluorescence and not every cell could be detected.

The results with RING-FISH polynucleotide probes indicated that this type of polynucleotide probes might be appropriate for the detection and identification of antibiotic resistance genes present in pathogens from clinical samples. Moreover, the effects showed that the hybridisations in different samples could lead to signal ranging from the halo or intermediate to whole cell fluorescence signal dependent on the composition of the clinical material. These data demonstrate again (see D.1.2.2., p. 138) that a defined protocol for one RING-FISH probe especially concerning the hybridisation time is not possible for all samples (pure culture, spiked and clinical material). The incubation times of hybridisations in spiked samples needed an additional hour in comparison to hybridisations in pure cultures (Table C.9, p. 120) because of reduced cell numbers and interfering components. In contrast, the incubation times of hybridisations in real clinical samples could be reduced (minus 1 h in comparison to incubation times in table C.9, p. 120) because of better permeabilisation of the bacterial cell envelope due to the immune response of the host. In summary, for the development of an adequate procedure, not only the hybridisation time and formamide concentrations play an important role but also the composition of the sample as well as the condition of bacterial cells (see D.1.2.2., p. 138).
Nevertheless, successful detection of resistance genes of pathogens by RING-FISH indicated that these polynucleotide probes could be also applied for genotypic analyses in combination with phenotypic investigations in the clinical routine.

D.2.3.3. The role of the GC content of the target region concerning the FISH protocol

The GC content of each RING-FISH polynucleotide probe was also considered in the evaluation of optimal FISH conditions on slides and in microplates. The hypothesis (Pavlekovic, unpublished) suggested when the GC content is higher than 60 mol % the hybridisation could not result in halo signals due to high formamide concentrations or increased temperatures. It was assumed that under such strong conditions an interaction of the polynucleotide probe with the target is impossible due to completely linearised nucleic acids. Thus, the molecules are not able to form secondary structures and a network. To perform hybridisations at 53°C at different formamide concentration, the artificial (high) temperature was calculated as described by McConaughy et al. (1969). 1 % formamide lowers the melting temperature by 0.72°C.

Despite the high GC content (66 mol %; Table C.9, p. 120) of the RING-FISH probe targeting *K. pneumoniae* the hybridisation resulted in halo signals. Probably, the denaturation at 80°C and hybridisation at 53°C contributed to partial linearisation of the probe as well as of the target region and enabled the halo formation. Increased formamide concentrations led to signal loss (see C.5.1.4., p. 116). In contrast, the methicillin resistance gene of *S. aureus* possesses a low GC content (31 mol %) hence the hybridisations were performed at lower temperature (50°C), too. The hybridisations at reduced temperature did not change the hitherto occurring appearance of the signal at 53°C. Intermediate and whole cell fluorescence signals of *S. aureus* were still detected. These results show that in the present study the GC content did not have an impact on the FISH protocol as to formamide concentration, denaturation time or temperature.

D.2.3.4. The impact of the probe length with regard to the protocol

The effect of the probe length on the formation of secondary structures was also determined. Table C. 9 (p. 120) comprises the length of the whole resistance genes as well as of the target regions for which RING-FISH probes were developed. The length of the probes varies from 188 (*aac(6´)* gene of *P. fluorescens*) to 1078 nucleotides (*ampC* gene of *E. aerogenes*).

The analysis of secondary structures (RNAdraw V1.1b2; Matzura et al., 1996) of the shortest probes (188, 282 nt) targeting a drug resistance gene of *P. fluorescens* and the 336 nt long probe targeting the antibiotic resistance gene of *Klebsiella oxytoca* indicated that secondary structures (Appendix, part F, pp. 177-180) can be generated under the applied hybridisation conditions (Table C.9, p. 120). Finally, the FISH results confirmed that halo signals could be also obtained with even 188 nt "short" polynucleotide probes. A positive side effect was the reduction of the incubation time for FISH with Gram-negative bacteria when shorter probes were used (with the exception of *E. aerogenes* described under D.2.3.1., p. 156). The application of these probes (targeting $bla_{OXY\text{-}2\text{-}5}$ gene of *K. oxytoca* or *aac(6´)* gene of *P. fluorescens*) allowed a shorter hybridisation time because the probes may penetrate the cell envelope faster. The reduced incubation time has some advantages. Inter alia, shorter hybridisation times lead to a more rapid identification of the pathogenic bacteria. Moreover, these findings suggested that short polynucleotide probes such as oligo-oligonucleotide probes (110 nucleotides) in addition to 23S rRNA domain III targeted polynucleotide probes could generate secondary structures, too. Thus, neither the length of the probe nor their GC content is solely responsible for the appearance of halo signals because also other factors may play a role in the signal formation.

D.2.3.5. Separation of bacteria from pure culture, spiked and real clinical material and possible explanations for failed immobilisation

The protocol that was developed for the cell sorting with oligo-oligonucleotide probes (see C.3.1., p. 101) was also applied for immobilisations using RING-FISH probes (see C.6.1., p. 128).

The immobilisation experiments carried out for pure cultures were also applied for spiked and for real clinical material. Table C.10 (p. 130) summarises the results obtained with RING-FISH probes from pure culture and spiked clinical samples. In general, all strains were successfully immobilised from pure culture showing that the enrichment with these probes is possible under the applied conditions. *E. coli* enriched from spiked blood samples, *P. fluorescens* and both *Enterococcus* spp. were separated from clinically relevant dilutions (10^{-3}, 10^{-4}). *A. baumannii*, *E. coli*, *K. oxytoca* and *K. pneumoniae* could not be enriched from spiked urine and plasma samples containing these bacteria in clinically realistic numbers. In some cavities, target cells could be found in the supernatant as shown by a positive PCR signal. Repeated immobilisation in fresh coated cavities increased the efficiency of the separation. Ineffective immobilisations of several bacteria from spiked and real clinical material were probably dependent on the composition of the sample. Possibly, eukaryotic cell components as well as the consistency of each specimen hampered the accessibility of the RING-FISH probe to its target cell. Another aspect is whether the polynucleotide probe really remained undamaged during the whole incubation time. It could be suggested that the polynucleotide probe was fragmented followed by hybridisation of shorter sequences to the target. That effect will make the network formation impossible. A further possible explanation is the loss of enriched cells due to subsequent washing steps.

The enrichments of pathogens from real samples were successful (Table C.11, p. 132). Altogether four real clinical samples were available containing *S. aureus* or *E. coli* as the only one pathogen or with coexistent bacteria used as natural non-target bacteria. Since both positive and negative control organisms were present, subsequent cloning and sequencing of respective PCR products were necessary to identify enriched and non-enriched pathogens. The sequencing data revealed a specific enrichment of *S. aureus* from the wound swab number 8 (Table B.4, p. 24)

by RING-FISH probes. In the case of ampicillin-resistant *E.coli* in the urine sample number 15 (Table B.4, p. 24), which also contained *Enterococcus faecalis,* the specific separation could be verified by sequencing. Additionally, the sequence analysis performed with BLAST (Basic Local Alignment Search Tool) and the ARB software indicated that some non-enriched target cells remained in the supernatant. The wound swab number 10 (Table B.4, p. 24) contained *E. coli* and *K. terrigena*, which are resistant to ampicillin. Consequently, both species were enriched indicating that antibiotic resistance genes can be detected by RING-FISH in bacteria belonging to different taxa. The reason for harbouring an antibiotic resistance gene with similar or the same sequence by these genera could be the high similarity of beta-lactamase genes among *Enterobacteriaceae* (Wiegand, 2003). Another explanation consists in horizontal gene transfer, which is likely between pathogens present in the same clinical sample. The successful immobilisations of pathogens from real clinical samples may be due to the effect of the immune system (see D.1.3., p. 144) on the permeabilisation of the cell envelope of pathogens.

The present study demonstrates the possible application of RING-FISH in clinical diagnostics if the particular material consistency and composition are considered. This technique combines a gene-dependent separation of pathogens with a subsequent investigation of the bacterial genome in one cavity. For the analysis of enriched or non-enriched pathogens, many other common molecular approaches are available such as several PCR-methods, FISH, FACS or antibody-based assays.

D.2.4. Future perspectives

The development of RING-FISH probes may be helpful for the genotypic detection of antibiotic resistance genes whereas the specificity spectrum, which is strongly correlated with the length of the probe, will still pose a challenge. Furthermore, a specific detection of resistant organisms based only on the RING-FISH probe is not possible because of high sequence similarity e.g. among the *ampC* beta-lactamase gene sequences of several *Enterobacteriaceae* (Wiegand, 2003). In this case, the detection of the antibiotic resistance gene is more important than the taxonomic identity of the pathogen. With regard to clinical medication nowadays, the identification of pathogens is not always performed up to the species level. It is not only important which bacterium is the infectious agent but rather which drug is effective against it. In the meantime, many sequences (of antibiotic resistance genes) are available for comparative sequence analyses. For the genotypic determination of pathogens, a polynucleotide probe targeting (a part of) the bacterial antibiotic resistance gene of several bacteria could be designed. Therefore, the target region should be highly similar to ensure a correct identification of resistant bacteria. Figure D.4 (p. 165) shows a part of *ampC* gene sequence alignment of some Gram-negative bacteria. This fragment (about 300 nt) could be appropriate for the generation of the resistance gene related RING-FISH probe targeting *ampC* genes of several *Enterobacteriaceae* and other pathogens causing nosocomial infections.
Based on this concept, the development of other types of polynucleotide probes could be realised. These probes could be also applied for the determination of pathogenic and apathogenic representatives of the same taxonomic level isolated from different clinical material e.g. *S. aureus* from skin or blood samples. Furthermore, the usage of gene related RNA polynucleotide probes for several examinations is possible. These probes can be availed in epidemiological studies to investigate the origin of resistance (or pathogenicity) and transmission e.g. via horizontal gene transfer. Additionally, chosen sequence fragments containing conserved and variable regions could serve for taxonomic analyses and probably enable a detection of bacteria comparable to the results obtained with 23S rRNA domain III targeted polynucleotide probes.

