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Polynucleotide Probe Based Enrichment of Bacterial Cells: Development of Probes for Species of Clinical Relevance

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ABBREVIATIONS

A	adenine
abs.	absolute
Ac	acetate
ampC	gene encoding an AmpC-type class C beta-lactamase
APS	ammoniumperoxodisulfate
bp	base pairs
BLAST	Basic Local Alignment Search Tool
°C	degree centigrade
C	cytosine
CIA	chloroform : isoamylalcohol = 24:1 (v/v)
cf.	confer
cm	centimetre
Cy	cyanine dye
d	day(s)
DAPI	4', 6'-Diamino-2-phenylindol-dihydrochloride
dATP	deoxyadenosinetriphosphate
dCTP	deoxycytidinetriphosphate
dH_2O	distilled water
DIG	digoxigenin
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
EDTA	ethylenediamintetraacetate
e.g.	exemplum gratiae
ermB	gene encoding an erythromycin resistance methylase
et al	et alteri
etc.	et cetera
EtBr	ethidiumbromide
$EtOH_{abs.}$	100% ethanol
FA	formamide
Fig.	figure
FISH	fluorescence *in situ* hybridissation
FLUOS	5, (6)-carboxyfluorescein-N-hydroxysuccinimidester
g	gram
G	guanine

GC	mol % guanine + cytosine
H_2O_{MQ}	ultra pure water (Millipore-system)
h	hour(s)
HD	yeast-dextrose medium
i.e.	id est
IPTG	isopropyl-D-thiogalactoside
kb	kilo bases
kDa	kilodalton
l	litre
LB	Luria-Bertani medium
LMG	Laboratorium voor Microbiologie Universiteit Gent, Belgien
M	molar
m	metre; milli(10^{-3})
min	minutes
mRNA	messenger RNA
µ	micro (10^{-6})
n	nano (10^{-9})
NaAc	sodium acetate
n.d.	no data, not determined
n.d.	no result
n.t.	not tested
nt	nucleotides
OD	optical density
p	pico (10^{-12})
Pa	pascal
parC	gene coding for ParC subunit of DNA topoisomerase IV
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFA	paraformaldehyde
pH	negative decadic logarithm of the proton concentration
q.v.	quod vide
resp.	respectively
RNA	ribonucleic acid
RNase	ribonuclease
rDNA	ribosomal DNA
rpm	revolutions per minute
rRNA	ribosomal RNA
RT	room temperature
SDS	sodiumdodecylsulfate
sec	seconds
SSC	standard saline citrate
sp	species (singular)
spp	species (plural)
ssp	subspecies

ss	single stranded
T	thymine
TAE	tris-acetate-EDTA
TE	tris-EDTA
TetAJ	gene(s) coding for tetracycline resistance protein
T_m	melting temperature of nucleic acids
Tab.	table
Taq	*Thermus aquaticus*
TE	tris-EDTA
TEMED	N,N,N',N'-tetramethylethylendiamine
Tris	tris-(hydroxmethyl-)aminomethane
U	Unit or uracil
UV	ultra violet
v.	vide
V	volt
v/v	volume / volume
W	watt
w/v	weight / volume
X-Gal	5-chlor-4-brom-3-indolyl-β-D-galactoside

A. Introduction

I. Diagnostics of infection in clinical microbiology

The classical determination of the identity and susceptibility for antibiotics of microorganisms in clinical routine laboratory is mainly based on culture methods that require more than 36 h or up to several days. Meanwhile, a rapid diagnosis would be necessary for successful therapy of the patient. Sometimes even without diagnosis the patient is treated with strong antibiotics. As a consequence, a controlled therapy often fails, as the applied antibiotic compound has influenced the infection-specific abundance and diversity of microorganisms in the particular case.
The conventional characterization system of pathogenic bacteria spends much time on the identification of pathogens: classical microbiological culture, microscopy, biochemical methods and susceptibility testing are included.

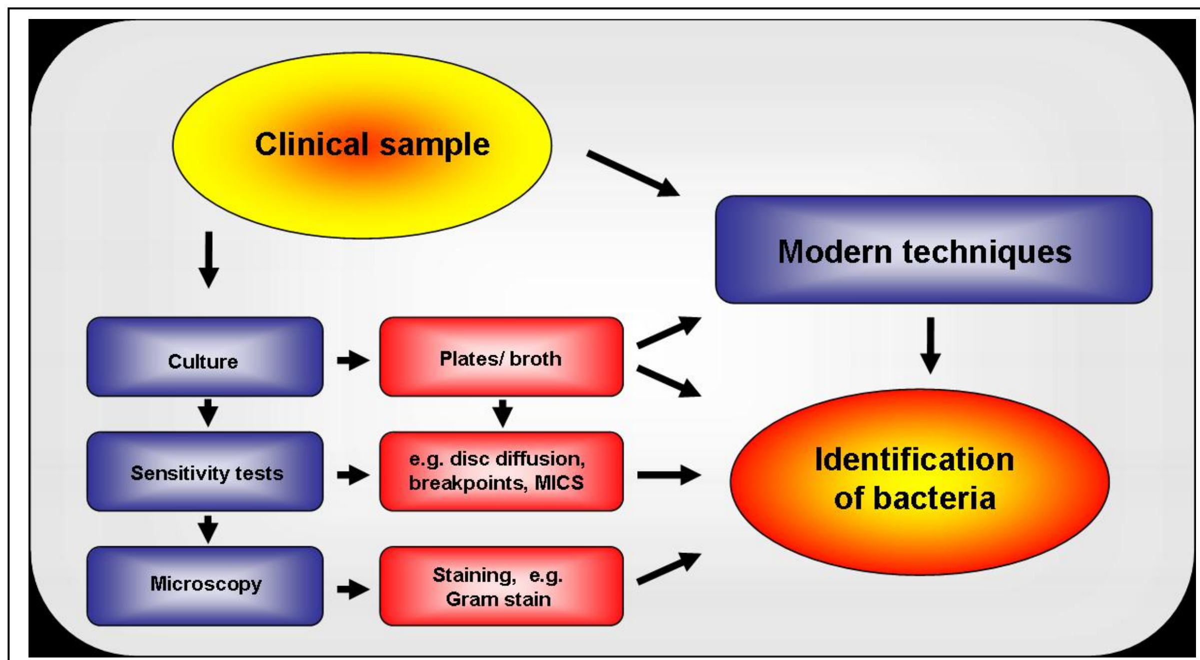

Fig A.1.: Diagram of the clinical laboratory diagnosis of infection

Especially in hospital care, there will be a strong demand for more rapid diagnoses in the future, because antibiotic resistances are exceedingly wide spread among hospital microorganisms, often pathogens, which can cause nosocomial infections (infections acquired during hospital stay). These nosocomial pathogens harbour

multidrug resistances and are known for rapid interhospital transfer of antibiotic resistance genes leading to greater patient morbidity and mortality.

With the rise of molecular biology techniques and laboratory automation during the last 20 years, important changes have taken place. Mediated by the development of many new analytical technologies, meanwhile the rapid determination of pathogenic bacteria in infectious diseases becomes possible.

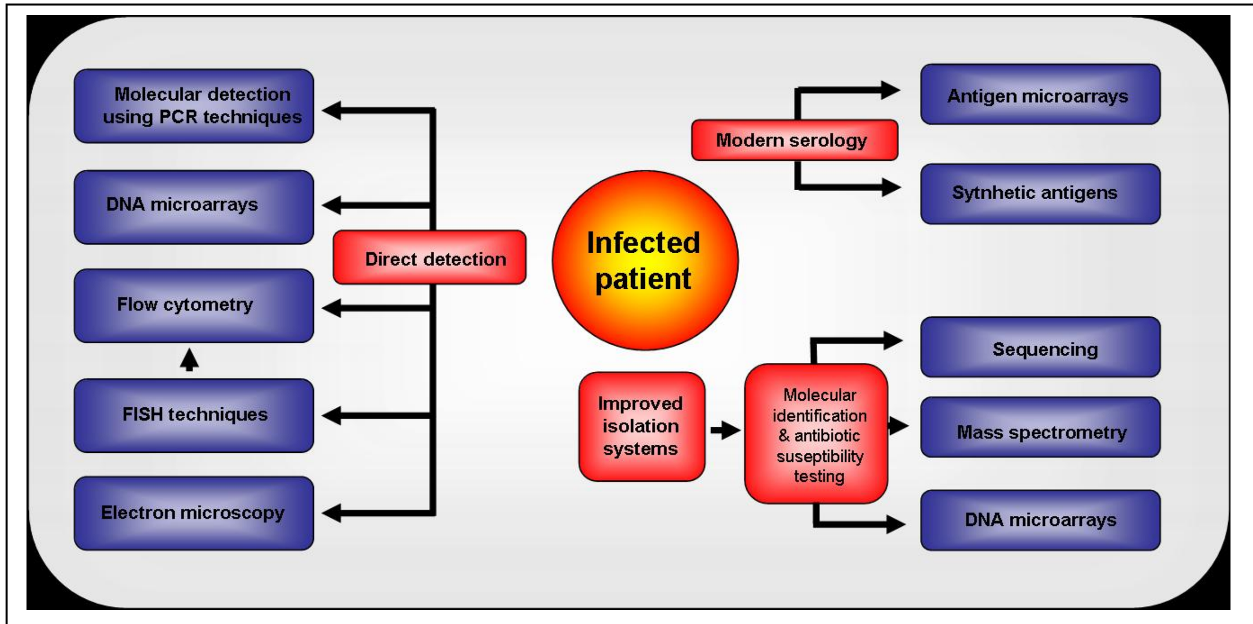

Fig A. 2 Modern techniques used to diagnose infectious diseases. Advances in a number of molecular and bacteriological techniques in recent years have significantly changed the way that clinical microbiology is carried out (Raoult, 2004, modified).

The diagram shown above exemplifies most of the methods, which are theoretically available nowadays. However, the operational availability of all mentioned techniques depends on further means, which may play a major role, such as economic resources and medical standard of the particular hospital and/or the developmental status of the country.

The advances in the modern techniques are mainly based on genetic analyses for detection and characterisation of unknown nucleic acids. Some PCR techniques, e.g. real-time PCR, can be used to amplify and detect target DNA in fully automated systems integrating the steps of sample preparation, amplification and detection in minutes, and are equally useful to or in combination with other techniques like quantitative DNA/DNA hybridisation or the development of specific DNA probes for the identification and detection of almost all medically important pathogens.

One of the main reasons for the boosting development in the sequence based identification of microorganisms is based in the accumulation of 16S rRNA sequences of bacteria and 18S rRNA of eukaryotes, which allows a customised design of specific PCR primers for application in bacterial or fungal detection systems.
These “universal” genes, though enabling a correct classification of many microorganisms, have the disadvantage of failing sometimes, when discrimination to the species level is required. Apart from 16S rRNA 75 further genes are known, which fulfil the premise of being present in all bacteria, while differing in sequence, e.g. elongation factor genes. Large databases of these genes are meanwhile available and can, depending on the gene, be used along for different levels of bacterial identification (Raoult et al, 2004).
Sometimes, a comparison of several genome sequences can be useful for the detection of the degree of relation between two strains by techniques such as Multilocus sequence typing (Maiden et al, 1998). Other techniques like antigen microarrays for the detection of specific antibodies against pathogens or flow cytometry, relying on the usage of fluorescently coated mono- or polycloncal antibodies meanwhile have found wide applications in the diagnosis of infectious diseases and become user-friendly and less expensive. Mass spectrometry has been reported to be useful not only in the field of proteomics and as an alternative sequencing method to Sanger, but also to be applicable for the specific detection of 16S rRNA gene fragments in complex samples (Wintzingerode et al, 2002).

II. FISH in general and in clinical microbiology

Another method included in the modern pool of diagnostic tools for rapid, exact and relatively cheap identification of pathogens sometimes applied in combination with other classical or modern techniques, is Fluorescence *In Situ* hybridisation (FISH), nowadays a standard technique. FISH enables localisation, identification and enumeration of bacteria in various fields of microbiology, e.g. environmental research, wastewater treatment plants, food processing industry, drinking water control and veterinary medicine. FISH is useful for fast clinical diagnosis in human medicine: oral cavity, gastro-intestinal flora, respiratory tract infections, pathogens in tissues or sterile compartments and blood cultures are often screened by FISH

(Moter et al, 2000). Used as stand-alone method or auxiliary to classical methods, it provides the opportunity not only to obtain information about growing and culturable organisms, but also about the presence of unculturable organisms, e.g. about organisms which grow very slowly or require fastidious nutrition resources for growth (Moter et al, 2000). Thus, it allows assessing the exact composition of mixed bacterial communities or microbial diversities in infections (Wagner et al, 1993, Choi et al, 1994).

The most common target molecule for FISH is 16S rRNA, 23S rRNA, intergenic spacer rRNA and mRNA. The reasons for the appropriateness of this molecule lie within its genetic stability, the ubiquitous presence amongst all bacteria and the high copy number in metabolically active and replicating microbial cells (up to 10^5 ribosomes per cell).

Especially the availability of conserved and varying parts allow development of probes for different taxonomic levels, starting from the design of species-specific probes through genus-, family-, order-, class- or phylum-specific hierarchies for differentiation (Woese,1987; Göbel, 1991; Amann et al, 1995; Ludwig et al 1994). The use of public or commercial databases, which contain steadily growing sequence data, facilitate probe design and probe match to expand accuracy and sensitivity of FISH probes more and more (e.g. ARB, ribosomal database project, probe base net and others).

Nevertheless, although rRNA offers the possibility for designing probes of highest specificity in relation to the different hierarchical levels, oligonucleotide probes targeting rRNA sequences often show decreased signal intensities when applied to environmental or clinical samples, or are often undetectable above background fluorescence in many cells (DeLong, 1999). Another problem that could hamper the success of FISH within clinical specimen is the decreased permeability of Gram-positive bacteria, often containing intra-strain varieties of the cell wall which cannot be treated with a similar protocol for equal success of permeabilisation (Jansen, et al 2000, Kempf et al, 2000).

Based on the often low metabolic activity of microbial cells, especially those originating from clinical samples, the signal intensities obtained using oligonucleotide probes are in many cases too low for valid and reliable diagnosis of the identity of the microorganisms enclosed.

Depending on the physiological state of microbial cells the content of rRNA can vary extremely, ranging from 10^3 up to 10^8 (DeLong, 1989, Kemp, 1993, Wallner, 1993, and Poulsen, 1993). The use of brighter fluorescent dyes such as Cy3 (Moter et al, 2000) or the application of two probes targeting one organism can increase the possibility of species-specific detection even with low signal intensities (Lee at al, 1993).

In addition, several systems are available that enhance the sensitivity of FISH and allow signal amplification, such as TSA (Tyramide signal amplification, Schönhuber et al, 1997, Schriml, 1999, Pernthaler et al, 2002 and 2004), implemented in Catalysed Reporter Deposition FISH (CARDFISH, Bobrow et al, 1989; Schmidt, 1997; Pernthaler et al, 2002), fluorochrome-conjugated antibody layering (Macechko, 1997) for amplifying FISH signals or usage of image-intensified video microscopy, application of chloramphenicol to a growing bacterial culture for increase of the rRNA content, use of helper oligonucleotide probes (Fuchs et al, 2000) and application of multilabelled polynucleotide probes (Trebesius et al, 1994; De Long et al, 1999; Pernthaler et al, 2002; Stoffels et al, 1999; Zimmermann et al, 2002; Zwirglmaier et al, 2004).

However, in natural samples the signal derived from fluorochrome-labelled oligonucleotide probes is often undetectable above background fluorescence in many cells (DeLong, 1999).

A possibility to circumvent this difficulty is the application of fluorochrome-labelled polyribonucleotide probes for identification and enumeration of bacteria as an approach to greatly enhance the sensitivity and applicability of FISH with environmental samples (DeLong, 1999).

These RNA probes are poly-labelled during the transcriptional generation and therefore independent of low signal intensities, as the signal amplification is already included by one single probe, randomly labelled during transcription.

For the first RNA polynucleotide probes, targeting the domain III (in all following parts termed as “DIII”) of the 23S rRNA, an previously unseen characteristic signal was obtained after polynucleotide probe hybridisations: a ring-shaped fluorescence in the area of the cell wall, with a dark inside of the cells, termed “HALO” (Trebesius et al, 1994, Stoffels et al 1999, see Fig.A3).

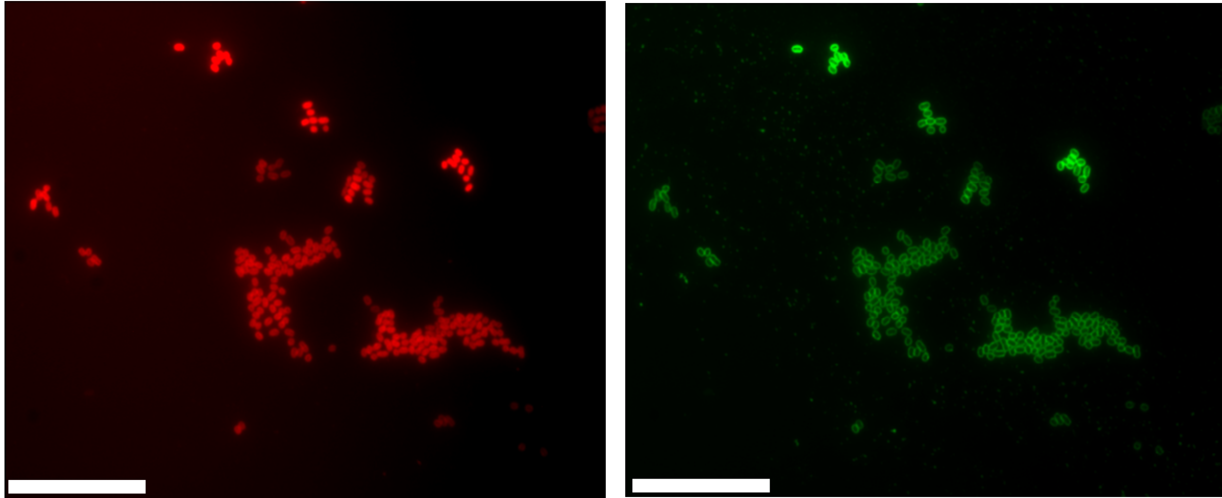

Fig. A.3. Example for the nature of signal obtained after EUB 338 Mix oligonucleotide hybridisation (whole cell fluorescence, Cy3, left) and 23S DIII rRNA targeted polynucleotide hybridisation (halo signal, FLUOS, right) with cells of *Klebsiella ornithinolytica*; bar 20 µm

This halo phenomenon led to formulation of several theories about its nascence. Trebesius et al (1991) and Stoffels et al (1999) proposed that the 250 nucleotide-long polynucleotide transcript probes are too long to enter the cell wall completely, but can penetrate the cell wall and partly bind to the ribosomal target structure, with the rest of the probes sticking out of the cell. These protruding parts of e.g. biotinylated probe molecules have attracted interest as being utilisable in a second hybridisation step, which relies on binding to streptavidin coated magnetic beads, in order to be used for MACS (Magnetic Activated Cell Sorting, e.g. Stoffels et al 1999).

The theory was extended by Zwirglmaier et al (2004) and resulted in the so called "network hypothesis": the polynucleotide probes form a three dimensional network around the whole cell, mediated by formation of inter-probe hybridisation between several probe molecules and intra-probe formation of secondary structures. Parts of the probes are partially linked to their target structures inside of the cell; partially they are embedded in secondary structures of the network in the area of the cell envelope. The cell sorting technique was further processed by introduction of microplates, which were coated with probe complementary nucleic acids, in advantage compared to the MACS cell sorting as a separation system (Zwirglmaier et al, 2004).

Meanwhile, DNA polynucleotide probes targeting the same region as the 23S DIII rRNA polyribonucleotide probes have been developed and led after hybridisations to the same special halo signal. For some applications, they turned out to be an alternative, as these probes may offer several advantages: higher stability, faster

generation, and seemingly less unspecific binding to non-target components, especially when applied to environmental samples (Zimmermann et al, 2001).

Given the signal amplification mediated by the poly-labelling, and the special nature of the signal obtained after hybridisations using polyribonucleotide probes, targets other than ribosomal structures have been considered. Sequences located on high, medium and low copy plasmids and finally chromosomal sequences have been chosen as targets for polyribonucleotide hybridisations. The signals obtained after hybridisations showed the same special characteristics as those observed with ribosomal targets, i.e. a halo. This new FISH technique was termed Recognition of Individual Genes (RING) FISH (Zwirglmaier et al, 2004). Cell sorting could be performed again in microplates, which were coated with complementary nucleic acids.

The development of RINGFISH and the extension of the enrichment technique to RINGFISH probes opened the door to a goal having seemed impossible before: the possibility not only to visualise the existence of single genes in particular bacterial cells, but also to use the special nature of the signal, the halo, evoked by the formation of a network of probe molecules in cell wall localisation for the enrichment of target cells via binding to complementary nucleic acids on different kinds of carrier surfaces. Thus, specifically immobilised target cells can be provided as whole cells for further genetic analysis.

In contrast, the method of chromosome painting, a technique used for microscopic detection of genetic material on subcellular level to identify regions on eukaryotic chromosomes, has also been adapted to identify bacteria (BCP, Bacterial Chromosome Painting, Lanoil et al 1997, Lanoil et al 2001), but is limited to the identification.

Up to now, polyribonucleotide probes and the related enrichment technique (Zwirglmaier et al 2004) have been shown to be applicable for enrichments in artificial mixtures of pure cultures. Cell sorting and enrichment or depletion from environmental or clinical samples based on ribosomal polynucleotide probes have been performed with cell sorting systems such as FACS (Fluorescence Activated Cell Sorting) and MACS (Magnetic Activated Cell Sorting, Stoffels et al, 1999).

Application of rRNA targeted polynucleotide probes and RINGFISH probes with relation to a clinical area and real clinical samples will be shown for the first time within this work.

III. Integration of the presented work within the GenoMik project (Genome Research on microorganisms) and short characterisation of the organisms relevant for the project

The presented work was part of the GenoMik (Genome Research on microorganisms) project, financed by the German federal Ministry of Education and Research (Bundesministerium für Bildung und Forschung, BMBF). The project title was “Rapid diagnosis of antibiotic resistances in medicine”.

In the course of the project, several methods for enrichment, depletion or cell sorting of relevant, bacterial target cells from clinical samples should be tested for their clinical applicability, including the cell sorting technique used in this work and a FACS based method. A subsequent screening for e.g. antibiotic resistance genes using DNA microarray techniques, developed by project partners should be possible after successful enrichment of target organisms from clinical specimen.

The global trend of the rise of antimicrobial resistance is well documented in literature (e.g. Livermore, 2003) and reveals wide variations at national levels (Albrich et al, 2004). The most important resistances occurring among Gram-negative bacteria are extended-spectrum ß-lactamases (ESBL) in *Klebsiella*, *E. coli* and *Proteus mirabilis*, high level third generation cephalosporin (AmpC) resistance, among Enterobacteriaceae like *Enterobacter* spp, *Citrobacter* spp; multidrug resistance among *Pseudomonas aeruginosa*, *Acinetobacter* spp and *Stenotrophomonas maltophilia*. Gram-positive bacteria relevant for the project are methicillin- (oxacillin) resistant *Staphylococcus aureus*, ß-lactam-resistant and multidrug resistant pneumococci and vancomycin-resistant *Enterococci* spp (Jones, 2001).

The organisms relevant for the project were chosen by clinical microbiologists. The list consisted of microorganisms, which were assigned under clinical aspects to five different groups:

1. Non-Fermenters, including inter alia *Pseudomonas* spp, *Acinetobacter* spp, *Stenotrophomonas maltophilia*

The importance of nosocomial infections caused by strictly aerobic Gram-negative bacilli, including *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia* and *Acinetobacte*r spp has been reported during the last 20 years (Bergogné-Bérézin and Towner, 1996).

Pseudomonaceae usually inhabit soil and water and can occur on the surface of plants, as they are true plant pathogens. *Pseudomonas aeruginosa* is the typical opportunistic pathogen of humans. It can cause severe nosocomial infections like uraemia, respiratory system infections, dermatitis, soft tissue infections, bacteremia, bone and joint infections, gastrointestinal infections and a variety of systemic infections. The bacterium is the main organism associated with cystic fibrosis infections. Its natural resistance to many antibiotics is caused by the permeability barrier afforded by its outer membrane lipopolysaccharides. Together with its biofilm associated growth, the cells are nearly impermeable to therapeutic concentrations of antibiotics Since its natural habitat is the soil where *Pseudomonas* is living in association with bacilli, actinomycetes and molds, it has developed resistance to a variety of their naturally-occurring antibiotics. Only a few antibiotics are effective against *Pseudomonas*, including fluoroquinolones, gentamicin and imipenem, and even those antibiotics are not effective against all strains. The futility of treating *Pseudomonas* infections with antibiotics is most dramatically illustrated in cystic fibrosis patients, virtually all of whom eventually become infected with a strain that is so resistant that it cannot be eradicated (www.textbookofbacteriology.net, Lengeler et al, 1999).

Acinetobacter spp are ubiquitously spread and inhabit soil, water and sewage. Although a normal inhabitant of human skin with a moderate low pathogenicity, *Acinetobacter* has consequently gained importance as cause or causative agent for numerous opportunistic infection processes, especially nosocomial infections in hospitals like respiratory infections, bacteraemia, meningitis, uraemia and other infections (Bergogné-Bérézin and Towner, 1996). Nevertheless, most *Acinetobacter* strain isolates from hospitalised patients, particularly those recovered from respiratory secretions and urine, represent colonisation rather than infection. *Acinetobacter baumannii* is a multiresistant aerobic Gram-negative bacillus sensitive

to relatively few antibiotics. Multidrug-resistant Acinetobacter is not a new or emerging phenomenon, but *Acinetobacter baumannii* has always been an organism inherently resistant to multiple antibiotics (www.emedicine.com, Lengeler et al, 1999).

Stenotrophomonas maltophilia is found in a variety of aquatic environments, infrequent in humans. Similar to *Acinetobacter* spp, low pathogenicity but frequent colonisation of hospital setting are characteristic. *S. maltophilia* usually is not capable of causing disease in healthy hosts without the assistance of invasive medical devices that bypass normal host defences. Especially in immunocompromised intensive care patients, and in patients with cystic fibrosis, *Stenotrophomonas* meanwhile has gained importance as causative organism of severe infections (Valdezate et al, 2001, Daporta et al, 2004). Multidrug resistance is reported for many strains isolated in hospitals nowadays, including resistance against ß-lactams, macrolides and aminoglycosides, imipenem and carbapenem (Valdezate et al, 2001).

2. *Enterobacteriaceae*, including inter alia *E. coli*, *Klebsiella* spp and *Enterobacter* spp

As causative organisms of pneumonia, sepsis, wound infection, cutaneous and subcutaneous infections, and uraemia, *Enterobacteriaceae* are known and wide spread as nosocomial pathogens. The natural environment of these partly opportunistic pathogens is usually the intestinal tract of humans and animals. Important factors of pathogenicity are their colonisation factors, including invasins, endo- and exotoxins. Resistance against antibiotics meanwhile is widely spread and complicates the fight against infections (Kayser et al, 2001).

Escherichia coli, a common inhabitant of the human intestine, normally does not cause disease. *E. coli* is an important indicator for faecal pollution of drinking water and food. Most strains are harmless, but several are known to produce toxins that can cause diarrhoea. One particular *E. coli* strain called O157:H7 can cause severe diarrhoea and kidney damage. Other strains, including enterohaemorrhagic E. coli (EHEC), enteropathogenic E. coli (EPEC), enterotoxigenic E. coli (ETEC), enteroinvasive E. coli (EIEC), enteroaggregative E. coli (EAggEC), uropathogenic E. coli (UPEC) and neonatal menigitis E. coli (NMEC) have gained much importance during the last 50 years as causatives of human infections such as meningitis,

septicaemia (15% of all cases of sepsis), urinary tract infections (70-80% of these infections are caused by *E. coli*), and intestinal infections (Kayser et al, 2001).

Klebsiella spp are ubiquitous in nature. In humans, colonisation of skin, pharynx, or gastrointestinal tract can occur. In recent years, *Klebsiella* spp have become important pathogens in nosocomial infections. Common sites include the urinary tract, lower respiratory tract, biliary tract, and surgical wound sites. Especially *K. pneumoniae* and *K. oxytoca* are responsible for most human infections. Extensive use of broad-spectrum antibiotics in hospitalised patients has led to both increased carriage of *Klebsiella* spp and, subsequently, the development of multidrug-resistant strains that produce an extended-spectrum beta-lactamase (ESBL). The first mutated form of beta-lactamases was isolated from a clinical strain in Germany 1983 (Shaw, 2004). ESBL strains are highly virulent, and have an extraordinary ability to spread. Infections with these species have become a major problem in most hospitals because of resistance to multiple antibiotics and potential transfer of plasmids to other organisms.

Enterobacter species, particularly *Enterobacter cloacae* and *Enterobacter aerogenes,* are important nosocomial pathogens responsible for a variety of infections, including bacteremia, lower respiratory tract infections, skin and soft tissue infections, urinary tract infections, endocarditis, intra-abdominal infections, septic arthritis, osteomyelitis, and ophthalmic infections. Multidrug resistance is wide spread: the prevalence of resistance to ß-lactam antibiotics, aminoglycosides, trimethoprim-sulfamethoxazole, and quinolones is common. Among the predisposing factors for such infections are prolonged hospitalization, in particular in intensive care units; prior treatment with antibiotics, general debilitation, and immunosuppression. Infection management is complicated by multiple antibiotic resistances. These bacteria possess inducible ß-lactamases, which are undetectable *in vitro* but are also responsible for resistance during treatment .

3. *Staphylococcus* spp

Staphylococci are normal inhabitants of the skin and/or mucosal surfaces of most warm-blooded animal and humans. The quantity of an individual *Staphylococcus* species (e.g. *S. aureus* versus *S. epidermidis*) depends on the host - some strains and species are particularly host-adapted (www.cvm.uiuc.edu). Species of

staphylococci, especially *S. aureus and S. epidermidis*, together with *E. coli* are the most prevalent causative organisms of infections in humans. Involved in nosocomial infections as well as in infections associated with medical devices, food born intoxications, pneumonia and wound infections (Kayser et al, 2001), *S. aureus* is the pathogen most frequently isolated pathogen from blood culture specimen. Clinical strains of staphylococci frequently remain undetected, because they have the capacity to reduce their growth rate considerably and to appear in so called small-colony variants (Krimmer et al, 1999). Thus, conventional microbiological techniques easily fail to detect them. Moreover, the formation of thick, multilayered biofilms associated to medical devices hampers detection. Since the 1960´s, the emergence of plasmid-encoded multiple resistant staphylococci (MRS, formerly termed methicillin resistant staphylococci) carrying resistance to methicillin and to most currently available antibiotics has dramatically narrowed the therapeutic arsenal to the exclusive use of glycopeptides such as vancomycin as main treatment in overuse, thus leading to MRSA (Multi Resistant *Staphylococcus aureus*) strains with decreased susceptibilities to glycopeptides (Francois, 2003).
Nowadays, many techniques have been designed for special detection of staphylcocci, e.g. commercially available agglutination kits, tests for detection of special enzymes produced only by *S. aureus* (Garcia et al, 2002) or development of novel media for exclusive growth of MRSA strains of staphylococci (Blanc et al, 2003). These techniques still rely on automated microbial detection systems (e.g. BacT/Alert, Biomerieux) of positive blood culture bottles or require culturing on plates. In contrast, the usage of FISH with oligonucleotide probes allows direct visualisation of staphylococci in clinical specimen (Krimmer et al, 1999). Flow cytometry and DNA microarrays can provide and facilitate high turnover rates.

4. *Streptococcus* spp

The genus *Streptococcus* is comprised of a wide variety of both pathogenic and commensal Gram-positive bacteria**.** They are found to inhabit a wide range of hosts, including humans and animals, often colonising the mucosal surfaces of the mouth, nares and pharynx. However, in certain circumstances, they may also inhabit the skin, heart or muscle tissue. Pathogenic streptococci of humans include *S. pyogenes, S. pneumoniae*, and *S. disagalactiae*. Among the pathogenic hemolytic streptococci, *S. pyogenes*, or group A streptococci, have been implicated as the

etiologic agent of acute pharyngitis ("strep throat"), impetigo, rheumatic fever, scarlet fever, glomerulonephritis, and invasive fasciitis (www.rockefeller.edu). Some Streptococci produce exotoxins and different enzymes, (e.g. streptolysine, streptokinase, hyaluronidase), leading to rapid spread and progression of infection (Kayser et al, 2001).

5. *Enterococcus* spp

Enterococci are widely distributed Gram-positive bacteria and can be found in air, water, sewage, soil and vegetation. They are also a dominant part of the microflora in many traditional fermented foods with a high influence on texture and taste and their application as starter cultures for special fermentations is common. As their primary source is the intestinal tract of humans and animals, enterococci exhibit only a marginal potential as pathogens (Lukasova et al, 2002). Even though, they have become an important cause of nosocomial infections (Kayser et al, 2001) and can lead to bacteraemia, uraemia, endocarditis and wound infections. Most of the clinical isolates belong to the species *E. faecalis* and *E. faecium*. Enterococci have been known to be resistant to most antibiotics used in clinical practice, as they are innately resistant to cephalosporines, aminoglycosides, clindamycin and probably to tetracyclines and erythromycin (Lukasova et al, 2002). Easy acquisition of antibiotic resistance and the capacity to pass on resistances to other species (Kühn et al, 2000) leads to the world wide increase of occurrence of strains, which harbour resistances against all current antibiotics. Especially vancomycin resistant enterococci are emerging as a global threat to public health (Lukasova et al, 2002).

IV. Aims of the thesis

The aims of this work were the transfer of the established technique for the polynucleotide probe based enrichment of bacterial cells in microplate cavities for its first time into a clinical setting, where a fast determination of identity, pathogenicity, and occurrence of antibiotic resistances of infectious organisms is the most important challenge during clinical routine diagnostics.

In the periphery of this, a number of modifications of already existing protocols had to be evaluated:

1. the time factor
2. adaptation to Gram-positive bacteria as a major task
3. transfer and adaptation to real clinical samples.

Furthermore, antibiotic resistance genes of clinical strains should be targeted with RINGFISH and the applicability of RINGFISH on real clinical samples should be tested.

The last aim was to examine, whether the advantages of rRNA targeted oligonucleotide probes (species-specificity, short hybridisation time) can be combined with the advantages of polynucleotide probes (halo-signal due to network formation, which can be used further for polynucleotide probe based enrichment of bacterial cells). Therefore, synthetic polynucleotide probes should be developed, consisting of repetitive stretches of specific oligonucleotide probe sequences.

B. Material and Methods

I. Bacterial strains and sample material

1. Organisms from culture collections

1.1. Gram-negative bacteria

Table B.1. Gram-negative bacteria in pure culture derived from culture collections

Organism	Origin*	Cultivation conditions
Escherichia coli	DSM 1103	LB, 37°C aerob
Neisseria canis	LMG 8383T	LB 30°C aerob
Pseudomonas aeruginosa	DSM 50071	HD/LB 30 °C aerob
Pseudomonas fluorescens	DSM 50124	Nutrient 30 °C aerob
Acinetobacter junii	ATCC 17908	R2A 30 °C aerob
Acinetobacter Iwoffii	ATCC 15309	R2A 30 °C aerob
Acinetobacter baumanii	ATCC 17978	R2A 30 °C aerob
Pseudomonas putida	DSM 291	Nutrient 30 °C aerob
Pseudomonas stutzeri	DSM 50238	Nutrient 30 °C aerob
Klebsiella oxytoca	DSM 5175	Nutrient 37°C aerob
Klebsiella terrigena = *Raoultella terrigena*	DSM 2687	Nutrient 37°C aerob
Klebsiella ozaenae = *Klebsiella pneumoniae ssp ozaenae*	LMG 3113	Nutrient 30°C aerob
Klebsiella ornithinolytica = *Raoultella ornithinolytica*	DSM 7464	Nutrient 37°C aerob
Klebsiella pneumoniae	DSM 30104	Nutrient 37°C aerob
Citrobacter freundii	DSM 30039	Nutrient 37°C aerob
Citrobacter youngae	NCIMB 13435	Nutrient 37°C aerob
Stenotrophomonas maltophilia	DSM 50170	Nutrient 37°C aerob
Pantoea agglomerans	DSM 3493	Nutrient 37°C aerob
Hafnia alvei	DSM 30163	Nutrient 37°C aerob
Enterobacter aerogenes	DSM 30053	LB, 37°C aerob
Enterobacter cloacae	DSM 30054	CasoAgar 37°C aerob
Serratia marcescens	DSM 30121	LB, 37°C aerob
Morganella morganii	DSM 30164	Nutrient 37°C aerob

* Origin see Table B.2.

1.2. Gram-positive bacteria

Table B.2. Gram-positive bacteria in pure culture derived from culture collections

Organism	Origin*	Cultivation conditions
Enterococcus faecium	DSM 20477[T]	BHI 37°C anaerob
Enterococcus faecalis ssp. liquefaciens	DSM 20380	BHI 37°C anaerob
Enterococcus durans	DSM 20633	BHI 37°C anaerob
Enterococcus gallinarum	DSM 30628	BHI 37°C anaerob
Staphylococcus aureus	DSM 20041	BHI 37°C anaerob
Staphylococcus saprophyticus	CCM 883	BHI 37°C anaerob
Staphylococcus haemolyticus	DSM 20263	BHI 37°C anaerob
Staphylococcus epidermidis	DSM 20044	BHI 37°C anaerob

* Origin:
DSM: DSMZ- Deutsche Staatssammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany: http://www.dsmz.de;
LMG: BCCM/LMG-Bacteria Collection, Laboratorium voor Mikrobiologie, Gent, Belgium: http://www.belspo.be/bccm/index.htm;
ATCC: American Type Culture Collection, Manassas, Virginia, USA: http://www.atc.org;
NCIMB: National Collections of Industrial, Marine and Food Bacteria, UK: http://www.ncimb.com/index.php
[T] = Type strain;

2. Organisms from Robert Bosch hospital in pure culture and spiked in real clinical samples

Table B.3. Organisms in pure culture and spiked in real clinical samples, derived from Robert Bosch hospital, Stuttgart, Germany

Organism	AF- Number*	Sample material / Fixation **
Enterococcus faecium	458387	EDTA blood, plasma, urine; PFA 4%
Enterococcus faecalis	no number	EDTA blood, plasma, urine; PFA 4%
Escherichia coli	441612	EDTA blood, plasma, urine; PFA 4%
Enterobacter aerogenes	427068	EDTA blood, plasma, urine; PFA 4%
Staphylococcus aureus	433088	EDTA blood, plasma, urine; PFA 4%
Pseudomonas aeruginosa	426470	EDTA blood, plasma, urine; PFA 4%
Stenotrophomonas maltophilia	484133	EDTA blood, plasma, urine; PFA 4%
Haemophilus influenzae	484476	EDTA blood, plasma, urine; PFA 4%
Acinetobacter baumannii	438775	EDTA blood, plasma, urine; PFA 4%
Burkholderia cepacia	RVB strain2	EDTA blood, plasma, urine; PFA 4%
Klebsiella pneumoniae	429457	EDTA blood, plasma, urine; PFA 4%
Klebsiella oxytoca	479983	EDTA blood, plasma, urine; PFA 4%
Staphylococcus epidermidis	no number	EDTA blood, plasma, urine; PFA 4%
Pseudomonas fluorescens	603085	EDTA blood, plasma, urine; PFA 4%
Streptococcus pyogenes	611333	EDTA blood, plasma, urine; PFA 4%
Proteus mirabilis	413702	pure culture

*AF: number for 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; * * PFA Fixation as described in B. III.1.

3. Real clinical samples and pure culture organisms isolated from the samples collected at Robert Bosch hospital

Table B.4. Real clinical samples and main interest pure culture organism isolated from the real clinical sample, collected at Robert Bosch hospital, Stuttgart, Germany

Sample number	Real sample specimens	AF- number of organism	Main interest pure culture organism isolated from real clinical sample	Fixation*
1	Urine	421100	*E. coli*	PFA 4%
2	Blood culture aerobic	430319	*E. coli*	PFA 4%
3	Wound smear	625811	*Pseudomonas aeruginosa*	PFA 4%
4	Tracheal swab	492106	*Pseudomonas aeruginosa*	PFA 4%
5	Wound smear	615234	*Staphlycoccus aureus*	PFA 4%
6	Urine	625857	*Enterococcus faecalis*	PFA 4%
7	Urine	422200	*Klebsiella oxytoca*	PFA 4%
8	Blood culture anaerobic	630073	*Staphlycoccus aureus*	PFA 4%
9	Tracheal swab	41162	*Klebsiella pneumoniae*	PFA 4%
10	Urine	415772	*Enterococcus faecium*	PFA 4%
11	Urine	49218	*Pseudomonas aeruginosa*	PFA 4%

* Fixation of sample and pure culture organism isolated from sample; PFA fixation as described in B. III.1.; the possibility to collect the real sample specimens at RBK was kindly provided by Prof. Dr. Cornelius Knabbe, Department of Clinical Chemistry and Laboratory Medicine, Robert Bosch hospital, Stuttgart

II. Cultivation of organisms

All organisms not obtained in fixed form were cultivated at conditions given in tables B.1. and B.2.

All listed media were prepared with dH_2O, sterilised by autoclaving for 20min, at 121 °C and a pressure of 1 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

BHI medium (Brain- Heart- Infusion)

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

HD Medium

peptone	12 g
glucose	5 g
yeast extract	5 g
NaCl	8 g
dH_2O	ad 1000 ml pH 7,2

R2A medium

peptone	0.5 g
yeast extract	0.5 g
casamino acids	0.5 g
glucose	0.5 g
starch, soluble	0.5 g
K_2HPO_4	0.3 g
$Na_4P_2O_7$	0.3 g
$MgSO_4$ x 7 H_2O	50 mg
dH_2O	ad 1000 ml pH 7.2

Caso agar

Peptone from casein	15.0 g
Peptone from soymeal	5.0 g
NaCl	5.0 g
Agar	15.0 g
dH_2O	ad 1000 ml pH 7.3

III. Fixation of organisms and sample material

1. PFA fixation

Reagents

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} 0.4-0.8) and centrifuge at 5000 – 12000 rpm for 2- 10min (depending on culture volume)
✦ resuspend cell pellet in PBS, (depending on cell pellet volume) and add 3 volumes of PFA 4%
✦ incubate at 4°C or on ice for 0.5- 12h
✦ centrifuge at 5000 – 12000 rpm for 2- 10min
✦ wash with appropriate amount of PBS
✦ resuspend in appropriate amount of PBS: $EtOH_{abs}$ in ratio 1:1
✦ store at -20°C up to 1 year

Procedure for fixation of spiked and real clinical samples

✦ a) centrifuge liquid sample material at 5000 – 12000 rpm for 2- 10min (depending on cell / sample volume) or
✦ b) directly (for highly viscous and granulous samples)
✦ for a): resuspend sample pellet in PBS, (depending on sample volume)
for a) +b): add 3 volumes of PFA 4%
✦ incubate at 4°C or on ice for 0.5- 12h
✦ centrifuge at 5000 – 12000 rpm for 2- 10min
✦ discard supernatant

✦ wash with appropriate amount of PBS

✦ resuspend in appropriate amount of PBS: $EtOH_{abs}$ in ratio 1:1

✦ store at -20°C up to 1 year

2. EtOH fixation

Reagents

PBS (see point III.1.)

$EtOH_{abs}$

Procedure for bacterial cells in pure culture

✦ harvest cells during exponential growth phase (OD 0.4-0.8) and centrifuge at 5000 – 12000 rpm for 2- 10min (dependent on cultivation volume)

✦ resuspend cell pellet in PBS, (depending on cell pellet volume) and add 1 vol $EtOH_{abs}$

✦ incubate at 4°C or on ice for 0.5- 12h

✦ centrifuge at 5000 – 12000 rpm for 2- 10min

✦ discard supernatant

✦ wash with appropriate amount of PBS

✦ resuspend in appropriate amount of PBS: $EtOH_{abs}$ in ratio 1:1

✦ store at -20°C up to 1 year

3. Bouin`s fixation

Reagents

Picric acid (Merck, Darmstadt, Germany)

Formaldehyde (Merck, Darmstadt, Germany)

Glacial acetic acid (Merck, Darmstadt, Germany)

Preparation of Bouin`s solution (50%):

✦ transfer 10 to 50 g picric acid to an airtight bottle

✦ fill up with H_2O_{MQ}

✦ incubate for 7 days while shaking several times

✦ remove 15 volumes of supernatant prior to use and mix with 5 volumes of 37% formaldehyde and 1 volume of glacial acetic acid

Procedure for fixation of bacterial cells and spiked clinical sample material

✦ transfer fixative and cultivated cells or sample material on a slide well with a ratio of 1:1

✦ air dry

✦ wash 3 times with H_2O_{MQ}

✦ continue with hybridisation or store at -20 °C

4. Formalin fixation

Reagents

PBS (see point III.1.)

$EtOH_{abs}$

Preparation of 4% formalin solution:

$NaH_2PO_4 \cdot 2\ H_2O$	10.0 g
$Na_2HPO_4 \cdot 7\ H_2O$	1.6 g
(Merck, Darmstadt, Germany)	
H_2O_{MQ}	ad 900 ml
formaldehyde 36-40%	100 ml

Procedure for fixation of bacterial cells in pure culture

✦ harvest cells during exponential growth phase (OD 0.4-0.8) and centrifuge at 5000 – 12000 rpm for 2- 10min (dependent on cultivation volume)

✦ resuspend cell pellet in 4% formalin solution

✦ incubate at 4°C for 4- 6h

✦ centrifuge at 5000 – 12000 rpm for 2- 10min

✦ discard supernatant

✦ wash with appropriate amount of PBS

✦ resuspend in appropriate amount of PBS: $EtOH_{abs}$ in ratio 1:1

✦ store at -20°C up to 1 year

IV. Isolation of nucleic acids

1. Genomic DNA

Isolation of genomic DNA (Wisotzkey et al, 1990, modified)

Reagents:

Saline-EDTA solution	0.15 M NaCl
	0.01 M EDTA, pH 8.0
20x SSC	3 M NaCl
	0.3 M tri-sodium-citrate, pH 7.0
lysozyme	10 mg/ml in 10mM Tris/HCl
Tris/HCl	10 mM Tris/HCl, pH 8.5
proteinase K	10 mg/ml proteinase K (Roche, Mannheim, Germany)
RNase A	10 mg/ml RNase A (Merck, Darmstadt, Germany) in H_2O_{MQ}
NaAc	5 M sodium acetate pH 5.5
SDS	25% (w/v) sodiumdodecylphosphate
CIA	chloroform: isoamylalcohol 24:1 (w/v)
$EtOH_{abs}$	
EtOH 70%	

Procedure

✦ centrifuge 5-30 ml overnight culture at 12000 rpm for 5min

✦ resuspend cell pellet in 1-6 ml Saline-EDTA, centrifuge at 12000 rpm for 3min

✦ resuspend cell pellet in 500 µl-3 ml Saline-EDTA

✦ add 20-120 µl of lysozyme and incubate at 37°C for 30min

✦ add 10-60 µl of RNase A and incubate at 37°C for 30min

✦ add 10-60 µl of proteinase K and incubate at 37°C for 30min

✦ add 40-240 µl of SDS and incubate at 60°C for 10min for cell lysis

✦ add 745 µl - 4470 µl CIA and 180-1080 µl NaAc, shake carefully several times

✦ centrifuge at 12000 rpm for 5-10min

✦ transfer upper phase to fresh tube and discard lower phase

✦ add 2 vol of $EtOH_{abs}$ and store at -20 °C for 2 h for precipitation of DNA

✦ centrifuge at 12000 rpm for 15min

- ✦ wash pellet with EtOH 70%
- ✦ resuspend DNA in 100-200 µl H_2O_{MQ} and store at -20 °C

2. Plasmid DNA

Isolation was carried out with QIAprep Spin Miniprep Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions.

V. Photometric measurement of nucleic acid concentration

Nucleic acids have an absorption maximum between 253 and 271 nm. For determination of the concentration of nucleic acids, the following approximate values are assumed:

Double stranded (ds) DNA: 1 OD 260 nm ≈ 50 µg/ml
Single stranded (ss) DNA: 1 OD 260nm ≈ 33 µg/ml (www.eppendorf.com)
(Single stranded (ss) RNA: 1 OD 260 nm ≈ 40 µg/ml)
The quotient between E260 nm/E230 nm and E260 nm/E280 nm can be used to assign potential contaminations with protein or RNA (Marmur, 1961):

$$E\ 260/E\ 230 > 2.2 \text{ and}$$
$$E\ 260/E\ 280 > 1.9$$

For calculating the concentration of ss oligonucleotide values from table B.5 were applied.

Table B.5. Extinction of coefficients of oligonucleotides

Nucleotide	extinction coefficient ε at 260nm [µmol/cm^2]	molecular weight [g/mol]
dAMP	15.20	312.2
dCMP	7.05	288.2
dGMP	12.01	328.2
dTMP	8.40	303.2

$$\frac{Vc \times OD260nm}{V_{Sample} \times \varepsilon \times d}$$

c: oligonucleotide concentration [mM]
Vc: cuvette volume [ml]
V_{sample}: sample volume [ml]
d: diameter of cuvette [cm]
ε: extinction coefficient [cm2/µmol] specific for oligonucleotide (see Table B.5)

VI. Agarose Gel Electrophoresis

Agarose gel electrophoresis is a standard method to estimate purity, size and concentration of nucleic acids.
Loaded on an agarose gel, nucleic acids will migrate towards the anode, due to their negative charge. Dependent on mass and conformation of the nucleic acid (circular, linear, supercoiled) it is possible to separate and visualise them under UV-light (302nm) using DNA intercalating Ethidiumbromide or SYBR-Green^{TM}.
A standard marker carried along allows obtaining information about size and amount of nucleic acids.

Reagents and equipment

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)
	Melted in 1x TAE
Loading buffer # 1	10 mm EDTA
	5% (w/v) Ficoll (Sigma-Aldrich, Steinheim, Germany)
	0.05% bromphenol blue
	0.05% xylene cyanol
	0.10% SYBR-GreenTM (FMC BioProducts, Rockland, ME, USA)
Loading buffer # 2	10 mm EDTA
	5% (w/v) Ficoll (Sigma, Steinheim, Germany)
	0.05% bromphenol blue

	0.05% xylene cyanol
Ethidiumbromide	1 µg/ml ethidiumbromide
Standard marker	1 µg 1kb standard (Invitrogen)
Gel chamber	Gel electrophoresis chamber (Gibco/BRL, Eggenstein, Germany) Type H3: 11x14 cm, 100 ml gel volume
Gel documentation	UV-transilluminator; 302 nm wavelength (Bachofer, Reutlingen, Germany) Cybertech CS1 Image Documentation (Cybertech, Berlin, Germany)

Electrophoresis using loading buffer # 1 with SYBR-GreenTM

✦ load gel with a mixture 1 vol of sample and one vol of loading buffer # 1
✦ start electrophoresis in 1x TAE and 90- 120 mA
✦ documentation using transilluminator

Electrophoresis using loading buffer # 2 without SYBR-GreenTM

✦ load gel with a mixture 1 vol of sample and one vol of loading buffer # 2
✦ start electrophoresis in 1x TAE and 90- 120 mA
✦ stain gel in EtBr solution for 20 minutes and wash with water
✦ documentation using transilluminator

VII. Primers

The tables below list all PCR and sequencing primers, oligonucleotide and polynucleotide probes that were used in this work.
All nucleotides have been synthesized and purified by MWG-Biotech AG (Ebersberg, Germany).
According to IUB, Nomenclature Committee, 1985, the following symbols for bases have been used:

M: A or C	**S:** C or G	**V:** A or C or G	**B:** C or G or T
R: A or G	**Y:** C or T	**H:** A or C or T	**N:** A, C, G or T
W: A or T	**K:** G or T	**D:** A or G or T	

1. Primers for amplification of 23S and 16S rDNA

1.1. Primers for 23S rDNA amplification

Table B.6. Primers for amplification of 23S rDNA; V= forward primer, R= reverse primer; the underlined part of the sequence shows the binding site for T3 polymerase

name	position*	sequence [5´-3´]	TD [C°]	GC [%]
313V	241	AGTAGCGGCG	43	70
317R	1601	ACC WGT GTC SGT TTH BGT AC	50	50
118V	115	CCG AAT GGG GRA ACC C	53	66
985R	2654	CCG GTC CTC TCG TAC T	52	63
1900VN	1366	MAD GCG TAG BCG AWG	58	63.6
317RT3	1601	ATA GGT ATT AAC CCT CAC TAA AGG ACC WGT GTC SGT TTH BGT AC	50	50

* (*E. coli*, Brosius at al, 1981)

1.2. Primers for 16S rDNA amplification

Table B.7. Primers for amplification of 16S rDNA; V= forward primer, R= reverse primer

name	position*	sequence [5´-3´]	TD [C°]	GC [%]
616Valt	8	AGA GTT TGA TYM TGG CTC AG	58	45
630R	1529	CAK AAA GGA GGT GAT CC	50	47

* (*E. coli*, Brosius at al, 1981)

2. Primers for amplification of antibiotic resistance genes

2.1. *ampC* gene of *E. coli*

Table B.8. Primers for amplification of the *ampC* gene of *E. coli;* V= forward primer, R= reverse primer; the underlined part of the sequence shows the binding site for T3 polymerase

name	sequence [5´-3´]	TD [C°]	GC [%]
EcoAmpC V3	ACG ATC TGA AAA TTC ACG TGC C	53	45.5
EcoAmpC RT3	ATA GGT ATT AAC CCT CAC TAA AGG GTT GCC AGT AGC GAG ATT GTG	51.8	50

The primers have been designed for the sequence **NCBI AF124204.**

2.2. *tetAJ* gene of *Proteus mirabilis*

Table B.9. Primers for amplification of the *tetAJ* gene of *Proteus mirabilis;* V= forward primer, R= reverse primer; the underlined part of the sequence shows the binding site for T3 polymerase

name	sequence [5´-3´]	TD [C°]	GC [%]
ProtTetAJ V1	ACG AAT AAT TGC GGG TAT	59	38.9
ProtTetAJ V2	TGC AGG TGA TCT TCG CAC	66	55.6
ProtTetAJ RT3	ATA GGT ATT AAC CCT CAC TAA AGG GCG GAT ATT TCA GTC GAA A	59	38.9

The primers have been designed for the original sequence **NCBI AF038993.**

2.3. *ermB* gene of *Enterococcus faecium*

Table B.10. Primers for amplification of the *ermB* gene of *Enterococcus faecium;* V= forward primer, R= reverse primer; the underlined part of the sequence shows the binding site for T3 polymerase

name	sequence [5´-3´]	TD [C°]	GC [%]
E.f.ermB V1	TTC AAT TCC CTA ACA AAC AGA GG	51.7	39,1
E.f.ermB RT3_1	ATA GGT ATT AAC CCT CAC TAA AGG GTG TTT ACT TTG GCG TGT TTC A	48.5	38,1
E.f.ermB V_2	GCC GTG CGT CTG ACA TCT AT	53.8	55
E.f.ermB RT3_2	ATA GGT ATT AAC CCT CAC TAA AGG GGC GTG TTT CAT TGC TTG ATG	66	45

The primers have been designed for the original **NCBI AF 229200.**

2.4. *parC* gene of *Enterococus faecalis*

Table B.11. Primers for amplification of the *parC* gene of *Enterococcus faecalis;* V= forward primer, R= reverse primer the underlined part of the sequence shows the binding site for T3 polymerase

name	sequence [5´-3´]	TD [C°]	GC [%]
E.f.l.ParC V	AGA CGG CAA TAC CTT TG	50	47
E.f.l. ParC RT3	ATA GGT ATT AAC CCT CAC TAA AGG GCG CCA TCC CTC CTT CC	52	56

The primers have been designed for the original sequence **NCBI AB 005036.**

3. Primers for amplification of plasmid DNA

Table B.12. Primers for amplification of plasmid DNA; V=forward primer, R=reverse primer

name	position	sequence [5´-3´]	TD [C°]	GC [%]
TEM F*	*bla*$_{TEM}$	ATG AGT ATT CAA CAT TTC CG	62	35
TEM R*	*bla*$_{TEM}$	TTA ATC AGT GAG GCA CCT AT	64	40
M13V	Multiple cloning site of plasmid pCR2.1**	GTA AAA CGA CGG CCA G	50	56
M13R	Multiple cloning site of plasmid pCR2.1**	CAG GAA ACA GCT ATG AC	50	47

* Grimm et al, 2004; **(Invitrogen, Karlsbad, CA, USA)

4. Sequencing primers

Table B.13. Primers for sequencing reactions, specific for 23S rDNA; V= forward primer, R=reverse primer

name	position*	sequence [5´-3´]	TD [C°]	GC [%]
M13V	Multiple cloning site of plasmid pCR2.1*	GTA AAA CGA CGG CCA G	50	56
M13R	Multiple cloning site of plasmid pCR2.1*	CAG GAA ACA GCT ATG AC	50	47
328V	1923	TCC TAA GGT AGC GAA ATT CCT TC	59	43
335V	23	GGT GGA TGC CYW GGC	55	70
992V	457	AGT ACC GTG AGG RAA	46	50
992R	457	TTC CCT CAC RGT ACT	46	50
1019V	803	TAG CTG GTT CTY YCC GAA	54	50
1019R	803	TTC GRR GAG AAC CAG CTA	54	50
1020R	976	TCT GGG YTS TTY CCC T	50	53
1027V	1608	AAA CCG ACA CAG GTR G	50	53
1036V	2492	TTG RYM CYT CGA TGT CG	52	53
1037R	1934	CTT ACC CGA CAA GGA ATT TCG	58	48

* *E. coli* position, Brosius at al, 1981; **Invitrogen, Karlsbad, CA, USA

VIII. Probes for Fluorescence *in situ* hybridisation (FISH)

1. Oligonucleotide probes for Fluorescence *in situ* hybridisation (oligo-FISH)

Table B.14. Oligonucleotide probes specific for 23S or 16S rRNA

Target organism	name		Position* and gene	Sequence [5´-3´]	TD [C°]	GC [%]
Bacteria	EUB 338[1]	EUB-Mix	338; 16S	GCTGCCTCCCGTAGGAGT	55	66.7
Applied as completion to EUB338; Planctomycetales	EUB 338 II[2]		338; 16S	GCAGCCACCCGTAGGTGT	66	66.7
Applied as completion to EUB338; Verrucomicrobiales	EUB 338 III[2]		338; 16S	GCTGCCACCCGTAGGTGT	66	66.7
Escherichia coli	1167[3]		1167; 23S	GCATAAGCGTCGCTGCCG	55	66.7
Enterococcus faecium	Efi58[4]		1475; 23S	TGACTCCTCTTCAGACTT	52	44.4
Enterococcus faecalis	Efe1473[4]		1473; 16S	TAACTCTACTCAAGACTCAT	54	35
Staphylococcus aureus	SAU 227[5]		227; 16S	AATGCAGCGCGGATCCAT	56	55.6
Pseudomonas aeruginosa	PseAerA[6]		1499; 23S	TCTCGGCCTTGAAACCCC	58	61.1
Pseudomonas fluorescens	PsAg1[7]		1520; 23S	GATAACTCGTCATCAGCTC	56	47.5
Enterobacter aerogenes	Eae 16S[8]		473; 16S	CGAGTAACGTCAATC GCC	55,6	56
Acinetobacter baumanii	Aba 79[9]		79;16S	TAGGTCCGGTAGCAAGCT	56	55
Stenotrophomonas maltophilia	Stemal[6]		633; 16S	GTCGTCCAGTATCCACTGC	53	57.9
Klebsiella pneumoniae	Kpn1721[5]		1721; 23S	CCACCCGGCAGGGGCTTC	66.7	60
Klebsiella species	Kpn1701[5]		1701; 23S	CCTACACACCAGCGTGCC	64	77,8

* (*E. coli*, Brosius et al, 1981);
[1]Amann et al, 1990; [2] Daims et al, 1999 ; [3] Neef at al, 1995; [4] Behr, 2002; [5] Santos, 2002, unpublished ; [6] Hogardt et al, 2000 ; [7]Boye et al, 1995 ; [8] Sandjong, 2003, unpublished; [9] Zimmermann, 2002

2. Polynucleotide probes for Fluorescence *in situ* hybridisation (poly-FISH)

2.1. 23S DIII rRNA targeted polynucleotide probes

All 23S DIII rRNA targeted probes have been generated by using the general primers 1900VN and 317RT3 (listed in Table B.6). The probes are usually approximately 250-300 bases long, depending on insertions or deletions typical for some DIII regions of different bacteria and can be labelled during transcriptional generation with e.g. Biotin-16-UTP and FLUOS-12-UTP as described in part X.1.

2.2. Recognition of individual genes (RING) FISH probes

The following probes have been generated using the specific primers included in the table:

Table B.15. RINGFISH probes generated using primers listed in Tables B.8.-B.11

name	Length of probe [nt]	primers used for generation
EcoAmpC 3	990	EcoAmpC V3 - EcoAmpC RT3
ProtTetAJ V1	259	ProtTetAJ V1 - ProtTetAJ RT3
ProtTetAJ V2	420	ProtTetAJ V2 - ProtTetAJ RT3
E.f.ermB V1	402	E.f.ermB V1 - E.f.ermB RT3_1
E.f.ermB V2	302	ermB V2 - ermB RT3_2
E. f.l. parC	182	parC V - parC RT3

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2.3. Synthetic construct probes

The following synthetic construct probes have been synthesized at MWG, Ebersberg, Germany.

Table B.16. DNA polynucleotide probes, consisting of a 4-time repetition of oligonucleotide probes listed in Table B.15.

Target organism	Name and labelling of probe	sequence of probe [5`-3`]	TD [°C]	GC [%]
Pseudomonas aeruginosa	PseAer_4Cy3	$(TCTCGGCCTTGA\ AACCCC)_4$	97,2	61.1
Pseudomonas fluorescens	PseFl_4*	$(GATAACTCGTCATCAGCTC)_4$	92	47.4
Klebsiella pneumoniae	Kpn_4 *	$(CCTACACACCAGCGTGCC)_4$	99.5	66.7

* labelled with BiotinChemLink (Roche, Mannheim, Germany)

The following probes have been generated via *in vitro* transcription X.1.2. using synthetic constructs as template.

Table B.17. RNA polynucleotide probes, consisting of a 4-time repetition of oligonucleotide probes listed in Table B.16, but divided by poly A – spacers (blue marked italics)

Target organism	Name of probe	sequence of probe 5`-3`direction	TD [°C]	GC [%]
Pseudomonas aeruginosa	PseAer4_sp	**UCUCGGCCUUGAAACCC***AAAAA***UCUCGGCCUUG AAACCCC***AAAAA***UCUCGGCCUUGAAACCCC***AAA AA***UCUCGGCCUUGAAACCCCC**	93,8	56,5
Pseudomonas fluorescens	Psefl4_sp	**CUCGACUACUGCUCAAUAG***AAAAA***CUCGACUAC UGCUCAAUAG***AAAAA***CUCGACUACUGCUCAAUA G***AAAAA* **CUCGACUACUGCUCAAUAG**	90.6	52,9

IX. Polymerase chain reaction

The enzymatic technique for specific amplification of defined DNA fragments, Polymerase Chain Reaction, includes a cyclic repetition of the following steps:

Denaturation: Thermal denaturation of the DNA to be amplified

Annealing: Oligonucleotide primers bind specifically to their complement target sequence

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

After the first cycle, the amplification starts to be exponential. The first synthesised strands are the templates for the following cycles. 10^6 fold amplification of the original sequence flanked by the primers is possible under optimal conditions of 25-30 cycles. Increasing the cycle number will end exponential amplification (Saiki et al, 1988).

Dependent on the diversity of used primers, a calculation of the specific dissociation temperature T_D is necessary. Suggs et al. (1981) propose an approximate value +/- 2°C for optimal annealing determined with the following formula:

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

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

All PCR reactions in this study were done with the following equipment and reagents:

Equipment

Primus 96 plus Thermal Cycler (MWG, Ebersberg, Germany)

Eppendorf Mastercycler Gradient (Eppendorf, Hamburg, Germany)

Reagents and reaction mixture

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

Reaction mixture per 100µl PCR reaction:

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

Expand High Fidelity^{TM} system (Roche, Mannheim, Germany)

Reaction mixture per 100µl PCR reaction:

Buffer (10x)	10 µl
dNTP-mix (10mM each)	2 µl
primer forward (50 µmol)	0.4 µl
primer reverse (50 µmol)	0.4 µl
DNA	50-100 ng
ExTaq^{TM}	0.4 µl
H_2O_{MQ}	ad 100 µl

Unless stated otherwise the following standard PCR programme has been used with varying annealing temperatures and elongation times:

Table B.18. Standard PCR programme; x: T_D of primer; y: ca. 1min per 1kb fragment length

Reaction	T [°C]	Time [min]	Nr. of cycles
initial denaturation	94°C	3min	1
denaturation	94°C	0.45min	30
annealing	x°C	0.45min	
elongation	72°C (ExTaq ™) 68°C (Expand High Fidelity ™)	ymin	
final denaturation	94°C	5min	1

X. Generation and labelling of probes

1. RNA probes generated from a ds PCR template

In vitro Transcription

For generation of ss RNA polynucleotide probes from a ds PCR template, *in vitro* transcription is a quite easy and effective method.

Reagents

NTP-Mix (Roche,Mannheim, Germany):

ATP [100mM] 3.9 µl

CTP [100mM] 3.9 µl

GTP [100mM] 3.9 µl

UTP [100mM] 1.4 µl

Biotin-16-UTP [10mM]

or Fluos-12-UTP [10mM] 25 µl

DNase I, RNase-free (Roche, Mannheim, Germany)

Ammoniumacetate NH_4- Acetate 10 M

EDTA 0.2 M, pH 8.0

$EtOH_{abs}$.

TE-buffer	Tris-HCl 10 mM, pH 8.0
	EDTA 1mM, pH 8.0

T3- RNA- Polymerase (Roche, Mannheim, Germany, transcription buffer included)
RNase-Inhibitor(Roche, Mannheim, Germany)

Reaction

NTP-Mix:	3 µl
Transcription buffer	3 µl
T3- RNA- Polymerase	3 µl
RNase-Inhibitor	1.5 µl
Template DNA	0.5-4 µg
ad H_2O_{MQ}	30 µl

Procedure

- ✦ incubate transcription reaction for 3 h at 37°C in thermocycler or water bath
- ✦ add 3 µl DNase I, for degradation of template DNA
- ✦ incubate 15min at 37°C in thermocycler or water bath
- ✦ add 3 µl EDTA 0,2 M
- ✦ add 16 µl NH_4- acetate and 156 µl $EtOH_{abs}$ for precipitation
- ✦ incubation 2 h at –80°C/o.n. at –20°C
- ✦ centrifugation at 4°C and14.000 rpm for 15min
- ✦ discard supernatant
- ✦ wash with 150 µl EtOH 70%
- ✦ centrifugation for 15min at 14.000 rpm, 4°C
- ✦ discard supernatant
- ✦ resuspension of pellet in 50 µl TE-Puffer and 1 µl RNase-Inhibitor
- ✦ storage at -20°C

2. RNA probes generated from a synthetic ss template

For generation of ss RNA probes, *in vitro* transcription from ss template DNA has been carried out. The DNA-dependent RNA polymerase is able to synthesize in 3´-

5´direction a ss RNA strand from different DNA templates. At least the promoter region has to be double stranded (http://www.ambion.com/).
Therefore, it is necessary to built up a ds T3 promoter region. This has been performed by annealing of a ss nucleic acid primer constituting the T3 promoter sequence to a reverse complementary T3 promoter sequence that is part of the 3` prime end of the synthetic construct template.

Annealing of ss nucleic acid primer

Material

template: ss synthetic template (containing reverse complementary T3 promoter sequence at 3`end)	50 pmol
primer: ss T3 promoter	50 pmol
thermocycler	MWG Primus 96

The following synthetic constructs have been used as template for *in vitro* transcription of synthetic construct probes:

Table B.19. Synthetic templates used for *in vitro* transcription; the underlined part shows the reverse complementary for T3 single and the binding site for T3 polymerase; italics: poly-T-spacer

Template	Name of template	sequence of template 5`-3`direction	TD [°C]	GC [%]
ss T3 promoter	T3 single	<u>ATA GGT ATTAAC CCT CAC TAA AG</u>	55,3	34,8
Pseudomonas aeruginosa	PseAer4_sp	GGGGTTTCAAGGCCGAGA*TTTT*GGGGTTTCAAGGCCGAGA*TTTTT*GGGGTTTCAAGGCCGAGA*TTTT*GGGGTTTCAAGGCCGAGA<u>CTTTAGTGAGGGTTAATACCTAT</u>	>75	47,3
Pseudomonas fluorescens	Psefl4_sp	GAGCTGATGACGAGTTATC*TTTTT*GAGCTGATGACGAGTTATC*TTTTT*GAGCTGATGACGAGTTATC*TTTTT*GAGCTGATGACGAGTTATC<u>CTTTAGTGAGGGTTAATACCTAT</u>	>75	38,6

Procedure

✦ denature equimolar amounts of template and promoter for 5min at 94°C
✦ incubate 20min at 55°C
✦ put on ice immediately
✦ perform *in vitro* transcription as described X.1.

XI. Hybridisations

Hybridisations are subjected to a number of influences. For optimal hybridisation results it is necessary to experimentally evaluate the special conditions for every single probe.
As a guideline, the following formulas and rules are given:

DNA-DNA-hybridisations

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

$$T_D = 2 \text{ x } (A+T) \; 4 \text{ x } (G + C)$$

Polynucleotides (Howley et al, 1979):

$$Tm = 81.5 + 16.6 \text{ x } \log M + 0.41 \text{ x } (\%GC) – 0.7 \text{ x } (\%FA)$$

RNA-RNA-hybridisations

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

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

RNA-DNA-hybridisations

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

,

$$T_D = 81.5 + 16.6 \text{ x } \log M + 0.4 \text{ x } \%GC - 820/n – 0.5 \text{ x } \%FA$$

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

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

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

1. FISH with oligonucleotide probes

Reagents

Hybridisation buffer

NaCl	900 mM
Tris-HCl	20 mM pH 8.0
SDS	0.01 %
Formamide	x %

Washing buffer

NaCl	x mM	(see further Table B.20)
Tris-HCl	20 mM	
SDS	0.01 %	
EDTA		

Hybridisation buffer

Table B.20. Compositions of hybridisation and washbuffers for oligonucleotide FISH

% formamide in hybridisation buffer	According Na+ - concentration [mM] in washing buffer	µl 5M NaCl for 50ml 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*	-*

*Addition of 5mM Na_2EDTA to washing buffer is required during the usage of more than 20% formamide in the hybridisation buffer.

Formamide is carcinogenic and therefore not part of the washing buffer. In contrast to the hybridisation buffer, in the washing buffer stringency is obtained by using higher concentrations of salt, corresponding to the particular concentration of formamide in the hybridisation buffer (Table B.20).

Procedure

✦ adjust temperature of hybridisation ovens to 46 °C for hybridisation and 48°C for washing
✦ prepare hybridisation buffer according to Table B.20
✦ use 2-8 µl of fixed material of cell or sample material per microscope slide well
✦ dry for 5min at 60°C
✦ dehydrate slide in increasing ethanol series (50%, 80%, 100%) for 3 minutes each
✦ pipette 9 µl of hybridisation buffer onto each well and add 1 µl probe solution (50-100 ng/µl)
✦ put slide into hybridisation tube lined with hybridisation-buffer-moisturised tissue and close it
✦ hybridise for 1.5-2h at 46°C
✦ rinse slide afterwards with dH_2O
✦ insert slide in tube filled with washing buffer (preheated to 48°C) for 15min
✦ rinse slide afterwards with dH_2O and air dry
✦ for microscope analysis, embed with mounting medium (Vectashield)

2. FISH with polynucleotide probes

2.1. Probes generated from a ds PCR template targeting rRNA

Hybridisation buffer:

NaCl	75 mM
Tris/HCl, pH 8.0	20 mM
SDS	0.01%
formamide	80-95%

Two hybridisation modes were applied for rRNA targeted polynucleotide probes:

Hybridisation on slides

✦ adjust temperature of hybridisation ovens to 80 °C for denaturation of polynucleotide probe molecules and 53°C for hybridisation
✦ use 2-8 µl of fixed bacterial cell or sample material per microscope slide well
✦ dry for 5min at 60°C
✦ dehydrate slide in increasing ethanol series (50%, 80%, 100%) for 3 min each
✦ pipette 10 µl of hybridisation buffer onto each well and add 3-6 µl probe solution
✦ put slide into hybridisation tube lined with hybridisation-buffer-moisturised tissue and close it
✦ denature polynucleotide probe molecules at 80°C for 20min
✦ hybridise at 53°C for 5-12h
✦ rinse slide afterwards with dH_2O and air dry
✦ store at -20°C or, if necessary, perform detection of biotin-16-UTP labelled probe molecules according to XI.3.3.2.

Hybridisation in 0.5 ml reaction tubes:

✦ use 5-20 µl of (pure culture cells) or 50-200 µl (bacterial cells containing sample material) of PFA fixed sample
✦ wash with 200-400 µl PBS
✦ add 3 vol $EtOH_{abs}$ and let stand for 3min at 25°C
✦ centrifuge at 12000 rpm for 2-5min to remove residual ethanol
✦ wash with 200-400 µl PBS
✦ centrifuge 2-5min at 12000 rpm
✦ resuspend cell pellet in 30µl (pure culture cells) or 30-100µl (bacterial cells containing sample material) hybridisation buffer
✦ add 5-15µl transcript probe (~4-5 µg)
✦ denature polynucleotide probe molecules at 80°C for 20min
✦ hybridise 5-12h at 53°C
✦ store at -20°C or if necessary, perform detection of biotin-16-UTP labelled probe molecules according to XI.3.3.2.

2.2. Probes generated from a PCR template targeting chromosomal DNA: RING-FISH probes

Hybridisation buffer:

NaCl	75 mM
Tris/HCl, pH 8.0	20 mM
SDS	0.01%
formamide	0-80%

Two hybridisation modes were applied for rRNA targeted polynucleotide probes:

Hybridisation on slides

✦ adjust temperature of hybridisation ovens to 80 °C for denaturation of polynucleotide probe molecules and target and 53°C for hybridisation

✦ use 2-8 µl fixed bacterial cell or sample material per microscope slide well

✦ dry for 5min at 60°C

✦ dehydrate slide in increasing ethanol series (50%, 80%, 100%) for 3 min each

✦ pipette 10 µl of hybridisation buffer onto each well and add 3-6 µl probe solution

✦ put slide into hybridisation tube lined with hybridisation-buffer-moisturised tissue and close it

✦ denature polynucleotide probe molecules at 80°C for 20min

✦ hybridise for 24-30h at 53°C

✦ rinse slide afterwards with dH_2O and air dry

✦ store at -20°C or if necessary, perform detection of biotin-16-UTP labelled probe molecules according to XI.3.3.2.

Hybridisation in 0.5 ml reaction tubes:

✦ use 5-20µl (pure culture cells) or 50-200 µl (sample material containing bacterial cells) of PFA fixed sample

✦ wash with 200-400 µl PBS

✦ add 3 vol $EtOH_{abs}$ and let stand at 25°C for 3min

✦ centrifuge 2-5min at 12000 rpm to remove residual ethanol

✦ wash with 200-400 µl PBS

✦ centrifuge 2-5min at 12000 rpm

✦ resuspend cell pellet in 30 µl (pure culture cells) or 30-100 µl (bacterial cells containing sample material) hybridisation buffer

✦ add 5-15 µl transcript probe (~4-5 µg)

✦ denature polynucleotide probe molecules at 80°C for 20min

✦ hybridise 24-30h at 53°C

✦ store at -20°C or if necessary, perform detection of biotin-16-UTP labelled probe molecules according to XI.3.3.2.

2.3. Synthetic construct probes

2.3.1. Hybridisations with synthetic DNA polynucleotide probes or with RNA polynucleotide probes generated from a synthetic template

The conditions for hybridisations with both types of probes consist of a combination of oligonucleotide probe FISH, and polynucleotide probe FISH: a step of denaturation at 80°C (for denaturation of secondary structures of probes and targets) is performed prior to the hybridisations at the given oligonucleotide probe FISH conditions, elongated for three hours.

2.3.2. Hybridisations

Reagents

Hybridisation buffer

NaCl	900 mM	
Tris-HCl	20 mM pH 8.0	
SDS	0.01 %	
Formamide	x %	

Washing buffer

NaCl	x mM	(see further Table B.20)
Tris-HCl	20 mM	
SDS	0.01 %	
EDTA		

The percentage of formamide used is the same as that used for the respective single oligonucleotide probe, of which the particular repetive-mer consists (hybridisation buffer and washing buffer see Table B.20).

Hybridisation on slides

✦ adjust temperature of hybridisation ovens to 80 °C for denaturation of polynucleotide probe molecules, to 46°C (DNA polynucleotide probes) or to 53°C (RNA polynucleotide probes)

✦ use 2-8 µl fixed bacterial cell or sample material per microscope slide well

✦ dry for 5min at 60°C

✦ dehydrate slide in increasing ethanol series (50%, 80%, 100%) for 3min each

✦ pipette 10 µl of hybridisation buffer onto each well and add 3-6 µl probe solution

✦ put slide into hybridisation tube lined with hybridisation-buffer-moisturised tissue and close it

✦ denature polynucleotide probe molecules at 80°C for 20min

✦ hybridise for 3h at 46°C (DNA polynucleotide probes) or at 53°C (RNA polynucleotide probes)

✦ rinse slide afterwards with dH_2O

✦ insert slide in tube filled up with washing buffer, preheated to 48°C (DNA polynucleotide probes) or to 55°C (RNA polynucleotide probes)

✦ rinse slide afterwards with dH_2O and air dry

✦ store at -20°C or if necessary, perform detection of biotin-16-UTP labelled probe molecules according to XI.3.3.2.

Hybridisation in 0.5 ml reaction tubes:

✦ use 5-10µl (pure culture cells) or 50-200µl (bacterial cells containing sample material) PFA fixed, per tube

✦ wash with 200-400 µl PBS

✦ add 3 vol $EtOH_{abs}$ and let stand for 3min at 25°C

✦ centrifuge at 12000 rpm for 2-5min to remove residual ethanol

✦ wash with 200-400 µl PBS

✦ centrifuge at 12000 rpm for 2-5min

✦ resuspend cell pellet in 30 µl (pure culture cells) or 30-100 µl (bacterial cells containing sample material) hybridisation buffer

✦ add 5-15 µl transcript probe (~4-5 µg)

✦ denature polynucleotide probe molecules at 80°C for 20min

✦ hybridise for 3h at 46°C (DNA polynucleotide probes) or at 53°C (RNA polynucleotide probes)

✦ rinse slide afterwards with dH_2O and air dry
✦ store at -20°C or if necessary, perform detection of biotin-16-UTP labelled probe molecules according to XI.3.3.2.

3. Pretreatment methods for Gram-positive bacteria and samples containing Gram-positive bacteria

To make sure that oligonucleotide and polynucleotide probes are able to hybridise to their target structure located on 16S, 23S rRNA or DNA, it is necessary to introduce different treatments to permeabilise the cell walls without destroying the shape and integrity of the bacterial cell.

3.1. Pretreatments for oligonucleotide probe based FISH

Reagents

Lysozyme	10 mg/ml in 50 mM Tris/HCl
Tris/HCl	50 mM

Procedure

✦ adjust temperature of hybridisation ovens to 46 °C for hybridisation and 48°C for washing
✦ prepare hybridisation buffer according to Table B.20
✦ use 2-8 µl of fixed material of cell or sample material per microscope slide well
✦ dry for 5min at 60°C
✦ dehydrate slide in first increasing ethanol series (50%, 80%, 100%) for 3min each
✦ apply 10 µl of lysoszyme on each slide well
✦ store on ice for 10min
✦ rinse slide with dH_2O
✦ dehydrate slide in second increasing ethanol series (50%, 80%, 100%) for 3min each
✦ go on as described above in point XI.1.

3.2. Pretreatments for polynucleotide probe based FISH and RINGFISH

Material

Lysozyme	10 mg/ml in 50 mM Tris/HCl
Tris/HCl	50 mM pH 8.0
Mutanolysine	20 U/ml in H_2O_{MQ}
Lysostaphine	1 U/ml in H_2O_{MQ}
Ultra-Turrax	Janke & Kimkel, IKA Labotechnik, Germany

3.2.1. Pure culture cells of enterococci or sample material containing enterococci

Hybridisations on slides

✦ use 2-8 µl of fixed bacterial cell or sample material per microscope slide well
✦ dry for 5min at 60°C
✦ dehydrate slide in first increasing ethanol series (50%, 80%, 100%) for 3min each
✦ apply 10 µl of lysoszyme on each slide well
✦ store on ice for 20min
✦ rinse slide with dH_2O
✦ (optional: incubate slide for 3 minutes at 200°C)
✦ dehydrate slide in second increasing ethanol series (50%, 80%, 100%) for 3min each
✦ continue according to point XI. 2.1.

Hybridisation in 0.5 ml reaction tubes:

✦ (optional: perform homogenisation of sample using 30sec of Ultra-Turrax)
✦ use 5-10 µl (pure culture cells) or 50-200 µl (bacterial cells containing sample material) of PFA fixed sample
✦ wash with 200-400 µl PBS
✦ add 3 vol $EtOH_{abs}$ and let stand for min at 25°C
✦ centrifuge 2-5min at 12000 rpm to remove residual ethanol
✦ wash with 200-400 µl PBS
✦ centrifuge 2-5min at 12000 rpm
✦ apply 10-50 µl of lysozyme and resuspend pellet
✦ incubate on ice or at 37°C for 20min

✦ centrifuge 2-5min at 12000rpm and discard supernatant

✦ add 200-400 µl PBS, centrifuge 2-5min at 12000 rpm and discard supernatant

✦resuspend cell pellet in 30µl (pure culture cells) or 30-100 µl (bacterial cells containing sample material) hybridisation buffer

✦ add 5-15 µl transcript probe (~4-5 µg)

✦ denature polynucleotide probe molecules at 80°C for 20min

✦ hybridise 5-12h at 53°C

✦ store at -20°C or if necessary, perform detection of biotin-16-UTP labelled probe molecules according to XI.3.3.2.