Figure D.4 A part of the alignment (ARB software) of *ampC* gene sequences of several Gram-negative bacteria which could be applied for the generation of novel about 300 nt long RING-FISH probes

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1101       1111       1121       1131       1141       1151       1161       1171       1181       1191       1200
|          |          |          |          |          |          |          |          |          |         |
ATTAAACCGC TGATGGCACA GCAGGATATT CCCGGGATGG CGG----TTG CCGTCTCCGT AAAGGGTAAG CCCTATTATT TCAATTAT-G GTTTTGCCGA   294   EscCo516
ATTAAACCGC TGATGGCACA GCAGGATATT CCCGGGATGG CGG----TTG CCGTCTCCGT AAAGGGTAAG CCCTATTATT TCAATTAT-G GTTTTGCCGA   295   KleOxyto
ATTAAACCGC TGATGGCACA GCAGGATATT CCCGGGATGG CGG----TTG CCGTCTCCGT AAAGGGTAAG CCCTATTATT TCAATTAT-G GTTTTGCCGA   299   KlePneu3
ATTAAACCGC TGATGGCACA GCAGGATATT CCCGGGATGG CGG----TTG CCGTCTCCGT AAAGGGTAAG CCCTATTATT TCAATTAT-G GTTTTGCCGA  1183   SalEnter
ATTAAACCGC TGATGGCACA GCAGGATATT CCCGGGATGG CGG----TTG CCGTCTCCGT AAAGGGTAAG CCCTATTATT TCAATTAC-G GTTTTGCCGA   742   MorMorga
ATCACGCCGT TAATGAAAGA ACAGGCGATA CCGGGCATGG CGG----TGG CGGTGATCTA TCACGGCAAA CCCTATTATT TCACCTGG-G GCCAGGCCGA   432   EntAerog
ATCACACCAC TGATGAAAGA ACAAGCGATT CCGGGTATGG CCG----TGG CAATCATCTA CCAGGGAAAA TCGTATTATT TTACCTGG-G GTAAAGCTGA   328   CitFreun
ATCACCCCGT TGATGCAGGA GCAGGCTATT CCGGGTATGG CCG----TTG CCGTTATCTA CCAGGGAAAA CCCTATTATT TCACCTGG-G GTAAAGCCGA   200   ProMirab
ATTACCCCGC TTATAGAGCA ACAAAAGATC CCCGGTATGG CGG----TAG CGGTAATTTA TCAGGGTAAA CCTTATTACT TTACCTGG-G GCTATGCGGA   188   EscColi
TTTAAACCGT TATTAGAAAA ATATGATGTG CCGGGTATGG CTG----TGG GTGTTATTCA AAATAATAAA AAGTATGAAA TGTATTAT-G GTCTTCAATC   212   AciBauma
TTTAAACCGT TATTAGAAAA ATATGATGTG CCGGGTATGG CTG----TGG GTGTTATTCA AAATAATAAA AAGTATGAAA TGTATTAT-G GTCTTCAATC   212   AciBaum2
ATCACCCCGT TAATGAAAGA ACAGGCGATA CCGGGCATGG CGG----TGG CGGTGATCTA TCACGGCAAA CCCTACTATT TCACCTGG-G GCCAGGCCGA   255   EntAero2
ATTACCCCGC TTATAGAGCA ACAAAAGATC CCCGGTATGG CGG----TAG CGGTAATTTA TCAGGGTAAA CCTTATTACT TTACCTGG-G GCTATGCGGA   188   EscColi|
GTTCCGCTGC TGCTGGCG-A GCGGCGCACT -----GTGGG CCA----GTA CCGATGCTAT CCATCAGAAG CTGACAGATC TCGAGAAG-C GTTCAGGCGG   235   KleOxyt2
TTAATTTTTC TAACGGATGG GTCGATGACA C--------- ---------G CAGCTATTTA CCGC--TTAA TTCGCTTCGT GCCTTCGA-A GCC-------   200   EntCloac
CGTGATGGTG GTGGCCGGCG TGCCGTTCAT GATCATCCCG GCGTTGTTGC CTTCAATCAA ACGCGACAGT TTCACTGGAT ACGGGTAGTT CTCCCACATC   347   SerMarce
ACGCCGCCGT ACAACCGGTG ATGAAGGCCA ATGACATTCC GGGCCTGGCC GTAGCCATCA GCCTGAAAGG AGAACCGCAT TACTTCAGCT ATGGGCTGGC   215   PseAe253

1201       1211       1221       1231       1241       1251       1261       1271       1281       1291       1300
|          |          |          |          |          |          |          |          |          |         |
TATTCAGGCA AAACAGCCGG TCACTGAAAA TACACTATTT GAGCTCGGAT CTGTAAGTAA AACTTTCACA GGTGTGCTGG GTGCGGTTTC TGTGGCGAAA   394   EscCo516
TATTCAGGCA AAACAGCCGG TCACTGAAAA TACACTATTT GAGCTCGGAT CTGTAAGTAA AACTTTCACA GGTGTGCTGG GTGCGGTTTC TGTGGCGAAA   395   KleOxyto
TATTCAGGCA AAACAGCCGG TCACTGAAAA TACACTATTT GAGCTCGGAT CTGTAAGTAA AACTTTCACA GGTGTGCTGG GTGCGGTTTC TGTGGCGAAA   399   KlePneu3
TATTCAGGCA AAACAGCCGG TCACTGAAAA TACACTATTT GAGCTCGGAT CTGTAAGTAA AACTTTCACA GGTGTGCTGG GTGCGGTTTC TGTGGCGAAA  1283   SalEnter
TGTTCAGGCA AAACAGCCTG TCACTGAAAA TACACTATTT GAGCTCGGAT CTGTAAGTAA AACTTTCACA GGTGTGCTGG GTGCGGTTTC CGTGGCGAAA   842   MorMorga
TGTTGCCGGG CAGCGTCCGG TGACGCGACA GACGTTGTTT GAGCTGGGCT CTGTTAGTAA GACTTTCACC GGCGTGCTGG GCGGCGATGC GGTGGCGCGC   532   EntAerog
TATTGCCAAT AATCGCCCTG TTACACAGCA AACGCTGTTT GAACTCGGAT CGGTCAGTAA AACCTTCAAT GGTGTGCTGG GTGGCGACGC TATCGCTCGC   428   CitFreun
TATCGCCAAT AACCACCCAG TCACGCAGCA AACGCTGTTT GAGCTAGGAT CGGTTAGTAA GACGTTTAAC GGCGTGTTGG GCGGCGATGC TATCGCCCGC   300   ProMirab
CATCGCCAAA AAGCAGCCCG TCACACAGCA AACGTTGTTT GAGTTAGGTT CGGTCAGCAA AACATTTACG GGCGTGCTTG GTGGCGACGC TATTGCTCGA   288   EscColi
TGTTCAAGAT AAAAAAGCCG TAAATAGCAG TACCATTTTT GAGCTAGGTT CTGTCAGTAA ATTATTTACT GCGACAGCAG GTGGATATGC AAAAAATAAA   312   AciBauma
TGTTCAAGAT AAAAAAGCCG TAAATAGCAG TACCATTTTT GAGCTAGGTT CTGTCAGTAA ATTATTTACT GCGACAGCAG GTGGATATGC AAAAAATAAA   312   AciBaum2
TGTTGCCGGG CAGCGTCCGG TGACGCGACA GACGTTGTTT GAGCTGGGCT CTGTTAGTAA GACTTTCACC GGCGTGCTGG GCGGCGATGC GGTGGCGCGC   355   EntAero2
CATCGCCAAA AAGCAGCCCG TCACACAGCA AACGTTGTTT GAGTTAGGTT CGGTCAGCAA AACATTTACG GGCGTGCTTG GTGGCGACGC TATTGCTCGA   288   EscColi|
CAGGTTGGGC GTGGCGCTAA TCAAC-ACGG CAGATAATT- ---CTCAAAT CTTATATCGC GGCGACGAGC GTTTTGCCAT GTGCAGCACC AGTAAAGTGA   330   KleOxyt2
-GCCGCAAGG CACC--TCAG TTTTACTAAT GCTGCCATTG AGCTGAATGT CACCCATTCA GCCATCAGTC AGCACGTTAA GACGC----T GGAACAACAT   293   EntCloac
AGATCCTGCG TGAACGCGCC CGCCTTGTAA TACCCGACGT GCGTCGCGGC CAGCGCGTCG CGCCATGGCT GCGCCACCTT CACCTGCTGC AGGTTGGCGT   447   SerMarce
CTCGAAAGAG GACGGCCGCC GGGTGACGCC GGAGACCCTG TTCGAGATCG GCTCGGTGAG CAAGACCTTC ACCGCCACCC TCGCCGGCTA TGCCCTGGCC   315   PseAe253

1301       1311       1321       1331       1341       1351       1361       1371       1381       1391       1400
|          |          |          |          |          |          |          |          |          |         |
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AAAGAGATGG CGCTGAATGA TCCGGCGGCA AAATACCAGC CGGAGCTGGC TCTGCCGCAG TGGAAGGGGA TCAC-ATTGC TGGATCTGGC TACCTATACC   494   KleOxyto
AAAGAGATGG CGCTGAATGA TCCGGCGGCA AAATACCAGC CGGAGCTGGC TCTGCCGCAG TGGAAGGGGA TCAC-ATTGC TGGATCTGGC TACCTATACC   498   KlePneu3
AAAGAGATGG CGCTGAATGA TCCGGCGGCA AAATACCAGC CGGAGCTGGC TCTGCCGCAG TGGAAGGGGA TCAC-ATTGC TGGATCTGGC TACCTATACC  1382   SalEnter
AAAGAGATGA CGTTGAATGA CCCGGCAGAA AAATACCAGC CGGAGCTGGC TCTGCCGCAG TGGAAGGGGA TCAC-ACTGC TGGATCTGGC TACCTACACC   941   MorMorga
GGTGAAATTA AGCTCAGCGA TCCGGCGCAG AAGTACTGGC CGCAGCTCAC CGGCCAGCAG TGGCAGGGGA TCTC-GCTGC TGCATCTCGC CACCTATACC   631   EntAerog
GGTGAAATCA AACTTAACGA TCCAGTCACC CGATACTGGC CCGAACTGAC AGGCAAACAG TGGCAGGGTA TCAG-CTTGC TGCACCTGGC CACTTACACA   527   CitFreun
GGCGAAATTA AGCTCAGCGA TCCGGTCACG AAATACTGGC CAGAACTGAC AGGCAAACAG TGGCAGGGTA TCCG-CCTGC TGCACTTAGC CACCTATACG   399   ProMirab
GGGGAAATCA AGTTAAGCGA TCCCACAACA AAATACTGGC CTGAACTTAC CGCTAAACAG TGGAATGGGA TCAC-ACTAT TACATCTCGC AACCTACACC   387   EscColi
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GGAAAAATCT CTTTTGACGA TACGCCTGGT AAATATTGGA AAGAACTAAA AAATACACCG ATTGACCAAG TTAA-CTTAC TTCAACTCGC GACGTATACA   411   AciBaum2
GGTGAAATTA AGCTCAGCGA TCCGGCGCAG AAGTACTGGC CGCAGCTCAC CGGCCAGCAG TGGCAGGGGA TCTC-GCTGC TGCATCTCGC CACCTATACC   454   EntAero2
GGGGAAATCA AGTTAAGCGA TCCCACAACA AAATACTGGC CTGAACTTAC CGCTAAACAG TGGAATGGGA TCAC-ACTAT TACATCTCGC AACCTACACC   387   EscColi|
TGGCCGCCGC CGCGGTATTA AAACAGAGCG AA-------- ----AG---- --C------A ATAAAGAGGT GGTAAATAAA AGGCTGGAGA T-----TAAC   401   KleOxyt2
CTGAACTGTC AGCTGTTTGT CCGGGTTTCG CGCGGATTGA TGCTGACGAC AGAGGGCGAG AATCTGCTGC CGGT-GCTGA ACGATTCGTT TGATCGTATC   392   EntCloac
CCAGATAGCG AATCAGATCG CGAGCGTTGG ACTTGATGCC GTAAGATTCG GCGTCCAGCG GGCCGGGGTT GACCCGCACC GGCTTATCGT CCTTGCTGTA   547   SerMarce
CAGGACAAGA TGCGCCTCGA CGACCGCGCC AGCCAGCACT GGCCGGCACT GCAGGGCAGC CGCTTCGACG GCATCAGCCT GCTCGACCTC GCGACCTATA   415   PseAe253
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E. Summary

In the present study, novel types of RNA polynucleotide probes (oligo-oligonucleotide and RING-FISH probes) were generated and hybridisations using these probes were successfully adapted to the FISH technique. These polynucleotide probes are useful for the identification of bacteria on slides as well as for immobilisations in coated microplates.

For the characterisation of bacteria causing nosocomial infections two strategies were chosen to design polynucleotide probes. The oligo-oligonucleotide probes consist of an alternating sequence of repetitive oligonucleotide fragments and spacer regions. These probes combine the advantages of specific short oligonucleotide probes and the potential of polynucleotide probes to form secondary structures. For the synthesis of monospecific probes (containing equal taxon-specific fragments) targeting one pathogen and multispecific probes (containing different taxon-specific fragments) targeting several pathogens, an effective procedure was established for the first time. In the course of this study, subsequent development, adaptation and improvement of former hybridisation protocols allowed the successful application of these probes for the identification and immobilisation of target cells. The approaches were focused on the evaluation of an efficient FISH technique and on the optimisation of an adequate pretreatment of the cell wall of Gram-positive bacteria. The established protocol could be successfully adapted to identify and enrich target bacteria from spiked and real clinical samples.