3.2.2. Pure culture cells of staphylococci or sample material containing staphylococci

The best hybridisation signals are obtained using the following pretreatment as standard:

Hybridisations on slides

✦ use 2-8 µl of fixed bacterial cell or sample material per microscope slide well

✦ dry for 5min at 60°C

✦ dehydrate slide in first increasing ethanol series (50%, 80%, 100%) for 3min each

✦ apply 10 µl of lysoszyme on each slide well

✦ store at 37°C for 10min

✦ rinse slide with dH_2O

✦ incubate at 200°C for 3-7min

or

✦ apply 1U / well of lysostaphine, incubate at 30°C for 5min, rinse with dH_2O and dry under airflow

✦ dehydrate slide in second increasing ethanol series (50%, 80%, 100%) for 3min each

✦ continue according to point XI.2.1.

Hybridisation in 0.5 ml reaction tubes:

✦ (optional: perform homogenisation of sample using 30sec of Ultra-Turrax)

✦ use 5-10 µl (pure culture cells) or 50-200 µl (bacterial cells containing sample material) of PFA fixed sample

✦ wash with 200-400 µl PBS
✦ add 3 vol $EtOH_{abs}$ and let stand 25°C for 3min at
✦ centrifuge 2-5min at 12000 rpm to remove residual ethanol
✦ wash with 200-400 µl PBS
✦ centrifuge at 12000 rpm for 2-5min
✦ apply 10-50 µl of lysozyme and resuspend pellet
✦ incubate on ice or at 37°C for 20min
✦ centrifuge 2-5min at 12000rpm and discard supernatant
✦ apply 10-50 µl of lysostaphine
✦ incubate at 30°C for 5min
✦ centrifuge 2-5min at 12000rpm and discard supernatant
✦ add 200-400 µl PBS, centrifuge 2- min at 12000rpm and discard supernatant
✦ resuspend cell pellet in 30µl (pure culture cells) 30-100µl (bacterial cells containing sample material) hybridisation buffer
✦ add 5-15 µl transcript probe (~4-5µg)
✦ denature polynucleotide probe molecules at 80°C for 20min
✦ hybridise 5-12h at 53°C
✦ store at -20°C or if necessary, perform detection of biotin-16-UTP labelled probe molecules according to XI.3.3.2.

3.2.3. Pure culture cells of streptococci or sample material containing streptococci

The best hybridisation signals are obtained using the following pretreatment as standard:

Hybridisations on slides

✦ use 2-8 µl of fixed bacterial cell or sample material per microscope slide well
✦ dry for 5min at 60°C
✦ dehydrate slide in first increasing ethanol series (50%, 80%, 100%) for 3min each
✦ apply 10 µl of lysoszyme on each slide well
✦ store at 37°C for 10min
✦ (optional: incubate slide for 3-7min at 200°C)
✦ rinse slide with dH_2O
✦ incubate at 200°C for 7min

or

✦ apply 1U/ well of lysostaphine, incubate at 30°C for 5min, rinse with dH_2O and dry under airflow

✦ (optional: incubate slide for 3-7min at 200°C)

✦ dehydrate slide in second increasing ethanol series (50%, 80%, 100%) for 3min each

✦ continue according to point XI.2.1.

Hybridisation in 0.5 ml reaction tubes:

✦ (optional: perform homogenisation of sample using 30sec of Ultra-Turrax)

✦ use 5-10 µl (pure culture cells) or 50-200 µl (bacterial cells containing sample material) of PFA fixed sample

✦ wash with 200-400 µl PBS

✦ add 3 vol $EtOH_{abs}$ and let stand at 25°C for 3min

✦ centrifuge at 12000rpm for 2-5min to remove residual ethanol

✦ wash with 200-400 µl PBS

✦ centrifuge at 12000 rpm for2-5min

✦ apply 10-50 µl of lysozyme and resuspend pellet

✦ incubate on ice or at 37°C for 20min

✦ centrifuge 2-5min at 12000rpm and discard supernatant

✦ apply 10-5 0µl of lysostaphine

✦ incubate at 30°C for 5min

✦ centrifuge 2-5min at 12000rpm and discard supernatant

✦ add 200-400 µl PBS, centrifuge 2-5min at 12000rpm and discard supernatant

✦resuspend cell pellet in 30µl (pure culture cells) or 30-100µl (bacterial cells containing sample material) hybridisation buffer

✦ add 5-15 µl transcript probe (~4-5 µg)

✦ denature polynucleotide probe molecules at 80°C for 20min

✦ hybridise 5-12h at 53°C

✦ store at -20°C or if necessary, perform detection of biotin-16-UTP labelled probe molecules according to XI.3.3.2.

XII. Cell sorting using DNA-coated microplates - enrichment procedures

Cell sorting consists of two different hybridisation steps. The first step is a hybridisation with a polynucleotide probe and its target cells, identically performed according to points X.2.
The second step is a hybridisation step for separating the already hybridised cells from non-target cells via binding to microplate cavities coated with complementary nucleic acids.

1. Coating of microplates with DNA

Material

Microplates	Maxisorp or Nucleolink (Nalge Nunc, Roskilde, Denmark)
Freshly prepared PCR product	
PCR film	Peqlab (Erlangen, Germany)
PBS	see point III.1.
PBS/$MgCl_2$	PBS containing $MgCl_2$ 0.1M

DNA binding in Maxisorp plates is realised by hydrophobic / hydrophilic interactions. In Nucleolink plates, DNA is bound covalently via carbodiimide condensation. The cell sorting process is performed equally with both types of microplates, but Nucleolink microplates have the advantage of being suitable for PCR detection afterwards.

Procedure

✦ amplify the probe sequence by PCR using probe specific primers (see part VII)
✦ for purification of the PCR product add 2.5 vol $EtOH_{abs}$., store 2h at –20°C
✦ centrifugation at 14000 rpm for 15min; resuspend in 100 µl H_2O_{MQ}
✦ for coating, use 1 µg/microplate cavity
✦ denature DNA for 10min at 94°C, place tube on ice directly afterwards to avoid renaturation
✦ add 50µl per well of freshly prepared ice cold PBS/$MgCl_2$
✦ disperse solution over microplate cavities, 50 µl per well on ice

✦ incubate 45min at 37°C

✦ discard supernatant and dry 1h at 60°C

✦ store coated microplates at 25°C up to several weeks in a dark and dry place

✦ before use, wash microplates with 100µl PBS to remove unbound DNA

2. Enrichment of bacterial target cells by cell sorting (Hybridisation in coated microplates – HYCOMP)

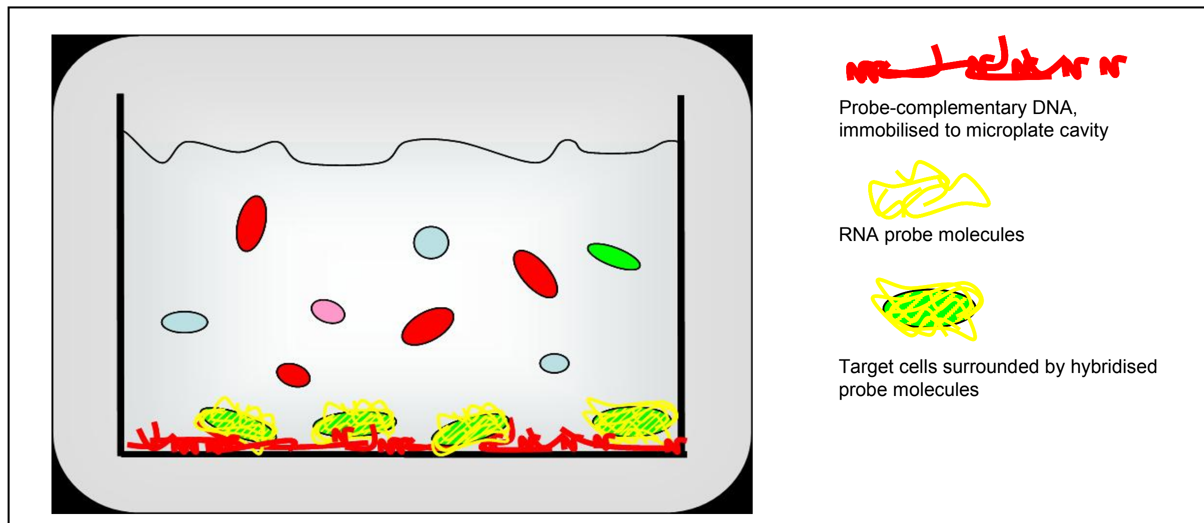


Fig.B.1. Diagram of cell sorting technique: after the first hybridisation of polynucleotide probes with the sample according to X.2.1., the sample is transferred to microplate cavities coated with complementary nucleic acids. The cell sorting or enrichment of target cells is achieved by a second hybridisation of the projecting, network-forming parts of the probe molecules from the first hybridisation to the complementary nucleic acids, immobilised on the bottom of the microplate cavities.

Material

Microplates	Maxisorp or Nucleolink (Nalge Nunc, Roskilde, Denmark)
Freshly prepared PCR product	
PCR Film	Peqlab (Erlangen, Germany)

Reagents

PBS	see point III.1.
MP buffer:	5x SSC
	0,02% SDS
	2% blocking

	0,1% N-laurylsarcosin
	33% formamide
Blocking :	10% (w/v) blocking reagent (Roche, Germany) in maleic acid buffer (0,15M NaCl, 0,1M maleic acid, pH 7,5)

Procedure

✦ *in situ* hybridisation in 0.5 ml tubes performed as described in XI.2.using labelled (biotin, digoxigenin, fluoresceine, Cy3) or unlabelled polynucleotide probes
✦ add 200-800 µl PBS
✦ centrifuge 5min at 12000 rpm
✦ resuspend in 50-100 µl MP buffer per µl PFA fixed cells or sample material used for hybridisation
✦ wash microplate cavities with 100µl PBS to remove unbound DNA
✦ prehybridise microplate cavities with 50 µl MP buffer 5min at 25°C
✦ apply 50 µl of solution per DNA coated microplate cavity
✦ put one time 50 µl of solution into uncoated microplate cavity (= negative control)
✦ cover microplate cavity with adhesive PCR film
✦ incubate 90min at 53°C (for rRNA targeted probes) or 37°C (for plasmid and chromosomal DNA targeted probes)
✦ remove the solution carefully without touching the walls of the microplate cavities
✦ if several microplate cavities were used, pool supernatants, centrifuge and resupend in 10-20 µl PBS; supernatant can be used for quantification of cell sorting efficiency, see XII.3.4.
✦ in microplate cavities immobilised target cells can be directly used for PCR analysis
or
✦ can be recovered by adding 100 µl H_2O_{MQ} per microplate cavity, incubation at 94°C for 5min and thorough rinsing to wash the cells off the walls of the microplate cavity

3. Detection of cells after cell sorting

3.1. Detection of biotin labelled cells in microplate cavities

Equipment

Microplate reader	Emax, MWG, Ebersberg, Germany

Reagents

PBS	see point III.1.
BM blue	POD substrate (Roche, Germany)
Blocking:	10% (w/v) blocking reagent (Roche, Germany) in maleic acid buffer (0,15M NaCl, 0,1M maleic acid, pH 7,5)
Blocking buffer	PBS containing 0.1% blocking
H_2SO_4	1M

Procedure

✦ remove supernatant from microplate cavities very gently and keep supernatant for microscope analysis (see XII.3.3.)
✦ wash with 100 µl PBS
✦ add 50 µl streptavidin-peroxidase conjugate, diluted 1:1000 in blocking buffer per microplate cavity
✦ incubate at 25°C for 30min
✦ discard supernatant
✦ wash 3x carefully with 100µl PBS
✦ add 100 µl BM blue
✦ incubate at 25°C for 5-15min; a colour change from colourless to blue indicates substrate reaction
✦ add 100µl 1M H_2SO_4 to stop enzyme reaction; colour changes from blue to yellow
✦ measure absorption in microplate reader at 450nm against 650nm reference

3.2. Detection using PCR

For PCR detection, NucleoLink Microplates (XII.2.) have to be used in cell sorting before.

✦ remove supernatant from microplate cavities
✦ keep supernatant for PCR detection or microscope analysis
✦ wash microplate cavities very carefully with 100µl PBS
✦ perform PCR with required primers and PCR conditions (Tables B.6, B.7, B.12)
✦ run agarose gel electrophoresis (see VI.)

PCR analysis of supernatant

✦ centrifuge supernatant from positive and negative controls at 12000 rpm for 5min
✦ resuspend pellet in 100-200µl PBS and centrifuge at 12000 rpm for 5min
✦ discard supernatant and resuspend in 10-20µl PBS
✦ perform PCR with 1µl of resuspensions and required primers and conditions (Tables B.6, B.7, B.12)

3.3. Detection for microscopic analysis

Reagents

DPBS	NaCl 137 mM
	Na_2HPO_4 8 mM
	KCl 2.7 mM
Streptavidi-Fluos / Cy3 / Cy5	Amersham Biosciences

Procedure

✦ use supernatant from microplate cavities after cell sorting
✦ centrifuge 5min at 12000 rpm
✦ wash 2x with 200-400 µl dH_2O
✦ resuspend in 10 µl dH_2O
✦ apply up to 5 µl on microscope slide, dry at 60°C
✦ add 30 µl streptavidin-fluoresceine or streptavidin-Cy3 diluted 1:200 in DPBS to each field of the slide
✦ incubate at 25°C for 45-60min in the dark

✦ rinse slide with 2 ml DPBS

✦ wash slide in 50 ml tube filled with DPBS for 20min in the dark

✦ dry slide under air flow

✦ for microscope analysis, embed with mounting medium

Storage of slides is possible at -20°C for several months in the dark.

3.4. Quantification of cell sorting efficiency

For quantification of the cell sorting efficiency, the supernatants from coated and uncoated (negative control, see X.3.2.) microplate cavities were analysed with regard to the percentages of target cells to non target cells. After pooling and washing of the supernatants (see point X.3.2.), the supernatants were resuspended in 10-20µl PBS and brought to microscopical fields. For the target cells, a species-specific oligonucleotide probe hybridisation is carried out according to point X.1. Afterwards, target cells are counted from 10 randomly chosen microscopic fields in epifluorescence modus, as they have been hybridised before with a species-specific oligonucleotide probe. Non target cells were counted in phase contrast modus from the same randomly chosen microscopic fields.

A comparison of the percentages before (microscopic fields containing the supernatant from uncoated microplate cavities) and after (microscopic fields containing the supernatant from cavities used for enrichment) cell sorting allows calculation of enrichment/depletion values using the following formula:

$$\text{Depletion value} = \frac{\text{\% target cells before-\% target cells after cell sorting)}}{\text{\% target cells before cell sorting}} \times 100$$

Example:

Target cells before cell sorting 80%

Target cells after cell sorting 40% → depletion value 50%

XIII. Cloning and Sequencing

1. Cloning with TOPO TA® Cloning Kit

For cloning of PCR products, the TOPO TA® Cloning Kit (Invitrogen, Carlsbad, USA) was used. The vector in this kit has overhangs of thymidine.

Due to the adenine overhangs, which are a result of terminal transferase activity of the ExTaq system polymerase used, the ligation of PCR products in the vector is possible.

The TOPO TA® Cloning Kit uses a topoisomerase for connecting of the overhangs, and the proper ligation takes place after heat shock transformation inside of the bacterial cell.

Reagents

LB medium	
X-Gal	(Sigma-Aldrich, Steinheim, Germany), 40 µl of 40 mg/ml X-Gal on one LB plate
ampicillin	(Sigma-Aldrich, Steinheim, Germany), 50 µg/ml

Reaction

Salt solution	1µl
Vector	1µl
PCR Product	1-4 µl
H_2O_{MQ}	ad 5 µl

The reaction and further procedure is carried out according to the manufacturer's instructions. 50-150 µl of the cloning reaction were afterwards transferred on LB-Amp-X-Gal plates and incubated over night at 37°C.

The selection relies on blue/white screening and results from an ampicillin and X-Gal selection.

2. Sequencing

Automated sequencing was carried out using a modified method of linearised amplification sequencing (Murray, 1989). The combined method of chain termination (Sanger et al 1977) and PCR amplification (Saiki et al., 1988) uses IR-labelled primers. After every incorporation of a ddNTP in an elongating sequence, the use of dideoxynucleotides (ddNTP's) in addition to normal nucleotides (dNTP's) will provoke chain breaks. The reason for the break is the hydrogen group on the 3`carbon instead of a hydroxyl group: after incorporation of a ddNTP it is not possible to form a phosphodiester bond between the ddNTP and the next dNTP.

The sequencing reaction was done with Sequitherm Excel™ II DNA Sequencing Kit-LC (66cm, Epicentre, Madison, Wisconsin), IRDye700/800 (LI-COR biosciences, Bad Homburg, Germany) as primer dyes.
The system is non-discriminatory between dNTPs and ddNTPs (Tabor and Richardson, 1995); thus, ddNTPs get incorporated into the nascent strands a 1000 fold better than e.g. with *Taq*- polymerase (Reeve and Fuller, 1995).
The separation of the resulting fragments was carried out in a LI-COR Global IR^2 DNA Sequencer (LI-COR Biosciences, Bad Homburg, Germany) and online detection subsequently via laser usage.
For analysis of the sequencing gels, *e*-Seq DNA Sequencing and Analysis Software (LI-COR Biosciences, Bad Homburg, Germany) was used.

Reaction and equipment

Sequencing kit	Sequitherm Excel™II DNA Sequencing Kit-LC (66cm, Epicentre, Madison, Wisconsin, USA)
Primers	5µM IR labelled (MWG, Ebersberg, Germany; see Table B.13)
Microtiterplate	polycarbonate, V- bottom (Biozym, Hess. Oldendorf, Germany)
Micro seals	Microseal™, A`-film (MJ Research Inc.,Waltham, USA)
Thermocycler	Type PTC-100™ (MJ Research Inc., Watertown, USA)

According to manufacturer's instructions, the components were added in given amounts into microplate cavities.

Reaction Mastermix for one reaction:

Sequitherm buffer	7.2 µl
Polymerase	1 µl
Primer (forward and reverse)	2 µl
DNA	250 ng
H_20_{MQ}	ad 17µl
ddNTPs	2 µl per microplate cavity

After covering the microplate with a seal, the following cycling programme has been used:

Table B.21. Cycling programme for sequencing reaction

Reaction	T [°C]	Time [min]	Nr. of cycles
initial denaturation	94°C	3min	1
denaturation	94°C	0.5min	25
Annealing/elongation	45°C	0.5min	
Final elongation	45°C	10min	1
cool down	4°C	forever	1

After the reaction, 3 µl of stop buffer has to be added.
Before loading the sequencing gel with the samples, a denaturation step of 5min at 94°C is necessary.

Polyacrylamidgelelectrophoresis

Reagents and equipment

1 x TBE (Tris- boric acid-EDTA- buffer)	134 mM Tris 44.5 mM boric acid 2.5 mM EDTA pH 8.0
Urea	
Long Ranger® Gel Solution	Cambrex Bio Science Rockland Inc., Rockland, ME, USA
DMSO	Dimethylsulfoxyde, J.T. Baker, Mallinckrodt Baker B.V., Devent, Netherlands

APS	10 % (w/v) ammoniumpersulfate (store aliquots at -20°C)
TEMED	N´-N´-N´-N´-tetramethylethylendiamine
Glass plates	66cm (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

✦ dissolve 21g of urea in 32 ml H_2O

✦ add 5 ml TBE and 500 µl DMSO

✦ add 4.3 ml Long Ranger Gel Solution

✦ degas mixture for 10min in an ultra sonic bath

✦ add 50 µl of TEMED and 350 µl of APS, stir, and filter sterilise

✦ prepare gel according to the manufacturer's instructions

✦ start prerun after placing the gel in LI-COR Global IR^2 DNA Sequencer

✦ denaturate sequencing reaction and load gel

✦ start electrophoresis (2000V, 25mA, 45W, 45°C)

✦ end electrophoresis after 10-12h

✦ Analysis with software (see above)

XIV. Software and equipment

1. Phylogenetic analysis of sequence data

The Blast Database (Altschul et al, 1997; http://www.ncbi.nlm.gov/cgi-bin/BLAST) was used in order to gain preliminary hints on similarities to known sequences (rDNA and non-ribosomal DNA sequences).
ARB was used for integration of sequences in an existing alignment of the ARB database. The alignment has been performed with the software package ARB (Ludwig, W. Strunk, O. et al 2004: ARB: a software environment for sequence data. Nucleic Acids Research 32 (4):1363-1371)

For further analysis of sequence data, the sequences have been arranged in order to similarity. In between the variable regions of the sequences, a secondary structure analysis or a small group of conserved bases allowed orientation for assignation of homologous positions.

2. Analysis of GC contents and secondary structures

- RNAdraw V1.1b2 (Matzura et al 1996); http://www.rnadraw.com
- *Ivo L. Hofacker*, Vienna RNA secondary structure server, Nucl. Acids Res. 31: 3429 3431 (2003); (http://rna.tbi.univie.ac.at)
- RNA structure 4.0, Mathews, Zuker, Turner 1996-2004
- pDRAW 32 1.0 (http://www.acaclone.com)
- XRNA 1.1.12.beta (http://rna.ucsc.edu/rnacenter/xrna/xrna.html)

3. Microscopy

Two different microscopes were used for image acquisition:

Epifluorescence microscope: Zeiss Axioplan, equipped with

- CCD camera, Princeton instruments CCD 1035x1317 (model RTE-CCD 1317-K/2)
- WinView software version 2.1.7.6., Princeton Instruments

Confocal Laser Scanning Microscope: Zeiss LSM 510 Ver.2.01 SP2
Quantification of probe signal intensities: DAIME software (Daims 2001, unpublished)

C. Results

In the work presented, different kinds of polynucleotide probes were examined with regard to their applicability in the area of clinical relevant bacteria and real clinical samples. In the course of the study, the method of cell fishing using the polynucleotide probe based enrichment technique (Zwirglmaier et al, 2004) was tested for its suitability to enrich bacterial target cells of clinical relevance from different kinds of clinical samples. Once enriched in microplate cavities, the genetic information of the whole cells can be provided for further screenings, such as antibiotic resistance genes or further phylogenetic analysis.
The work consists of three different parts. The first part is a general evaluation of the possibilities and limits as well as necessary adjustments of 23S rRNA DIII targeted polynucleotide probe based enrichment technique for all bacterial strains relevant for the project, especially for Gram-positive bacteria, spiked clinical sample material and real clinical samples. The second part deals with the relatively new method of RINGFISH (Zwirglmaier et al, 2004) for examples of clinical relevance. The third part exemplifies the application of new synthetic polynucleotide probes that combine different advantages of oligo- and polynucleotide probes.

I. 23S rRNA DIII targeted polynucleotide probes

1. General features of 23S rRNA DIII targeted polynucleotide probes

1.1. Spectrum of specificity

For evaluation of the particular specificity of all DIII targeted RNA polynucleotide probes used in this study, the probes and the binding regions of the microorganisms relevant for the project were examined by two different approaches: direct *in situ* hybridisations with all relevant bacterial strains, and rRNA sequence analysis by similarity matrix methods using an ARB database.

1.1.1. FISH Analysis

For all pure culture organisms from Tables B.1 and B.2, a DIII polynucleotide probe hybridisation with the probe generated by *in vitro* transcription from the DNA of each organism was performed with all other project relevant organisms to examine the specificity of the rRNA targeted probes *in situ* (see Table C.1).
All Gram-positive bacteria were subjected to special pretreatment protocols as described in B.XI.3. before hybridisation. If Gram-negative and positive bacteria were applied in mixtures, the Gram-negative bacteria were added to the mixture after the pretreatment of the Gram-positive bacteria to protect them from cell lysis.

1.1.1.1.Gram-negative bacteria versus Gram-positive bacteria

No hybridisation using DIII targeted polynucleotide probes of members of the Gram-negative bacteria applied to cells of Gram-positive bacteria allowed detection of signals and vice versa (marked red in Table C.1).

1.1.1.2. Gram-negative bacteria

All probes generated for Gram-negative bacteria belonging to the families of *Enterobacteriaceae* (including here *E. coli*, *Enterobacter* spp, *Klebsiella* spp, *Citrobacter* spp, *Pantoea* spp, *Hafnia* spp, all γ-Proteobacteria) showed positive hybridisation signals in hybridisations with all members of *Enterobacteriaceae* (marked green in Table C.1).

Only *Stenotrophomonas maltophilia* could be differentiated from all other bacteria tested. The family of *Pseudomonaceae* (including the here examined *Pseudomonas* spp, γ-Proteobacteria) showed positive hybridisation signals in hybridisations with all probes from members of *Enterobacteriaceae* and vice versa.

The members of *Moraxellaceae* examined, including all species of *Acinetobacter* examined here, γ-Proteobacteria, could be differentiated from *Enterobacteriaceae* using 23S DIII rRNA targeted polynucleotide probes, but not from *Pseudomonas* spp.

1.1.1.3. Gram-positive bacteria

The Gram-positive bacteria examined belong to low GC-Firmicutes, and include species from the genera *Staphylococcus*, *Enterococcus* and *Streptococcus*.
Here, differentiation was possible between Enterococci and Staphylococci, whereas Enterococci and streptococci did not allow differentiation, meaning, that in repeated hybridisations the results varied (marked blue in Table C.1). Staphylococci and streptococci otherwise allowed differentiation.

Table C.1 summarises the results of *in situ* analysis in combination with the results of *in silico* sequence analysis of the DIII polynucleotide probe target region by a calculation of the similarity matrix of all examined bacteria (see further part 1.1.2).

Similarity matrix analysis of the whole target sequence (*E. coli* position 1366-1601)*

Table C.1. Similarity matrix of DIII 23S rDNA sequences of project relevant organisms in combination with poly-FISH results of hybridisations using the respective probes;
colour index: **green**: positive FISH result; **red**: negative FISH result; **blue**: positive and negative results in repetitive polynucleotide probe FISH experiments; * Brosius et al 1981

	Organism / probe DIII	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1	*Escherichia coli*	100	97	93,6	97,4	93,6	93,2	84,3	93,6	91,9	70,2	74,9	74,8	73,6	69,4	68,1	69,8	60,4	59,6	61,3	63,9	61,9	61,3	57,5
2	*Klebsiella pneumoniae*	97	100	94	99,1	94	93,6	84,7	94	92,3	68,9	74,9	73,5	73,2	70,2	68,1	69,8	62,6	61,3	62,2	62,6	62,4	61,8	59,2
3	*Klebsiella ozaenae*	93,6	94	100	94,5	98,3	97,9	85,2	98,7	97	69,3	75,7	74,8	76,2	70,6	69,4	70,2	61,3	60,9	62,6	64,8	63,7	62,7	59,7
4	*Klebsiella terrigena*	97,4	99,1	94,5	100	94,4	94	84,7	94,5	92,8	69,3	75,3	73,9	73,6	70,6	68,5	70,2	62,6	61,3	62,6	63	62,8	62,2	59,2
5	*Citrobacter freundii*	93,6	94	98,3	94,4	100	99,6	86,3	98,3	97,9	70,1	76,1	75,1	77,4	70,2	68,9	69,8	62	62	63,8	65,1	64,6	63,6	59,9
6	*Citrobacter youngae*	93,2	93,6	97,9	94	99,6	100	86	97,9	97,9	70,2	75,7	74,8	77	69,9	68,6	69,5	61,7	61,7	63,5	64,8	64,3	63,3	59,7
7	*Hafnia alvei*	84,3	84,7	85,2	84,7	86,3	86	100	86,1	85,1	70,7	78,3	76,9	77	69,8	68,1	69,4	61,3	60	60,4	61,7	62,4	61,8	60,1
8	*Enterobacter aerogenes*	91,9	92,3	98,7	94,5	98,3	97,9	86,1	100	97	70,2	76,2	75,2	76,6	70,2	68,9	69,8	61,7	61,3	63,5	64,8	63,7	62,7	60,5
9	*Enterobacter cloacae*	93,2	93,6	97	92,8	97,9	97,9	85,1	97	100	70,2	77	75,6	78,3	71,2	69,1	69,9	60,4	60,9	62,6	63,9	65,2	64,2	59,2
10	*Stenotrophomonas maltophilia*	70,2	68,9	69,3	69,3	70,1	70,2	70,7	70,2	70,2	100	72,4	73,7	73,3	66,7	68,4	68	55,7	57,5	54,8	55,7	61,8	60,5	53,6
11	*Pseudomonas aeruginosa*	74,9	74,9	75,7	75,3	76,1	75,7	78,3	76,2	77	72,4	100	94,1	89,9	78,1	75,9	77,2	62,2	73,9	64,3	63,9	68,1	65,8	61,8
12	*Pseudomonas fluorescens*	74,8	73,5	74,8	73,9	75,1	74,8	76,9	75,2	75,6	73,7	94,1	100	88,6	78	75,8	77,1	65,9	63,9	65,9	67,7	69,6	67,3	62,9
13	*Pseudomonas stutzeri*	73,6	73,2	76,2	73,6	77,4	77	77	76,6	78,3	73,3	89,9	88,6	100	80,2	78,1	79,3	60	63,5	63	62,2	67,2	65,4	60,1
14	*Acinetobacter baumanii*	69,4	70,2	70,6	70,6	70,2	69,9	69,8	70,2	71,2	66,7	78,1	78	80,2	100	93,3	95,8	55,2	57	57,4	54,3	61,6	61,8	60,1
15	*Acinetobacter junii*	68,1	68,1	69,4	68,5	68,9	68,6	68,1	68,9	69,1	68,4	75,9	75,8	78,1	93,3	100	96,6	53,5	55,2	55,7	52,6	59,8	60,1	57,5
16	*Acinetobacter lwoffii*	69,8	69,8	70,2	70,2	69,8	69,5	69,4	69,8	69,9	68	77,2	77,1	79,3	95,8	96,6	100	53,9	55,7	56,1	53	60,3	60,5	57,9
17	*Enterococcus faecium*	60,4	62,6	61,3	62,6	62	61,7	61,3	61,7	60,4	55,7	62,2	65,9	60	55,2	53,5	53,9	100	88,4	92,1	90,1	73,5	72,4	74,8
18	*Enterococcus faecalis*	59,6	61,3	60,9	61,3	62	61,7	60	61,3	60,9	57,5	63,9	66,4	63,5	57	55,2	55,7	88,4	100	88	85,1	73,9	72,8	76,5
19	*Enterococcus durans*	61,3	62,2	62,6	62,6	63,8	63,5	60,4	63,5	62,6	54,8	64,3	65,9	63	57,4	55,7	56,1	92,1	88	100	90,1	73,1	72,4	75,2
20	*Enterococcus gallinarum*	63,9	62,6	64,8	63	65,1	64,8	61,7	64,8	63,9	55,7	63,9	67,7	62,2	54,3	52,6	53	90,1	85,1	90,1	100	69,7	69,8	70,9
21	*Staphylococcus aureus*	61,9	62,4	63,7	62,8	64,6	64,3	62,4	63,7	65,2	61,8	68,1	69,6	67,2	61,6	59,8	60,3	73,5	73,9	73,1	69,7	100	92,1	68,9
22	*Staphylococcus epidermidis*	61,3	61,8	62,7	62,2	63,6	63,3	61,8	62,7	64,2	60,5	65,8	67,3	65,4	61,8	60,1	60,5	72,4	72,8	72,4	69,8	92,1	100	67,3
23	*Streptococcus pyogenes*	57,5	59,2	59,7	59,2	59,9	59,7	60,1	60,5	59,2	53,6	61,8	62,9	60,1	60,1	57,5	57,9	74,8	76,5	75,2	70,9	68,9	67,3	100

1.1.2. Similarity matrix

The target sequences of DIII probes were compared by calculating similarity matrices of all examined species using 23S rRNA sequences of an existing ARB database alignment. The probe binding region, *(E. coli* position 1366-1601, including helices 52 to 59 to 51) is quite variable, particularly Helix 58, whereas the helices upstream and downstream of Helix 58 are usually more conserved. Therefore, the probe binding region was divided in three sections: the region upstream of Helix 58 (helices 52-57), Helix 58 and the region downstream of Helix 58 (helices 59-51) as visualised in Fig.C.1. Four different matrices were calculated thereof, using filters generated by ARB implemented tools to cover the different sections: one matrix containing the whole binding region (see Table C.1), one for the upstream of Helix 58, one for Helix 58 and one for the region downstream of Helix 58. Only the matrix covering the whole target sequence for all bacterial species is shown here in Table C.1., whereas the matrices over the single sections are found in part F. I.

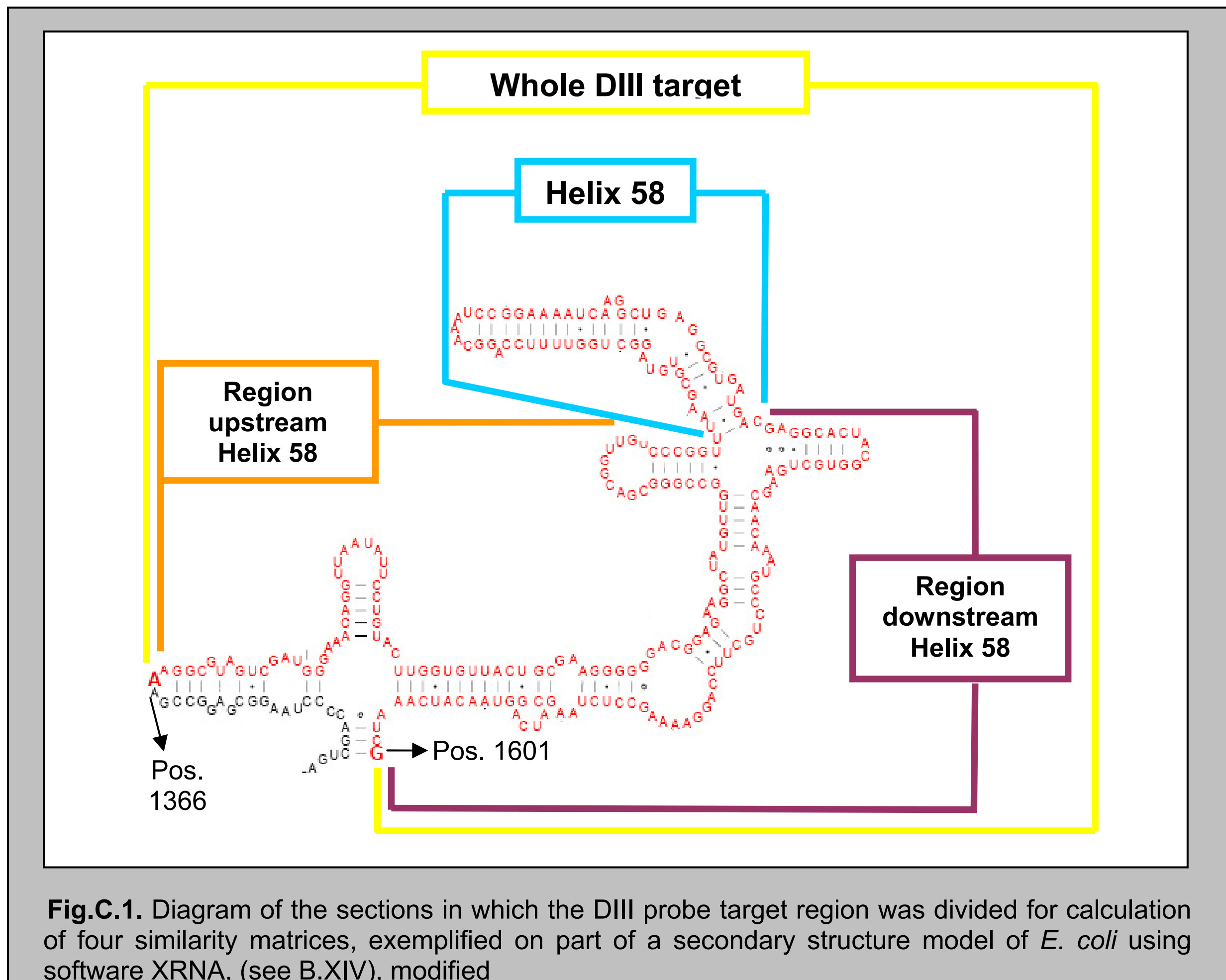


Fig.C.1. Diagram of the sections in which the DIII probe target region was divided for calculation of four similarity matrices, exemplified on part of a secondary structure model of *E. coli* using software XRNA, (see B.XIV), modified

In combination with the *in situ* data, a dissimilarity of **24-30%** is necessary for discrimination of non target organisms during DIII targeted polynucleotide FISH (see Tables C.1., C.2. and in F.I.). In other words, **70-76%** sequence similarity calculated over the whole DIII target region has to be regarded as the cut off for positive and negative hybridisation signals. All compared organisms which shared more than 76% of similarity in the target sequence could not be differentiated by application of DIII targeted polynucleotide probes, whereas sequence similarity below 70% allowed differentiation.

TableC.2. Bacterial group-combined values from matrices calculated over whole target sequence, Helix 58 and region upstream and downstream of Helix 58; colour index: **green**: + FISH result; **red**: - FISH result; **blue**: + and – results in repeated FISH experiments; vs.: versus, compared to each other

Groups of bacteria	Range of similarity values for			
	Whole DIII target region	Upstream Helix 58	Helix 58	Downstream Helix 58
Length of sequence in the different matrix sections*	233	100	58	75
Enterobacteriaceae (without 10)*	84-99.6%	99-100%	78-96.6%	94-100%
***Pseudomonas* spp**	88-94%	87-91%	89-93%	87-92%
***Acinetobacter* spp**	93-96%	96-100%	86-95%	96-100%
Enterobacteriaceae **vs.** *Pseudomonas* spp	73-78%	82-86%	65-72%	69-73%
Enterobacteriaceae **vs.** *Acinetobacter* spp	68-70%	79-81%	50-65%	61-67%
Pseudomonas spp **vs.** *Acinetobacter* spp	75-80%	81-84%	70-75%	73-77%
***Enterococcus* spp**	85-92-100%	88-95%	72-83%	92-95%
***Streptococcus* spp**	100%	100%	100%	100%
***Staphylococcus* spp**	92-100%	92-100	93.1-100%	92-100%
Enterococcus spp **vs.** *Streptococcus* spp	70-76%	72-80%	65-74%	73-79%
Enterococcus spp **vs.** *Staphylococcus*	69-73%	70-82%	60-74%	69-74%
Streptococcus spp **vs.** *Staphylococcus*	67-68%	69-72%	65-69%	63-66%
Enterobacteriaceae **vs.** *Enterococcus* spp	60-65%	69-72%	50-61%	54-63%
Enterobacteriaceae **vs.** *Streptococcus* spp	57-60%	66-68%	54-58%	51-54%
Enterobacteriaceae **vs.** *Staphylococcus spp*	61-63%	66-69%	71-79%	49-53%
Pseudomonas spp **vs.** *Enterococcus* spp	60-67%	68-74%	49-57%	61-70%
Pseudomonas spp **vs.** *Streptococcus* spp	60-61%	65-68%	67-73%	61-66%
Pseudomonas spp **vs.** *Staphylococcus spp*	65-68%	66-74%	50-54%	60-65%
Acinetobacter spp **vs.** *Enterococcus* spp	53-63%	61-64%	40-52%	50-56%
Acinetobacter spp **vs.** *Streptococcus* spp	59-61%	65-66%	47-52%	57-60%
Acinetobacter spp **vs.** *Staphylococcus spp*	57-60%	64-66%	57-63%	54-61%

* without 10: Similarity values from organism 10, *Stenotrophomonas maltophilia*, Table C.1, not included

The following results focus primarily on the similarity matrix calculated over the complete target sequence of all examined bacteria and the combination with the FISH data, but not on the inter-helical differences between the target sequences shown also in Table C.2. A detailed description of all findings regarding especially the role of Helix 58 and the regions upstream and downstream of Helix 58 will be done in D.I.1.2.

Gram-positive bacteria and Gram-negative bacteria can be differentiated using DIII targeted polynucleotide probes (see part C.1.1.1.). The grade of similarity over the whole target sequence ranges from 53-68%.

The similarity regarding the group of *Enterobacteriaceae* versus all Gram-positive bacteria varies from 57-65%. As mentioned in C.1.1.1. no FISH signal can be obtained by hybridisation of *Enterobacteriaceae* with probes of all Gram-positive bacteria. *Acinetobacter* spp examined versus all Gram-positive bacteria show a similarity ranging from 53-63%, and also no FISH signal, as also not from hybridisations between *Pseudomonas* spp and all Gram-positive bacteria listed, exhibiting a value of 61-68% of similarity.

Among all tested Gram-positive bacteria, the resolution is limited between the genera of enterococci and streptococci, as they have a similarity ranging from 70-76%. In repeated hybridisations under identical conditions positive signals in FISH were often detected (marked blue in C.1 and C.2). Possible reasons for this ambiguity in FISH results are discussed later in D.I.2.

Staphylococci in comparison to streptococci show a range of 67%-68% similarity over the whole sequence. FISH hybridisations between these genera using the opposite probes are negative. Between enterococci and staphylococci, similarities of 69-73% can be found and differentiation by FISH is possible.

None of the *Enterobacteriaceae* included in the Table (number 1-9 in Table C.1.) allows differentiation using the respective probes targeting DIII, with a range from 84-100% similarity between the different species. Only *Stenotrophomonas maltophilia* stands out, as its similarity to all other organisms ranges from 53-73%. This extraordinary position is confirmed by the FISH results, where *Stenotrophomonas maltophilia* can be differentiated from all the others.

The *Pseudomonas* species examined here have a similarity ranging from 73-78% compared to *Enterobacteriaceae* and 75-80% compared to *Acinetobacter* spp. FISH results were positive. *Acinetobacter* spp otherwise allow differentiation from all

examined *Enterobacteriaceae* using FISH. The similarity value is 68-70% over the whole target sequence.

1.2. Role of secondary structures and hybridisation conditions

The abilities to form secondary structures and to allow hybridisation of several probe molecules play a major role in the formation of a network and the resulting halo character of the signal after hybridisations.

Although DIII targeted polynucleotide probes are limited in specificity as shown in part C.1.1. they have some peculiar advantages.

The target structure rRNA is present in high copy numbers of up to 10^5 in growing bacterial cultures, and still of up to 10^3 copies in resting and low metabolic activity cells (i.e. in environmental samples and clinical samples). Considering the signal amplification based on the formation of the network of multilabelled probe molecules, detection is still easily possible. The resulting halo signal is obtained consistenly even for Gram-positive bacteria, as long as an appropriate pretreatment protocol has been applied (B.XI.3. and C.2.1.1.).

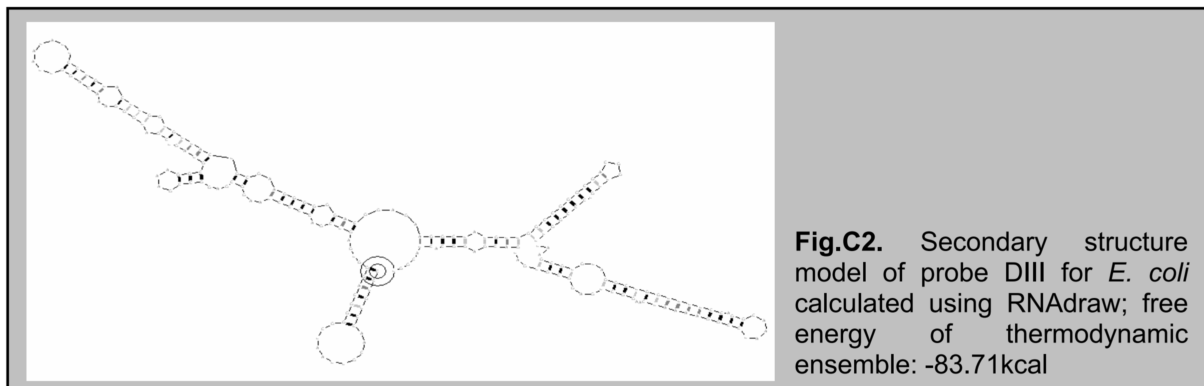
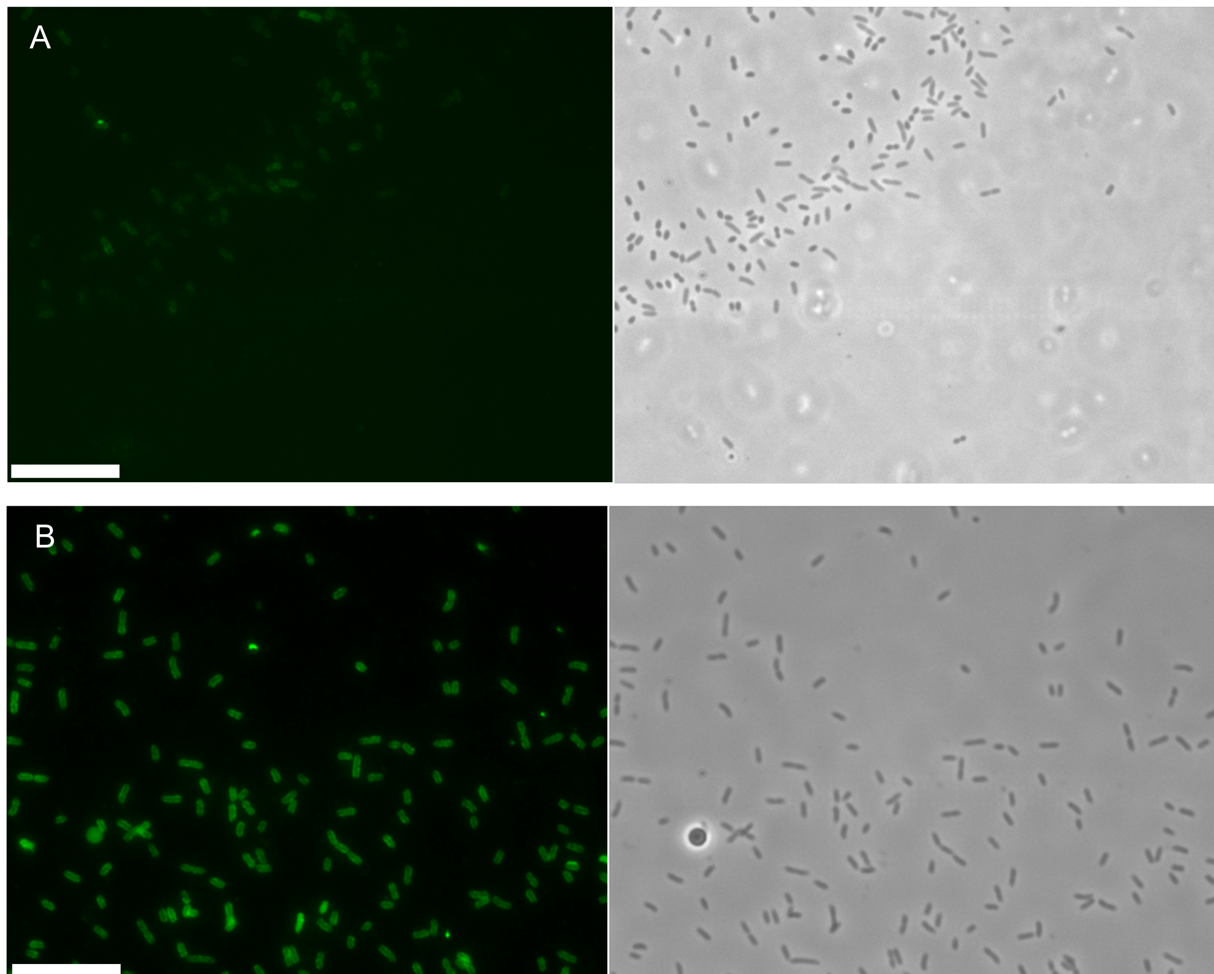


Fig.C2. Secondary structure model of probe DIII for *E. coli* calculated using RNAdraw; free energy of thermodynamic ensemble: -83.71kcal

Generally, DIII targeted probes can be used over a range of formamide from 40- 90% in the hybridisation (see Figs.C.3 A-D). At 40% of formamide in the hybridisation buffer, the signals are very weak and seem somehow blurred, as can be seen in Fig.C.3 A. The specificity, however, is not reduced. Below 40% of formamide, no more signals can be obtained. Above 55 % formamide, high signal intensities can be observed (Fig.C.3 B), which augment with increasing concentrations of formamide (65%, Fig.C.3 C). Optimal signal intensities are achieved with 80% formamide in the

hybridisation buffer, (Fig.C.3 D) resulting in stable, clear and very strong halo signals. The specificity is given in the scope as listed in Tables C.1 and C.2.

It is not possible to trigger a higher specificity for DIII targeted polynucleotide probes by further increasing the formamide concentration in the hybridisation buffer as the signal intensities weaken just as well as the enrichment capacities of the polynucleotide probe based enrichments by loosing network due to break down of secondary structures and inter-probe hybridisations.



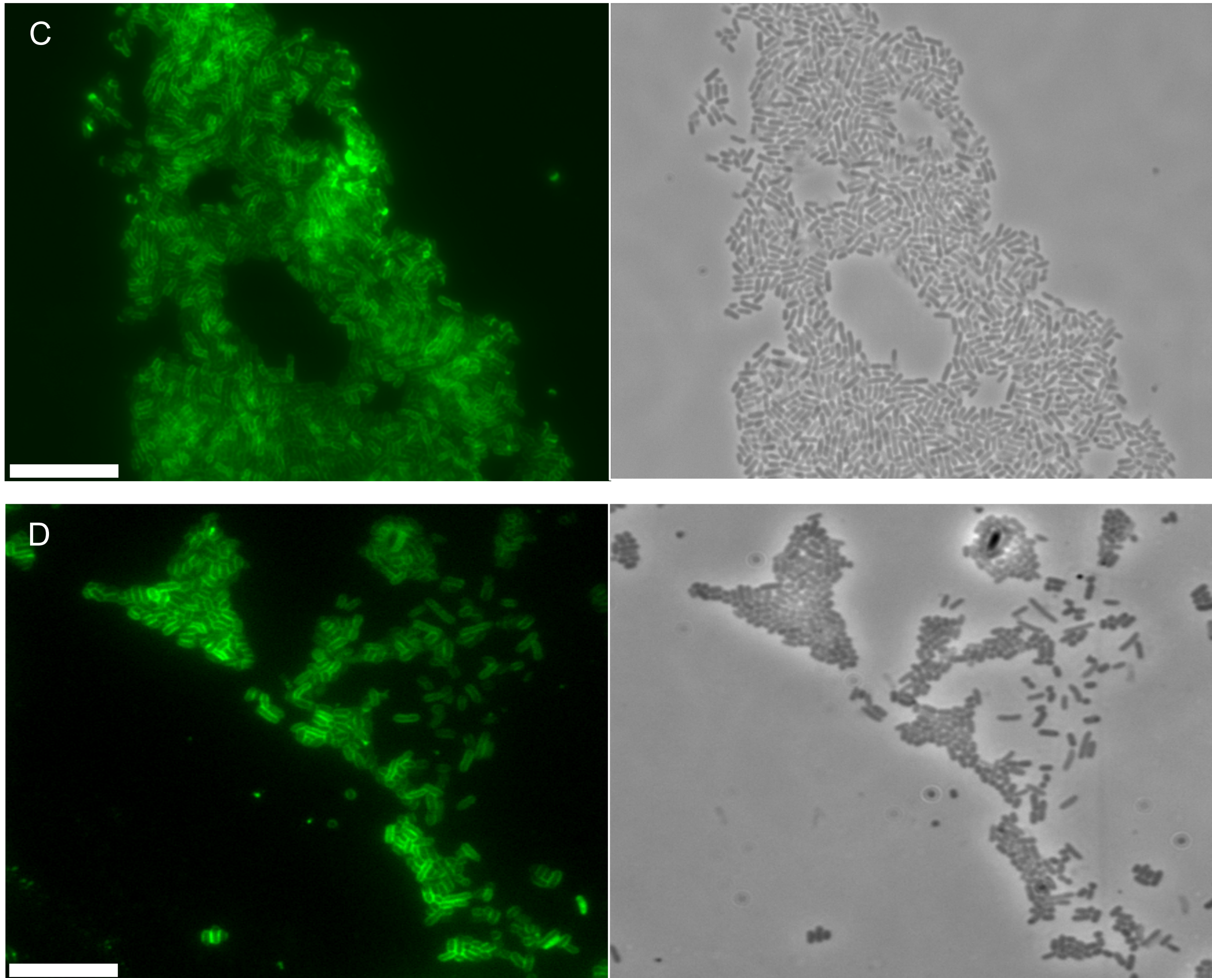


Fig.C.3. Fluorescence *in situ* hybridisation using probe DIII for *Pseudomonas aeruginosa*, epifluorescence (left), phase contrast (right image); bar 10µm.
A: in mixture with cells of *Neisseria canis* and **40%** of FA in the hybridisation; (see page 67)
B: **55%** of FA in the hybridisation buffer; (see page 67)
C: **65%** of FA in the hybridisation buffer
D: **80%** of FA in the hybridisation buffer

2. Adaptation of the protocol

2.1. Use of different pretreatments to enable or enhance probe binding

2.1.1. Gram-positive bacteria

The Gram-positive bacterial cell wall is, due to its thick peptidoglycan layer of 20-80nm, impervious to polynucleotide probes. To enable probe binding to target structures, a pretreatment, more powerful than the standard pretreatment used for

application of oligonucleotide probe FISH with Gram-positive bacteria (see B.XI.) is necessary.

The protocols proposed in Table C.3 were developed during this work and in cooperation with two diploma theses (Sandjong 2003, Ludyga 2004). For the three project relevant groups of Gram-positive bacteria enterococci, staphylococci and streptococci (Tables B.2 to 4), the methods allow controlled permeabilisation of the cell walls using cell wall lysing enzymes, exposition to heat or a combination of both. The stable formation of the probe network, necessary for subsequent enrichment hybridisations, results in a homogeneous halo signal which is similar to that observed for Gram-negative bacteria. The cells still show integrity without losing shape or even bursting (see Fig. C.4, C.5).

The individual incubation times required for the lysozyme step, enzymatic combination and heat treatment are not listed, due to the fact that they are different for all species examined; in fact, every new strain can require smaller or larger modifications in the duration of the particular pretreatment to obtain optimal results.

Detailed protocols that have been applied in this study, especially for application on real clinical samples, are described in B.XI.2.

Table C.3. Pretreatment protocols for permeabilisation of Gram-positive bacterial cell walls before polynucleotide hybridisations in comparison to Gram-negative bacterial cells; +: necessary; (+) optional, -: not necessary; * after hybridisation with polynucleotide probes

Organism		1st EtOH series	Enzymatic pretreatment	200°C pretreatment	2nd EtOH series	Halo signals*
Gram-negative bacteria		+	-	-	-	+
Gram-positive bacteria	Enterococci	+	Lysozyme 0°C	(+)	+	+
	Staphylococci	+	Lysozyme & Lysostaphin 30°C	(+)	+	+
			Lysozyme 0°C	-		
	Streptococci	+	Mutanolysin 37°C	+	+	+

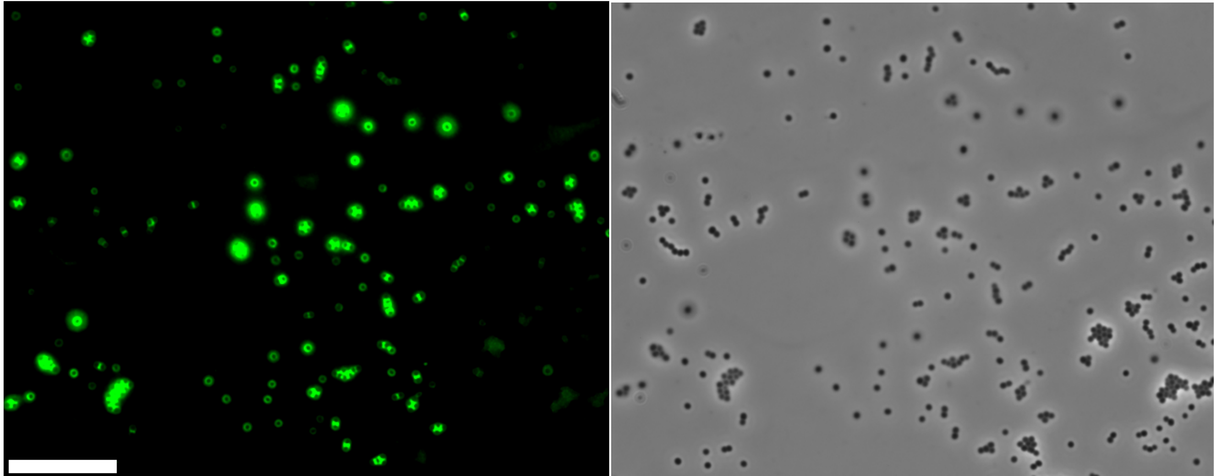


Fig.C.4. Fluorescence *in situ* hybridisation using probe DIII for *Staphylococcus aureus*; **80%** of FA in the hybridisation buffer, epifluorescence (left) and phase contrast (right image); bar 10 µm
Pretreatment: in between two ethanol series incubation using lysozyme (10mg/ml) for 20 minutes on ice and subsequent heat exposition for 3min at 200°C

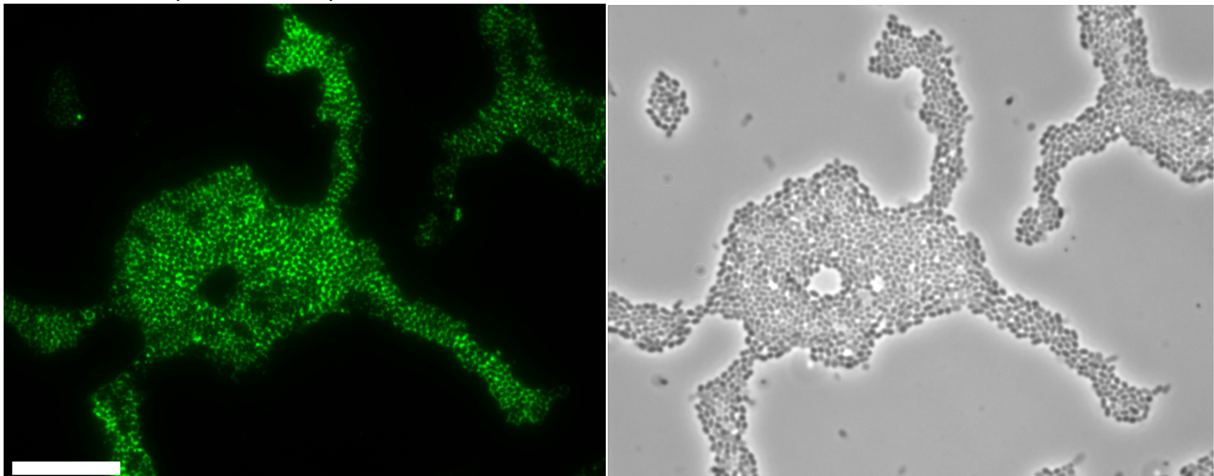


Fig.C.5. Fluorescence *in situ* hybridisation using probe DIII for *Enterococcus faecium*; **80%** of FA in the hybridisation buffer, epifluorescence (left) and phase contrast (right image); bar 10 µm
Pretreatment: in between two ethanol series incubation using lysozyme (10mg/ml) for 10 minutes on ice and subsequent heat exposition for 3min at 200°C

The proposed pretreatment protocols are also suitable for hybridisations in solution. To prove the impact of the pretreatment, binding capacities to complementary nucleic acids during the enrichment hybridisation of not pretreated Gram-positive bacterial cells have been examined in comparison to Gram-positive bacterial cells that have been subjected to a pretreatment.

The given examples for species of enterococci show the average values of signal intensities obtained after enrichment hybridisations, based on a streptavidin peroxidase system for biotin-labelled probes (see B.XII.). One fraction of cells (each appointed 1a, 1b for 1st. and 2nd. hybridisation) was not pretreated, the other fraction (2a, 2b, analogous to 1a, 1b) was subjected to a pretreatment of 10 min with

lysozyme (see B.XI.). In both cases, the average values of uncoated cavities as negative control are included.

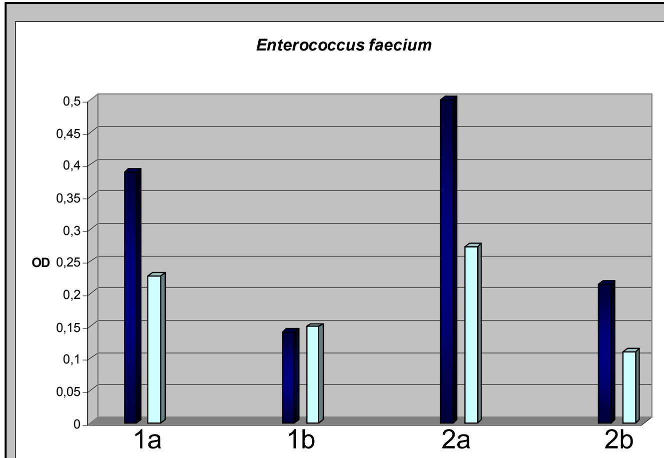


Fig.C.6. 23S DIII rRNA based enrichment hybridisation with cells of *Enterococcus faecium* untreated (1a, 1b) and treated with 10 min of lysozyme (2a, 2b) before hybridisation.
1a, 2a: average signal intensities of 1st enrichment hybridisation, each on 7 cavities coated with complementary nucleic acids, dark blue columns; neg. control uncoated cavities: light blue columns;
1b, 2b: 2nd enrichment hybridisation of transferred supernatant of 1st enrichment hybridisation on fresh cavities, dark blue columns; neg. controls analog 1a, 2a.

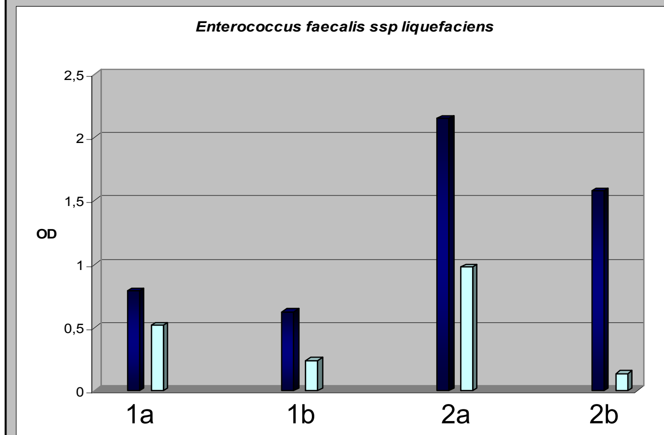


Fig.C.7. 23S DIII rRNA based enrichment hybridisation with cells of *Enterococcus faecalis ssp liquefaciens* treated (1a, 1b) and treated with 10 min of lysozyme (2a, 2b) before hybridisation.
1a, 2a: average signal intensities of 1st enrichment hybridisation, each on 7 cavities coated with complementary nucleic acids, dark blue columns; neg. control uncoated cavities: light blue columns;
1b, 2b: 2nd enrichment hybridisation of transferred supernatant of 1st enrichment hybridisation on fresh cavities, dark blue columns; neg. controls analog 1a, 2a.

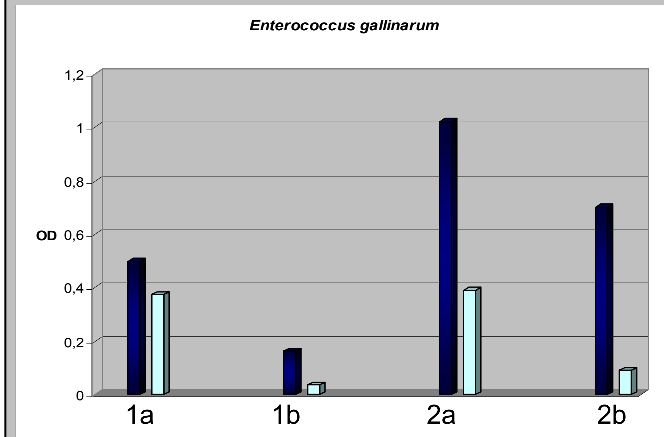


Fig.C.8. 23S DIII rRNA based enrichment hybridisation with cells of *Enterococcus gallinarum* untreated (1a, 1b) and treated with 10 min of lysozyme (2a, 2b) before hybridisation.
1a, 2a: average signal intensities of 1st enrichment hybridisation, each on 7 cavities coated with complementary nucleic acids, dark blue columns; neg. control uncoated cavities: light blue columns;
1b, 2b: 2nd enrichment hybridisation of transferred supernatant of 1st enrichment hybridisation on fresh cavities, dark blue columns; neg. controls analog 1a, 2a.

The diagrams depict the highly increased immobilisation of target cells in microplate cavities after application of the particular pretreatment protocol in comparison to the average values obtained from enrichments of cells that had not been subjected to pretreatments. The values of the columns indicating first and second enrichment hybridisation after application of the pretreatment protocol to the cells are up to 100-150% higher than their comparatives. In the case of *E. faecalis* (Fig.C.7), the average

values of the enrichments from cells without pretreatment exhibit almost no difference to the negative control which was uncoated. This indicates that obviously no succesful hybridisation of probe molecules to target structures had happened during hybridisation in solution, based on the fact that the polynucleotide probes simply could not enter the cell due to the lack of permeabilty.

2.1.2. Gram-negative bacteria

Although not necessary in the case of polynucleotide hybridisations with Gram-negative bacteria, a pretreatment of the cells can quantitatively enhance the cell binding capacities of hybridised cells to complementary nucleic acids immobilised to microplate cavities in enrichment hybridisations. Figs.C.8, 9 and 10 indicate the signal intensities obtained after enrichment hybridisations for examples of *Acinetobacter* species that have been subjected to a pretreatment in comparison to cells without application of a pretreatment.

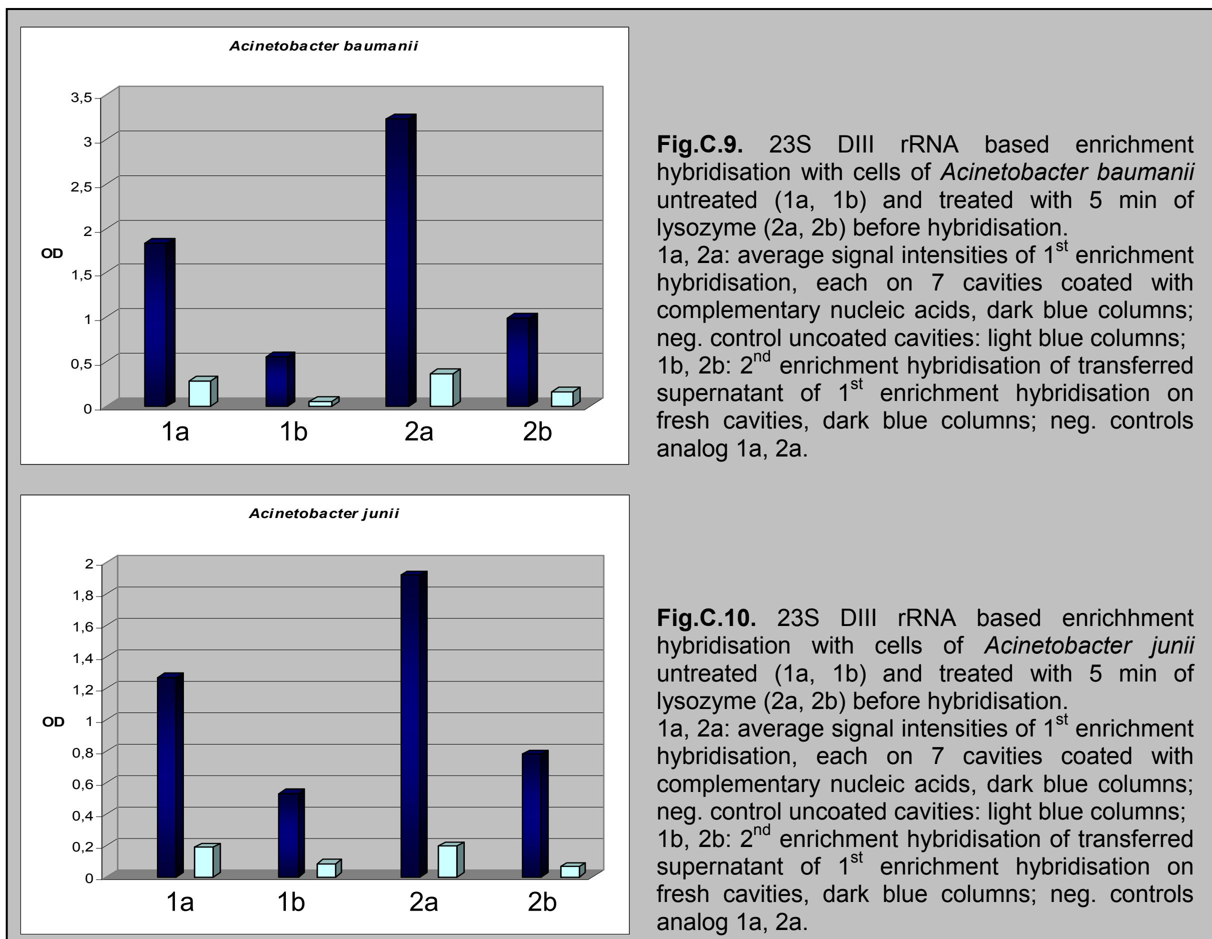


Fig.C.9. 23S DIII rRNA based enrichment hybridisation with cells of *Acinetobacter baumanii* untreated (1a, 1b) and treated with 5 min of lysozyme (2a, 2b) before hybridisation.
1a, 2a: average signal intensities of 1st enrichment hybridisation, each on 7 cavities coated with complementary nucleic acids, dark blue columns; neg. control uncoated cavities: light blue columns; 1b, 2b: 2nd enrichment hybridisation of transferred supernatant of 1st enrichment hybridisation on fresh cavities, dark blue columns; neg. controls analog 1a, 2a.

Fig.C.10. 23S DIII rRNA based enrichhment hybridisation with cells of *Acinetobacter junii* untreated (1a, 1b) and treated with 5 min of lysozyme (2a, 2b) before hybridisation.
1a, 2a: average signal intensities of 1st enrichment hybridisation, each on 7 cavities coated with complementary nucleic acids, dark blue columns; neg. control uncoated cavities: light blue columns; 1b, 2b: 2nd enrichment hybridisation of transferred supernatant of 1st enrichment hybridisation on fresh cavities, dark blue columns; neg. controls analog 1a, 2a.

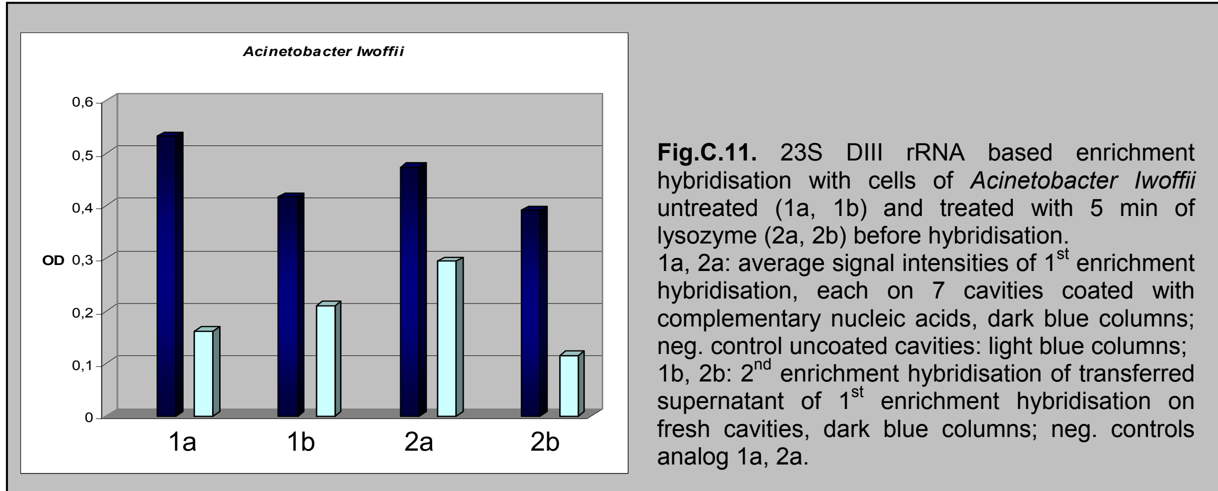


Fig.C.11. 23S DIII rRNA based enrichment hybridisation with cells of *Acinetobacter lwoffii* untreated (1a, 1b) and treated with 5 min of lysozyme (2a, 2b) before hybridisation.
1a, 2a: average signal intensities of 1st enrichment hybridisation, each on 7 cavities coated with complementary nucleic acids, dark blue columns; neg. control uncoated cavities: light blue columns;
1b, 2b: 2nd enrichment hybridisation of transferred supernatant of 1st enrichment hybridisation on fresh cavities, dark blue columns; neg. controls analog 1a, 2a.

The results of this comparison of signal intensities obtained after biotin labelled polynucleotide probe hybridisation demonstrate that after application of a pretreatment to pure culture cells of *Acinetobacter baumannii and Acinetobacter junii* with lysozyme for 5min at 37°C (Figs. C.9 and 10) it is possible to obtain significant higher signal intensities in comparison to not pretreated cells. Only for *Acinetobacter lwoffii* the enrichment values were lower for cells that had been pretreated (Fig.C.11). However, microscopic analysis of the pretreated Gram-negative cells, demonstrate great differences in nature and intensity of the signals.

Figs.C.12, C.13 and C.14 show the results after DIII targeted polynucleotide *in situ* hybridisations of three closely related species, *Acinetobacter baumannii, Acinetobacter junii* and *Acinetobacter lwoffii* with the respective DIII targeted polynucleotide probes after an identical pretreatment with lysozyme for 5 minutes at 37°C before hybridisation.

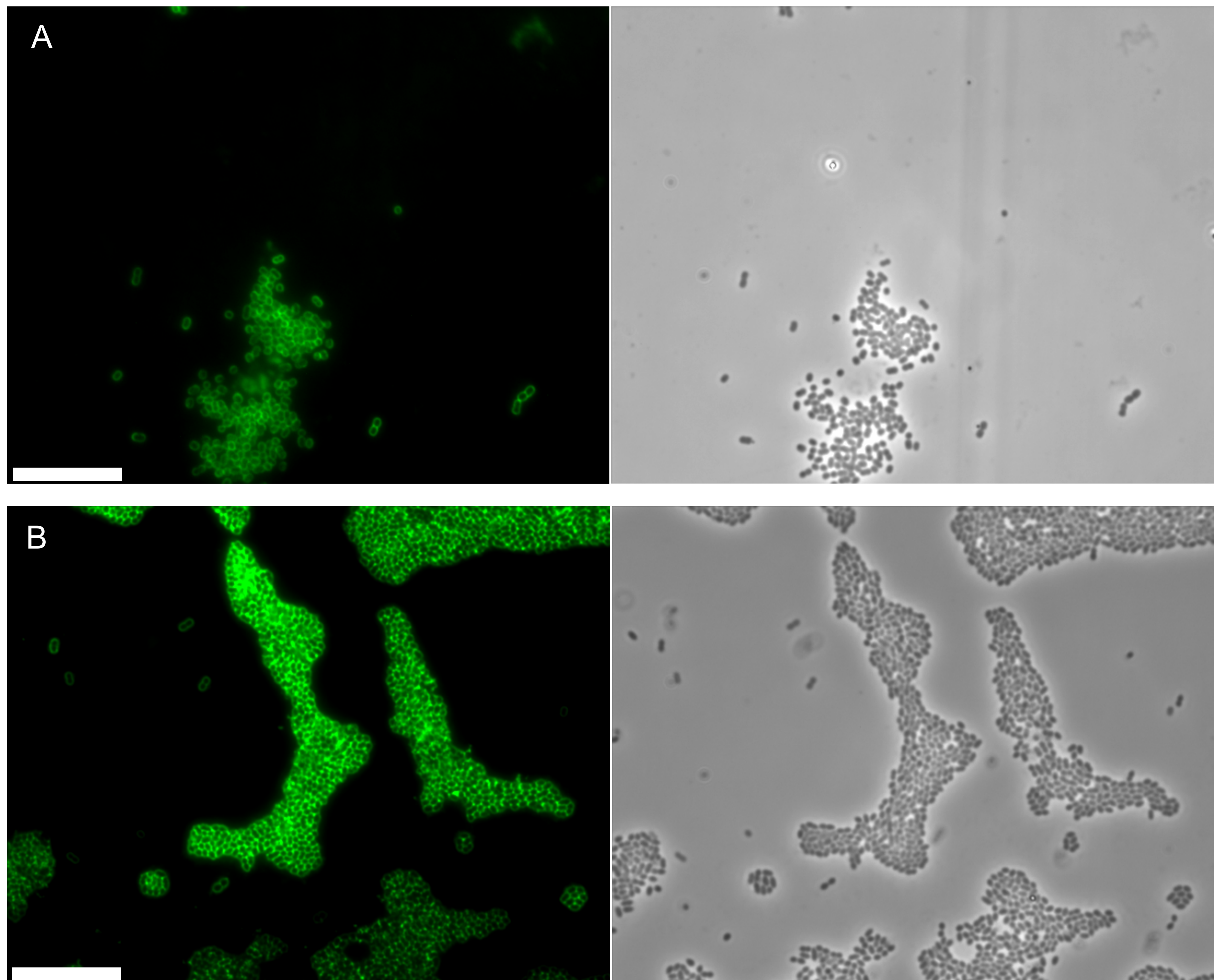


Fig.C.12. Fluorescence *in situ* hybridisation using probe DIII for *Acinetobacter baumanii*, epifluorescence (left) and phase contrast (right image); bar 10 µm
A: without any pretreatment;
B: Pretreatment 5min of lysozyme before fluorescence *in situ* hybridisation

Untreated cells of *Acinetobacter baumannii* (Fig.C.12 A) presented the usual halo signal after hybridisation. In contrast, the signal intensities after a pretreatment seem to be increased as indicated in Fig.C.12 B, confirming the results of quantitative enrichment measurements. Yet all cells on the slide showed conservation of cellular integrity and strong halo signals.

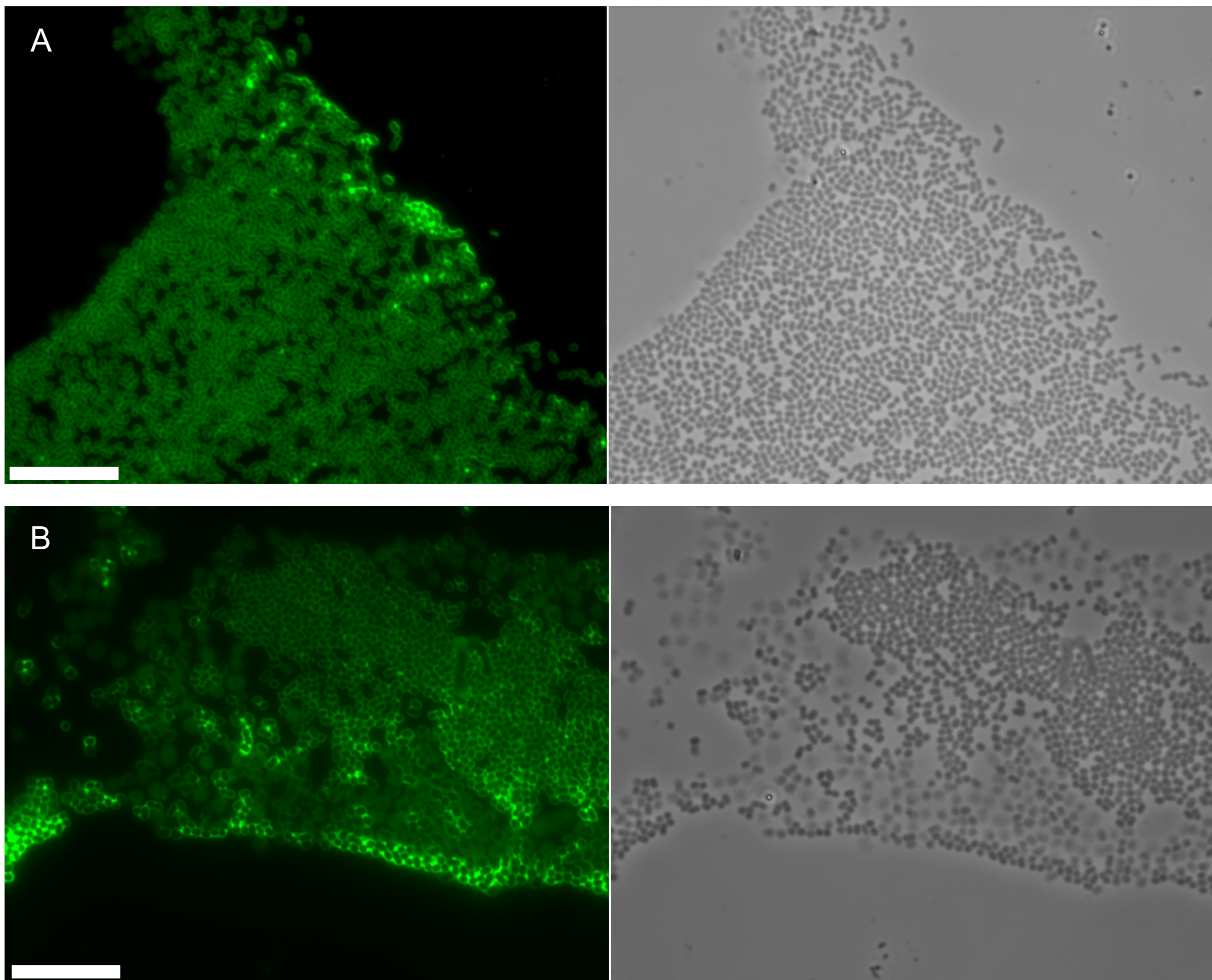


Fig.C.13. A, B: Pretreatment 5min of lysozyme before fluorescence *in situ* hybridisation using probe DIII for *Acinetobacter junii*, epifluorescence (left) and phase contrast (right image); bar 10 µm

The pretreatment with lysozyme for 5 minutes at 37°C applied to cells of *Acinetobacter junii* before hybridisation had different effects. The cellular integrity of approximately 75% of all cells remained, resulting in the typical strong halo signals (Fig.C.13 A), whereas about 25% of the cells seem to have been affected too harshly. These cells look burst, showing complete loss of cellular integrity and resulting in a signal of whole cell fluorescence, thus being not utilisable for further examination or application (Fig.C.13 B).