The second attempt dealt with RING-FISH probes, which were applied for the detection of antibiotic resistance genes harboured by these pathogens. Concerning the generation of RING-FISH probes, modifications of the protocol, sample treatment and appropriate permeabilisation of the cell envelope of Gram-positive bacteria were also necessary for a successful performance. Moreover, the separation of bacteria from different spiked and real clinical material could be realised using oligo-oligonucleotide and RING-FISH probes.

In both approaches, the major aspects were concentrated on a situation, which is typical for real clinical specimens. For this purpose, the selection of potential target and non-target cells was adapted to the possible coexistence in real clinical material. Furthermore, mostly spiked clinical samples were used which contained the positive

control organism in an authentic bacterial count when real specimens were not available. It was important to see that the sample composition and consistency as well as the host-pathogen interplay have a great impact on the hybridisation result. All these essential factors were integrated in the entire concept with respect to develop adequate protocols.
Finally, efficient immobilisations of pathogens from spiked and real clinical samples demonstrate their applicability in the clinical diagnostics. Further advantages of the immobilisation-technique consist in the combination of the separation followed by subsequent analyses of the genome from enriched or non-enriched bacteria by additional molecular methods.

E. Zusammenfassung

Im Rahmen dieser Forschungsarbeit wurden neuartige RNS Polynukleotidsondentypen (Oligo-oligonukleotid- und RING-FISH-Sonden) entwickelt und die Hybridisierungen unter Verwendung dieser Sonden erfolgreich an die FISH-Technik angepasst. Diese Polynukleotidsonden eignen sich sowohl zur Identifizierung von Bakterien auf Objektträgern als auch zur Immobilisierung in beschichteten Mikrotiterplatten.

Zur Bestimmung von Hospitalkeimen wurden zwei Strategien zur Entwicklung von Polynukleotidsonden ausgewählt. Die Oligo-oligonukleotidsonden bestehen aus einer alternierenden Sequenz aus repetitiven Oligonukleotidfragmenten und Spacerregionen. Diese Sonden vereinen die Vorteile von kurzen spezifischen Oligonukleotidsonden und die Eigenschaft von Polynukleotidsonden, Sekundärstrukturen auszubilden. Für die Herstellung von monospezifischen Sonden (enthalten gleiche taxonspezifische Fragmente), die gegen einen Krankheitserreger gerichtet sind und von multispezifischen Sonden (enthalten verschiedene taxonspezifische Fragmente), die gegen mehrere Krankheitserreger gerichtet sind, wurde zum ersten Mal ein effektives Verfahren etabliert. Im Zuge der Forschungsarbeit erlaubte die anschließende Entwicklung, Anpassung und Verbesserung bisheriger Hybridisierungsprotokolle die erfolgreiche Anwendung dieser Sonden zur Identifizierung und Immobilisierung von Zielzellen. Die Ansätze konzentrierten sich auf die Evaluierung einer effizienten FISH-Technik und die Optimierung einer entsprechenden Vorbehandlung der Zellwand Gram-positiver Bakterien. Das etablierte Protokoll eignete sich ebenfalls, um Zielorganismen aus versetzten Proben und realem klinischen Material erfolgreich nachzuweisen und zu immobilisieren.
Der zweite Ansatz befasste sich mit RING-FISH-Sonden, die der Detektion von Antibiotikaresistenzgenen dieser Krankheitserreger dienen. Hinsichtlich der Entwicklung von RING-FISH-Sonden und der erfolgreichen Durchführung waren Protokollmodifizierungen, Probenbehandlung und eine angemessene Permeabilisierung der Zellhülle Gram-positiver Bakterien erforderlich. Zusätzlich konnte die Separierung von Bakterien aus unterschiedlichen versetzten und realen

klinischen Proben unter Einsatz der Oligo-oligonukleotid- und RING-FISH-Sonden verwirklicht werden.
In beiden Ansätzen waren die Hauptaspekte auf die typischen Bedingungen in realen klinischen Proben ausgerichtet. Aus diesem Grund erfolgte die Auswahl der Ziel- und Nichtzielorganismen entsprechend der möglichen Koexistenz in realem klinischem Material. Darüber hinaus wurden meistens versetzte klinische Proben verwendet, die die Positivkontrolle in authentischer Keimzahl enthielten, wenn reales Material nicht vorhanden war. In diesem Zusammenhang war es wichtig, zu erkennen, dass sich sowohl die Probenzusammensetzung und Konsistenz als auch die Wirt-Pathogen-Wechselwirkung auf den Hybridisierungserfolg stark auswirkt. All diese grundlegenden Faktoren wurden bezüglich der Ermittlung eines geeigneten Protokolls in das gesamte Konzept mit einbezogen.
Schließlich zeigen die effizienten Immobilisierungen der Krankheitserreger aus versetzten und realen klinischen Proben, dass diese Sonden in klinischen Diagnostikverfahren einsetzbar sind. Weitere Vorteile der Anreicherungstechnik beruhen auf der Vereinigung der Separierung mit anschließenden Analysen des Genoms angereicherter beziehungsweise nicht angereicherter Bakterien mit Hilfe von zusätzlichen molekularbiologischen Methoden.

F. Appendix

F.1. Secondary structure models of oligo-oligonucleotide probes under denaturation and hybridisation conditions

Since the secondary structure models of all oligo-oligonucleotide probes are similar only some selected models are presented here. All models were calculated under denaturation and hybridisation conditions with respective formamide concentrations in the buffer (McConaughy et al., 1969).

F.1.1. Monospecific oligo-oligonucleotide probes

Figure F.1.1 Secondary structure model (RNAdraw V1.1b2, Matzura et al., 1996) of monospecific RNA oligo-oligonucleotide probe PseaerB_GC (Table C.2, p. 75) with 20 % formamide in the buffer (Table C.4, p. 89)
A: under denaturation conditions at 80°C (94°C), **B:** under hybridisation conditions at 53°C (67°C)

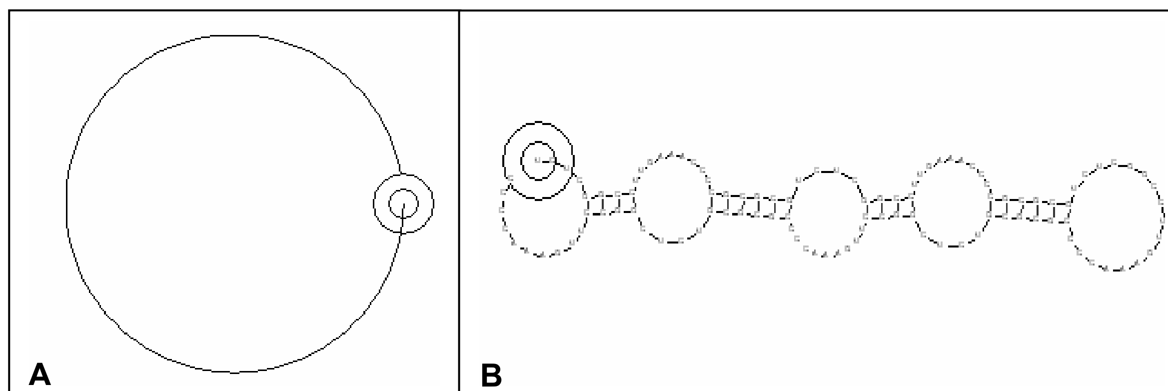


Figure F.1.2 Secondary structure model (RNAdraw V1.1b2, Matzura et al., 1996) of monospecific RNA oligo-oligonucleotide probe Kpn_GC (Table C.2, p. 75) with 20 % formamide in the buffer (Table C.4, p. 89) **A:** under denaturation conditions at 80°C (94°C), **B:** under hybridisation conditions at 53°C (67°C)

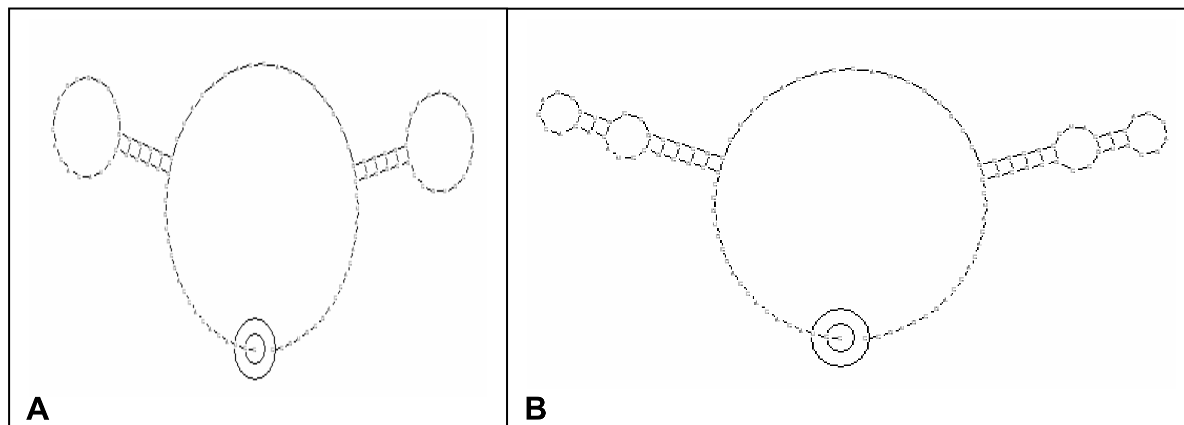
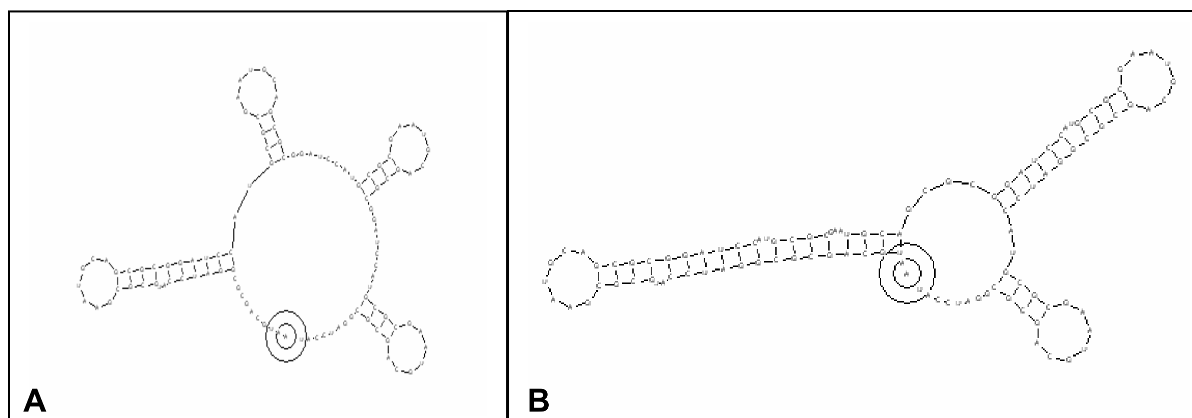
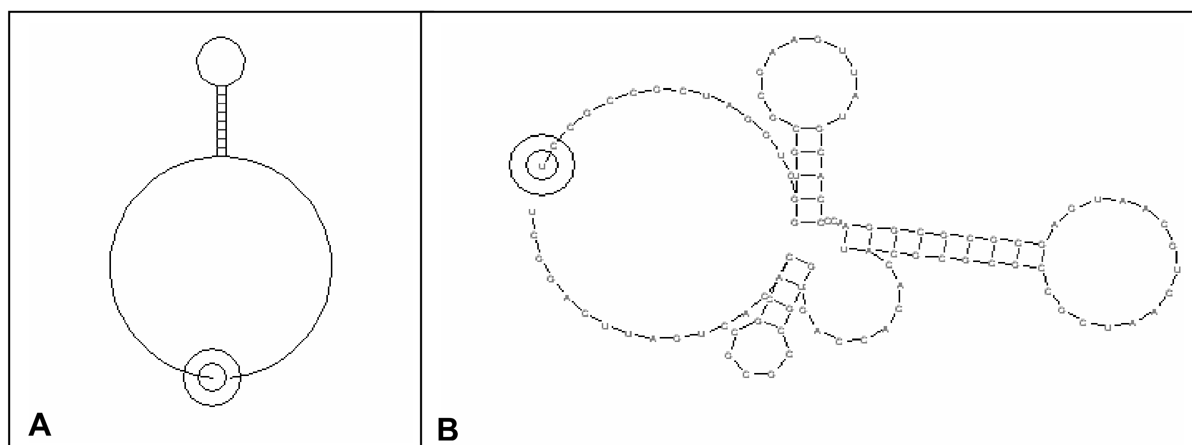