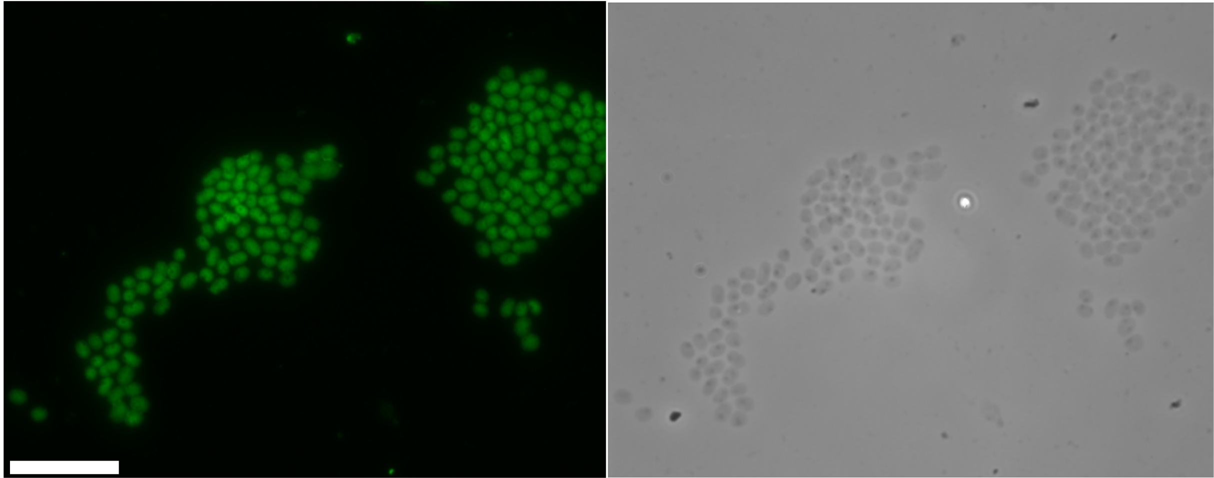


Fig.C.14. Pretreatment 5min of lysozyme before fluorescence *in situ* hybridisation using probe DIII for *Acinetobacter lwoffii*, epifluorescence (left) and phase contrast (right image); bar 10 µm

In the case of application of the same pretreatment to cells of *Acinetobacter lwoffii*, 100% of the cells on the same slide showed complete loss of cellular integrity and shape, resulting in weak whole cell fluorescence. Moreover, the cells of *Acinetobacter lwoffii* were completely burst and bloated. Whole cell fluorescence of the cell bodies with lost cellular integrity could be observed for all cells on the whole slide (Fig.C.14). This result is consistent with the results obtained from comparative measurement of the signal intensities after enrichment hybridisation of pretreated and not preteated cells of *A. lwoffii*.

In summary, the result is that exact control of the pretreatment degree is not possible in the case of Gram-negative bacteria. This impact could weigh even higher when applied to environmental or clinical sample material, where the cells are in different states of growth, conditions and environments in contrast to pure culture cells. Partial permeabilisation and/or complete lysis of target cells is easily possible, resulting in cells that are not suitable any more for enrichment hybridisations.

Due to this general drawback, for all following hybridisations with Gram-negative cells, or sample material containing Gram-negative cells as target organism, no pretreatment was performed to permeabilise the cell walls for an increased enrichment success.

2.2. Development and evaluation of the time-reduced protocol

In the context of the project the keyword "reduction of time" played a major role (see also introduction). If the minimum and maximum standard time prescriptions are followed for every single step, all steps together require a time effort ranging from 11-23 h. The initial step consists of the fixation of the cells or the sample material, followed by the first hybridisation in solution and the second hybridisation for the enrichment of target cells. In this work, it was examined whether an abbreviation of single steps within the whole approach still allows an enrichment of target cells, which is high enough to enable subsequent utilisation of the immobilised cells for further screenings like phylogenetic analyses or screening for antibiotic resistance genes. The results obtained from cells processed under time reduced conditions should be equivalent to the results obtained from cells that have been processed under standard conditions. Special regard should be taken on cell conservation and integrity, specificity of the used polynucleotide probes, signal intensity of the halo signal and enrichment capacities.

2.2.1. First reduction: Fixation time

The first step that attracted attention as being a potential target for possible time reduction was the method of cell or sample fixation and duration. The general protocol for PFA fixation proposes 5-9h of fixation time. The signal intensity and the cell integrity after a 23S DIII rRNA targeted polynucleotide hybridisation should not be weakened for cells that have been fixed for shorter than the standard times. Therefore, pure culture cells of *Klebsiella ornithinolytica* as example for all Gram-negative bacterial cells and *Enterococcus faecium* as example for all Gram-positive bacterial cells relevant for the project were grown to exponential phase. After harvesting they were fixed as described in B.II.3.1. for different periods of time (0.5, 1, 2, 3, 4, 5h and standard maximum of 9h).

2.2.2.1. Hybridisation on slides

The hybridisations using the cells that had been fixed for reduced times with the respective DIII targeted polynucleotide probes were carried out as usual for 9h

(see B.XI.2.). Figs.C.15 A, B and C indicate that there is no loss of signal intensity and cell integrity.

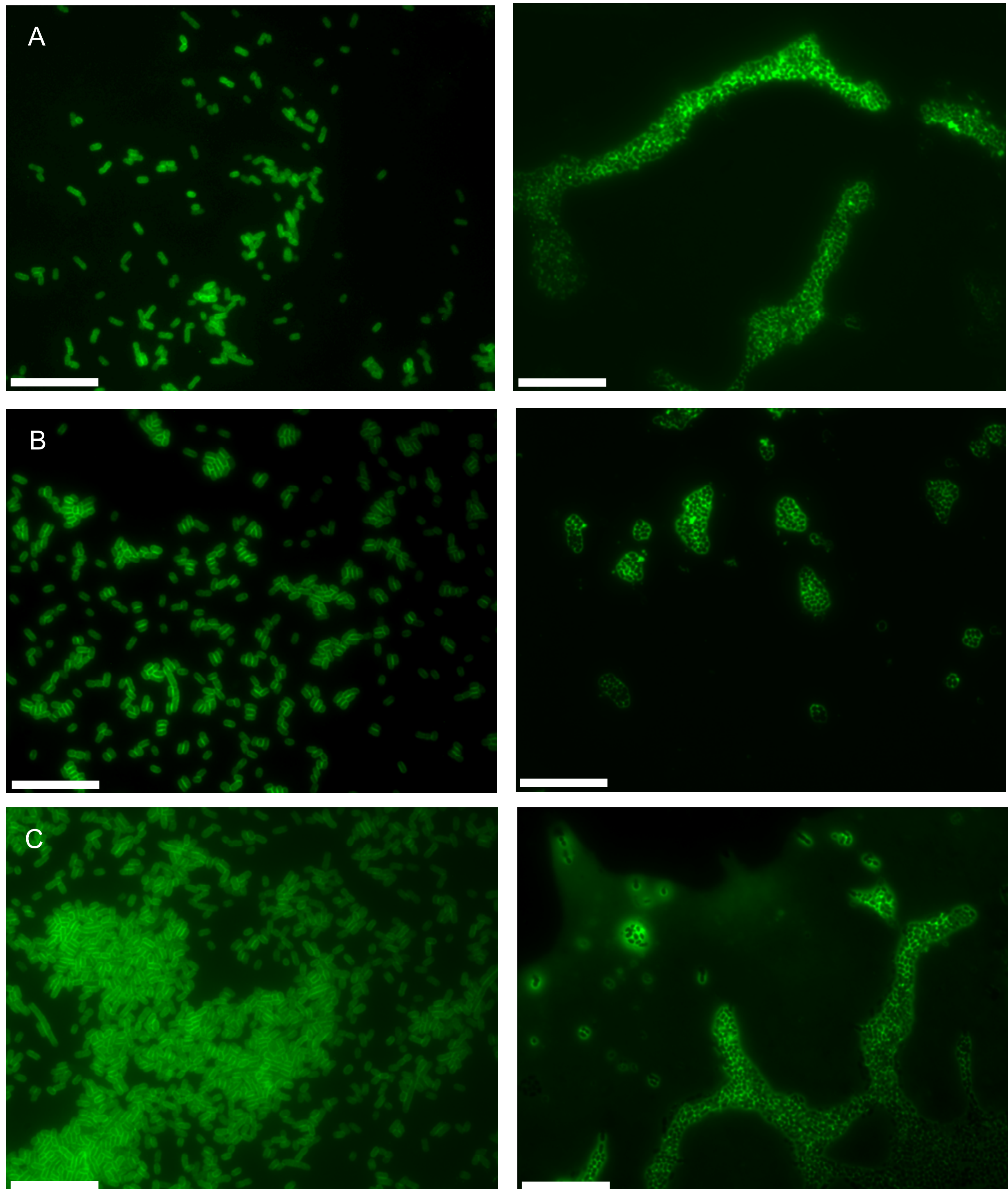


Fig.C.15. DIII - Fluorescence *in situ* hybridisation using respective probes DIII for cells of *Klebsiella ornithinolytica* (left) and *Enterococcus faecium* (right images).
A: Cells were fixed using 4% PFA for **0.5** hours; bar 10 µm
B: Cells were fixed using 4% PFA for **3** hours; bar 10 µm
C: Cells were fixed using 4% PFA for **5** hours; bar 10 µm

2.2.1.2. Enrichment hybridisations

The applicability of cells that have been fixed for shorter periods of time for enrichment hybridisations was tested by hybridisations in solution, followed by subsequent enrichment hybridisation. Therefore, the signal intensities, obtained from uncoated microplate cavities (negative control) or cavities coated with complementary nucleic acid were measured and PCR detection of immobilised cells after the first and second enrichment hybridisation was carried out as described in B.XII.

2.2.1.2. a) Comparison of signal intensities

The comparison of the signal intensities obtained from enrichment of cells, which were fixed for 0.5, 1, 2, 3, 4 and 5h were quite similar for both Gram-negative and Gram-positive bacteria (Fig.C.16). However, the signal intensities obtained from Gram-positive bacterial enrichments are lower than those of Gram-negative bacterial enrichments.

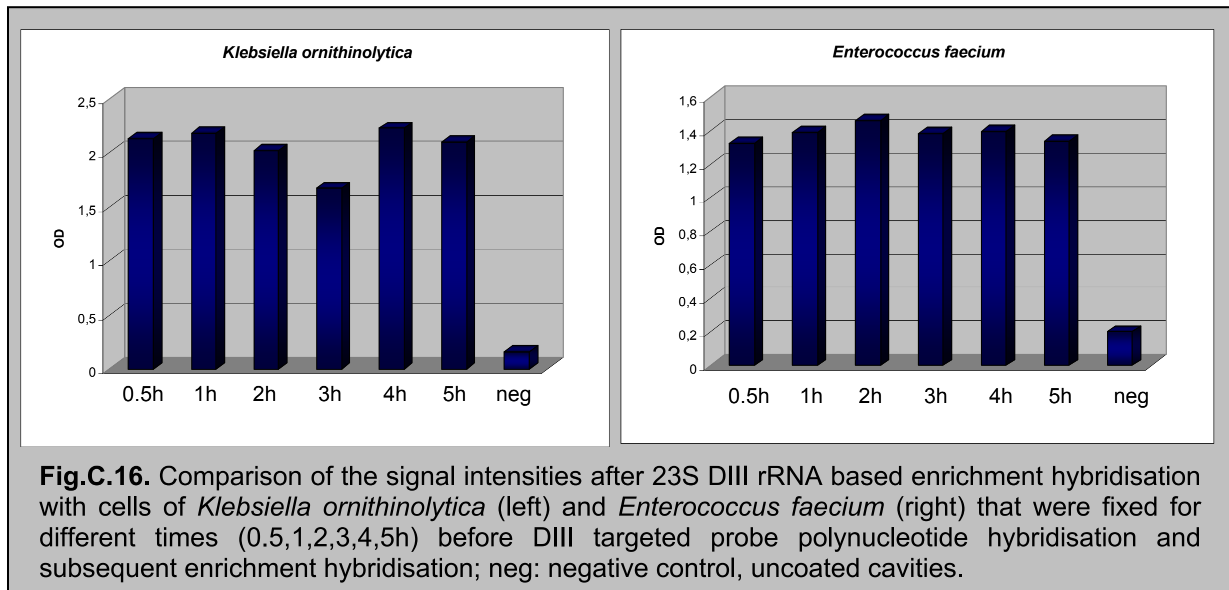


Fig.C.16. Comparison of the signal intensities after 23S DIII rRNA based enrichment hybridisation with cells of *Klebsiella ornithinolytica* (left) and *Enterococcus faecium* (right) that were fixed for different times (0.5,1,2,3,4,5h) before DIII targeted probe polynucleotide hybridisation and subsequent enrichment hybridisation; neg: negative control, uncoated cavities.

2.2.1.2. b) PCR based detection

The detection of cells via PCR using the conserved primers 118F-985R for amplification of 23S rDNA confirmed the results from the comparison of the signal intensities. All PCR products are present and more or less equally strong (Fig. C.17). The overall result is, that no notable loss of cells, cellular integrity and binding capacities of hybridised cells to complementary nucleic acids in microplate cavities occurs when the cells used were fixed for decreased periods of time.

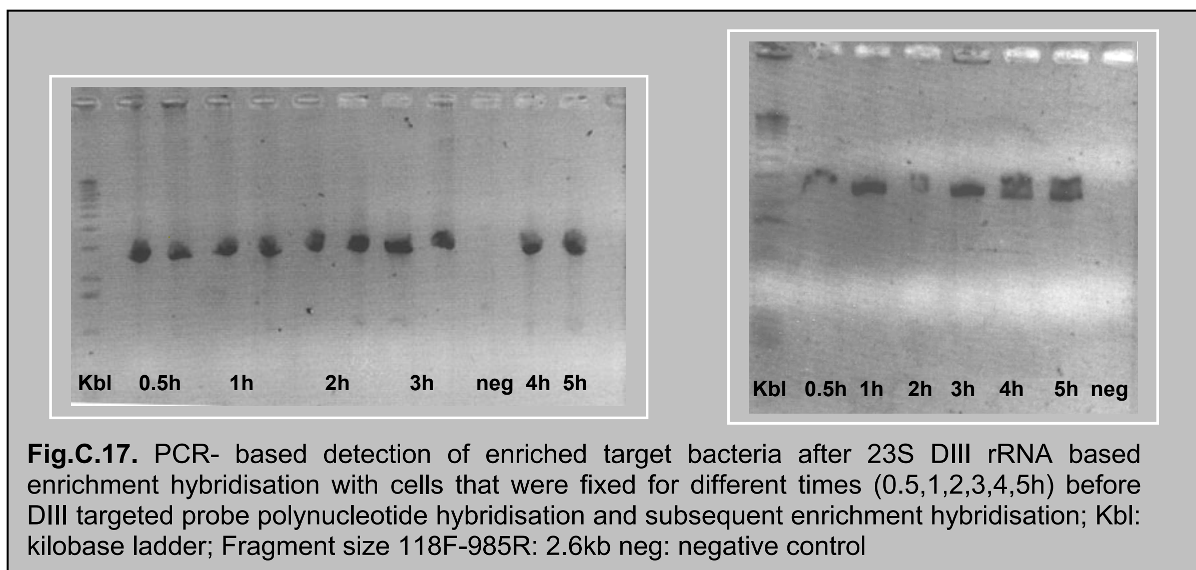


Fig.C.17. PCR- based detection of enriched target bacteria after 23S DIII rRNA based enrichment hybridisation with cells that were fixed for different times (0.5,1,2,3,4,5h) before DIII targeted probe polynucleotide hybridisation and subsequent enrichment hybridisation; Kbl: kilobase ladder; Fragment size 118F-985R: 2.6kb neg: negative control

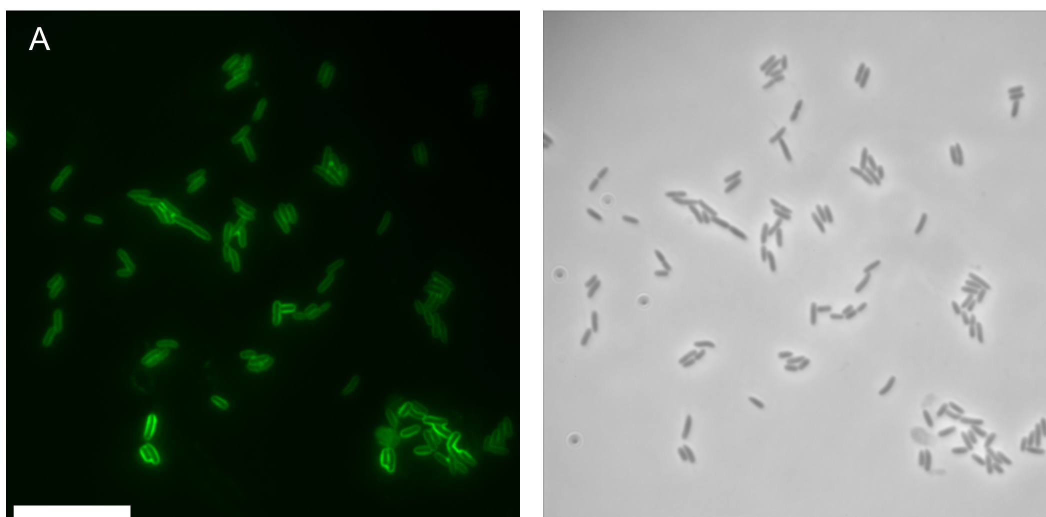
2.2.2. Second reduction: hybridisation time

The second step of a possible time reduction was hybridisation time using standard fixed cells. The signal intensity, specificity and cell integrity as obtained with standard hybridisation time should be ensured. Therefore, a step of microwaving microbial cells before hybridisations was performed. Microwaving sample material before FISH is a widely used method in different areas of FISH applications, e.g. histochemistry and pathology (Lan et al, 1996), but also in bacterial FISH techniques. Just as other treatments used for the permeabilisation of Gram-positive cell walls, microwaving the bacterial cells enhances the accessibility of the target structure, permeabilises the cell walls and triggers the specificity of the probe (Franks et al, 1998).

Table C.4. Fixation method, conditions for microwave step, hybridisation and resulting nature of signal; mw: microwaving

Organism	Fixation	Probe	sec of mw	min of hybridisation	Nature of signal
Gram-positive bacteria	EtOH	DIII	120	20, 40, 60	Halo
	PFA 4%	DIII	120	20, 40, 60	Halo
Gram-negative bacteria	PFA 4%	DIII	30	20, 40, 60	Halo

The reduction of hybridisation time was not showing a loss of halo signal, cellular integrity and signal intensity for examples on Gram-negative and Gram-positive bacteria (Figs.C.18 A, B and C. 19).



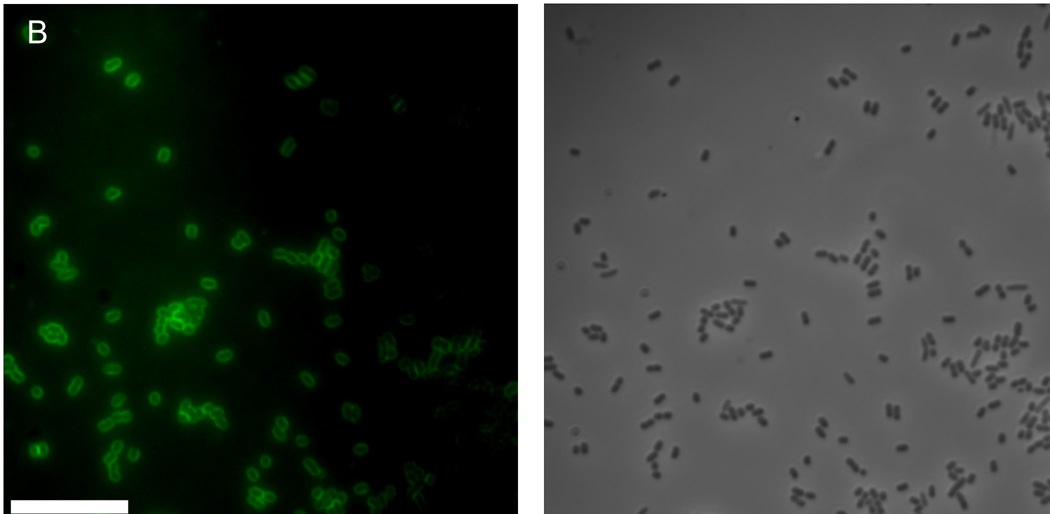


Fig.C.18. DIII - Fluorescence *in situ* hybridisation, 20minutes hybridisation time after 30 seconds of microwaving; epifluorescence (left) and phase contrast (right image); bar 10 µm
A: *Pseudomonas aeruginosa* (see page 81)
B: *Klebsiella ornithinolytica*

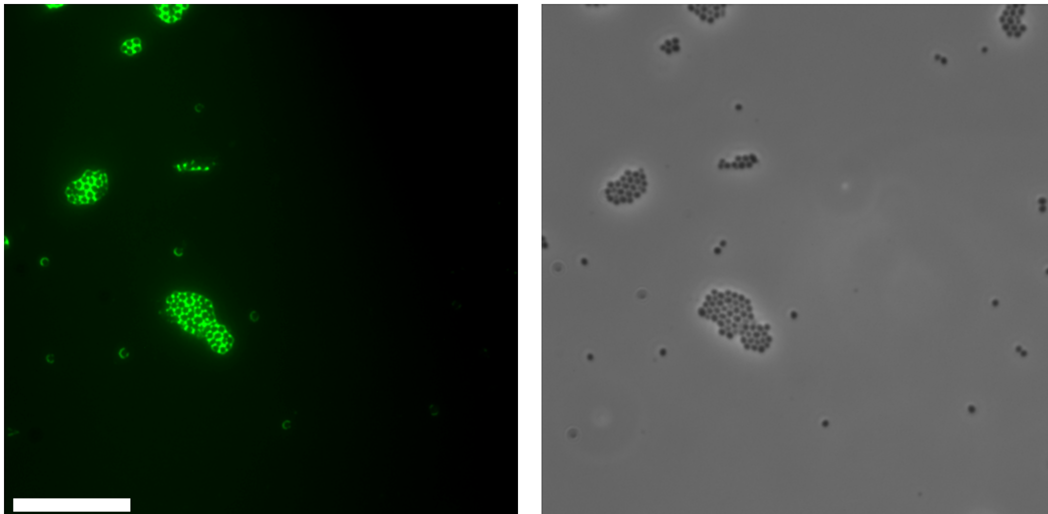


Fig.C19. DIII - Fluorescence *in situ* hybridisation using DIII targeted polynucleotide probe for *Enterococcus faecium,* 20min of hybridisation after 120 sec of microwaving; epifluorescence (left) and phase contrast (right image); bar 10 µm

2.2.3. Combination of first and second reduction

In order to find out if a combination of both, reduction of fixation time and hybridisation time applied in one approach has any influence on the cell integrity or signal intensity, a comparative analysis of signal intensities using the DAIME software package (Daims, unpublished) was performed. Using this software, it is possible to quantify the intensities of probe signals obtained after image acquisition using a Confocal Laser Scanning Microscope (CLSM). Therefore, 10-20 images have to be taken randomly from every single well containing the hybridised cells to be examined. The epifluorescence channel which is necessary for the detection of the fluorochrome used as label for the probe has to be used. The presence of about 100 cells per image should be assured. The values obtained from cells stated to be the standard are taken under the optimal settings using the CLSM with regard to signal intensity and focus. All further image acquisitions from the wells to be examined have to be taken under identical standard settings to ensure comparability. The software analysis later allows the quantification of all signal intensities obtained in comparison to standard signal intensities.

In this study, the signal intensities obtained using cells that were fixed for different periods of time (0.5, 1, 2, 3, 4 and 5h) and each hybridised for reduced hybridisation times (20, 40, 60min) using DIII targeted polynucleotide probes were quantitatively compared to the signal intensities obtained using cells fixed for maximum standard time (9h) and hybridised for standard hybridisation time (9h). As representative example for Gram-negative bacteria and Gram-positive bacteria *Klebsiella ornithinolytica* and *Enterococcus faecium* were chosen. Before hybridisation, a step of microwaving the cells for 30 seconds (Gram-negative bacteria) or 120 seconds (Gram-positive bacteria) respectively, was performed (see parts C.2.1. and C.2.2.).

The signal intensity obtained from applied standard conditions (cell fixation 9h and hybridisation time 9h) was set to 100%. The signal intensities obtained from cells of all other conditions were set in relation to this standard.

2.3.3.1. Klebsiella ornithinolytica

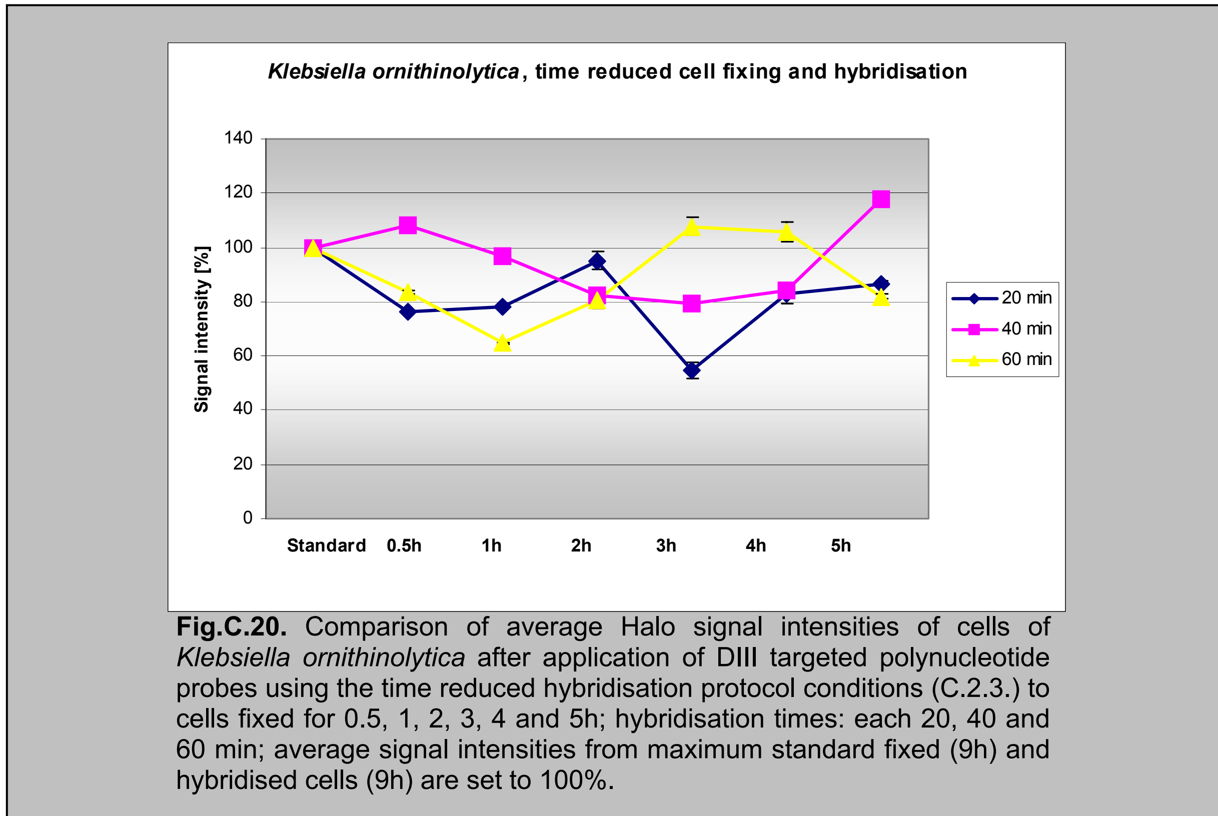


Fig.C.20. Comparison of average Halo signal intensities of cells of *Klebsiella ornithinolytica* after application of DIII targeted polynucleotide probes using the time reduced hybridisation protocol conditions (C.2.3.) to cells fixed for 0.5, 1, 2, 3, 4 and 5h; hybridisation times: each 20, 40 and 60 min; average signal intensities from maximum standard fixed (9h) and hybridised cells (9h) are set to 100%.

In the case of *Klebsiella ornithinolytica* (Fig.C.20), the values of the signal intensities obtained with 20 minutes of hybridisation vary from 95.15% (duration of cell fixation 2h) as the highest value to 54.7% (cell fixation duration 3h) as lowest value in comparison to standard 100% signal intensity. The values for hybridisation time of 40 and 60min vary from 64.7 to 118.04%. The comparison of the signal intensity values obtained after 20min, 40min and 60min of hybridisation time reveal no remarkable loss for any of the conditions. Microscopic analysis of the cells did not exhibit any difference in the macroscopic nature of the signal, either. The halo signal of cells fixed for 0.5h, hybridised for 20, 40 or 60min did not differ from the halo signal of cells fixed and hybridised after application of all other conditions.

2.3.3.2. Enterococcus faecium

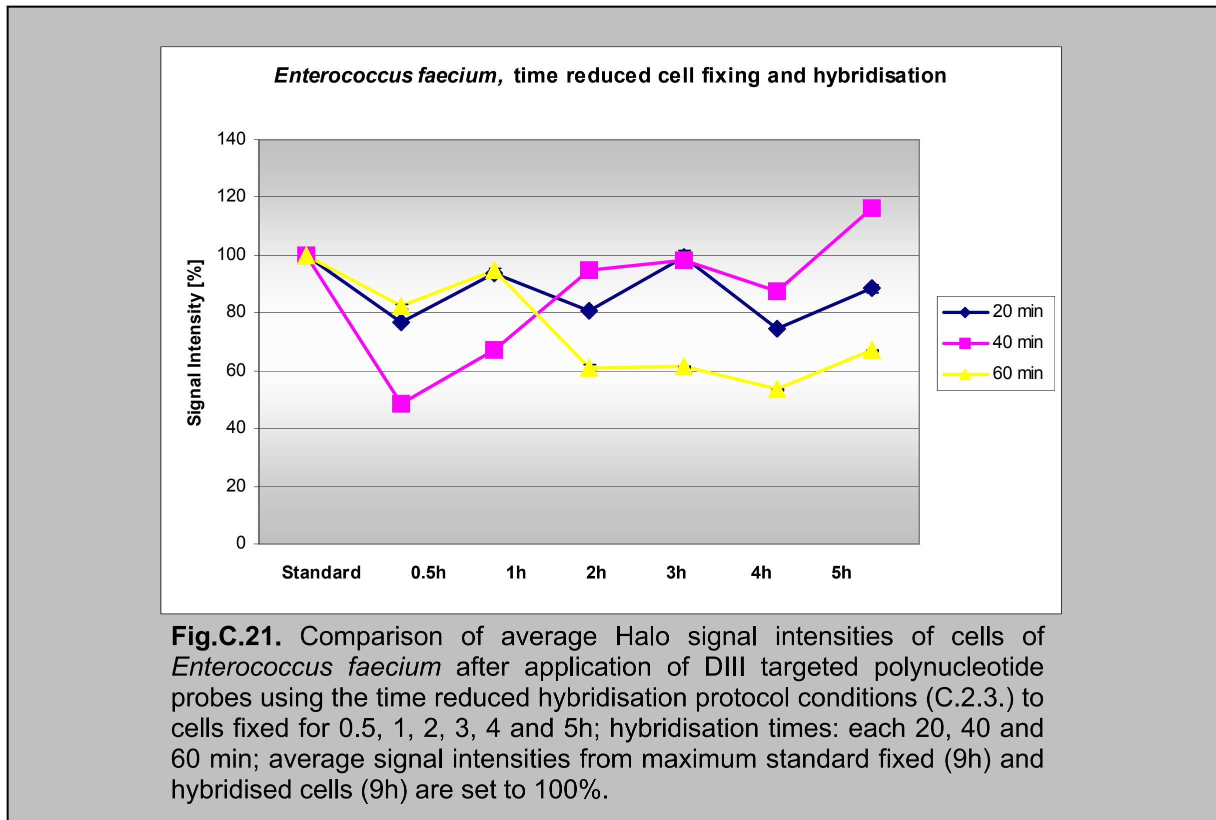


Fig.C.21. Comparison of average Halo signal intensities of cells of *Enterococcus faecium* after application of DIII targeted polynucleotide probes using the time reduced hybridisation protocol conditions (C.2.3.) to cells fixed for 0.5, 1, 2, 3, 4 and 5h; hybridisation times: each 20, 40 and 60 min; average signal intensities from maximum standard fixed (9h) and hybridised cells (9h) are set to 100%.

The analysis of the signal intensities after hybridisations with cells of the Gram-positive bacterium *Enterococcus faecium* (Fig.C.21) allows confirmation of the results obtained with the cells of Gram-negative bacterium *Klebsiella ornithinolytica*. Although the values obtained with 20 and 60min of hybridisation time respectively, were always below the 100% mark of cells fixed and hybridised using standard conditions and show minima below 60% of signal intensities (see columns for 60min of hybridisation, cells fixed for 2, 3, 4 and 5h), the microscopic analysis did not reveal a loss of halo signals or cell integrity. The column depicting 40min of hybridisation for *Enterococcus faecium* shows low level intensities for 0.5h and 1h of cell fixation, but very high intensities for 2, 3, 4 and especially 5h of fixation time.

The comparison of signal intensities obtained after DIII targeted polynucleotide probe hybridisation using the time reduced protocol demonstrates the applicability of the protocol for both Gram-negative and Gram-positive bacterial cells. Time saving of 9h is possible by application of every time reducing step (see Table C.5).

Table C.5. Comparison of time consumption for the whole procedure of enrichment hybridisation of old protocol and new protocol

procedure/ time	old protocol	new protocol
fixation of cells	5-9h	0.5h
hybridisation in solution	5-12h	0.33-1h
enrichment hybridisation	1-2h	1-2h
Summa summarum	**11-23h**	**1.5-3.5h**

3. Application of 23S rRNA DIII targeted polynucleotide probes to spiked clinical samples

3.1. Evaluation of fixation method for spiked clinical samples

Real clinical samples of EDTA blood, urine and plasma, spiked in different dilutions (10^{-1}-10^{-4}) with pure culture organisms as target bacteria were available. To obtain first experience whether an application of DIII targeted polynucleotide probes in this natural clinical environment leads to positive hybridisations or if it is potentially hampered by the natural conditions provided, all three different specimens were examined by FISH using 23S DIII rRNA targeted polynucleotide probes, before being applied to enrichment hybridisations. The potential impact of natural sample components on hybridisation success is discussed in detail in chapter D.I.4.1.

Due to the unknown behaviour and outcome of *in situ* hybridisations targeting bacteria in real samples, different fixation methods (Ethanol, Formalin, Bouin, and PFA) were tested with regard to several demands: presence of halo signal, duration of fixation and effort (meaning straightforwardness) necessary for fixation.

The fixations were performed as described in parts B.III. Table C.6 documents the findings:

Table C.6. Comparison of fixation methods with regard to special demands on halo signal, endurance of fixation and work; -: improper; (+): insufficient; +: good; ++: very good

Characteristics / Fixation	Bouin	Formalin	Ethanol	PFA
Halo- Signal	(+)	-	(+)	++
Fixation time	-	-	++	+
Effort	-	+	++	+

The most important demand for polynucleotide probe hybridisation in the context of this study the occurrence of a halo signal, as this is the precondition for subsequent enrichment hybridisations. After hybridisation with the respective DIII targeted polynucleotide probes, the best halo signals could be observed within the three different spiked sample materials EDTA blood, urine, and plasma after fixation using 4% of PFA (B.III.1.), equally for Gram-negative and Gram-positive bacterial cells.

Cell fixation using ethanol, though very short in time and associated with very low effort, led to halo signals that were a) limited in homogeneity, b) insufficient in signal intensity or c) not present at all. The further methods of fixation like formalin, a standard clinical fixation method for different kinds of clinical isolates and microbial cells (Broadaway et al, 2003; Luna et al, 2002; Lanoil et al, 1997) and Bouin`s fixation, also known from histology and surgical pathology in clinical areas and protozoology (Fried et al, 2002), turned out to be highly more time (6-24h) and effort consuming in comparison to PFA and Ethanol fixation. In addition, the halo signal was significantly weaker or inhomogeneous, even rather not visible. Therefore, for all future hybridisations with clinical samples, the PFA fixation was used as standard fixation, especially since the development of the time reduced protocol allowed shortening of the PFA fixation in relation to time by fulfilling all special demands on the nature of the signal (see part C.I.2).

3.2. Application of the time-reduced hybridisation protocol to spiked clinical samples

The development of the time reduced protocol allows enormous time saving. The applicability could be shown with pure cultures in part C.I.2.

The spiked clinical samples were provided in fixed form from Robert Bosch hospital, Stuttgart. The time reduced hybridisation protocol was applied for all spiked clinical samples. Hybridisation time in solution was generally 1h; the enrichment hybridisation was carried out as usual (see Table C.5).

3.3. Calculation of enrichment efficiency

The enrichment efficiency was analysed exemplarily for two clinical urine samples spiked with *E. coli* AF 441612 and *Enterococcus faecium* AF 458387 as representatives of Gram-positive and Gram-negative bacteria, respectively. Therefore, the supernatant was examined after enrichment hybridisation for obtaining the ratio of target organisms to non target organisms according to point B.XII.4. The supernatant of a microplate cavity which was not coated with complementary nucleic acids was taken as reference for obtaining the original ratio of target to non target organism in the native urine samples which were spiked with the target organism.

Target cells were labelled using the species-specific oligonucleotide probes Eco 1167 (*E. coli*) and Efi 58 (*Enterococcus faecium*, see Table B.14) in oligonucleotide FISH before cell counting. Because the particular urine samples held very few non target organisms, a defined amount of further non-target cells, morphologically distinct from the target organisms, was added artificially: cells of *Pseudomonas stutzeri*, a comparatively long rod. All target cells were counted in epifluorescence modus on ten randomly chosen fields, whereas the non target cells were counted from the same fields in phase contrast for comparison. The calculation of the percentage values were carried out as described in part B.XII.4. Table C.7 shows the obtained percentages of enrichment success for all dilutions.

Table C.7. Comparison of enrichment efficiency on examples *E. coli* and *Enterococcus faecium*

Enrichment value from dilution of spiked organism in urine sample / organism	*E. coli*			*Enterococcus faecium*		
	% of target organism in reference ST (=before enrichment)*	% of target organism after enrichment**	% of enrichment success***	% of target organism in reference ST (=before enrichment)*	% of target organism after enrichment**	% of enrichment success***
10^{-1}	74.42	51.12	65.62	67.08	34.64	48.36
10^{-2}	52,09	36.27	30.37	51.76	16.68	67.78
10^{-3}	45.01	11.14	75.24	46.55	16.51	65.94
10^{-4}	33.95	11.01	67.57	40.51	16.82	58.48

* target organism relative to non target organism ** relative to target organism ***calculated percentage of target cell enrichment in comparison to target cells before enrichment; ST= supernatant of cavities, where enrichment hybridisation was carried out (see B.XII), here reflecting status before enrichment

The enrichment success for *E. coli* from spiked urine samples varies from 30.37-75.24% between the different dilutions. In the case of *Enterococcus faecium*, the variation between the different dilutions is not so ostentatious and ranges from 48.4-67.8%. Zwirglmaier (2003) described that the percentage of enrichment success (*** in Table C.7) of target cells increases in relation to the percentage of target cells in the reference supernatant (* in Table C.7), which is comparable to the status of the ratio target cells-non target cells before an enrichment hybridisation. The fewer target cells present before enrichment in relation to non target cells, the higher is the enrichment success afterwards in relation to the initial target cells. This trend could not be observed in the given quantifications, though the % of decrease of target cells in the dilution from 10^{-1} to 10^{-4} can clearly be seen for both species.

3.4. Enrichment hybridisations with spiked clinical samples

Enrichment hybridisations using spiked clinical samples served as experiments for evaluation of hybridisation conditions in the complex and differing matrices of all clinical sample material, before applying the method to real clinical samples. Usually, the amount of PFA fixed material for starting the first hybridisation necessary for probe binding to target structures was 150 µl, processed according to B.XI.2. and resuspended in 60 µl of hybridisation buffer, with generally 6µl (~6µg) of probe solution. After the first hybridisation, the enrichment hybridisation was carried out on microplate cavities coated with complementary nucleic acids, according to point B.XII.2. The transfer of the supernatant to fresh cavities after the first enrichment allows further enrichment of remaining cells, which could not be enriched on the first due to the fact that the surface of the cavity was already saturated.

Fig.C.21 exemplifies the PCR based detection of enriched bacterial cells using conserved primers 118F and 985R from urine samples, spiked in dilutions 10^0-10^{-4} with *E. coli* (left side) and *E. faecalis* (right side).

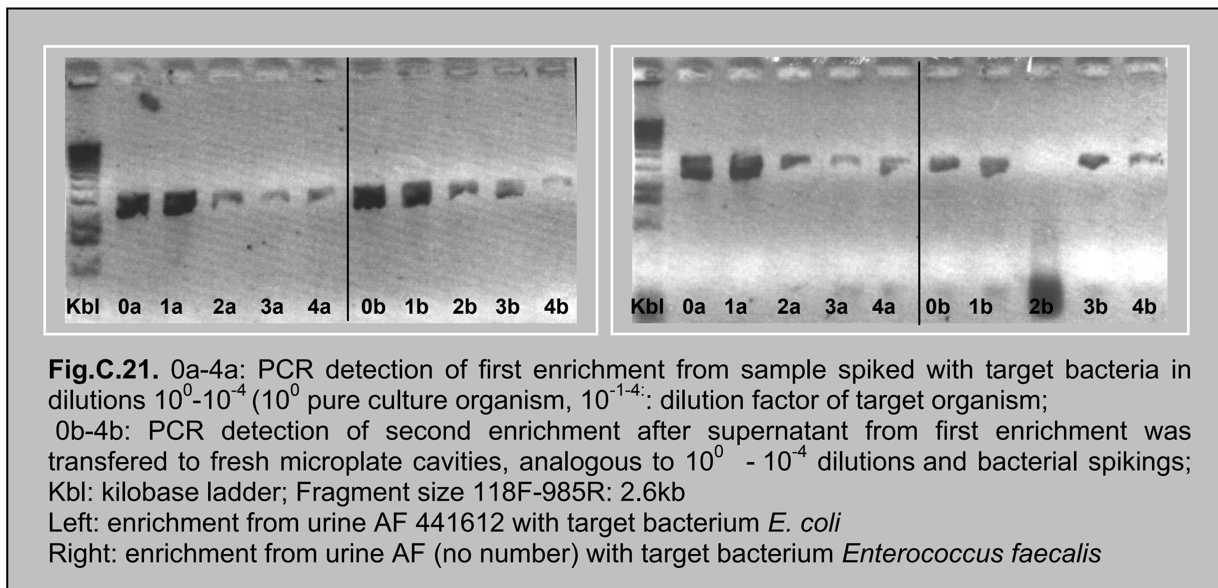


Fig.C.21. 0a-4a: PCR detection of first enrichment from sample spiked with target bacteria in dilutions 10^0-10^{-4} (10^0 pure culture organism, 10^{-1-4}: dilution factor of target organism;
0b-4b: PCR detection of second enrichment after supernatant from first enrichment was transfered to fresh microplate cavities, analogous to 10^0 - 10^{-4} dilutions and bacterial spikings;
Kbl: kilobase ladder; Fragment size 118F-985R: 2.6kb
Left: enrichment from urine AF 441612 with target bacterium *E. coli*
Right: enrichment from urine AF (no number) with target bacterium *Enterococcus faecalis*

In this example, the PCR based detection was possible up to the highest dilution of 10^{-4} in the first and the second enrichment (after transfer of the supernatants from the first cavities used for enrichment to fresh cavities). The decrease of the intensity of the PCR product with increasing dilution factor can clearly be seen. Only one cavity

of the second enrichment of cells of *Enterococcus faecium* (Fig C.21 B-2b) failed in PCR detection.

Of all three kinds of spiked sample materials, urine samples turned out to be the least complicated. The enrichment success from urine samples was given for all microorganisms spiked in urine that were tested, though not in every case a detection of the organism using PCR after enrichment hybridisation was possible for the highest dilutions. In this spiking, a dilution of 10^{-4} is comparable to $8x10^3$ cells. This cell number is similar to what can be found in natural infected urine, where cell numbers of 10^3-10^4 are usual for samples from infected patients.

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Table C.8 sums up the results of the enrichments from all spiked samples (each spiked in dilutions from 10^{-1}-10^{-4} with the target bacterium) that were tested.

Table C.8. Successful enrichments from spiked clinical sample material; n.r.: no result; n.t.: not tested

Spiked organism / sample material	Enrichment successful in dilution		
	EDTA blood	urine	plasma
E. coli	10^{-1} - 10^{-2}	10^{-1}	10^{-1} - 10^{-3}
Pseudomonas aeruginosa	10^{-1} - 10^{-4}	10^{-1} - 10^{-4}	10^{-1} - 10^{-4}
Enterobacter aerogenes	10^{-1} - 10^{-2}	n.r.	10^{-1} - 10^{-4}
Stenotrophomonas maltophilia*	10^{-2} - 10^{-4}	10^{-1}	n.r.
Haemophilus influenzae*	n.t.	10^{-1} - 10^{-2}	n.t.
Klebsiella oxytoca	n.t.	10^{-1} - 10^{-2}	n.t.
Enterococcus faecium	n.r.	10^{-1} - 10^{-4}	n.r.
Enterocccus faecalis	n.r.	10^{-1} - 10^{-4}	n.r.

* Enrichment success confirmed by PCR, but not by cloning and sequencing

It was possible to successfully perform enrichments from spiked blood samples for Gram-negative bacteria, allowing detection of immobilised cells using PCR up to the last dilution. For Gram-positive bacteria, enrichment from blood samples was not possible. In the case of the spiked plasma samples, Gram-positive bacteria also did not allow successful enrichment hybridisations, whereas again the tested Gram-negative bacteria, except for *Stenotrophomonas maltophilia*, again allowed successful detection of enriched bacteria up to the highest dilution.

3.5. Cloning and sequencing of PCR products from enrichment hybridisations

All PCR products obtained from successful enrichment hybridisations using conserved primers 118F and 985R were cloned for separating the PCR products. In the spiked clinical samples, the spiked bacteria constitute the majority in contrast to the natively present bacteria, but enrichment of non target organism in the scope of the specificity of DIII targeted polynucleotide probes can be possible. After cloning of the PCR product (according to point B.XIII), clones were chosen arbitrarily and in numbers of 5-10 per cloning plate. Positive clones with the right size of insert in the plasmid were sequenced, followed by similarity comparison of nucleic acid sequences using BLAST (data for every single clone not shown), and examination of the diagnostic regions of sequences after alignment in an ARB sequence data base, to confirm the identity of the enriched organism. All sequences and partial sequences obtained turned out to be the target organism, which was supposed to be enriched out of the particular sample (Table C.9).

Table C.9. Result of sequence annotation of cloned and sequenced PCR products obtained from successful enrichments of target organisms from spiked clinical sample material.

Spiked organism	sample	Number of clones sequenced	Result of sequence annotation	
			ARB*	Blast similarity
E. coli	EDTA blood	11	*E. coli*	*E. coli* 23SrRNA
	Urine	8	*E. coli*	*E. coli* 23SrRNA
	plasma	2	*E. coli*	*E. coli* 23SrRNA
Pseudomonas aeruginosa	EDTA blood	3	*Pseudomonas aeruginosa*	*P. aeruginosa* 23SrRNA
	Urine	4	*Pseudomonas aeruginosa*	*P. aeruginosa* 23SrRNA
	plasma	4	*Pseudomonas aeruginosa*	*P. aeruginosa* 23SrRNA
Enterobacter aerogenes	plasma	7	*Enterobacter aerogenes*	*E. aerogenes* 23SrRNA
Klebsiella oxytoca	Urine	3	*Klebsiella species*	*K. oxytoca* 23SrRNA
Enterococcus faecium	Urine	6	*Enterococcus faecium*	*E. faecium* 23SrRNA
Enterococcus faecalis	Urine	7	*Enterococcus faecalis*	*E. faecalis* 23SrRNA

*ARB: confirmed by examination of the diagnostic regions in a rRNA ARB database alignment

4. Application of 23S rRNA DIII targeted polynucleotide probes to real clinical samples

4.1. Application of standard protocol

All real clinical samples were fixed directly after receipt, using the time reduced protocol (see C.I.2.2.). Duration of fixation was 1h. For all enrichment hybridisations with real clinical samples, the standard enrichment protocol was performed, except for *E. coli* AF 421100 from real clinical sample number 1, AF 421100, where in addition to the standard hybridisation protocol for one enrichment hybridisation, the time reduced hybridisation protocol was applied (see point C.4.2.2.). Real clinical samples provide very different and complex conditions which can not be estimated for their impact on polynucleotide hybridisations and polynucleotide probe based enrichment hybridisations. The amount of sample available varies strongly and is in most of the cases very low. In relation to that, application of the time reduced protocol for polynucleotide probe hybridisations in the initial phase for testing the transfer of the technique on real samples may lead to unnecessary loss of cells or hybridisation signals.

4.1.1. Detection of DIII generated signal in real clinical samples

The real clinical samples available are listed in Table B.4. They vary in origin. The samples contained different organisms, indicating existing or potential infection of the patient they originated from. Identity and abundance of the organisms present in the samples were revealed by clinical microbiological routine screening at the Robert Bosch hospital, Stuttgart, which consists of sample collection, culturing and plating on selective agar plates as recommended for the special specimens, identification and susceptibility to antibiotics of the isolated organism via different standards such as physiological tests and agar diffusion tests. The dominant and infectious organisms in the particular samples were thus available as pure culture organism for every sample.

The 11 samples consisted of 5 different clinical specimens: one blood culture, aerobic, one blood culture, anaerobic, five different urines, two different wound smears and two different tracheal swabs.
They differed in many parameters: volume, natural composition, components, number, abundance and accessibility of microorganisms, viscosity. All these factors may have an unknown impact and influence, even inhibitory influence, on the success of hybridisation.

The general question to be answered primarily was:
Is it possible to obtain the halo signal after hybridisations with polynucleotide probes considering the complex conditions and the special environment of real clinical samples?
Therefore, all real clinical samples were tested with the respective DIII targeted probes in hybridisations on slides *in situ* before performing any enrichment reactions. For samples containing Gram-positive bacteria as target organisms, a pretreatment protocol as described in (B.11.3.) for permeabilisation of the cell walls was carried out before hybridisation.
In addition, all samples were screened for the existence of the target organism with species-specific oligonucleotide probes.
Due to relatively limited specificity of DIII targeted polynucleotide probes, (see Tables C.1 and C.2) unspecific binding to non target bacteria which are a natural part or a contaminant of the particular sample may occur. In all samples examined, the target bacterium was dominant, except for samples numbers 6 and 9, where infection with the target organism was stated as sporadic among several others in the patient's record. The occurrence of few non-target organisms present did not lead to false positive hybridisation signals and enrichments. Figs.C.22-C.27 indicate that applicability of DIII targeted polynucleotide probes was possible, and therefore enrichment hybridisations could be applied in the following.

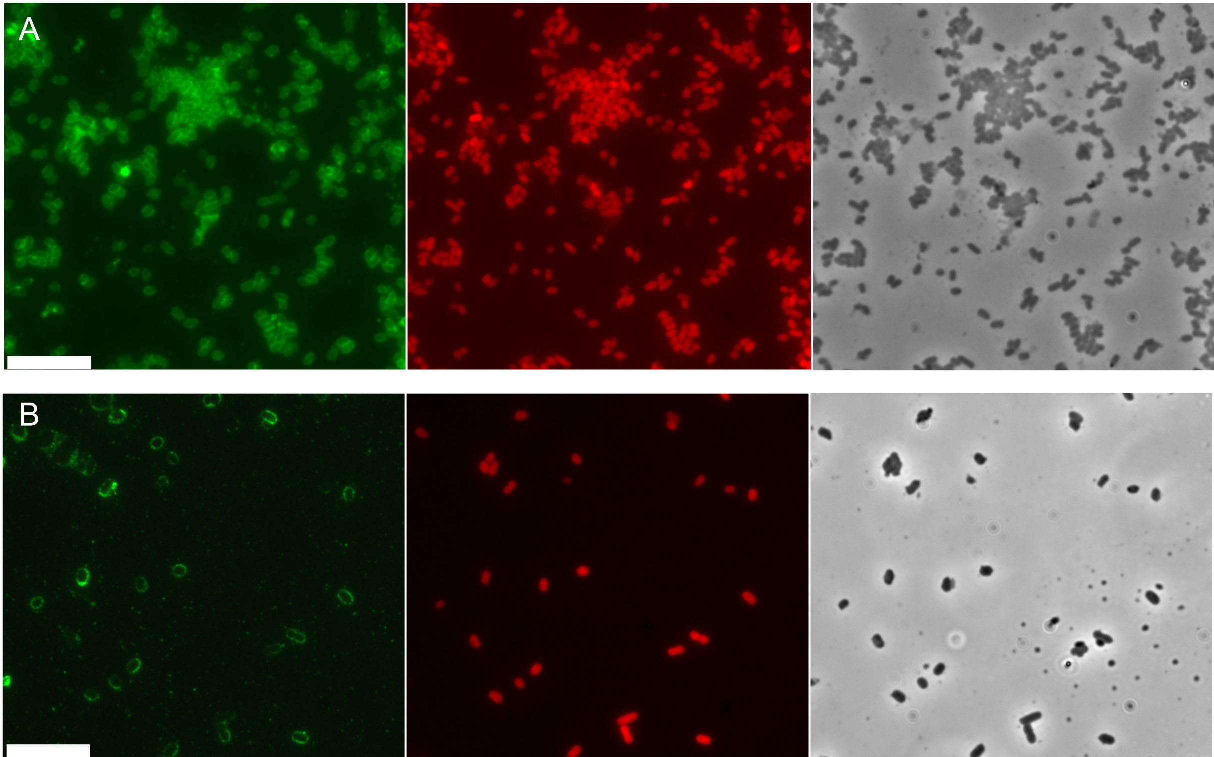
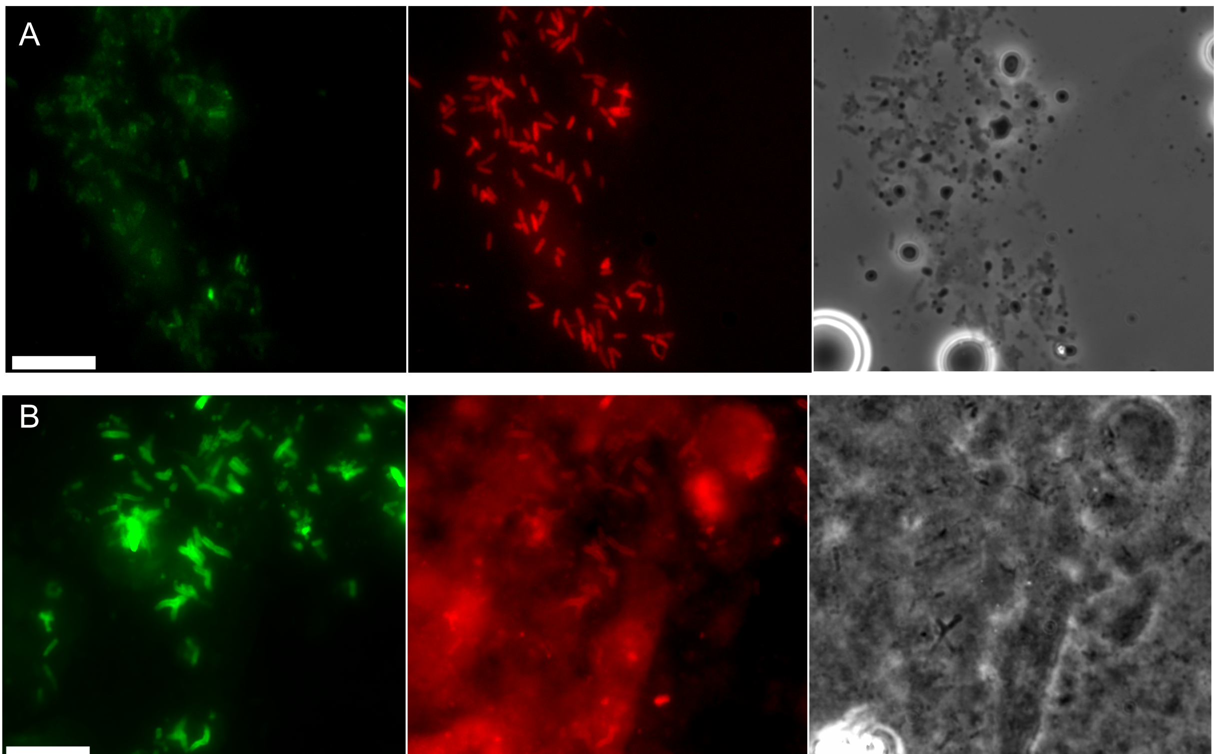


Fig.C.22. Fluorescence *in situ* hybridisation using DIII targeted polynucleotide probe for cells of *E. coli*, Fluos-12-UTP labelled (left) and *E. coli* specific probe Eco 1167 Cy3 (middle), phase contrast (right image), bar 10µm
A: clinical specimen number 1 (urine)
B: clinical specimen number 2 (aerobic blood culture)



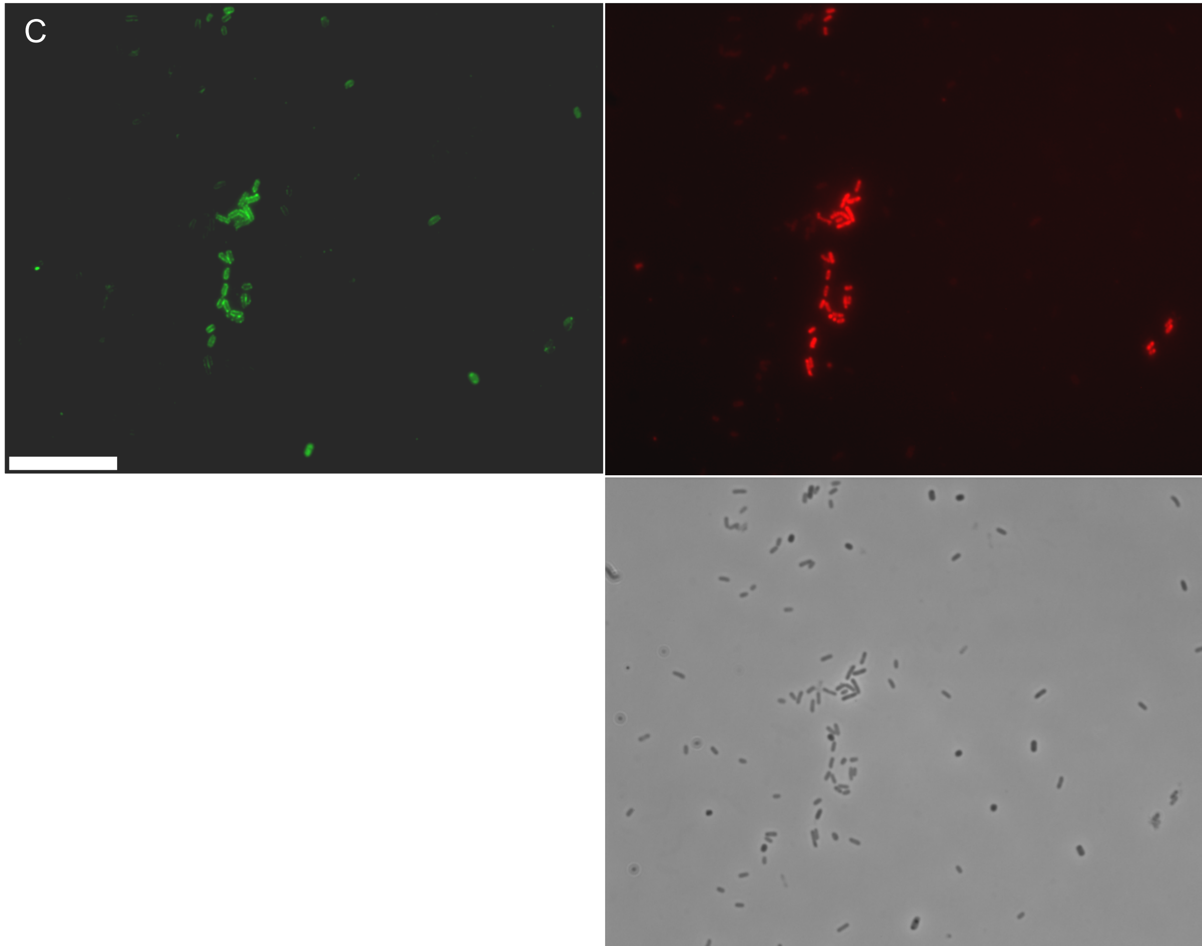


Fig.C.23. Fluorescence *in situ* hybridisation with clinical specimen, DIII targeted polynucleotide probe for *Pseudomonas aeruginosa*, Fluos-12-UTP labelled (left) and *Pseudomonas aeruginosa* specific probe PseAer Cy3 (middle), phase contrast (right image), bar 10µm
A: clinical specimen number 3 (wound smear), see page 95
B: clinical specimen number 4 (tracheal swab), see page 95
C: clinical specimen number 11 (urine)

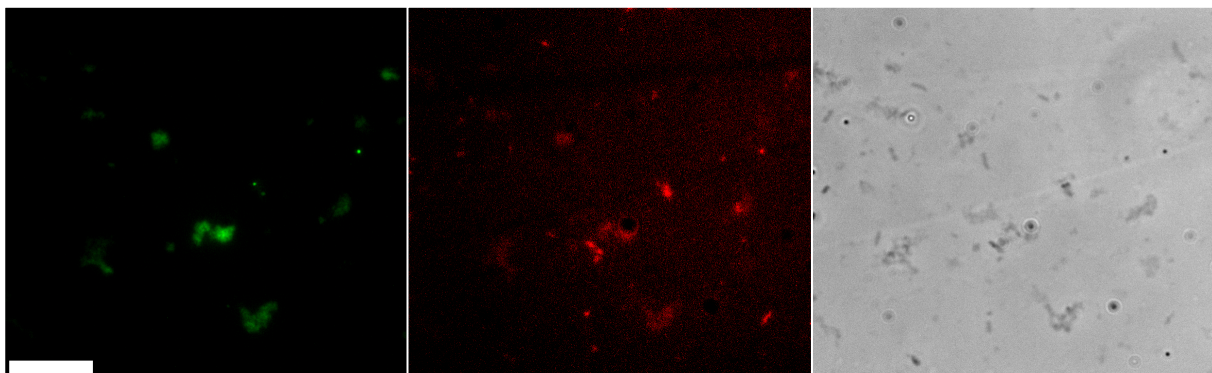


Fig.C.24. Fluorescence *in situ* hybridisation with clinical specimen number 8 (wound smear), DIII targeted polynucleotide probe for *Staphylococcus aureus*, Fluos-12-UTP labelled (left) and *Staphylococcus aureus* specific probe SAU 227 (middle), phase contrast (right image), bar 10µm

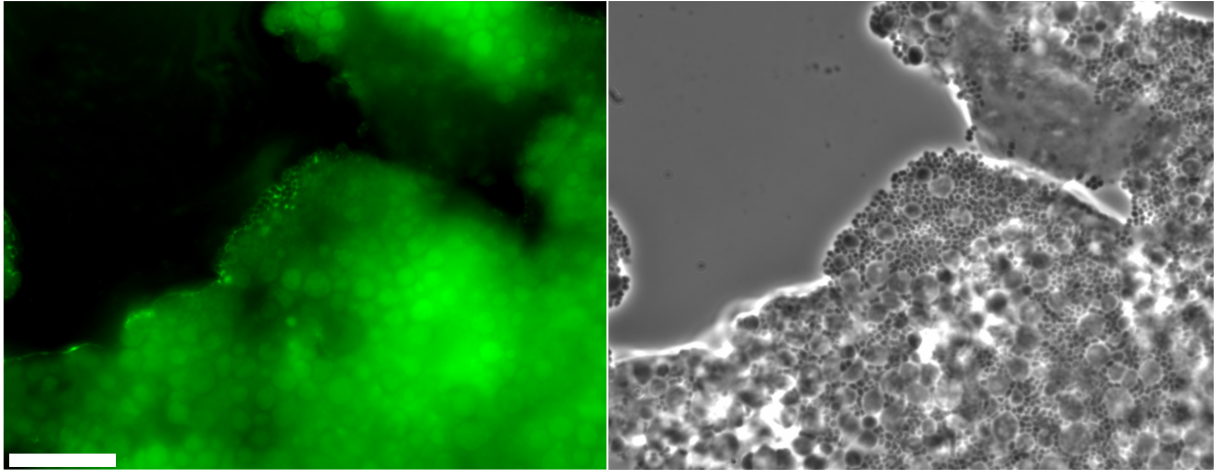


Fig.C.25. Fluorescence *in situ* hybridisation with clinical specimen number 10 (urine), DIII targeted polynucleotide probe for *Enterococcus faecium*, Fluos-12-UTP labelled (left) for *Enterococcus faecium*, phase contrast (right image), bar 10µm

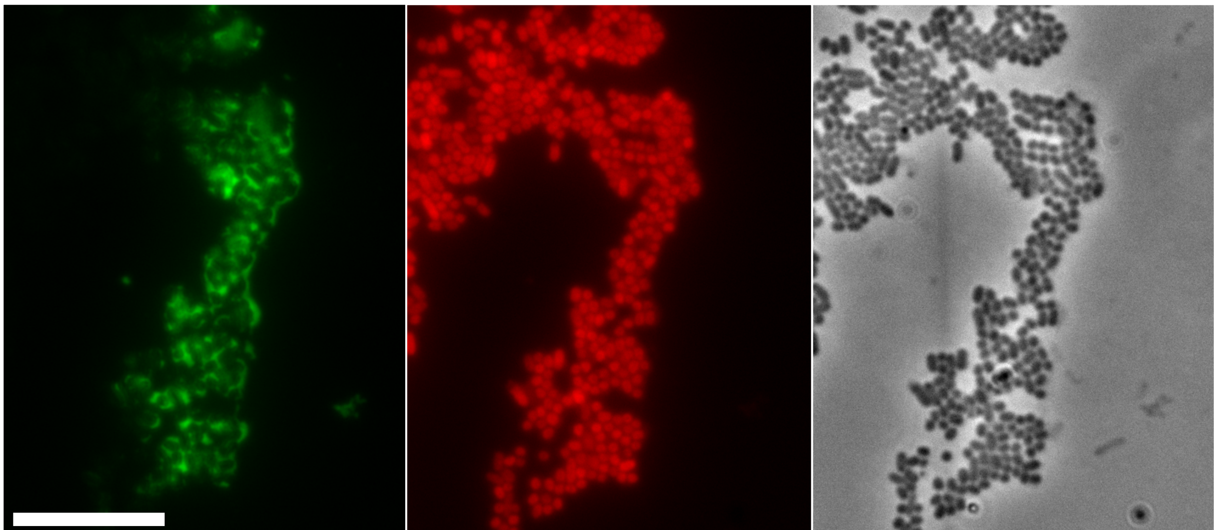


Fig.C.26. Fluorescence *in situ* hybridisation with clinical specimen number 9 (tracheal swab), DIII targeted polynucleotide probe for *Klebsiella pneumoniae*, Fluos-12-UTP labelled (left) and *Klebsiella species* specific probe Kpn (middle), phase contrast (right image), bar 10µm

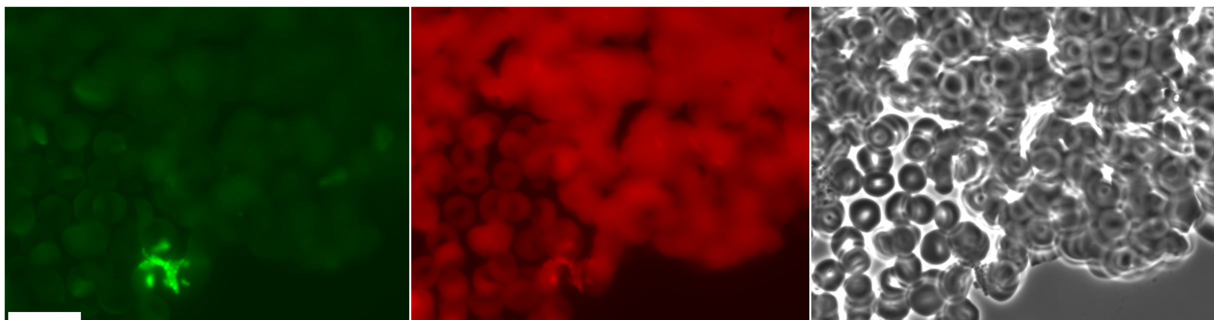


Fig.C.27. Fluorescence *in situ* hybridisation with clinical specimen number 8 (anaerobic blood culture), DIII targeted polynucleotide probe for *Staphylococcus aureus*, Fluos-12-UTP labelled (left) and *Staphylococcus aureus* specific probe SAU 227 (middle), phase contrast (right image), bar 10µm

4.2. Enrichment hybridisations with real clinical samples

The success of DIII targeted polynucleotide probe hybridisations on slides demonstrated the applicability of the probes for the detection of bacteria in clinical samples. The same reasons which hamper successful polynucleotide hybridisations and especially enrichment hybridisations in spiked clinical samples are also true for polynucleotide FISH with real samples. The resulting additional challenges are discussed in chapter D.I.4.

Table C.10 visualises the results of all enrichment hybridisations (performed according to point B.XI. and B.XII and depending on sample amount and specimens) with subsequent PCR detection of immobilised target cells using universal primers 118F-985R, followed by cloning and sequencing.

Table C.10. Results of enrichment hybridisations from real clinical samples containing target bacteria (Table B.4); dark blue marked fields: enrichment and sequencing based confirmation of identity; light blue marked fields: enrichment successful, no sequencing based confirmation of identity; white fields: no enrichment hybridisation successful

Sample Nr.	Sample	AF-Nr.	Fixation	Target organism in sample	Number of target organism in sample*
1	urine	421100	PFA 4%	*E. coli*	10^6
2	blood culture aerobic	430319	PFA 4%	*E. coli*	no data
3	wound smear	625811	PFA 4%	*Pseudomonas aeruginosa*	$>10^6$
4	tracheal swab	492106	PFA 4%	*Pseudomonas aeruginosa*	moderate
5	wound smear	615234	PFA 4%	*Staphylococcus aureus*	$>10^6$
6	urine	625857	PFA 4%	*Enterococcus faecalis*	sporadic
7	urine	422200	PFA 4%	*Klebsiella species*	10^5
8	blood culture anaerobic	630073	PFA 4%	*Staphylococcus aureus*	no data
9	Tracheal swab	41162	PFA 4%	*Klebsiella pneumoniae*	sporadic
10	urine	415772	PFA 4%	*Enterococcus faecium*	no data
11	urine	49218	PFA 4%	*Pseudomonas aeruginosa*	no data

* as documented in patients records from Robert Bosch hospital, Stuttgart

From real clinical samples number 1, 2, 3, 7, 8, and 10 (marked in dark blue) successful enrichment hybridisations with PCR based detection were possible. In

these cases, subsequent analysis of the cloned and sequenced PCR products confirmed the identity of the immobilised organism from the given real clinical samples (see further Table C.11.).

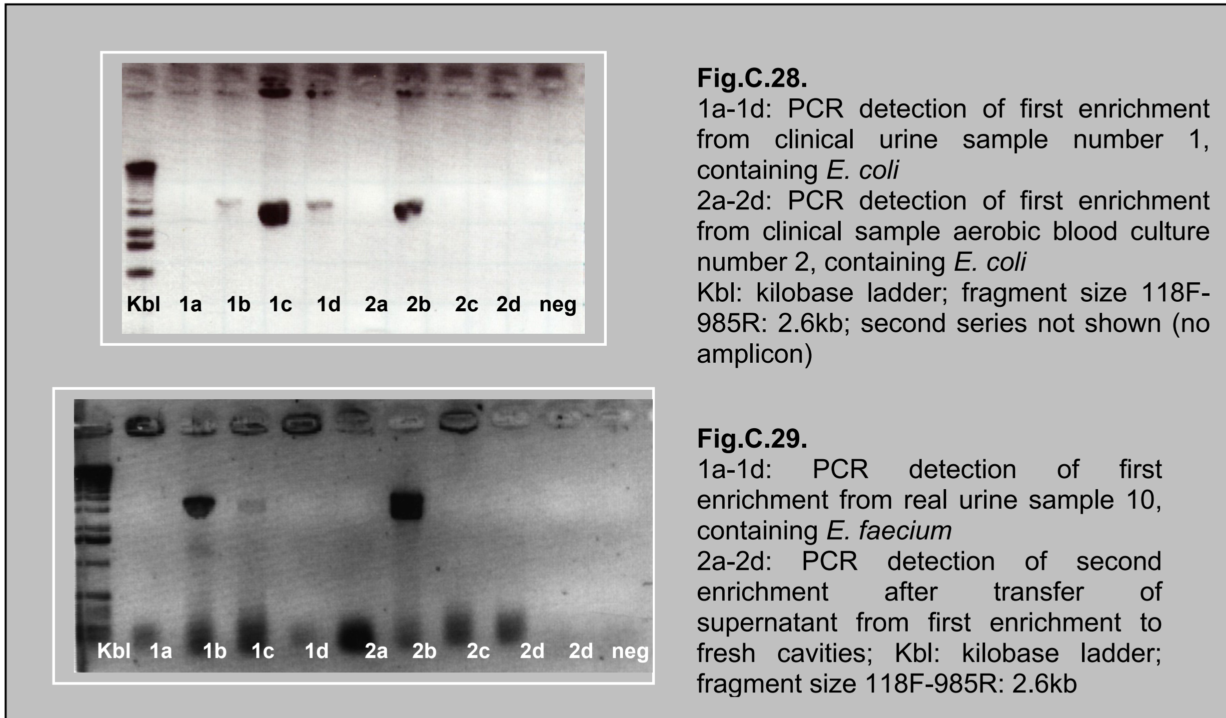


Fig.C.28.
1a-1d: PCR detection of first enrichment from clinical urine sample number 1, containing *E. coli*
2a-2d: PCR detection of first enrichment from clinical sample aerobic blood culture number 2, containing *E. coli*
Kbl: kilobase ladder; fragment size 118F-985R: 2.6kb; second series not shown (no amplicon)

Fig.C.29.
1a-1d: PCR detection of first enrichment from real urine sample 10, containing *E. faecium*
2a-2d: PCR detection of second enrichment after transfer of supernatant from first enrichment to fresh cavities; Kbl: kilobase ladder; fragment size 118F-985R: 2.6kb

Fig.C.28 exemplifies the PCR based detection of enrichment success after enrichment hybridisations from real clinical sample urine number 1, and aerobic blood culture number 2, both containing *E. coli* as dominant, infectious organism. In the case of real sample number 1, it was possible to obtain PCR products from immobilised cells in three cavities. Sample number 2, the aerobic blood culture, led to only one positive PCR result. The supernatants of both first enrichments were transferred to fresh cavities afterwards to allow further immobilisation of target cells. The PCR detection was negative here (image not shown).

Fig.C.29 exemplifies the PCR based detection of enrichment success after enrichment hybridisations from real clinical urine sample number 10, containing *Enterococcus faecium* as infectious organism, and showing positive PCR results for two out of four cavities of the first enrichment series and one for the second enrichment series after the transfer of the supernatant of the first series to fresh microplate cavities.

The confirmation of the identity of the enriched target organism based on sequencing is listed in Table C.11.

For samples number 5 and 9 in Table C.8 (marked in light blue), it was possible to enrich microbial cells out of the particular samples in microplate cavities and detect them with PCR. But in contrast to the above cases, it was not possible to obtain clones with the correct insert size after cloning of the PCR product. Thus, identity of the bacterial cells enriched could not be confirmed by sequence analysis. The target organism *Enterococcus faecalis* present as infectious organism in urine sample number 5, and *Klebsiella pneumoniae* present in tracheal swab sample number 9, have been reported in the patient record to be present in the sample only very sporadically. Furthermore, FISH based detection of bacteria is generally problematic in tracheal swab samples (see further point sample number 4 and parts D.I.4.1.2., D.I.4.1.3

It was not possible to enrich the special target organisms from the real clinical samples numbers 4, 6 and 11 (unmarked fields of the Table C.10). There are different evident and potential reasons for this:

Sample number 4 is a tracheal swab containing the *Pseudomonas aeruginosa*. In tracheal swab samples, generally and naturally inhibiting components of the sampling components play a major role. Although the detection of *Pseudomonas aeruginosa* in the sample number 4 was possible on slides using DIII targeted polynucleotide probes as well as using PseAer oligonucleotide probe (see Figs. C.23 B), the enrichment failed. As demonstrated in Fig.C. 23 B, the bacteria are embedded in a thick and highly viscous matrix, which could be the reason for impossible hybridisation during the enrichment hybridisation (see also D.I.4.1.2. and D.I.4.3.)

Sample number 6, a wound smear containing *Enterococcus faecalis* as target bacterium, was only less than 200 µl of sample volume. This was not enough for many steps of enrichment, because the loss of bacteria was too high.

Sample 11, an urine sample with *Pseudomonas aeruginosa* as target bacterium, was available in a too small volume to perform more than one step of enrichment hybridisation. Also, the occurrence of the target bacterium was stated as very sporadic (see Table C.10).

4.3 Application of the time reduced protocol

For one enrichment hybridisation targeting *E. coli* from real urine sample number 1, AF 421100, the complete time reduced protocol was applied. In addition to the cell fixation, which was done for 1h, the hybridisation time of the first hybridisation in solution for primary probe binding to the target region on the ribosomes was 1h here. The enrichment hybridisation was carried out as usual (see Table C.6).

The PCR based detection allowed visualisation of two amplicons (1a and 1d), shown in Fig.C.30. The second enrichment on fresh cavities did not allow further immobilisation of cells.

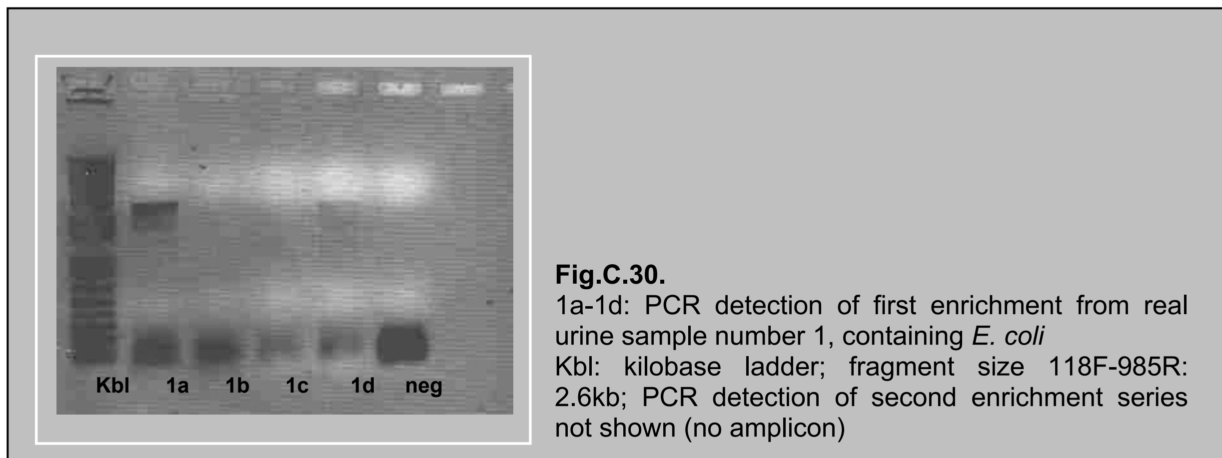


Fig.C.30.
1a-1d: PCR detection of first enrichment from real urine sample number 1, containing *E. coli*
Kbl: kilobase ladder; fragment size 118F-985R: 2.6kb; PCR detection of second enrichment series not shown (no amplicon)

4.4. Cloning and sequencing of PCR products from enrichment hybridisations of real clinical samples

All PCR products obtained after successful enrichment by using conserved primers 118F and 985R were cloned (see B.XIII) unless stated otherwise. Clones were chosen randomly and in numbers of 5-10 per cloning plate. Cloning, sequencing and analysis of the obtained sequences was performed as mentioned in B.X.III.

Table C.11. Result of sequence annotation of cloned and sequenced PCR products obtained from successful enrichments of target organisms from real clinical sample material

Sample material and number	Primers used for PCR	Name of clones	Result of sequence allocation	
			ARB*	Blast similarity
Urine number 1 containing *E. coli*	118F-985R	Ua[1]	*E. coli*	97% to *E.coli* 23SrRNA AJ 278710
		Ub[1]	*E. coli*	99% to *E.coli* 23SrRNA AJ 278710
		Ud[1]	*E. coli*	99% to *E.coli* 23SrRNA AJ053964
		1D/2	*E. coli*	98% to *E.coli* 23SrRNA to AE005174
		1C/1	*E. coli*	97% to *E.coli* 23SrRNA AJ 278710
		1C/2	*E. coli*	97% to *E.coli* 23SrRNA AJ 278710
		1D/1	*E. coli*	95% to *E.coli* 23SrRNA AJ 278710
	TEM F – TEM R	b	*E. coli*	99% *E. coli* TEM 1 to AY 794946
		d	*E. coli*	98% *E. coli* TEM 1 to AY 794946
		e	*E. coli*	93% *E. coli* TEM 1 to AY 794946
		4c	*E. coli*	99% *E. coli* TEM 1 to AB194682
		4d	*E. coli*	99% *E. coli* TEM 1 to AB194682
		4e	*E. coli*	99% *E. coli* TEM 1 to AB194682
		2c	*E. coli*	99% *E. coli* TEM 1 to AB194682
Aerobic blood culture number 2 containing *E. coli*	118F-985R	B2	*E. coli*	98% to *E.coli* 23SrRNA V00348
		2B/1	*E. coli*	98% to *E.coli* 23SrRNA to AE005174
		2B/2	*E. coli*	96% to *E.coli* 23SrRNA V00348
		2B/3	*E. coli*	99% to *E.coli* 23SrRNA V00348
Wound smear number 3 containing *Pseudomonas aeruginosa*	118F-985R/ 313F-317R	4/3a	*P. aeruginosa*	98% to 23SrRNA PAO 1 AE004949
Urine number 7 containing *Klebsiella oxytoca*	118F-985R	4a	*K. species*	94% Kl. species 23S rRNA
		4b	*K. species*	92% Kl. species 23S rRNA
		4c	*K. species*	94% Kl. species 23S rRNA
Urine number 10 containing *Enterococcus faecium*	118F-985R	P 1/2c	*E. faecium*	97% *E. faecium* 23SrRNA to X79341
		T 1/4 a	*E. faecium*	96% *E. faecium* 23SrRNA to X79341
		T1/4 e	*E. faecium*	95% *E. faecium* 23SrRNA to X79341
		T2/4 a	*E. faecium*	98% *E. faecium* 23SrRNA to AJ295305
		T2/4b	*E. faecium*	98% *E. faecium* 23SrRNA to AJ295305
		Tc	*E. faecium*	97% *E. faecium* 23SrRNA to X79341
		Td	*E. faecium*	97% *E. faecium* 23SrRNA to X79341
		Te	*E. faecium*	97% *E. faecium* 23SrRNA to X79341
Anaerobic Blood culture number 8 containing *Staphylococcus aureus*	118F-985R	T1a	*S. aureus*	99% to *S. aureus* 23SrRNA BX571857
		T1c	*S. aureus*	97% to *S. aureus* 23SrRNA BX571857
		T2b	*S. aureus*	99% to *S. aureus* 23SrRNA BX571857
		T2c	*S. aureus*	98% to *S. aureus* 23SrRNA BX571857
		T3d	*S. aureus*	99% to *S. aureus* 23SrRNA BX571857

*ARB: confirmed by examination of the diagnostic regions in a rRNA ARB database alignment; clones marked with [1] were obtained after enrichment using the time reduced protocol

All sequenced clones belonged to the group of the target organism, which was supposed to be enriched out of the particular sample. The detection of immobilised

E. coli using TEM primers (Table B.12) was performed in order to send amplicons to the project partner ITB (Institute of Technical Biochemistry) Stuttgart for analysis on a microarray, developed for the detection of all current known SNPs (single nucleotide polymorphisms) occurring in TEM beta-lactamases (Grimm et al, 2004). The applicability of whole cells for further antibiotic resistance screening could be shown, as the array analysis detected TEM1 for the analysed amplicon. This result fits well with the analysis of the TEM sequences obtained in this work, revealing a similarity of 93-99% to public database entries for TEM1 beta-lactamase.