Figure F.1.3 Secondary structure model (RNAdraw V1.1b2, Matzura et al., 1996) of monospecific RNA oligo-oligonucleotide probe Sau227_GC (Table C.2, p. 75) with 10 % formamide in the buffer (Table C.4, p. 89)
A: under denaturation conditions at 80°C (87°C), **B:** under hybridisation conditions at 53°C (60°C)



F.1.2. Multispecific oligo-oligonucleotide probes

Figure F.1.4 Secondary structure model (RNAdraw V1.1b2, Matzura et al., 1996) of multispecific RNA oligo-oligonucleotide probe MO1_GC with 30 % formamide in the buffer (Table C.6, p. 99)
A: under denaturation conditions at 80°C (101°C), **B:** under hybridisation conditions at 53°C (74°C)



F.2. Primer binding sites and sequences of antibiotic resistance genes of respective pathogens

vanB gene sequence of NCBI AY786179 and *Enterococcus faecium* AF 458387 and *Enterococcus faecalis* Staph/Strep28 (Table B.12, p. 34)

Figure F.2.1 AY786179 represents the reference sequence of *vanB* gene of *E. faecium*.
VanB409 shows the fragment length of 409 nt of *E. faecium* AF 458387 and *E. faecalis* Staph/Strep28 obtained after amplification and sequencing. The red box demonstrates the forward primer binding site, the blue box the reverse primer binding site.

```
                 1          11         21         31         41         51         61         71         81         91        100
                 |          |          |          |          |          |          |          |          |          |          |
AY786179    1   TGAAAGAAAG CGAATACGAA ACACGGCGTA TTGATGTGGC TTTCCCGGTT TTGCATGGCA AATGCGGGGA GGATGGTGCG ATACAGGGGC TGTTTGTATT  100
 VanB409    1   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---CAGGGGC TGTTTGTATT   17

                 101        111        121        131        141        151        161        171        181        191        200
                 |          |          |          |          |          |          |          |          |          |          |
AY786179  101   GTCTGGTATC CCCTATGTGG GCTGTGATAT TCAAAGCTCC GCAGCTTGCA TGGACAAATC ACTGGCCTAC ATTCTTACAA AAAATGCGGG CATCGCCGTT  200
 VanB409   18   GTCTGGTATC CCCTATGTAG GCTGCGATAT TCAAAGCTCC GCAGCTTGCA TGGACAAATC ACTGGCCTAC ATTCTTACAA AAAATGCGGG CATCGCCGTC  117

                 201        211        221        231        241        251        261        271        281        291        300
                 |          |          |          |          |          |          |          |          |          |          |
AY786179  201   CCCGAATTTC AAATGATTGA TAAAGGTGAC AAGCCGGAGG CGGGTGCGCT TACCTACCCT GTCTTTGTGA AGCCGGCACG GTCAGGTTCG TCCTTTGGCG  300
 VanB409  118   CCCGAATTTC AAATGATTGA AAAAGGTGAC AAACCGGAGG CGAGGACGCT TACCTACCCT GTCTTTGTGA AGCCGGCACG GTCAGGTTCG TCCTTTGGCG  217

                 301        311        321        331        341        351        361        371        381        391        400
                 |          |          |          |          |          |          |          |          |          |          |
AY786179  301   TAACCAAAGT AAACGGTACG GAAGAACTTA ACGCTGCGAT AGAAGCGGCA GGACAATATG ATGGAAAAAT CTTAATTGAG CAAGCGATTT CGGGCTGTGA  400
 VanB409  218   TAACCAAAGT AAACAGTACG GAAGAACTAA ACGCTGCGAT AGTAGCAGCA GGACAATATG ATGGAAAAAT CTTAATTGAG CAAGCGATTT CGGGCTGTGA  317

                 401        411        421        431        441        451        461        471        481        491        500
                 |          |          |          |          |          |          |          |          |          |          |
AY786179  401   GGTCGGGTGT GCGGTCATGG GGAACGAGGA TGATTTGATT GTCGGCGAAG TGGATCAAAT CCGGCTGAGC CACGGTATCT TCCGCATCCA TCAGGAAAAC  500
 VanB409  318   GGTCGGCTGC GCGGTCATGG GAAACGAGGA TGATTTGATT GTCGGCGAAG TGGATCAAAT CCGGTTGAGC CACGGTATCT TCCGCATCCA TC--------  409

                 501
                 |
AY786179  501   GA                                                                                                             502
 VanB409  410   --                                                                                                             409
```

bla$_{OXY-2-5}$ gene sequence of NCBI AY303806 and *Klebsiella oxytoca* AF 479983 (Table B.9, p. 32)

Figure F.2.2 AY303806 represents the reference sequence of *bla*$_{OXY-2-5}$ gene of *K. oxytoca*. KoCef336 and KoCef587 show the fragment length of 336 or 587 nt of *K. oxytoca* AF 479983 obtained after amplification and sequencing. The red box demonstrates the forward primer binding site, the blue box the reverse primer binding site. The dashed boxes symbolise the binding sites for the primer pair of the longer fragment.

```
                 1          11         21         31         41         51         61         71         81         91         100
                 |          |          |          |          |          |          |          |          |          |          |
AY303806      1  GCGCAGCGAG GGGATCGCCG CAGCGGCAGG ATGGCGCGGT TTCGGTAACT GTGACGGGAA CATAGCGGCT CCTTATTTTT ATGGGTCTGG CGAGACACTA   100
KoCef336      1  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------     0
KoCef587      1  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------     0

                 101        111        121        131        141        151        161        171        181        191        200
                 |          |          |          |          |          |          |          |          |          |          |
AY303806    101  TAAGCAGGCG CTGGAAGGAC AAATATCGAA TTGATGATGG AATTCCATCA TCAATAAATT GCTTGTCAAA ATAGCGGGAG TCGCATATAC TCCGCTGCGA   200
KoCef336      1  TAAGCAGGCG CTGGAAGGAC AAATATCGAA TTGATGATGG AATTCCATCA TCAATAAATT GCTTGTCAAA ATAGCGGGAG TCGCATATAG TCCGCTGCGA   100
KoCef587      1  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------     0

                 201        211        221        231        241        251        261        271        281        291        300
                 |          |          |          |          |          |          |          |          |          |          |
AY303806    201  CTTATCACTC TCAAGGAATC AGAAATGATA AAAAGTTCGT GGCGTAAAAT TGCAATGCTA GCCGCCGCCG TTCCGCTGCT GCTGGCGAGC GGCGCACTGT   300
KoCef336    101  CTTATCACTC TCAAGGAATC AGAGATGATA AAAAGTTCGT GGCGTAAAAT TGCAATGCTA GCCGCCGCCG TTCCGCTGCT GCTGGCGAGC GGCGCACTGT   200
KoCef587      1  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------     0

                 301        311        321        331        341        351        361        371        381        391        400
                 |          |          |          |          |          |          |          |          |          |          |
AY303806    301  GGGCCAGTAC CGATGCTATC CATCAGAAGC TGACAGATCT CGAGAAGCGT TCAGGCGGCA GGTTGGGCGT GGCGCTAATC AACACGGCAG ATAATTCTCA   400
KoCef336    201  GGGCCAGTAC CGATGCTATC CATCAGAAGC TGACAGATCT CGAGAAGCGT TCAGGCGGCA GGTTGGGCGT GGCGCTAATC AACACGGCAG ATAATTCTCA   300
KoCef587      1  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------     0

                 401        411        421        431        441        451        461        471        481        491        500
                 |          |          |          |          |          |          |          |          |          |          |
AY303806    401  AATCTTATAT CGCGGCGACG AGCGTTTTGC CATGTGGAGC ACCAGTAAAG TGATGGCCGC CGCCGCGGTA TTAAAACAGA GCGAAAGCAA TAAAGAGGTG   500
KoCef336    301  AATCTTATAT CGCGGCGACG AGCGTTTTGC CATGTG---- ---------- ---------- ---------- ---------- ---------- ----------   336
KoCef587      1  ---------- ------GACG AGCGTTTTGC CATGTGGAGC ACCAGTAAAG TGATGGCCGC CGCCGCGGTA TTAAAACAGA GCGAAAGCAA TAAAGAGGTG    84

                 501        511        521        531        541        551        561        571        581        591        600
                 |          |          |          |          |          |          |          |          |          |          |
AY303806    501  GTAAATAAAA GGCTGGAGAT TAACGCAGCC GATTTGGTGG TCTGGAGCCC GATTACCGAA AAACATCTCC AGAGCGGAAT GACGCTGGCT GAGCTAAGCG   600
KoCef336    337  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------   336
KoCef587     85  GTAAATAAAA GGCTGGAGAT TAACGCAGCC GATTTGGTGG TCTGGAGTCC GATTACCGAA AAACATCTCC AGAGCGGAAT GACGCTGGCT GAGCTAAGCG   184

                 601        611        621        631        641        651        661        671        681        691        700
                 |          |          |          |          |          |          |          |          |          |          |
AY303806    601  CGGCGACGCT GCAATATAGC GACAATACGG CGATGAATCT GATCATCGGC TACCTTGGCG GGCCGGAAAA AGTCACCGCC TTCGCCCGCA GTATCGGCGA   700
KoCef336    337  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------   336
KoCef587    185  CGGCGACGCT GCAATATAGC GACAATACGG CGATGAATCT GATCATCGGC TACCTTGGCG GGCCGGAAAA AGTCACCGCC TTCGCCCGCA GTATCGGCGA   284

                 701        711        721        731        741        751        761        771        781        791        800
                 |          |          |          |          |          |          |          |          |          |          |
AY303806    701  TGCCACCTTT CGTCTCGATC GTACGGAGCC CACGCTGAAT ACCGCCATCC CGGGCGATGA GCGTGATACC AGCACGCCGC TGGCGATGGC TGAAAGCCTG   800
KoCef336    337  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------   336
KoCef587    285  TGCCACCTTT CGTCTCGATC GTACGGAGCC CACGCTGAAT ACCGCCATCC CGGGCGATGA GCGTGATACC AGCACGCCGC TGGCGATGGC TGAAAGCCTG   384

                 801        811        821        831        841        851        861        871        881        891        900
                 |          |          |          |          |          |          |          |          |          |          |
AY303806    801  CGCAAGCTGA CGCTTGGCGA TGCGCTGGGC GAACAGCAAC GCGCCCAGTT AGTCACCTGG CTGAAAGGCA ATACCACCGG CGGGCAAAGC ATTCGCGCGG   900
KoCef336    337  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------   336
KoCef587    385  CGCAAGCTGA CGCTTGGCAA TGCGCTGGGC GAACAGCAAC GCGCCCAGTT AGTCACCTGG CTGAAAGGCA ATACCACCGG CGGGCAAAGC ATTCGCGCGG   484

                 901        911        921        931        941        951        961        971        981        991        1000
                 |          |          |          |          |          |          |          |          |          |          |
AY303806    901  GCCTGCCTGA AAGCTGGGTG GTCGGCGATA AAACCGGCTC CGGAGATTAC GGCACCACCA ATGATATTGC GGTTATCTGG CCGGAAGATC ACGCTCCGCT  1000
KoCef336    337  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------   336
KoCef587    485  GCCTGCCTGA AAGCTGGGTG GTCGGAGATA AAACCGGCGC CGGAGATTAC GGCACCACCA ATGATATTGC GGTTATCTGG CCGGAAGATC ACGCTCCGCT   584

                 1001       1011       1021       1031       1041       1051       1061       1071       1081       1091
                 |          |          |          |          |          |          |          |          |          |
AY303806   1001  GGTATTAGTC ACCTACTTTA CCCAGCCGCA GCAGGATGCG AAAAACCGCA AAGAGGTGTT AGCCGCAGCG GCAAAAATCG TGACCGAAGG GCTTTAA      1097
KoCef336    337  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -------       336
KoCef587    585  GGT------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -------       587
```

bla$_{SHV-1}$ gene sequence of NCBI AF124984 and *Klebsiella pneumoniae* AF 429457 (Table B.10, p. 33)

Figure F.2.3 AF124984 represents the reference sequence of *bla*$_{SHV-1}$ gene of *K. pneumoniae.* KpPip365 and KpPip536 show the fragment length of 365 or 536 nt of *K. pneumoniae* AF 429457 obtained after amplification and sequencing. The red box demonstrates the binding site for the forward primer, the blue box the binding site for the reverse primer. The dashed boxes symbolise the binding sites for the primer pair of the longer fragment

```
                1          11         21         31         41         51         61         71         81         91         100
AF124984     1  ACTTTCTTAT AGTTCATCAC GGCCTTGAGT CAAAAAATAG CGTGCTTAGG CAGGGCTAGA TATTGATTAT TCGAAATAAA AGATGAAAAA TGATGAAGGA   100
KpPip365     1  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------     0
KpPip536     1  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------     0

                101        111        121        131        141        151        161        171        181        191        200
AF124984   101  AAAAAGAGGA ATTGTGAATC AGCAAAACGC CGGGTTATTC TTATTTGTCG CTTCTTTACT CGCCTTTATC GGCCCTCACT CAAGGATGTA TTGTGGTTAT   200
KpPip365     1  ---------- ---------- ---------- ---------- ---------- ---------- --------TC GGCCCTCACT CAAGGATGTA TTGTGGTTAT    32
KpPip536     1  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------     0

                201        211        221        231        241        251        261        271        281        291        300
AF124984   201  GCGTTATATT CGCCTGTGTA TTATCTCCCT GTTAGCCACC CTGCCGCTGG CGGTACACGC CAGCCCGCAG CCGCTTGAGC AAATTAAACT AAGCGAAAGC   300
KpPip365    33  GCGTTATATT CGCCTGTGTA TTATCTCCCT GTTAGCCACC CTGCCGCTGG CGGTACACGC CAGCCCGCAG CCGCTTGAGC AAATTAAACT AAGCGAAAGC   132
KpPip536     1  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------     0

                301        311        321        331        341        351        361        371        381        391        400
AF124984   301  CAGCTGTCGG GCCGCGTAGG CATGATAGAA ATGGATCTGG CCAGCGGCCG CACGCTGACC GCCTGGCGCG CCGATGAACG CTTTCCCATG ATGAGCACCT   400
KpPip365   133  CAGCTGTCGG GCCGCGTAGG CATGATAGAA ATGGATCTGG CCAGCGGCCG CACGCTGACC GCCTGGCGCG CCGATGAACG CTTTCCCATG ATGAGCACCT   232
KpPip536     1  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------     0

                401        411        421        431        441        451        461        471        481        491        500
AF124984   401  TTAAAGTAGT GCTCTGCGGC GCAGTGCTGG CGCGGGTGGA TGCCGGTGAC GAACAGCTGG AGCGAAAGAT CCACTATCGC CAGCAGGATC TGGTGGACTA   500
KpPip365   233  TTAAAGTAGT GCTCTGCGGC GCAGTGCTGG CGCGGGTGGA TGCCGGTGAC GAACAGCTGG AGCGAAAGAT CCACTATCGC CAGCAGGATC TGGTGGACTA   332
KpPip536     1  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------     0

                501        511        521        531        541        551        561        571        581        591        600
AF124984   501  CTCGCCGGTC AGCGAAAAAC ACCTTGCCGA CGGCATGACG GTCGGCGAAC TCTGCGCCGC CGCCATTACC ATGAGCGATA ACAGCGCCGC CAATCTGCTA   600
KpPip365   333  CTCGCCGGTC AGCGAAAAAC ACCTTGCCGA CGG------- ---------- ---------- ---------- ---------- ---------- ----------   365
KpPip536     1  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------     0

                601        611        621        631        641        651        661        671        681        691        700
AF124984   601  CTGGCCACCG TCGGCGGCCC CGCAGGATTG ACTGCCTTTT TGCGCAGAT CGGCGACAAC GTCACCCGCC TTGACCGCTG GGAAACGGAA CTGAATGAGG   700
KpPip365   366  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------   365
KpPip536     1  ---------- ---------- ---------- ---------- -----CAGAT CGGCGACAAC GTCACCCGCC TTGACCGCTG GGAAACGGAA CTGAATGAGG    55

                701        711        721        731        741        751        761        771        781        791        800
AF124984   701  CGCTTCCCGG CGACGCCCGC GACACCACTA CCCCGGCCAG CATGGCCGCG ACCCTGCGCA AGCTGCTGAC CAGCCAGCGT CTGAGCGCCC GTTCGCAACG   800
KpPip365   366  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------   365
KpPip536    56  CGCTTCCCGG CGACGCCCGC GACACCACTA CCCCGGCCAG CATGGCCGCG ACCCTGCGCA AGCTGCTGAC CAGCCAGCGT CTGAGCGCCC GTTCGCAACG   155

                801        811        821        831        841        851        861        871        881        891        900
AF124984   801  GCAGCTGCTG CAGTGGATGG TGGACGATCG GGTCGCCGGA CCGTTGATCC GCTCCGTGCT GCCGGCGGGC TGGTTTATCG CCGATAAGAC CGGAGCTGGC   900
KpPip365   366  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------   365
KpPip536   156  GCAGCTGCTG CAGTGGATGG TGGACGATCG GGTCGCCGGA CCGTTGATCC GCTCCGTGCT GCCGGCGGGC TGGTTTATCG CCGATAAGAC CGGAGCTGGC   255

                901        911        921        931        941        951        961        971        981        991        1000
AF124984   901  GAGCGGGGTG CGCGCGGGAT TGTCGCCCTG CTTGGCCCGA ATAACAAAGC AGAGCGCATT GTGGTGATTT ATCTGCGGGA TACCCCGGCG AGCATGGCCG  1000
KpPip365   366  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------   365
KpPip536   256  GAGCGGGGTG CGCGCGGGAT TGTCGCCCTG CTTGGCCCGA ATAACAAAGC AGAGCGCATT GTGGTGATTT ATCTGCGGGA TACCCCGGCG AGCATGGCCG   355

                1001       1011       1021       1031       1041       1051       1061       1071       1081       1091       1100
AF124984  1001  AGCGAAATCA GCAAATCGCC GGGATCGGCG CGGCGCTGAT CGAGCACTGG CAACGCTAAC CCGGCGGTGG CCGCGCGCGT TATCCGGCCC GCAGCACCTC  1100
KpPip365   366  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------   365
KpPip536   356  AGCGAAATCA GCAAATCGCC GGGATCGGCG CGGCGCTGAT CGAGCACTGG CAACGCTAAC CCGGCGGTGG CCGCGCGCGT TATCCGGCCC GCAGCACCTC   455

                1101       1111       1121       1131       1141       1151       1161       1171       1181       1191       1200
AF124984  1101  GCAGGCGTGC CGGGCGATAT GACTGGCGGC GGCATCGGAA AGATGCCGGT CGGTAATGAT GGTGGTGAAC CGGGTCAAAG GTAACGCCAT AAACGTGGCC  1200
KpPip365   366  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------   365
KpPip536   456  GCAGGCGTGC CGGGCGATAT GACTGGCGGC GGCATCGGAA AGATGCCGGT CGGTAATGAT GGTGGTGAAC CGGGTCAAAG G--------- ----------   536

                1201       1211       1221       1231       1241       1251       1261       1271       1281       1291       1300
AF124984  1201  ACCTGATTGT ATTTCGAACT GTCGCACAGC AGGATGCTTC TCGCGCTGAC CTGGCTGACG GTCTCCTTGA CGGTAACCTT GTTCTCATCA GGGGTGAATA  1300
KpPip365   366  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------   365
KpPip536   537  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------   536

                1301       1311       1321       1331       1341
AF124984  1301  TCCCGCGACT GTCCCAGCCG CTGGCGGAGA TAAAGGCCGT AT                                                                 1342
KpPip365   366  ---------- ---------- ---------- ---------- --                                                                  365
KpPip536   537  ---------- ---------- ---------- ---------- --                                                                  536
```

mecA gene sequence of NCBI AY786579 and *Staphylococcus aureus* AF 678848 (Table B.13, p. 34)