II. RING-FISH probes

1. Special features of RING-FISH probes

RINGFISH (*R*ecognition of *In*dividual *G*enes, Zwirglmaier et al, 2004) allows the detection of single genes located on plasmids or chromosomal nucleic acids. The signal obtained with these special polynucleotide probes is often similar to the halo signal already known from rRNA targeted polynucleotide probe FISH. Equally to rRNA polynucleotide probes, the halo signal can be used for enrichment of target cells via binding to complementary nucleic acids on different kinds of carrier surfaces.

2. Role of secondary structures

Especially for RINGFISH probes, the abilities to form secondary structures and to allow hybridisation of several probe molecules is important as it enables signal amplification and therefore allows visualisation of successful binding of the probe molecules to the target sequence. Apart from that, the formation of secondary structures and inter-probe hybridisations are, among other factors, special preconditions for the formation of a network, resulting in the halo signal, which allows utilisation for enrichment hybridisations.

3. Choice of targets

The target organisms present in the real clinical samples are opportunistic pathogens and can potentially cause many nosocomial infections in hospitals (see Table B.4 and Introduction). The real clinical strains listed in Table B.4 harboured resistances against the listed antibiotics (Table C.12), which were ascertained in clinical routine diagnostics and documented as clinical findings in the patient record.

RINGFISH probes were designed targeting the following genes of the given target organisms in Table C.12.

Table C.12. Target genes of RINGFISH probes and based antibiotic resistance against antibiotic

Target organism	Target gene	Resistance against antibiotic
Proteus mirabilis AF 413702	*tetAJ*	Tetracyclin (tetracycline)
E. coli AF 421100	*ampC*	Ampicillin (ß-lactam antibiotic)
E. coli AF 430319	*ampC*	Ampicillin (ß-lactam antibiotic)
Enterococcus faecium AF625857	*ermB*	Erythromycin (macrolide)
Enterococcus faecalis AF415772	*parC*	Levofloxacin (fluoroquinilone)

All PCR products obtained after amplification using the special designed primers (Tables B.8- B.11) for each target gene were sequenced to confirm the identity of the amplified sequence, before the amplicon was used as a template for *in vitro* transcriptions.
Table C.13 below shows the similarity of the obtained sequences in comparison to the original sequences from NCBI, listed with respective accession numbers. The sequences for the single genes as well as the respective primer binding sites are mapped in F.II.

Table C.13. Similarity comparison of resistance gene sequences from NCBI to the respective resistance gene sequences obtained after amplification of the particular target gene (using the respective primers as listed in Table B.8-B.11) from the clinical strain DNAs and sequencing; sequence similarity was calculated using tools implemented in ARB.

Similarity original sequence	Sequence of amplicon of target organism	Sequence of amplicon of target organism
NCBI AF 339200	**ermB (V1) of *Enterococcus faecium* AF625857**	***ermB* (V2) of *Enterococcus faecium* AF625857**
	99%	99%
NCBI AB 005036	***parC* of *Enterococcus faecalis* AF415772**	
	99,45%	
NCBI AF 038993	***tetAJ* (V1) of *Proteus mirabilis* AF**	***tetAJ* (V2) of *Proteus mirabilis* AF**
	97,62%	97,62%
NCBI AF124204	***ampC* (V3) of *E. coli* AF421100**	***ampC* (V3) of *E. coli* AF430319**
	96,87%	96,87%

4. Application of RINGFISH probes

4.1. Evaluation of hybridisation conditions

Every newly designed RINGFISH probe requires evaluation of the special hybridisation conditions with regard to specificity, nature of signal and signal intensity.

The probes that were designed for application of RINGFISH are listed in Table B.15. All RINGFISH probes were first tested with the pure culture organism harbouring the gene of interest (see Table C.12). According to the protocol that was described previously (Zwirglmaier et al, 2004), the RINGFISH hybridisation for all probes was tested primarily under the given formamide concentrations of 5-15%. Only two of the probes tested (TetAJ V1 and TetAJ V2) showed appearance of weak halo signals or halo-like signals (see Table C14).

Surprisingly, it was not possible to achieve any kind of signal with the rest of the probes.

Changes in the hybridisation protocol, however, allowed detection of halo signal for the rest of the probes. The main difference to the given protocol consists in the highly increased concentration of formamide. Considering the temperature required for complete denaturation of artificially calculated secondary structures of the respective probe molecules (see D.II.4. and F.III.), it is evident, that in these cases the denaturation of the secondary structures requires a higher temperature or adequate effect for denaturation, namely increased concentration of formamide, for optimal denaturation of the probe sequence stretch. In combination with 20 minutes of denaturation at 80°C prior to hybridisation, the complete denaturation of the probe can be achieved by using different formamide concentrations.

Table C.14 summarises the different conditions which are necessary to obtain positive hybridisation signals after hybridisations using the developed probes targeting antibiotic resistance genes.

Table C.14. Conditions for the different RINGFISH hybridisations and nature of signal obtained after hybridisation; +: yes; -: no.

Probe	GC[%]	FA [%] hybridisation	Hybridisation time [h]	Tested in		Signal
				pure culture	clinical samples	
ermB1+2	36	0	20	**+**	**+**	Halo signal / whole cell fluorescence app. 90% target cells
TetAJV1+2	38	10	24	**+**	**-**	Halo signal app. 70% target cells
parC	41	20-60	20	**+**	**+**	Halo signal / whole cell fluorescence app. 90% target cells
EcoAmpCV3	50	80	24	**+**	**+**	Halo signal app. 50% target cells

As also reported by Zwirglmaier et al (2003) not all target cells allow detection of signals due to several facts like decreased accessibility of the target region in different states of cell cycles.

4.2. Hybridisations with RING-FISH probes

After the special evaluation of the hybridisation conditions and enhanced specificity conditions it was possible to detect the signals resulting from successful binding of the probe molecules to their plasmid or chromosomal target. The specificity was proven in mixed (target and non-target bacteria applied together) hybridisations as well as in pure culture hybridisations with non-target organisms.

4.2.1. *Proteus mirabilis* TetAJ V1 and TetAJ V2 RINGFISH probes

Proteus mirabilis is an enteric bacterium often mentioned in the context of nosocomial infections. The strain used in this study (see Table B.3) harbours a known resistance against tetracycline antibiotics. Usually, the resistance against tetracyclines is located on plasmids. Tetracycline antibiotics are broad spectrum antibiotics that affect almost all Gram-positive and Gram-negative bacteria. The action is localised at the 30S subunit of the ribosomes and based on inhibiting the binding of aminoacyl-t-RNA to the ribosomal A-site during translation. The mechanisms of resistance are based partly on genes encoding efflux pumps, partly on genes encoding ribosomal protection proteins (Chopra et al, 2001).
The polynucleotide probes developed for the given strain of *Proteus mirabilis* target the *tetAJ* gene located on plasmids and encoding efflux pumps, which transport the antibiotic directly out of the microbial cell after crossing of the membrane (Chopra et al 2001). The sequence of the templates for *in vitro* transcription to generate the polynucleotide probes and the binding sites of the primers for amplification of the target gene are mapped in F.II.
Two probes targeting the same gene, but differing in length (see Tables B.15 and D.8) were tested for *Proteus mirabilis*. Both probes allowed detection of halo signals after hybridisation for 25 hours at 10% of formamide. The specificity is given for *Proteus mirabilis* as long as the negative control does not harbour tetracycline resistance genes. The images below show the findings described (Figs.C.31-33).

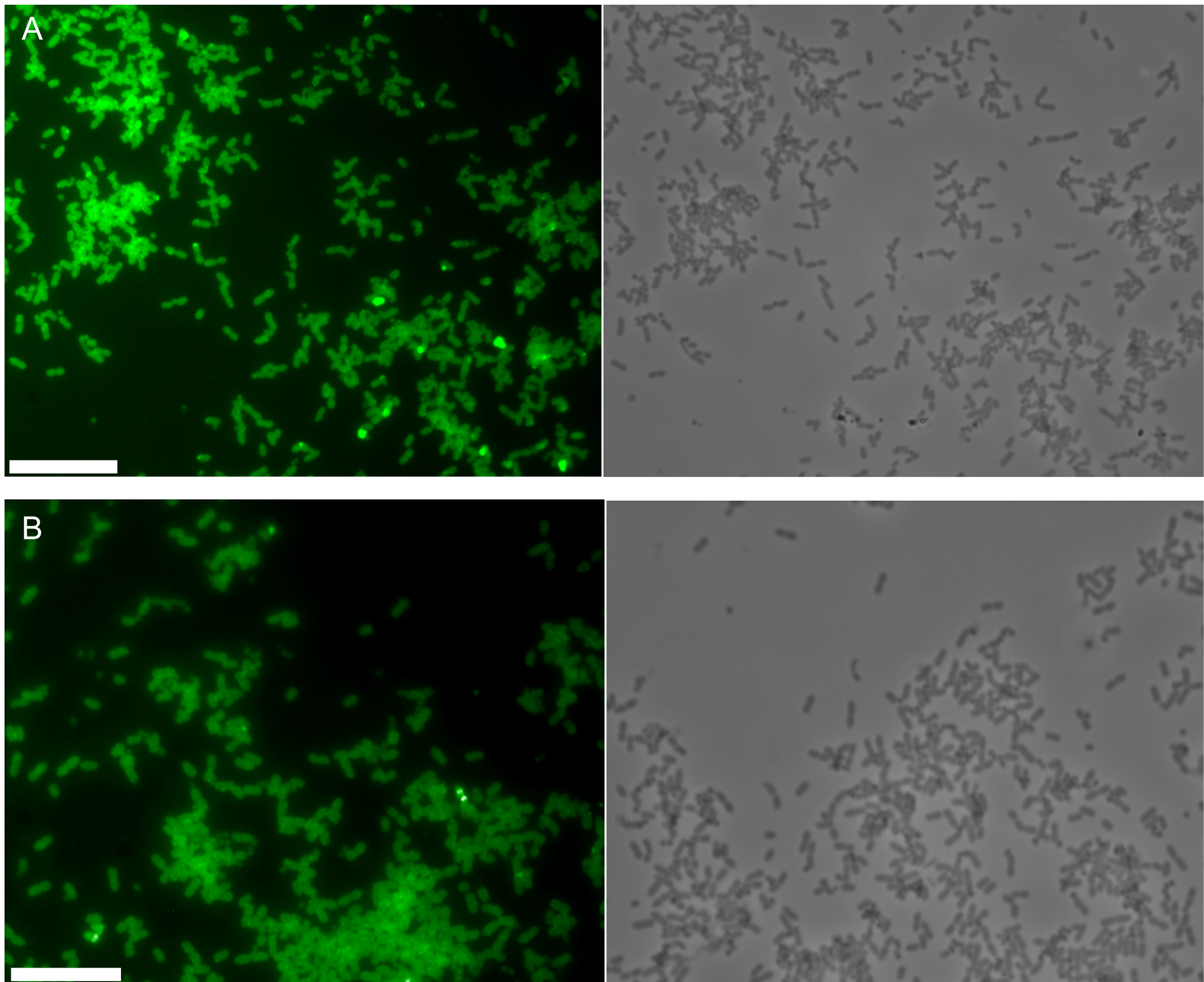
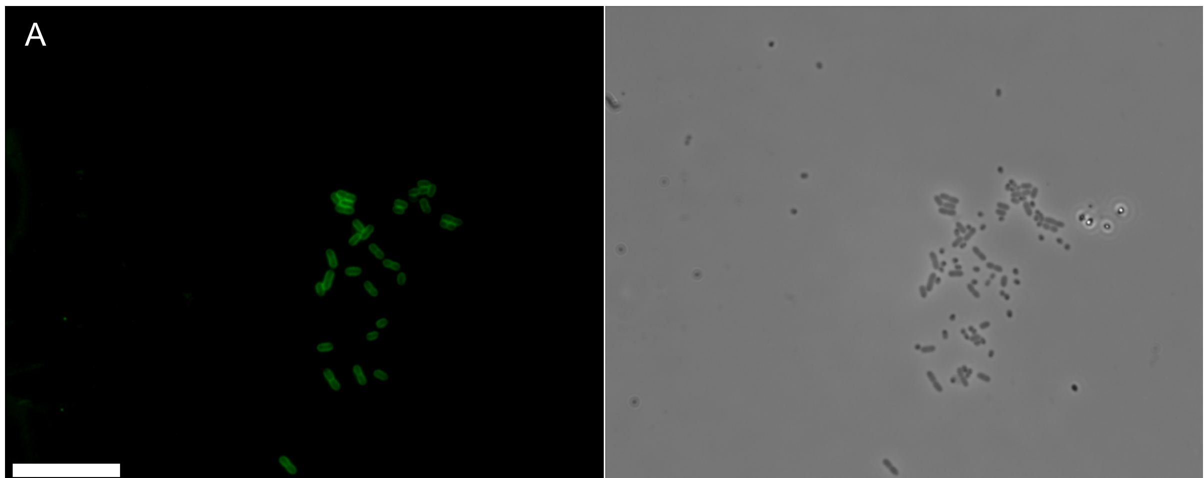


Fig.C.31. Fluorescence *in situ* hybridisation with RINGFISH probe **TetAJ V1 and TetAJ V2** on pure culture cells of the clinical strain *Proteus mirabilis*, 10% formamide in the hybridisation buffer, Fluos-12-UTP labelled (left) and phase contrast (right image), bar 10µm
A: TetAJ V1
B: TetAJ V2



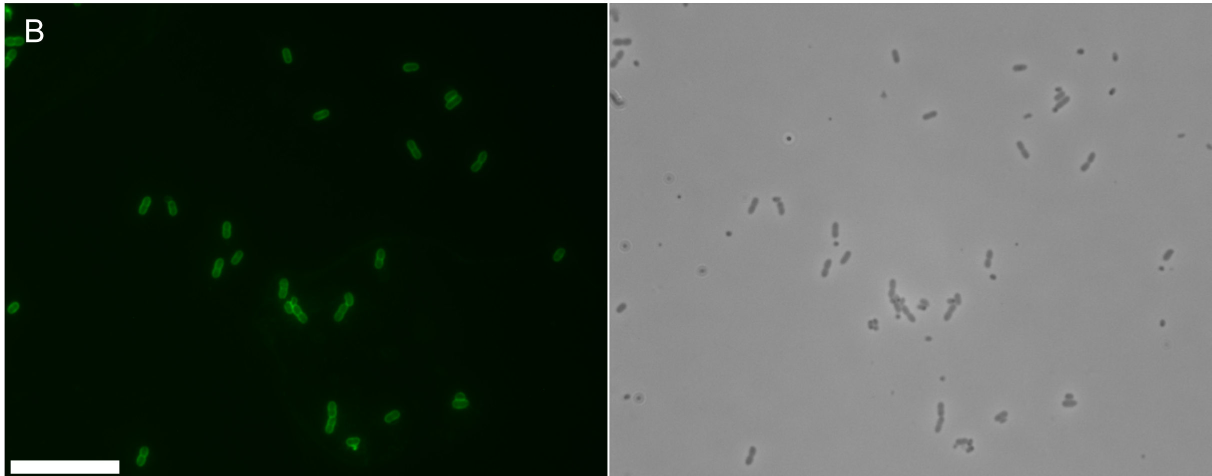


Fig.C.32. Fluorescence *in situ* hybridisation with RINGFISH probe **TetAJ V2** and **TetAJ V2** on cells of the clinical strain *Proteus mirabilis* (rods) in mixture with cells of *Neisseria canis* (negative control, small cocci), 10% formamide in the hybridisation buffer, Fluos-12-UTP labelled (left) and phase contrast (right image), bar 10µm
A: probe TetAJ V1 (see page 108)
B: probe TetAJ V2

There was no visible difference with regard to signal intensity or specificity for probe TetAJV1 and TetAJ V2. The negative control, cells of the coccoid Gram-negative bacterium *Neisseria canis* applied in control experiments did not show any kind of signals (see FigC.32 in mixture with signal-giving cells of target organism *Proteus mirabilis*) for both of the probes. Target cells sometimes appeared to show an ambiguous nature of signals: halo signals, fragmented halo signals, spot like signals occurring only in one area of the cell were observed during identically performed repetitions of hybridisations. This phenomenon had also already been reported from Zwirglmaier et al (2004). For probe TetAJV1, the shorter probe, measuring 278 nucleotides in length, weak whole cell fluorescence (Fig.C.34) could sometimes be observed, probably due to the length, which under the special hybridisation conditions might enable a complete entering into the cell and thus lead to whole cell fluorescence. Despite this ambiguity of the signals, the specificity was not lost. Small changes in the hybridisation conditions, e.g. fluctuations in temperature or slight changes in salt concentrations in the hybridisation buffer and the procedure, might cause the diversity of signals; apart from that, the growth state of the cells could also play a role.

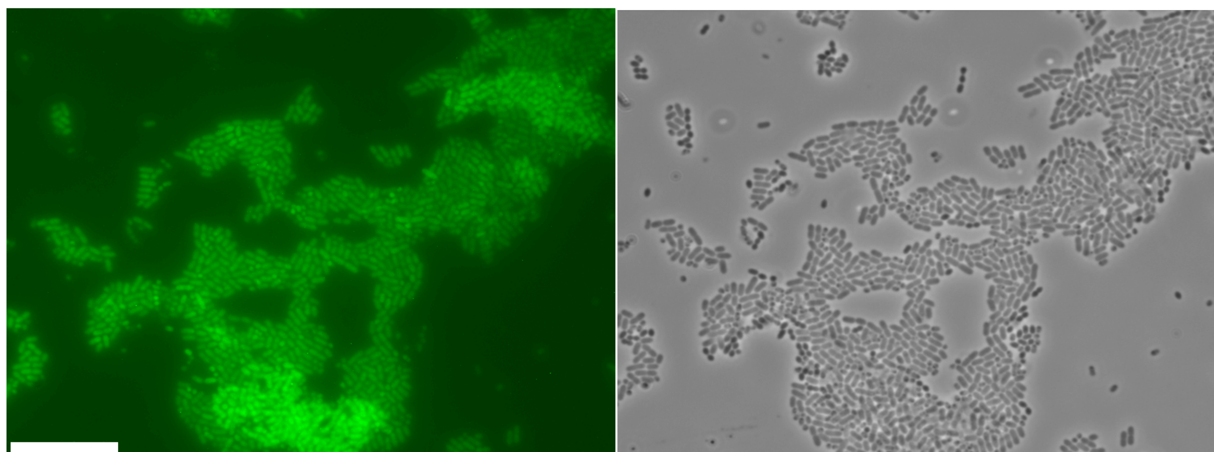


Fig.C.34. Fluorescence *in situ* hybridisation with RINGFISH probe TetAJV2 on cells of the clinical strain *Proteus mirabilis* (rods) in mixture with cells of *Neisseria canis* (negative control, small cocci), 10% formamide in the hybridisation buffer, Fluos-12-UTP labelled (left) and phase contrast (right image), bar 10µm

4.2.2. *E. coli* AmpCV 3 RINGFISH probe

Enterobacterial *ampC* is a chromosomal encoded resistance gene that codes for a ß-lactamase that is able to hydrolyse ß-lactam antibiotics (Volkmann et al 2003).

The *ampC* gene product of *E. coli* is responsible for and involved in resistance to ampicillin and other ß-lactam antibiotics. The probe presented here measured 990 nucleotides in length and was constructed to target an *ampC* gene located on the chromosome of *E. coli*. The sequence of the templates for *in vitro* transcription to generate the polynucleotide probes and the binding sites of the primers for amplification of the target gene are mapped in F.II.

The conditions necessary for these long probes are 80% formamide and 25h of hybridisation. Figs.C.35-37 visualise the signals derived from *in situ* hybridisations.

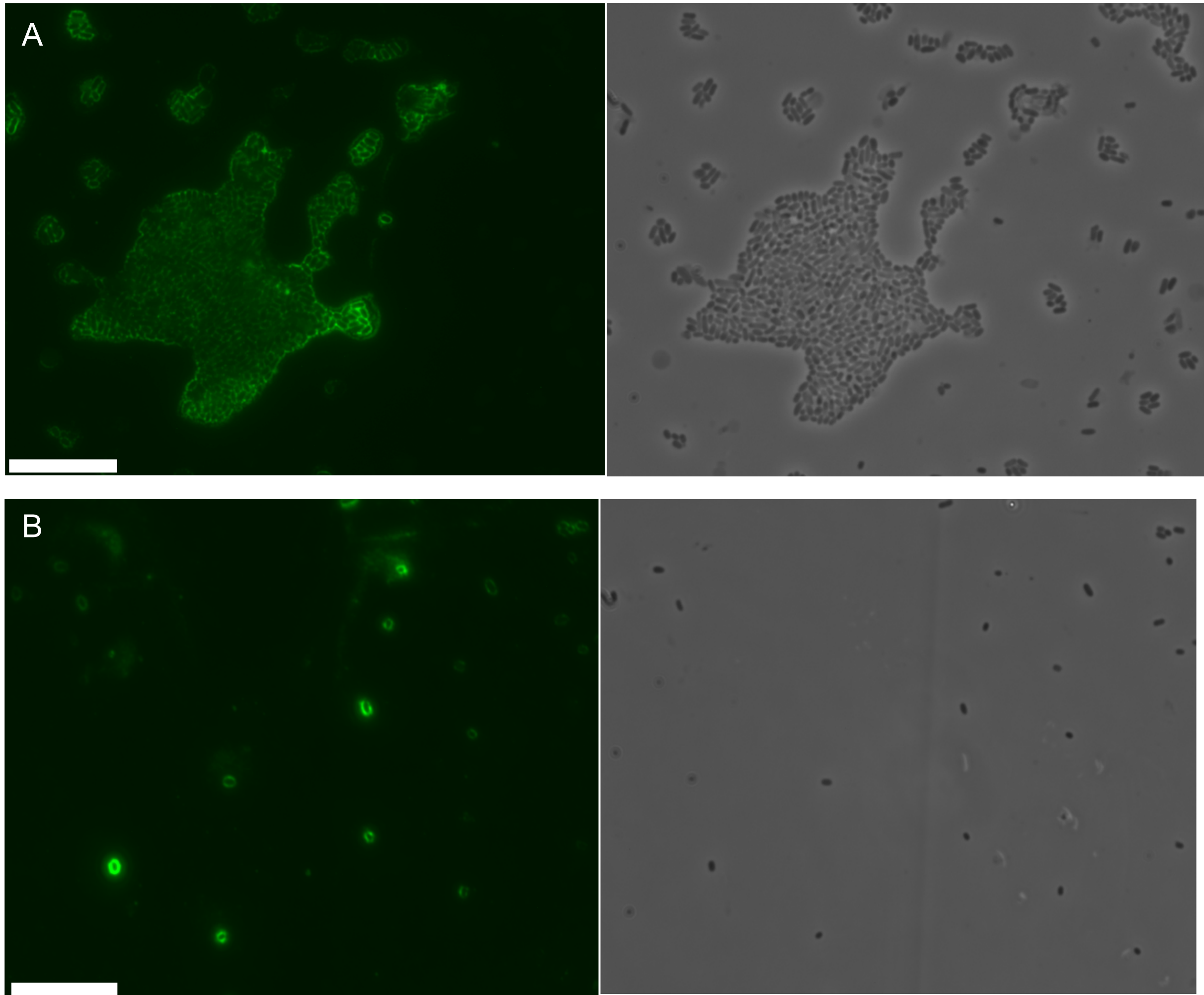
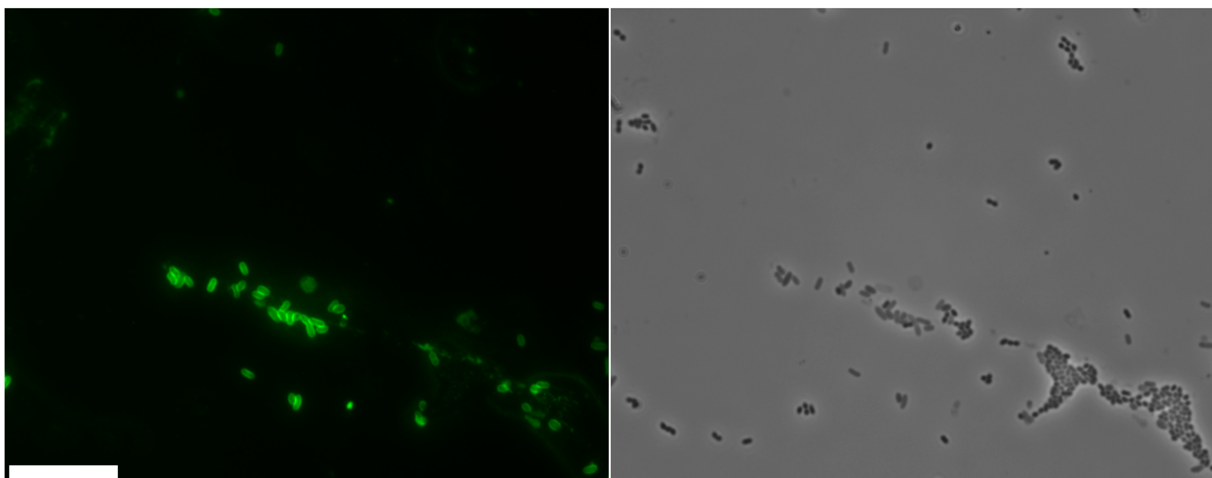


Fig.C.35. Fluorescence *in situ* hybridisation with RINGFISH probe EcoAmpCV3, 80% formamide in the hybridisation buffer, Fluos-12-UTP labelled (left) and phase contrast (right image), bar 10µm
A: on pure culture cells of the clinical strain *E. coli* AF421100
B: on pure culture cells of the clinical strain *E. coli* AF430319



FigC.36. Fluorescence *in situ* hybridisation with RINGFISH probe EcoAmpCV3 on cells of the clinical strain *E. coli* AF421100 in mixture with cells of *Neisseria canis* (negative control, small cocci), 80% formamide in the hybridisation buffer, Fluos-12-UTP labelled (left) and phase contrast (right image), bar 10µm

The probe for *E. coli* AF 421100 and *E. coli* AF 430319 led to more or less similar halo signals. The negative control, cells of the coccoid Gram-negative bacterium *Neisseria canis* applied in control experiments did not allow observation of any kind of signals (Fig.C.36) in mixture with signal-giving cells of the target organism *E. coli*. As already mentioned in C.II.4.2.1 for *Proteus mirabilis*, target cells sometimes appeared to have an ambiguous nature of signals: halo signals, fragmented halo signals, spot like signals occurring in only one area of the cell were observed during identically performed repetitive hybridisations.

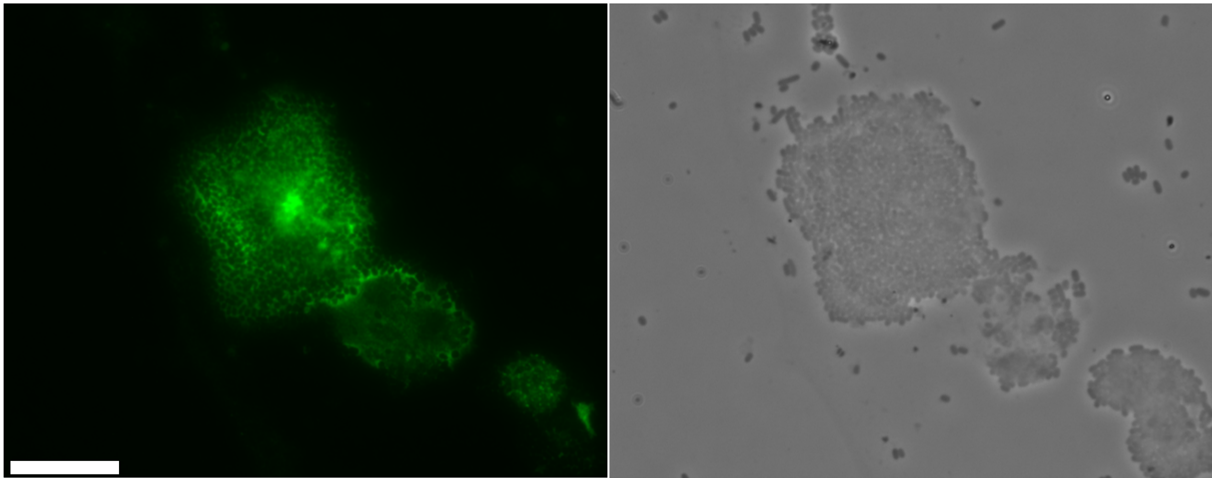


Fig.C.37. Fluorescence *in situ* hybridisation with RINGFISH probe EcoAmpCV3 on real clinical sample urine number 1, containing cells of *E .coli*, 80% formamide in the hybridisation buffer, Fluos-12-UTP labelled (left) and phase contrast (right image), bar 10µm

Fig.C.37 shows the result of application of probe EcoAmpCV3 to real clinical sample number 1, urine AF421100 containing *E. coli* AF 421100. The signal is weaker compared to the signals obtained from the pure culture organism *E. coli* AF 421100.
Real clinical sample number 2, AF 430319 aerobic blood culture, natively containing *E. coli* AF430319, from which it was isolated, did not allow microscopic detection of the target organism using probe EcoAmpCV3 designed for *E. coli* AF430319. However, after application of the probe to real clinical sample 2 in an enrichment hybridisation, it was possible to detect immobilised target cells after the enrichment with PCR, see further C.II.4.3.

4.2.3. *Enterococcus faecium* ermB1 and ermB2 RINGFISH probes

The clinical strain *Enterococcus faecium* AF 415772 harbours resistances against macrolide antibiotics, among ohers against erythromycin. This resistance refers to methylases, encoded by the *erm* (erythromycine ribosome methylation) gene. Mutations of the target site of macrolides diminish the binding affinity of the antibiotics to the ribosome, and protein synthesis is not affected strongly. Macrolide antibiotics like erythromycin interfere during early protein synthesis: the drug blocks the growth of the nascent peptide chain by inhibition of the movement of the peptidyl-t-RNA from the A-site to the P-site of the ribosome (translocation); the assembly of new large ribosomal subunits is inhibited, and a gradual depletion of functional ribosomes in the cell follows (Vester et al, 2001). Two probes targeting the same gene, *ermB*, were designed for *Enterococcus faecium*, differing in length (see Table B.10). The sequence of the templates for *in vitro* transcription to generate the polynucleotide probes and the binding sites of the primers for amplification of the target gene are mapped in F.II.

Figs.C.38- C.44 depict the findings of application of probe ermB1 and ermB2 to pure culture cells, real clinical samples, and negative controls.

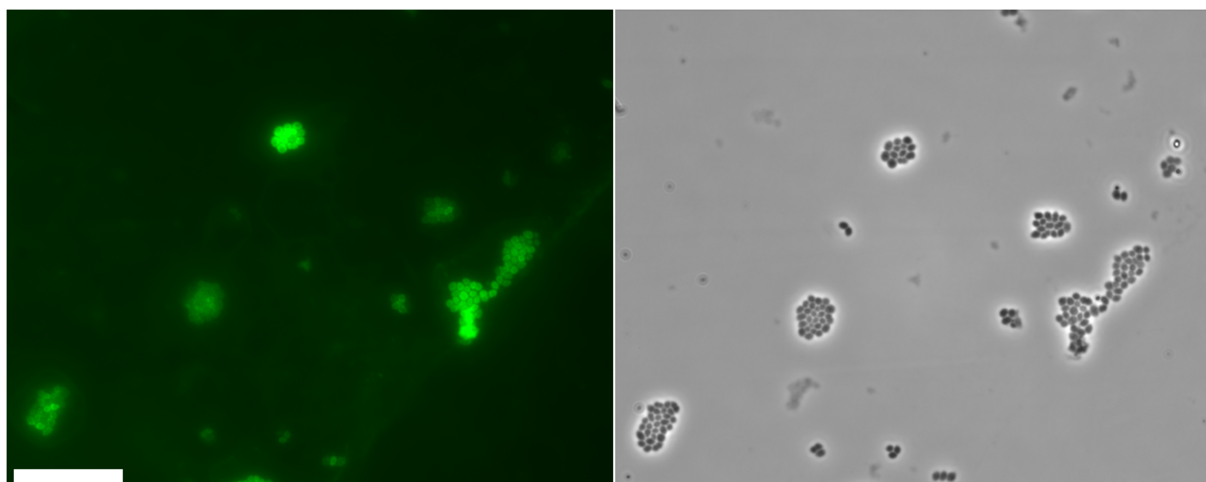
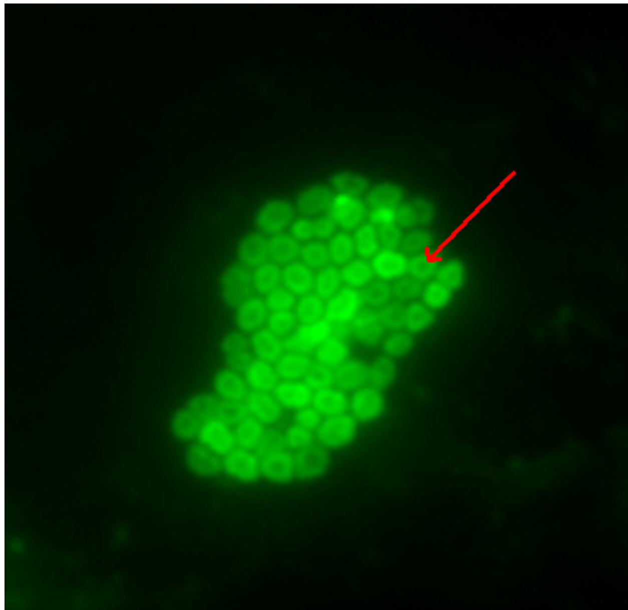


Fig.C.38. Fluorescence *in situ* hybridisation with RINGFISH probe ermB2 on cells of clinical strain *of Enterococcus faecium* AF 415772, 0% formamide in the hybridisation buffer, Fluos-12-UTP labelled (left) and phase contrast (right image), bar 10µm

Hybridisations with RINGFISH probe ermB1 and ermB2 led to halo-like signals after application of an appropriate pretreatment protocol (B.XI.3.). There was no difference observed between the two probes with regard to signal intensity, nature of signal and

specificity. One remarkable difference to usual halo signals, however, is the thickness of the halo (Fig.C.39).



FigC.39. Fluorescence *in situ* hybridisation with RINGFISH probe ermB2 on cells of the clinical strain *of Enterococcus faecium*, AF 415772, 0% formamide in the hybridisation buffer, Fluos-12-UTP labelled; the arrow indicates the special thickness of the Halo signal

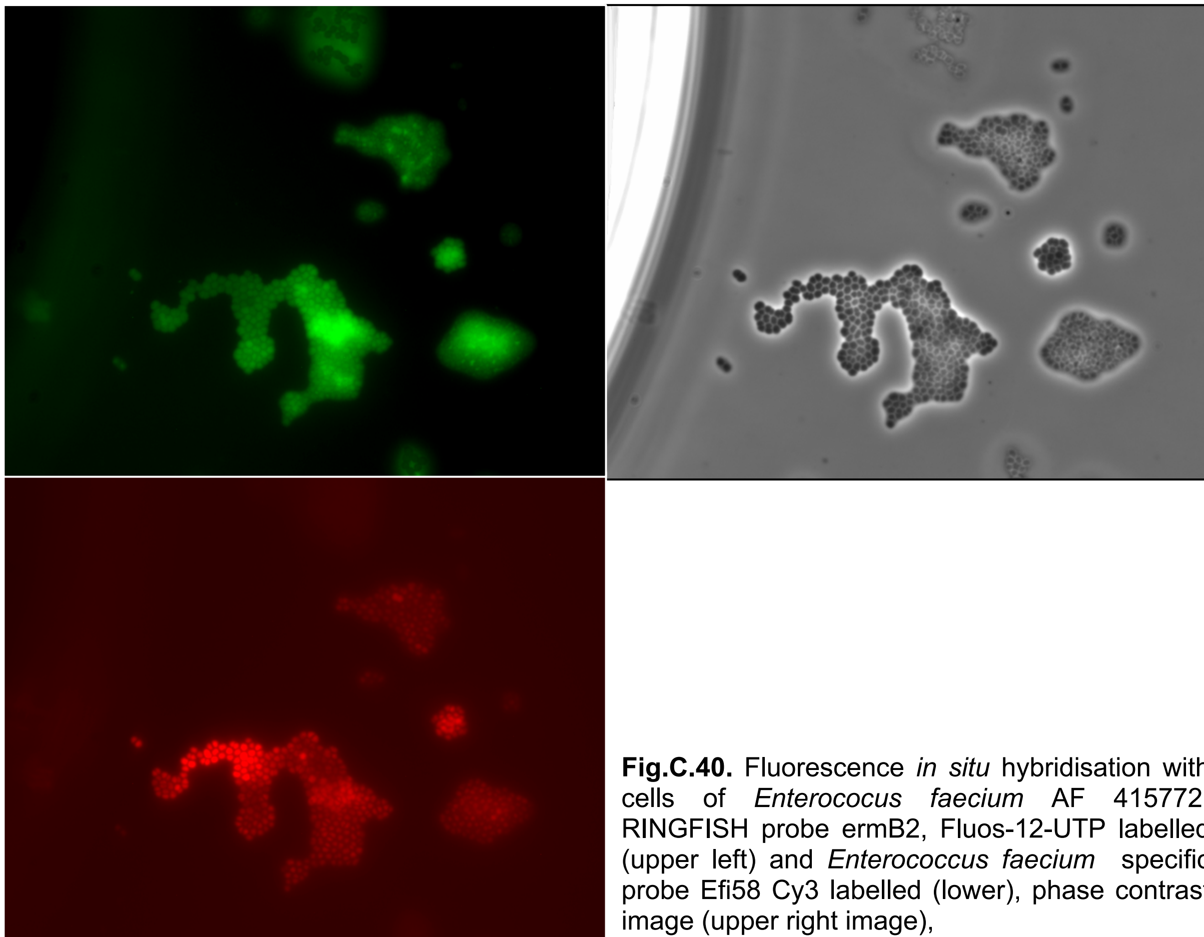


Fig.C.40. Fluorescence *in situ* hybridisation with cells of *Enterococus faecium* AF 415772, RINGFISH probe ermB2, Fluos-12-UTP labelled (upper left) and *Enterococcus faecium* specific probe Efi58 Cy3 labelled (lower), phase contrast image (upper right image),

The conditions for oligonucleotide probe and RINFISH probe hybridisations are highly different. Thus, it is difficult to obtain both signals in one session. Performing the oligonucleotide probe FISH before the RINGFISH leads to very weak or missing oligonucleotide probe signals, because of the initial step of 20 min at 80°C, which is crucial for the denaturation of the secondary structures of the polynucleotide probes. On the other hand, performing the RINGFISH before the oligonucleotide probe FISH leads to complete loss of the RINGFISH signal, since the conditions for this probes are 0% formamide (for probe ermB), but the oligonucleotide probe FISH requires 30% (in case of probe Efi 58). The presented images (C.40. and C.41) were achieved by microscopic analysis of the cells on the slides directly after the first hybridisation with the oligonucleotide probe Efi 58 (see Table B.14), specific for *E. faecium*, before performing the RINGFISH. In order to find the same cell structure again on the slide for detection of the RINGFISH signals, the position of the slide on the mechanical stage of the microscope was noted down.

The RINGFISH signals obtained after the successive double hybridisation differed slightly from the earlier hybridisations, as they were kind of blurry, with many cells showing whole cell fluorescence signal in addition to signals in the area of the usual “halo-area”. An overlay of both pictures of oligonucleotide and RINGFISH probe showed, that the signal obtained from the RINGFISH probe covered a slightly larger area than the cell envelope, whereas the oligonucleotide probe signal was limited to the inner parts of the cells. On the other hand, a dark field could be observed in the middle of the cell area, indicating, that the RINGFISH probe was mainly located in the “halo-area” (see Fig.C.41).