Figure F.2.4 AY786579 represents the reference sequence of *mecA* gene of *S. aureus.* MecA500 shows the fragment length of 500 nt of *S. aureus* AF 678848 obtained after amplification and sequencing. The red box demonstrates the binding site for the forward primer, the blue box the binding site for the reverse primer.

```
               1          11         21         31         41         51         61         71         81         91         100
               |          |          |          |          |          |          |          |          |          |
AY786579    1  ATGAAAAAGA TAAAAATTGT TCCACTTATT TTAATAGTTG TAGTTGTCGG GTTTGGTATA TATTTTTATG CTTCAAAAGA TAAAGAAATT AATAATACTA   100
 MecA500    1  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------     0

               101        111        121        131        141        151        161        171        181        191        200
               |          |          |          |          |          |          |          |          |          |
AY786579  101  TTGATGCAAT TGAAGATAAA AATTTCAAAC AAGTTTATAA AGATAGCAGT TATATTTCTA AAAGCGATAA TGGTGAAGTA GAAATGACTG AACGTCCGAT   200
 MecA500    1  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------     0

               201        211        221        231        241        251        261        271        281        291        300
               |          |          |          |          |          |          |          |          |          |
AY786579  201  AAAAATATAT AATAGTTTAG GCGTTAAAGA TATAAACATT CAGGATCGTA AAATAAAAAA AGTATCTAAA AATAAAAAAC GAGTAGATGC TCAATATAAA   300
 MecA500    1  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------     0

               301        311        321        331        341        351        361        371        381        391        400
               |          |          |          |          |          |          |          |          |          |
AY786579  301  ATTAAAACAA ACTACGGTAA CATTGATCGC AACGTTCAAT TTAATTTTGT TAAAGAAGAT GGTATGTGGA AGTTAGATTG GGATCATAGC GTCATTATTC   400
 MecA500    1  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------     0

               401        411        421        431        441        451        461        471        481        491        500
               |          |          |          |          |          |          |          |          |          |
AY786579  401  CAGGAATGCA GAAAGACCAA AGCATACATA TTGAAAATTT AAAATCAGAA CGTGGTAAAA TTTTAGACCG AAACAATGTG GAATTGGCCA ATACAGGAAC   500
 MecA500    1  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------     0

               501        511        521        531        541        551        561        571        581        591        600
               |          |          |          |          |          |          |          |          |          |
AY786579  501  AGCATATGAG ATAGGCATCG TTCCAAAGAA TGTATCTAAA AAAGATTATA AAGCAATCGC TAAAGAACTA AGTATTTCTG AAGACTATAT CAAACAACAA   600
 MecA500    1  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------     0

               601        611        621        631        641        651        661        671        681        691        700
               |          |          |          |          |          |          |          |          |          |
AY786579  601  ATGGATCAAA ATTGGGTACA AGATGATACC TTCGTTCCAC TTAAAACCGT TAAAAAAATG GATGAATATT TAAGTGATTT CGCAAAAAAA TTTCATCTTA   700
 MecA500    1  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------     0

               701        711        721        731        741        751        761        771        781        791        800
               |          |          |          |          |          |          |          |          |          |
AY786579  701  CAACTAATGA AACAGAAAGT CGTAACTATC CTCTAGGAAA AGCGACTTCA CATCTATTAG GTTATGTTGG TCCCATTAAC TCTGAAGAAT TAAAACAAAA   800
 MecA500    1  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------     0

               801        811        821        831        841        851        861        871        881        891        900
               |          |          |          |          |          |          |          |          |          |
AY786579  801  AGAATATAAA GGCTATAAAG ATGATGCAGT TATTGGTAAA AAGGGACTCG AAAAACTTTA CGATAAAAAG CTCCAACATG AAGATGGCTA TCGTGTCACA   900
 MecA500    1  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------     0

               901        911        921        931        941        951        961        971        981        991        1000
               |          |          |          |          |          |          |          |          |          |
AY786579  901  ATCGTTGACG ATAATAGCAA TACAATCGCA CATACATTAA TAGAGAAAAA GAAAAAAGAT GGCAAAGATA TTCAACTAAC TATTGATGCT AAAGTTCAAA  1000
 MecA500    1  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------     0

               1001       1011       1021       1031       1041       1051       1061       1071       1081       1091       1100
               |          |          |          |          |          |          |          |          |          |
AY786579 1001  AGAGTATTTA TAACAACATG AAAAATGATT ATGGCTCAGG TACTGCTATC CACCCTCAAA CAGGTGAATT ATTAGCACTT GTAAGCACAC CTTCATATGA  1100
 MecA500    1  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------     0

               1101       1111       1121       1131       1141       1151       1161       1171       1181       1191       1200
               |          |          |          |          |          |          |          |          |          |
AY786579 1101  CGTCTATCCA TTTATGTATG GCATGAGTAA CGAAGAATAT AATAAATTAA CCGAAGATAA AAAAGAACCT CTGCTCAACA AGTTCCAGAT TACAACTTCA  1200
 MecA500    1  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------     0

               1201       1211       1221       1231       1241       1251       1261       1271       1281       1291       1300
               |          |          |          |          |          |          |          |          |          |
AY786579 1201  CCAGGTTCAA CTCAAAAAAT ATTAACAGCA ATGATTGGGT TAAATAACAA AACATTAGAC GATAAAACAA GTTATAAAAT CGATGGTAAA GGTTGGCAAA  1300
 MecA500    1  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------     0

               1301       1311       1321       1331       1341       1351       1361       1371       1381       1391       1400
               |          |          |          |          |          |          |          |          |          |
AY786579 1301  AAGATAAATC TTGGGGTGGT TACAACGTTA CAAGATATGA AGTGGTAAAT GGTAATATCG ACTTAAAACA AGCAATAGAA TCATCAGATA ACATTTTCTT  1400
 MecA500    1  -------ATC TTGGGGTGGT TACAACGTTA CAAGATATGA AGTGGTAAAT GGTAATATCG ACTTAAAACA AGCAATAGAA TCATCAGATA ACATTTTCTT    93

               1401       1411       1421       1431       1441       1451       1461       1471       1481       1491       1500
               |          |          |          |          |          |          |          |          |          |
AY786579 1401  TGCTAGAGTA GCACTCGAAT TAGGCAGTAA GAAATTTGAA AAAGGCATAA AAAAACTAGG TGTTGGTGAA GATATACCAA GTGATTATCC ATTTTATAAT  1500
 MecA500   94  TGCTAGAGTA GCACTCGAAT TAGGCAGTAA GAAATTTGAA AAAGGCATGA AAAAACTAGG TGTTGGTGAA GATATACCAA GTGATTATCC ATTTTATAAT   193

               1501       1511       1521       1531       1541       1551       1561       1571       1581       1591       1600
               |          |          |          |          |          |          |          |          |          |
AY786579 1501  GCTCAAATTT CAAACAAAAA TTTAGATAAT GAAATATTAT TAGCTGATTC AGGTTACGGA CAAGGTGAAA TACTGATTAA CCCAGTACAG ATCCTTTCAA  1600
 MecA500  194  GCTCAAATTT CAAACAAAAA TTTAGATAAT GAAATATTAT TAGCTGATTC AGGTTACGGA CAAGGTGAAA TACTGATTAA CCCAGTACAG ATCCTTTCAA   293

               1601       1611       1621       1631       1641       1651       1661       1671       1681       1691       1700
               |          |          |          |          |          |          |          |          |          |
AY786579 1601  TCTATAGCGC ATTAGAAAAT AATGGCAATA TTAACGCACC TCACTTATTA AAAGACACGA AAAACAAAGT TTGGAAGAAA AATATTATTT CCAAAGAAAA  1700
 MecA500  294  TCTATAGCGC ATTAGAAAAT AATGGCAATA TTAACGCACC TCACTTATTA AAAGACACGA AAAACAAAGT TTGGAAGAAA AATATTATTT CCAAAGAAAA   393

               1701       1711       1721       1731       1741       1751       1761       1771       1781       1791       1800
               |          |          |          |          |          |          |          |          |          |
AY786579 1701  TATCAATCTA TTAACTGATG GTATGCAACA AGTCGTAAAT AAAACACATA AAGAAGATAT TTATAGATCT TATGCAAACT TAATTGGCAA ATCCGGTACT  1800
 MecA500  394  TATCAATCTA TTAACTGATG GTATGCAACA AGTCGTAAAT AAAACACATA AAGAAGATAT TTATAGATCT TATGCAAACT TAATTGGCAA ATCCGGTACT   493

               1801       1811       1821       1831       1841       1851       1861       1871       1881       1891       1900
               |          |          |          |          |          |          |          |          |          |
AY786579 1801  GCAGAACTCA AAATGAAACA AGGAGAAACT GGCAGACAAA TTGGGTGGTT TATATCATAT GATAAAGATA ATCCAAACAT GATGATGGCT ATTAATGTTA  1900
 MecA500  494  GCAGAAC--- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------   500

               1901       1911       1921       1931       1941       1951       1961       1971       1981       1991       2000
               |          |          |          |          |          |          |          |          |          |
AY786579 1901  AAGATGTACA AGATAAAGGA ATGGCTAGCT ACAATGCCAA AATCTCAGGT AAAGTGTATG ATGAGCTATA TGAGAACGGT AATAAAAAAT ACGATATAGA  2000
 MecA500  501  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------   500

               2001
               |
AY786579 2001  TGAATAA                                                                                                       2007
 MecA500  501  -------                                                                                                        500
```

aac(6´) gene sequence of NCBI L06163 and *Pseudomonas fluorescens* AF 603085 (Table B.11, p. 33)

Figure F.2.5 L06163 represents the reference sequence of the *aac(6´)* gene of *P. fluorescens.* PsflGtm409 shows the fragment length of 409 nt of *P. fluorescens* AF 603085 obtained after amplification and sequencing. The red box demonstrates the binding site for the forward primer, the blue box the binding site for the reverse primer. The orange box symbolizes the binding site for the reverse primer for the fragment of 188 nt, the violet box symbolises the binding site for the reverse primer used for the amplification of the 282 nt long fragment.

```
                 1          11         21         31         41         51         61         71         81         91         100
   L06163     1  GGATCGCCCG TTACCCGCGG CGCGGAACGA GCAATTGCAG CCCCAGCAAC AATTGCTGGC GGTGCTCCAG GCGAGCGAGA TCCGTGCACA GCACCTTGCC   100
PsflGtm409    1  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -------GCC     3

                 101        111        121        131        141        151        161        171        181        191        200
   L06163   101  GTAGAAGAAC AGCAAGGCCG CCAATGCCTG ACGATGCGTG GAGACCGAAA CCTTGCGCTC GTTCGCCAGC CAGGACAGAA ATGCCTCGAC TTCGCTGCTG   200
PsflGtm409    4  GTAGAAGAAC AGCAAGGCCG CCAATGCCTG ACGATGCGTG GAGACCGAAA CCTTGCGCTC GTTCGCCAGC CAGGACAGAA ATGCCTCGAC TTCGCTGCTG   103

                 201        211        221        231        241        251        261        271        281        291        300
   L06163   201  CCCAAGGTTG CCGGGTGACG CACACCGTGG AAACGGATGA AGGCACGAAC CCAGTGGACA TAAGCCTGTT CGGTTCGTAA ACTGTAATGC AAGTAGCGTA   300
PsflGtm409  104  CCCAAGGTTG CCGGGTGACG CACACCGTGG AAACGGATGA AGGCACGAAC CCAGTGGACA TAAGCCTGTT CGGTTGGTAA GCTGTAATGC AAGTAGCGTA   203

                 301        311        321        331        341        351        361        371        381        391        400
   L06163   301  TGCGCTCACG CAACTGGTCC AGAACCTTGA CCGAACGCAG CGGTGGTAAC GGCGCAGTGG CGGTTTTCAT GGCTTGTTAT GACTGTTTTT TTGTACAGTC   400
PsflGtm409  204  TGCGCTCACG CAACTGGTCC AGAACCTTGA CCGAACGCAG CGGTGGTAAC GGCGCAGTGG CGGTTTTCAT GGCTTGTTAT GACTGTTTTT TTGTACAGTC   303

                 401        411        421        431        441        451        461        471        481        491        500
   L06163   401  TATGCCTCGG GCATCCAAGC AGCAAGCGCG TTACGCCGTG GGTCGATGTT TGATGTTATG GAGCAGCAAC GATGTTACGC AGCAGGCCAG TCGCCCTAAA   500
PsflGtm409  304  TATGCCTCGG GCATCCAAGC AGCAAGCGCG TTACGCCGTG GGTCGATGTT TGATGTTATG GAGCAGCAAC GATGTTACGC AGCAGGCCAG TCGCCCTAAA   403

                 501        511        521        531        541        551        561        571        581        591        600
   L06163   501  ACAAAGTTAG GCAGCACGGA GACACTTCAG CATGCATCCC GGCGTTGTTA CTCTGCGTCC GATGACCGAA GACGACATCG GTATGCTTCA CGAATGGTTG   600
PsflGtm409  404  ACAAAG.... .......... .......... .......... .......... .......... .......... .......... .......... ..........   409

                 601        611        621        631        641        651        661        671        681        691        700
   L06163   601  AATCGGCCGC ACATTGTCGA ATGGTGGGGT GGTGAGCGGC CCTCGCTCGA AGAGGTGAAA GAGGACTATC GGCCCAGCGC GTTGGCCGAA GAAGGAGTGA   700
PsflGtm409  410  .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........   409

                 701        711        721        731        741        751        761        771        781        791        800
   L06163   701  CGCCGTACAT CGGTTTGCTT GACGGAACTC CATTCGCGTT CGCACAGTCG TACGTTGCGC TCGGGTCGGG TGGTGGATGG TGGGAGGAAG AGACCGATCC   800
PsflGtm409  410  .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........   409

                 801        811        821        831        841        851        861        871        881        891        900
   L06163   801  TGGTGTCCGC GGAATCGATC AATCAATCGC CGATTCCGGG CTTCTCGGAA GAGGTTACGG CACTCGGCTG GTGCAGGCGC TTGTTGATTT GCTGTTCGCC   900
PsflGtm409  410  .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........   409

                 901        911        921        931        941        951        961        971        981        991        1000
   L06163   901  GACCCGCAGG TATCCAAGGT TCAGACGGAC CCCTCCCCGA ACAACATGCG CGCGATACGC TGCTATGAGA AGGCAGGCTT CCGGAAGGTC AAGGTCGTTT  1000
PsflGtm409  410  .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........   409

                 1001       1011       1021       1031       1041       1051       1061       1071       1081       1091       1100
   L06163  1001  CAACACCGGA TGGGCCGGCC ATGTACATGT TGCACGAGCG TCCGTTGGTG AACGGTTTGC GCAGTGCGGC CTAACTTTTC GCTCCAACGG ACGCTTGACC  1100
PsflGtm409  410  .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........   409

                 1101       1111       1121       1131       1141       1151       1161       1171       1181       1191       1200
   L06163  1101  CGTCGGCCCG GCCGCTGCCG CAGCCGGACC GCCGCGTCAA CCGCCGCTGA CCTCCGGAGT TAAACATCAT GAGGGAAGCG GTGATCGCCG AAGTATCGAC  1200
PsflGtm409  410  .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........   409

                 1201       1211       1221       1231       1241       1251       1261       1271       1281       1291       1300
   L06163  1201  TCAACTATCA GAGGTAGTTG GCGTCATCGA GCGCCATCTC GAACCGACGT TGCTGGCCGT ACATTTGTAC GGCTCCGCAG TGGATGGCGG CCTGAAGCCA  1300
PsflGtm409  410  .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........   409

                 1301       1311       1321       1331       1341       1351       1361       1371       1381       1391       1400
   L06163  1301  CACAGTGATA TTGATTTGCT GGTTACGGTG ACCGTAAGGC TTGATGAAAC AACGCGGCGA GCTTTGATCA ACGACCTTTT GGAAACGTCG GCTTCCCCTG  1400
PsflGtm409  410  .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........   409

                 1401       1411       1421       1431       1441       1451       1461       1471       1481       1491       1500
   L06163  1401  GAGAGAGCGA GATTCTCCGC GCTGTAGAAG TCACCATTGT TGTGCACGAC GACATCATTC CGTGGCGTTA TCCAGCTAAG CGCGAACTGC AGTTTGGAGA  1500
PsflGtm409  410  .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........   409

                 1501       1511       1521       1531       1541       1551       1561       1571       1581       1591       1600
   L06163  1501  ATGGCAGCGC AATGACATTC TTGCAGGTAT CTTCGAGCCA GCCACGATTG ACATTGATCT GGGTGCTGCC GCTGGTGGCC CTGGCCATCG GTGCCTGGCT  1600
PsflGtm409  410  .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........   409

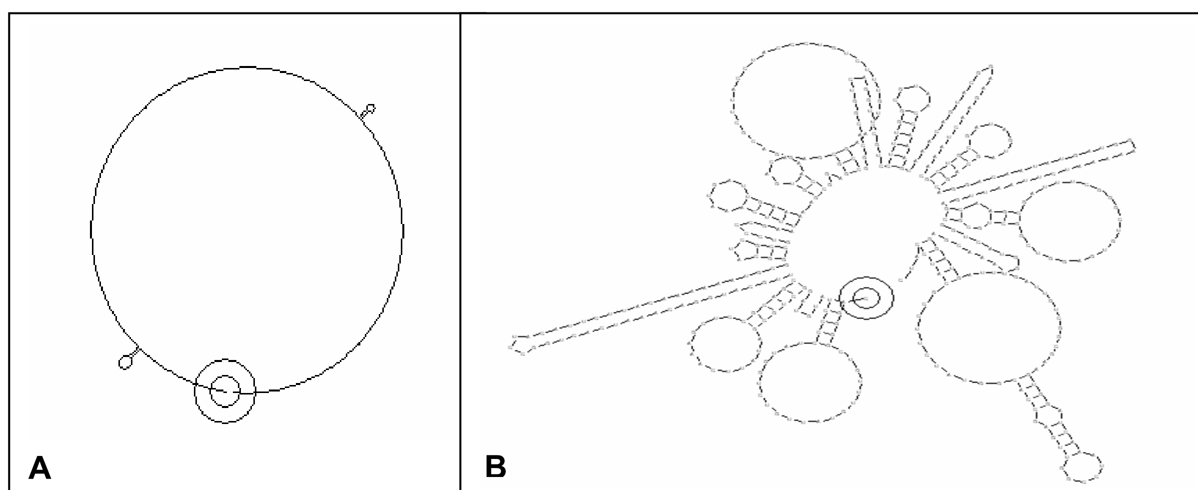
                 1601       1611       1621       1631       1641       1651       1661       1671       1681       1691       1700
   L06163  1601  GGCGTGGCGT GCCTTCGACC AGGCTGGCGT GGACATCCAG GTTCGCTTCG AGAGCGGCGA CGGGATCCTG AGCATCCTGC TCTCCGACCT GCCCCCCGAC  1700
PsflGtm409  410  .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........   409

                 1701
   L06163  1701  CAGATCC                                                                                                       1707
PsflGtm409  410  .......                                                                                                        409
```

F.3. Secondary structure models of RING-FISH polynucleotide probes under denaturation and hybridisation conditions

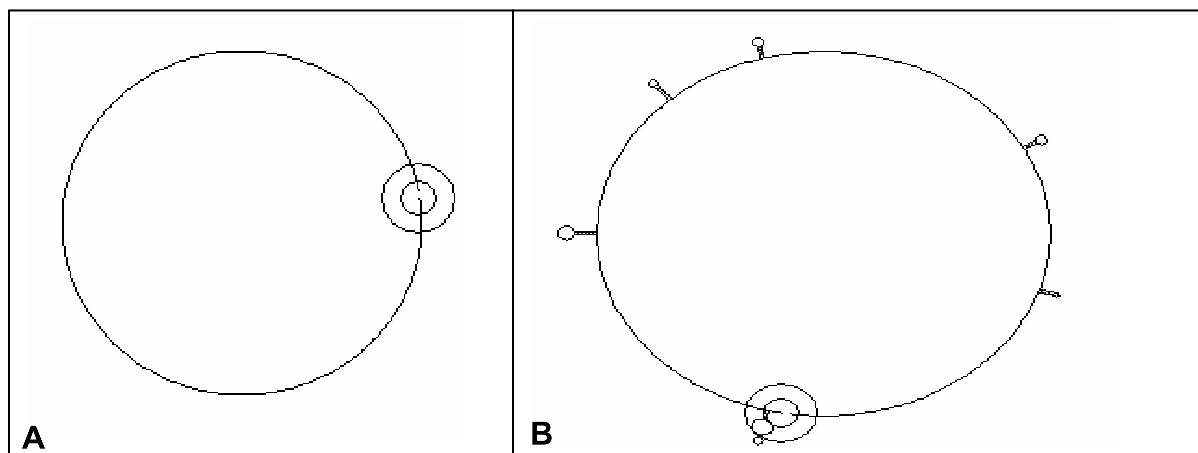
vanB gene targeted RING-FISH probe

Figure F.3.1 Secondary structure model (RNAdraw V1.1b2, Matzura et al., 1996) of RING-FISH polynucleotide probe (409 nt, p. 172) targeting *vanB* gene of *Enterococcus faecium* AF458387 and *Enterococcus faecalis* Staph/Strep28 with 0 % formamide in the buffer (Table C. 9, p. 120)
A: under denaturation conditions at 80°C, **B:** under hybridisation conditions at 53°C



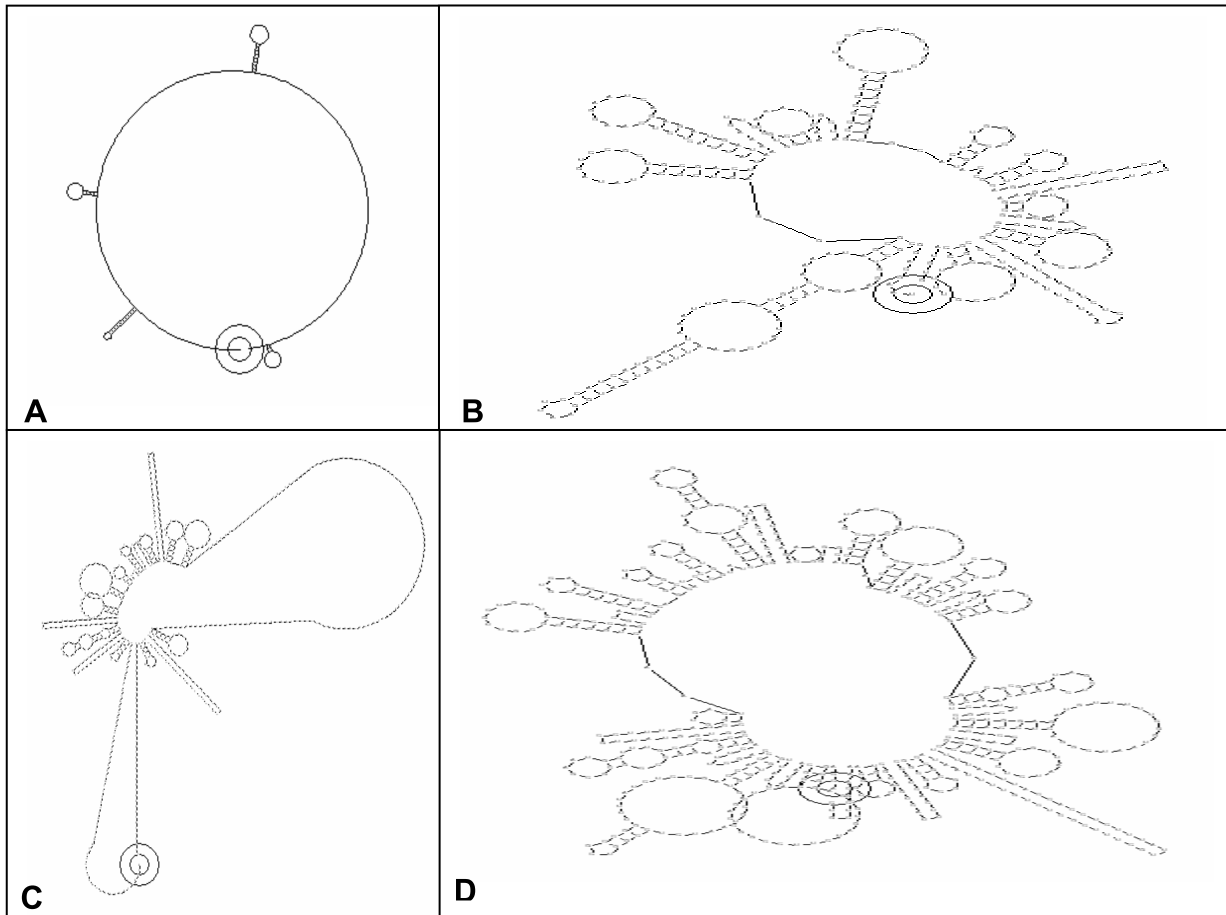
mecA gene targeted RING-FISH probe

Figure F.3.2 Secondary structure model (RNAdraw V1.1b2, Matzura et al., 1996) of RING-FISH polynucleotide probe (500 nt, p. 175) targeting *mecA* gene of *Staphylococcus aureus* with 0 % formamide in the buffer (Table C.9, p. 120)
A: under denaturation conditions at 80°C, **B:** under hybridisation conditions at 53°C



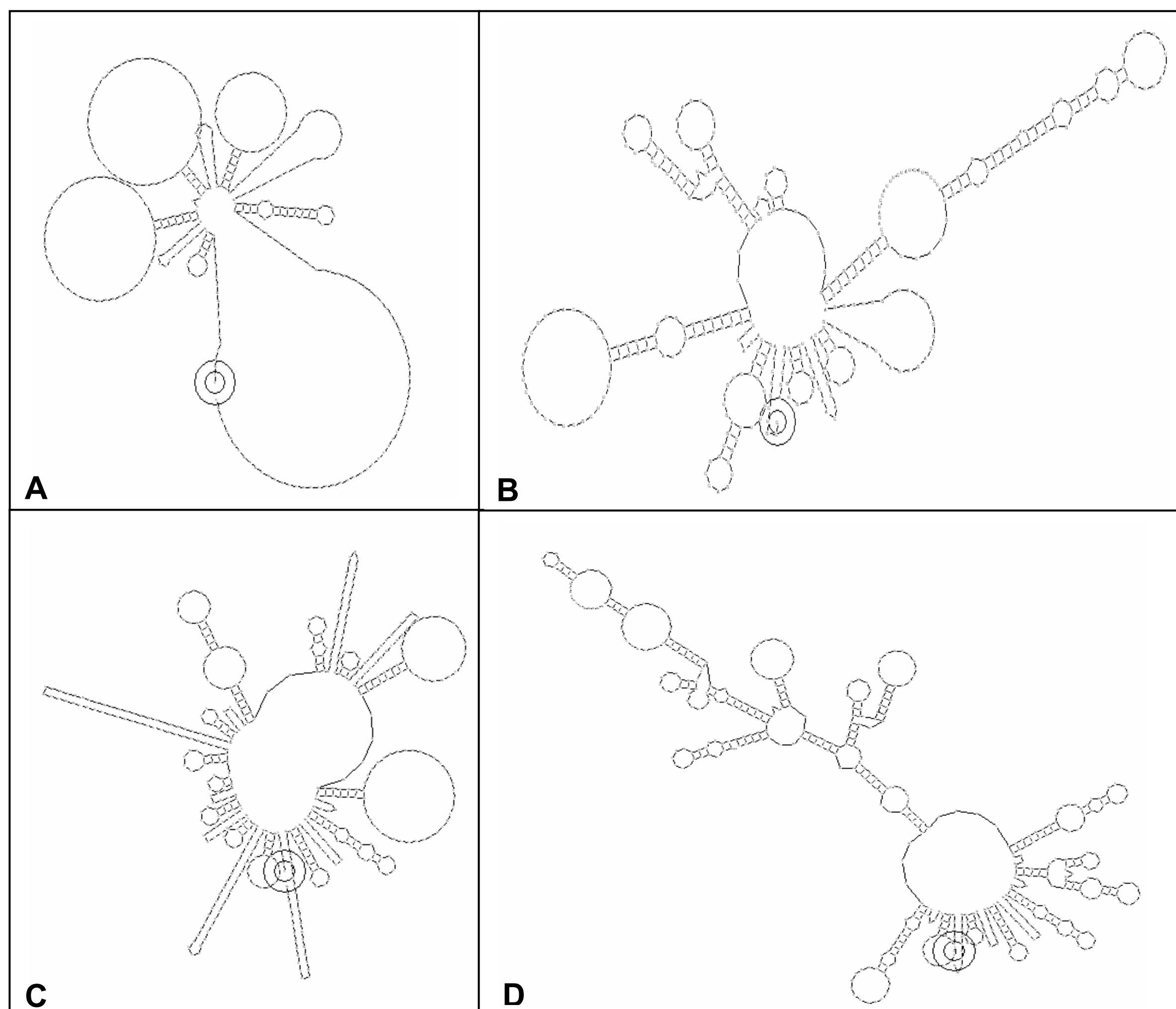
bla$_{OXY\text{-}2\text{-}5}$ gene targeted RING-FISH probe

Figure F.3.3 Secondary structure model (RNAdraw V1.1b2, Matzura et al., 1996) of RING-FISH polynucleotide probe (p. 173) targeting *bla*$_{OXY\text{-}2\text{-}5}$ gene of *Klebsiella oxytoca* AF 479983 with 0 % formamide in the buffer (Table C.9, p. 120); **A+B:** secondary structure model of the 336 nt long RING-FISH probe
C+D: secondary structure model of the 587 nt long RING-FISH probe
A, C: under denaturation conditions at 80°C, **B, D:** under hybridisation conditions at 53°C



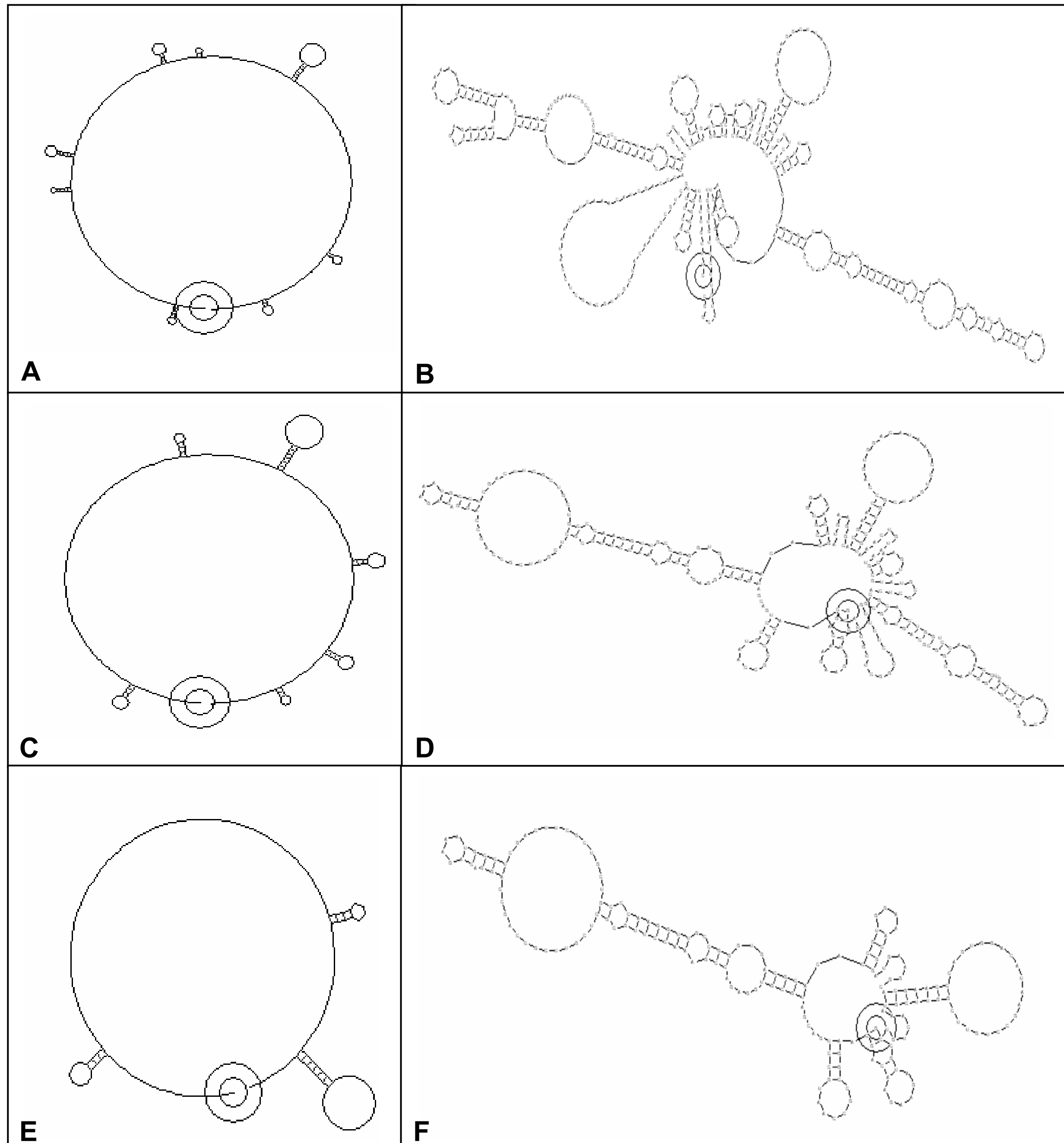
bla$_{SHV-1}$ gene targeted RING-FISH probe

Figure F.3.4 Secondary structure model (RNAdraw V1.1b2, Matzura et al., 1996) of RING-FISH polynucleotide probe (p. 174) targeting *bla*$_{SHV-1}$ gene of *Klebsiella pneumoniae* AF 429457 with 0 % formamide in the buffer (Table C.9, p. 120); **A+B:** secondary structure model of the 365 nt long RING-FISH probe
C+D: secondary structure model of the 536 nt long RING-FISH probe
A, C: under denaturation conditions at 80°C, **B, D:** under hybridisation conditions at 53°C



aac(6´) gene targeted RING-FISH probe

Figure F.3.5 Secondary structure model (RNAdraw V1.1b2, Matzura et al., 1996) of RING-FISH polynucleotide probe (p. 176) targeting *aac(6´)* gene of *Pseudomonas fluorescens* AF 603085 with 0 % formamide in the buffer (Table C.9, p. 120).
A+B: secondary structure model of the 409 nt long RING-FISH probe,
C+D: secondary structure model of the 282 long RING-FISH probe,
E+F: secondary structure model of the 188 nt long RING-FISH probe
A, C, E: under denaturation conditions at 80°C, **B, D, F:** under hybridisation conditions at 53°C



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Die vorliegende Arbeit wurde am Lehrstuhl für Mikrobiologie der Technischen Universität München unter der Leitung von Herrn Prof. Dr. K.-H. Schleifer im Zeitraum von November 2004 bis April 2007 angefertigt. Diese Arbeit wurde vom BMBF (Bundesministerium für Bildung und Forschung, Deutschland) im Rahmen des PathoGenoMik Teilprojektes „Schnelldiagnose von Antibiotikaresistenzen in der Medizin" gefördert.

An dieser Stelle möchte ich mich bedanken bei:

Prof. Dr. K.-H. Schleifer für die Möglichkeit die Arbeit an seinem Lehrstuhl durchführen zu können und das Interesse an dieser Arbeit

Dr. W. Ludwig für die fachliche Betreuung, die viel Freiraum für eigene Ideen zuließ und eigenständiges Arbeiten erforderte

Katrin Fichtl für viele Tipps auch innerhalb des Projektes

Manuela Hartmann für die gute Zusammenarbeit, die wertvollen Ratschläge und eine schöne Zeit in „unserem" Labor sowie das Korrekturlesen

Meinen Praktikantinnen Romy Laugks und Kristina Gramlich für die erfolgreiche Zusammenarbeit und meinen Hiwis Margot und Fiona

Barbara Wunner-Füssl, Sybille Schadhauser, Beate Schuhmacher und Helga Gaenge für die Hilfe in Angelegenheiten wie Sequenzieren, Bestellen, Stämme-Animpfen, Organisieren und vieles mehr

Silvia Weber, Sybille Stindl, Kristina Rappl (Korrekturlesen), Daniela Meisinger, Martin Pilhofer, Andi Bauer, Marko Pavlekovic und Andi Hofmann für eine angenehme Atmosphäre und schöne zwei Jahre

Nadja Schmider und Regina Setzer fürs Korrekturlesen

Allen lieben Freunden, die mich stets moralisch unterstützt haben, insbesondere Birgit Hundmaier, die immer an mich geglaubt und meine Arbeit mit großem Interesse verfolgt hat

Natürlich meinen Eltern und meiner Schwester für ihre unaufhörliche Unterstützung jeglicher Art und medizinisches Fachwissen