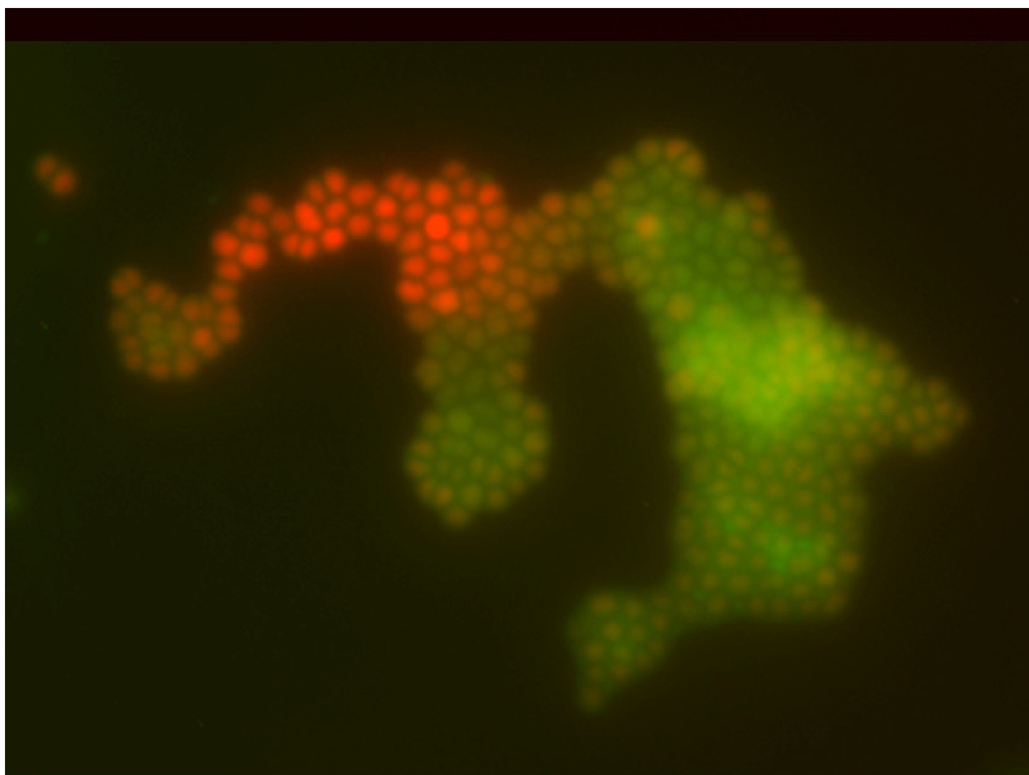


Fig.C.41. Cells of *Enterococcus faecium* after double hybridisation using RINGFISH probe ermB2, Fluos-12-UTP labelled (green) and *Enterococcus faecium* specific probe Efi58 Cy3 labelled (red), overlay of both images, bar 10µm

The reasons for these differing signals may be the increased permeability of the target cells after the oligonucleotide probe hybridisation with 30% formamide. The formation of a network in the area of the cell wall may be inhibited or disturbed by the oligonucleotide probe molecules that have bound to their target sites on the ribosomes. The blurriness of the signal could be caused by the cell wall, which might be bloated by the whole pretreatment and hybridisation procedures.

In the real clinical sample urine AF 625857, containing the target bacterium *Enterococcus faecium* AF 625857 with the known resistance against erythromycin, red blood cells and other epithelial cells show a strong autofluorescent background signal (Fig.C.42).

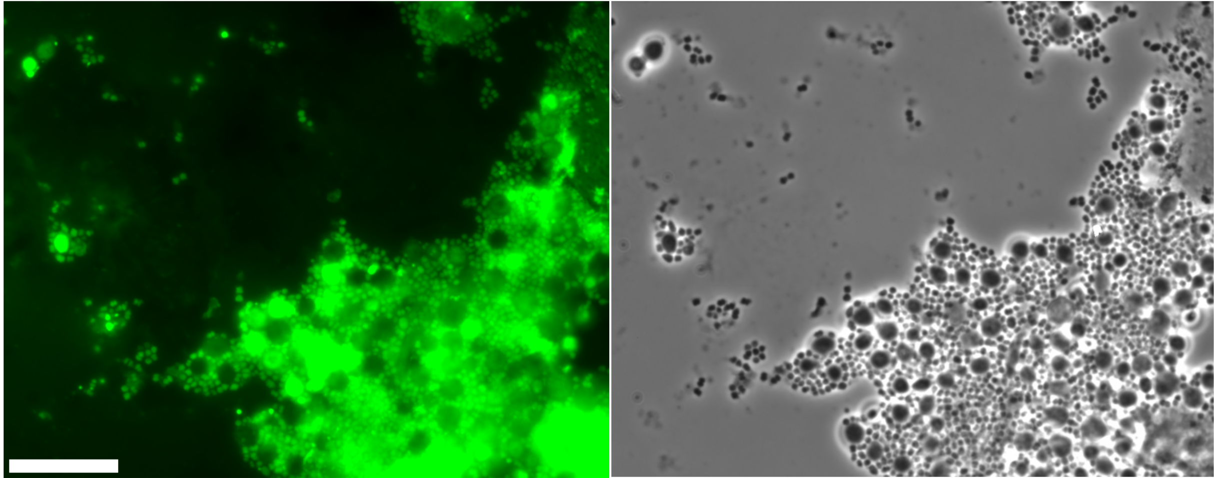
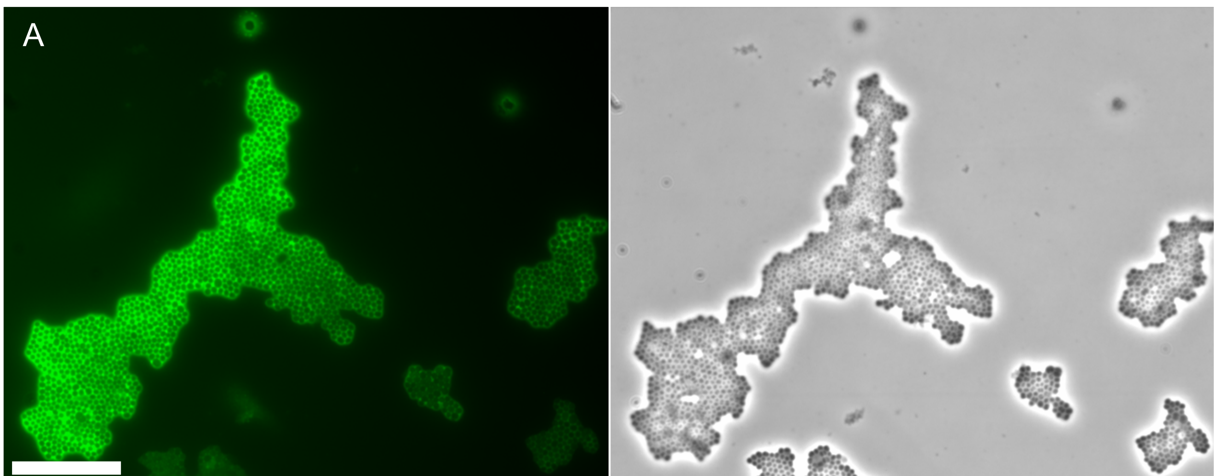


Fig.C.42. Fluorescence *in situ* hybridisation with RINGFISH probe ermB2 on real clinical sample number 10, urine, containing cells of *Enterococcus faecium*, 0% formamide in the hybridisation buffer, Fluos-12-UTP labelled (left) and phase contrast (right image), bar 10µm

The primers generated for amplification of the *ermB* gene (ermB V2 and R2) could also be used to amplify the *ermB* genes of the clinical strains number 5 *Staphylococcus aureus*, AF 615234, number 8 *Staphylococcus aureus, AF 630073* and number 6, *Enterococcus faecalis*, AF 625857.

According to the patient records of these samples, an occurrence of erythromycin resistance was reported for all these organisms presented. Comparative sequence analysis of public BLAST database entries for gene *ermB* of *Staphylococcus aureus* and *Enterococcus faecalis* exhibited a similarity of 99-100 % in comparison to the ermB gene of *Enterococcus faecium* AF 415772. Application of the ermB2 RINGFISH probe generated from DNA of *Enterococcus faecium* AF 4157721 on the other strains harbouring the gene showed the same signal as with cells of *Enterococcus faecium* AF 415772 as expected (Figs.C.43 A, B and C).



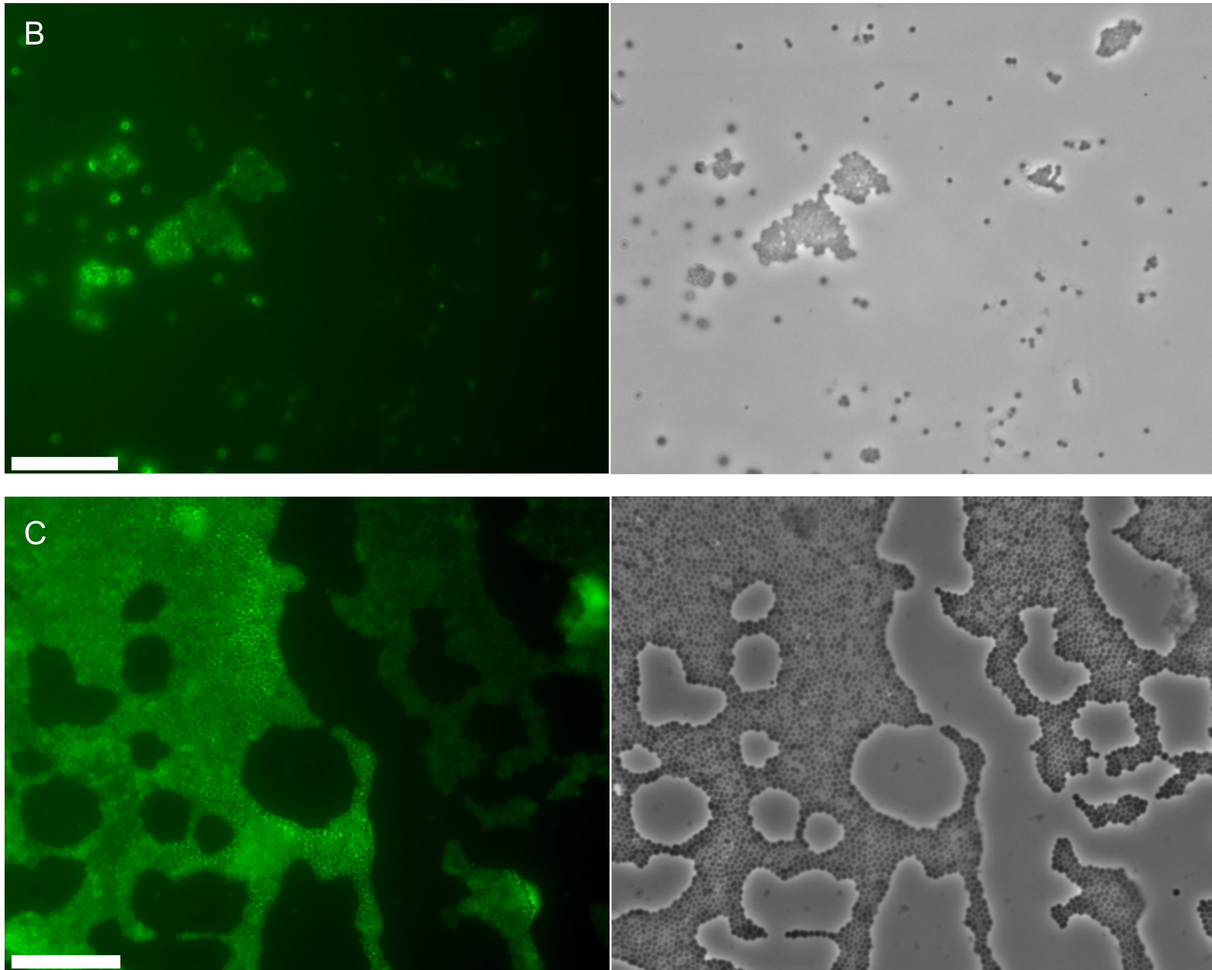


Fig.C.43. Fluorescence *in situ* hybridisation with different pure culture cells RINGFISH probe ermB2, Fluos-12-UTP labelled (left) phase contrast (right image), bar 10µm
A: *Staphylococcus aureus* AF 615324 (see page117)
B: *Staphylococcus aureus* AF 630073
C: *Enterococcus faecalis* AF 625857

Strains of *E. coli* DSM 1103 and *Enterococcus faecium* DSM 20477^T (see Tables B.1 and B.2), were used as negative controls. No PCR amplification of the *ermB* gene fragment was possible using the primers designed for amplification of the *ermB* gene of *Enterococcus faecium*. The following images of *in situ* hybridisations of the named species with the ermB2 probe depict the specificity of the ermB2 probe only for species, which harbour the gene (Figs.C.44 A and B), as no hybridisation signal could be obtained here. Double hybridisations of the RINGFISH probe ermB2 and a specific oligonucleotide probe, targeting the RINGFISH target organisms or the non target organism, did not lead to a satisfactory detection of both signals in the cases of negative control experiments with mixed cultures.

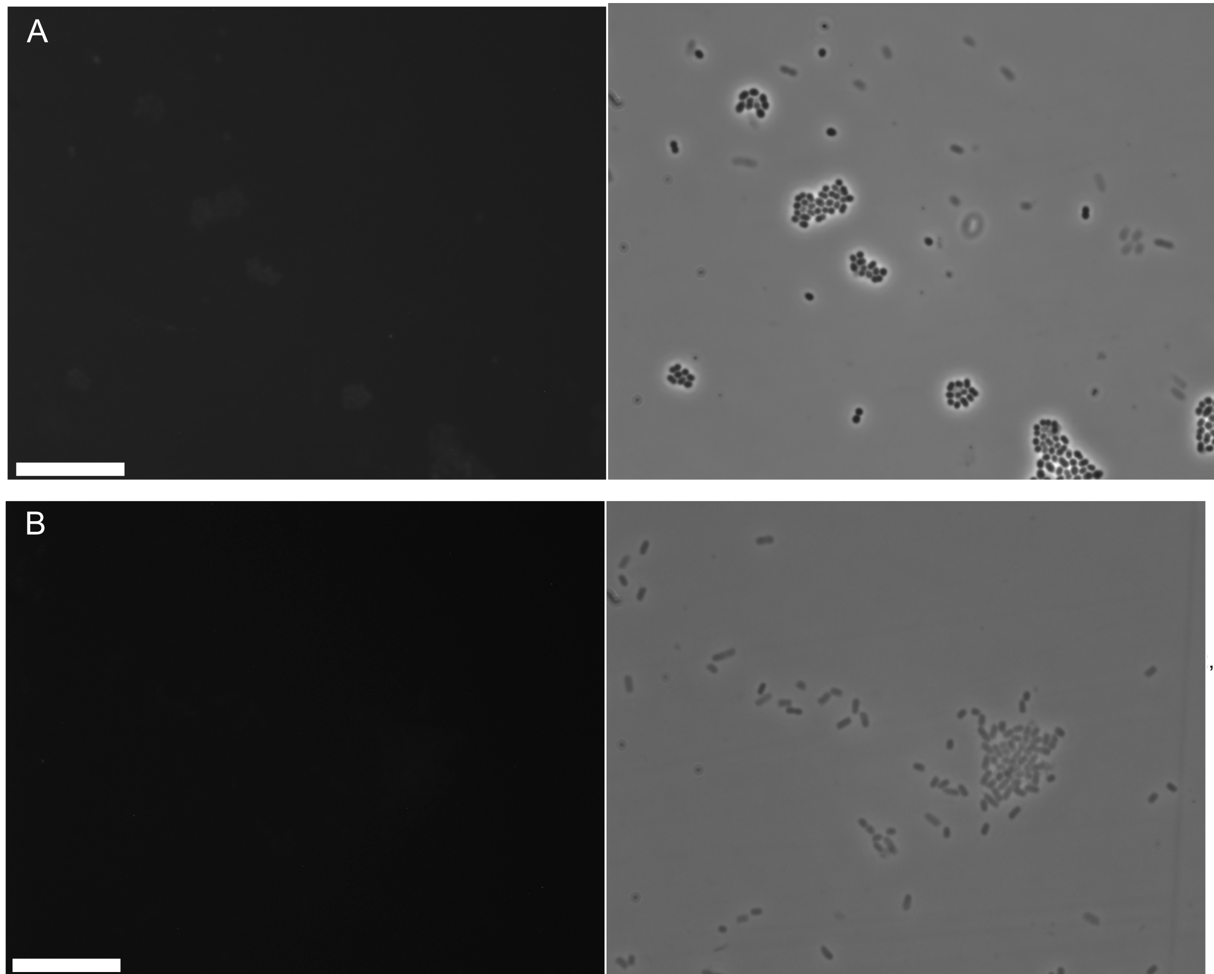
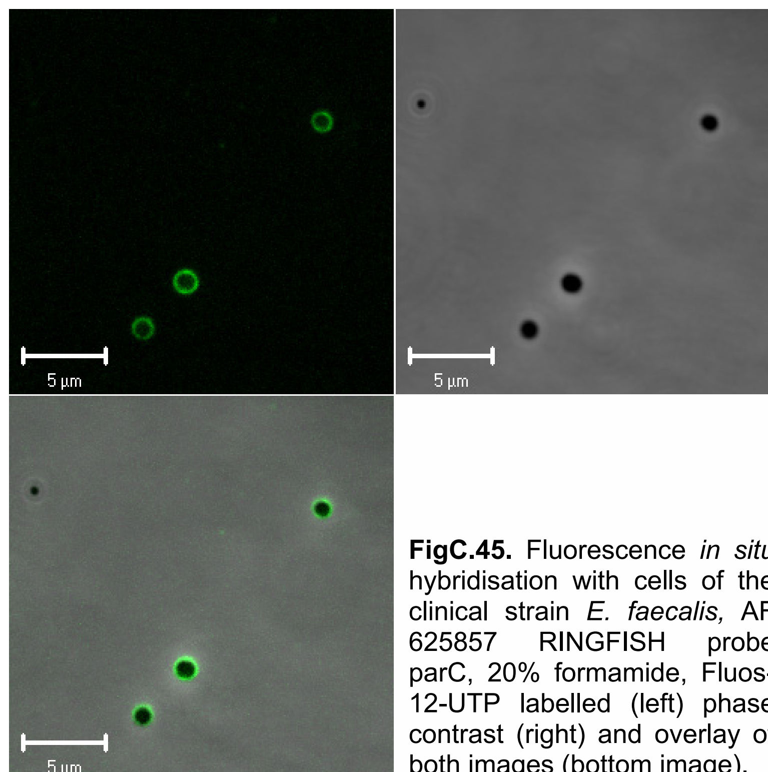


Fig.C.44. Fluorescence *in situ* hybridisation with different pure culture cells as negative controls, RINGFISH probe ermB2, Fluos-12-UTP labelled (left) phase contrast image (right image), bar 10µm
A: *Enterococcus faecium* DSM 20477^{T}
B: *E. coli DSM* 1103

4.2.4. *Enterococcus faecalis* parC RINGFISH probe

The gene *parC* is part of the topoisomerase IV, responsible e.g. for supercoiling of DNA and present in every bacterial cell. Mutations in the nucleotide sequence can result in an exchange of amino acids, which can result in resistance to fluoroquinolones as levofloxacin and others. The *parC* gene of *Enterococcus faecalis* shows great similarity to that of closely related species, e.g*. Enterococcus faecium*, and even a high similarity to other Gram-positive bacteria, such as *Staphylococcus* spp (95-100%), in contrast to the *parC* genes of e.g. *E. coli* (no significant similarity). The sequence of the templates for *in vitro* transcription to generate the polynucleotide probes and the binding sites of the primers for amplification of the target gene are mapped in F.II.

Hybridisations with RINGFISH probe parC led after application of an appropriate pretreatment protocol (B.XI.3.), similar to the ermB probes for to halo signals. Similar to the halo signals obtained after hybridisations using the ermB probes for *Enterococcus faecium* AF 415772, sometimes the halo seemed to appear thicker than the halos known from hybridisations with rRNA targeted polynucleotide probes. The signals could be obtained from 20% of formamide on, up to 60% but not below 20% (Figs.C.45-C.49).



FigC.45. Fluorescence *in situ* hybridisation with cells of the clinical strain *E. faecalis,* AF 625857 RINGFISH probe parC, 20% formamide, Fluos-12-UTP labelled (left) phase contrast (right) and overlay of both images (bottom image).

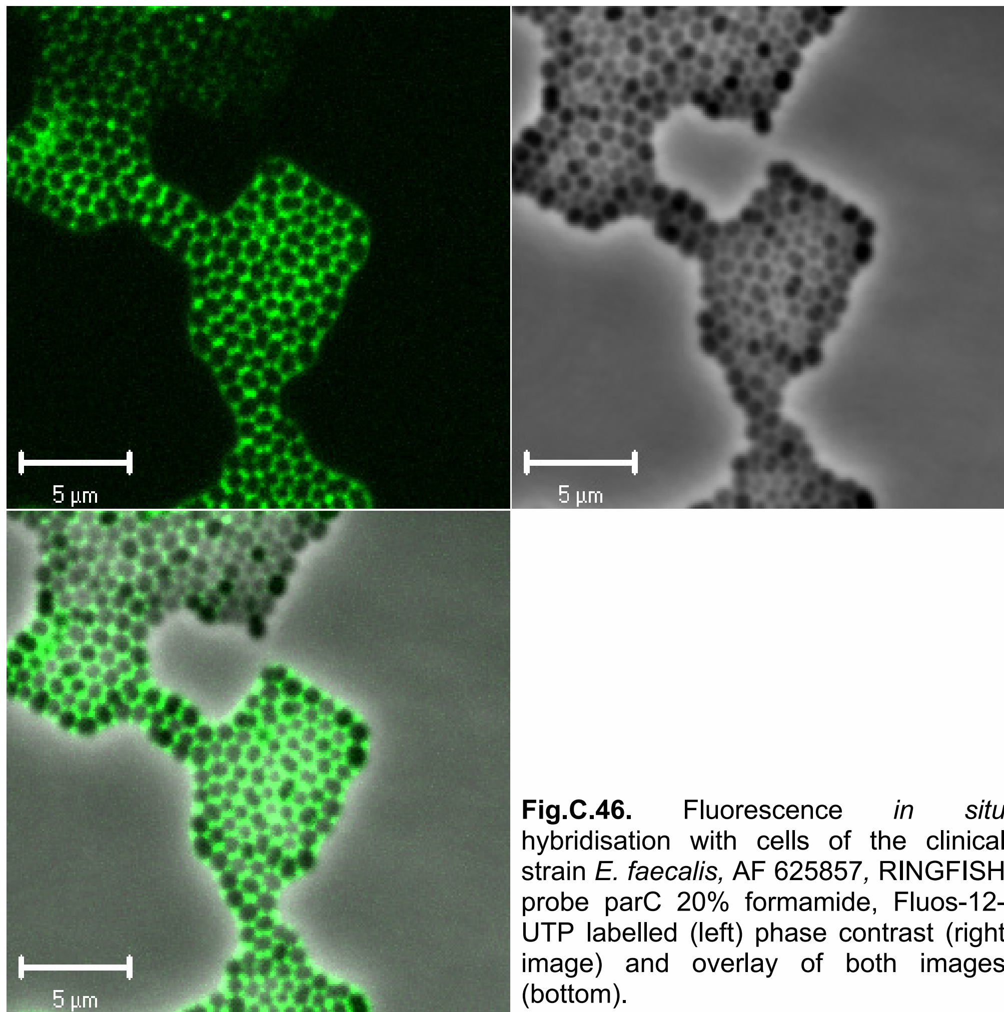


Fig.C.46. Fluorescence *in situ* hybridisation with cells of the clinical strain *E. faecalis,* AF 625857, RINGFISH probe parC 20% formamide, Fluos-12-UTP labelled (left) phase contrast (right image) and overlay of both images (bottom).

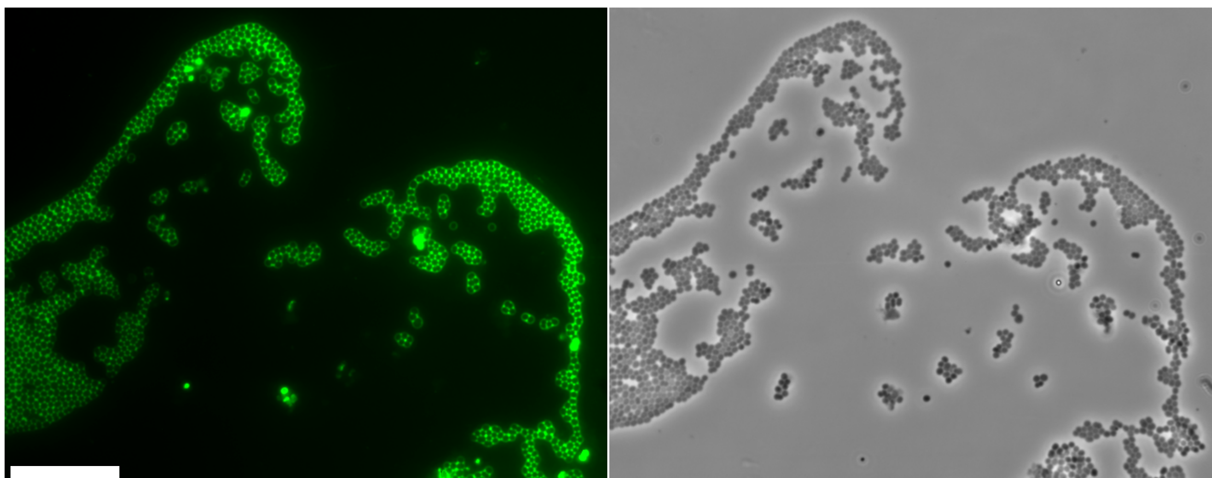


Fig.C.47. Fluorescence *in situ* hybridisation with cells of the clinical strain *E. faecalis,* AF 625857, RINGFISH probe parC 20% formamide, Fluos-12-UTP labelled (left) phase contrast image (right image); bar 10µm

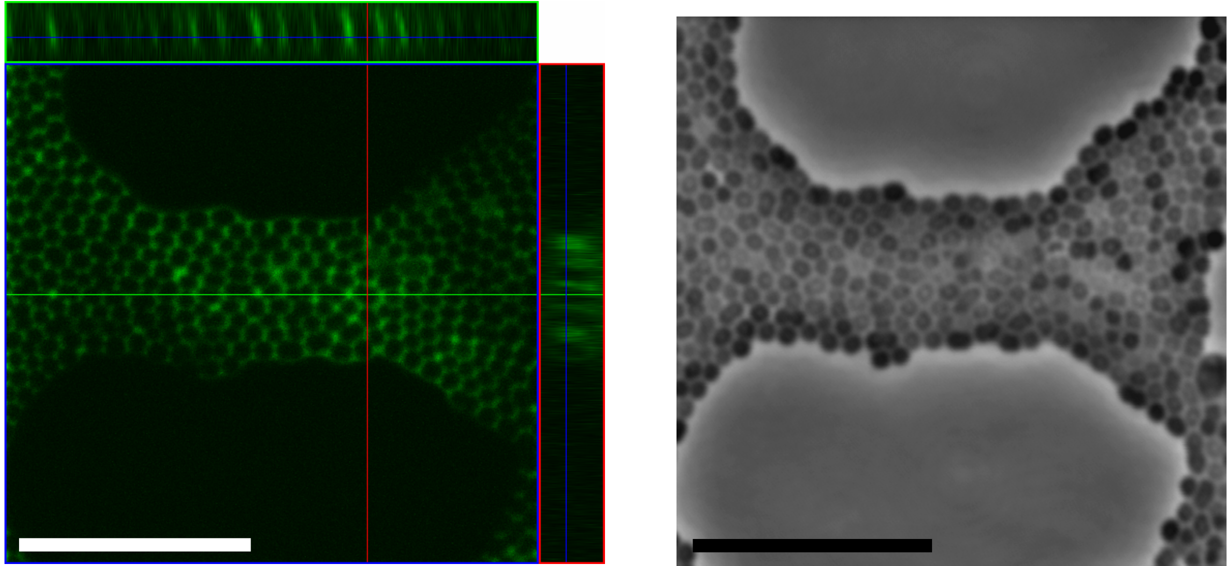


Fig.C.48. Fluorescence *in situ* hybridisation with cells of the clinical strain *E. faecalis,* AF 625857, RINGFISH probe parC 60% formamide, Fluos-12-UTP labelled (left) phase contrast image (right image), stack of 6 images, left; bar 10µm

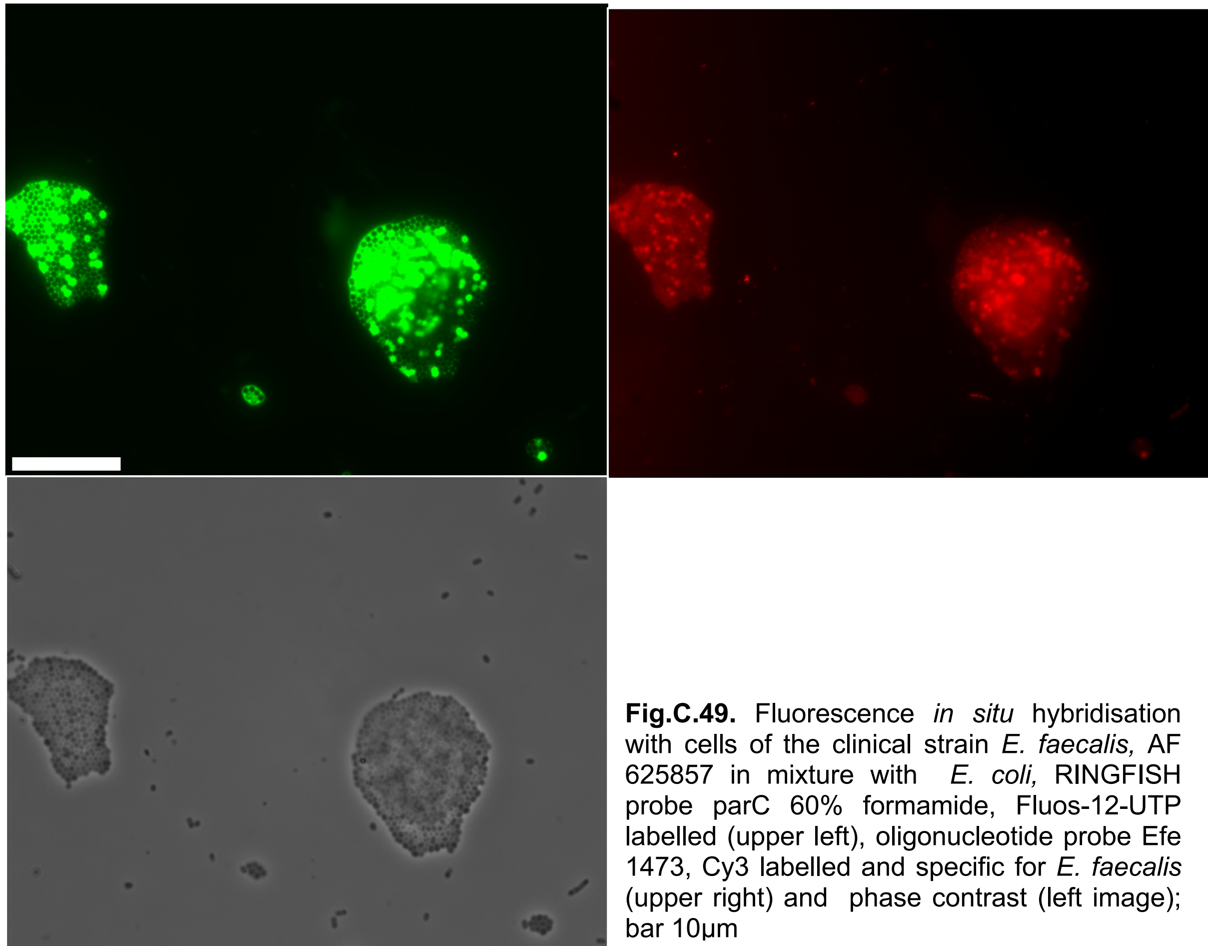


Fig.C.49. Fluorescence *in situ* hybridisation with cells of the clinical strain *E. faecalis,* AF 625857 in mixture with *E. coli,* RINGFISH probe parC 60% formamide, Fluos-12-UTP labelled (upper left), oligonucleotide probe Efe 1473, Cy3 labelled and specific for *E. faecalis* (upper right) and phase contrast (left image); bar 10µm

For probe parC, it was also very difficult to obtain double hybridisation signal with both oligo- and RINGFISH probes. This could be achieved again by microscopic

analysis of the cells on the slides directly after the first hybridisation with the oligonucleotide probe, specific for *E. faecalis*, before performing the RINGFISH. For finding the same cell structure again on the slide for detection of the RINGFISH signals, the position of the slide on the mechanical stage of the microscope was noted. The signals obtained here still remained halo-like, in contrast to the findings for probe ermB for *Enterococcus faecium* (Fig.C.49).

The specificity of probe parC generated for *Enterococcus faecalis* AF 625857 is limited, as mentioned above. Application of the probe parC on species of staphylococci and enterococci allowed detection of the typical halo signal, similar to the signals obtained after hybridisation with cells of *Enterococcus faecalis* (Fig.C.50).

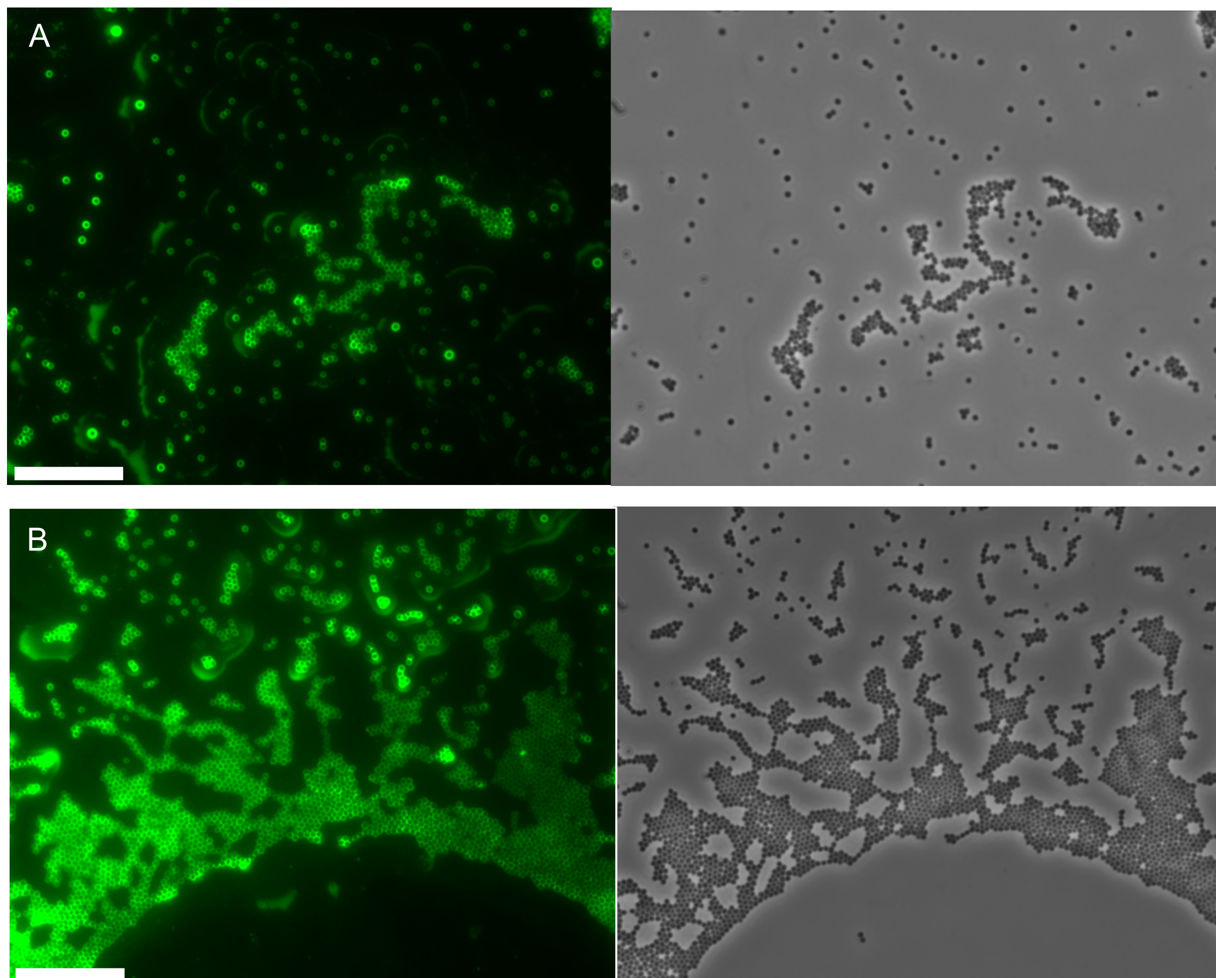


Fig.C.50. Fluorescence *in situ* hybridisation with clinical strains of *Staphylococcus aureus*, RINGFISH probe parC from *Enterococcus faecalis* AF 625857,60% formamide, Fluos-12-UTP labelled (left) phase contrast image (right image); bar 10µm
A: cells of *Staphylococcus aureus* AF 615234
B: cells of *Staphylococcus aureus* AF 630073

The clinical strain *E. coli*, AF 421100 (Table B.4) was applied as a negative control. According to the patients record, this strain was sensitive to levofloxacin (targets the *par*C gene). These cells do not allow detection of any kind of signals after the respective hybridisations (Figs.C.51 A and B below). Double hybridisations of the RINGFISH probe parC and a specific oligonucleotide probe, targeting *Enterococcus faecalis*, with both the halo signal and the oligonucleotide probe derived whole cell fluorescence signal are shown (Figs.C.49 and C.51 B).

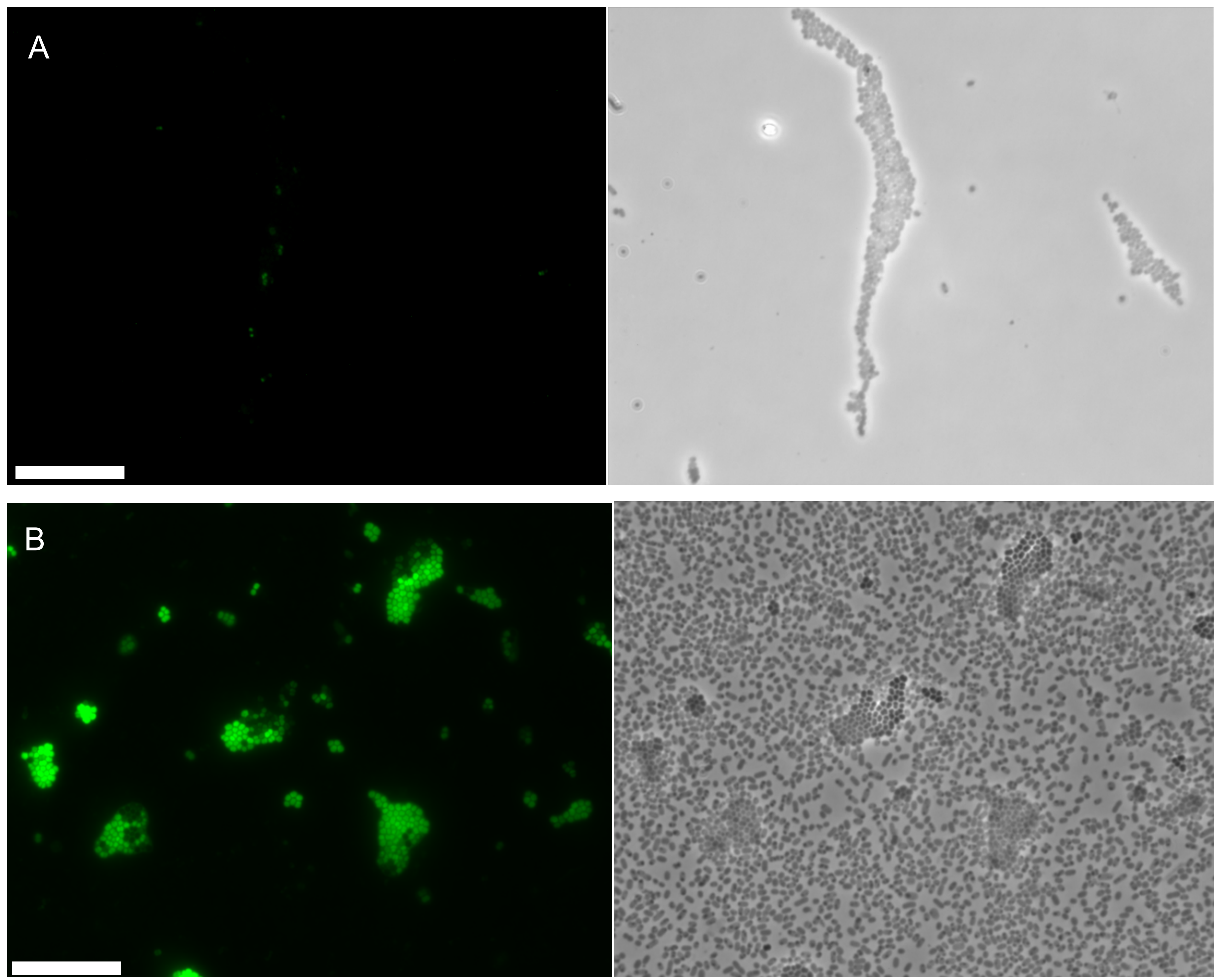


Fig.C.51. Fluorescence *in situ* hybridisation with pure culture cells, RINGFISH probe parC 60% formamide, Fluos-12-UTP labelled (left) phase contrast (right image); bar 10µm
A: cells of the clinical strain *E. coli* AF 421100
B: cells of the clinical strain *E. faecalis,* AF 625857 in mixture with *E. coli* AF 421100

The application of probe parC to real clinical urine sample number 6, from which the strain *Enterococcus faecalis* AF 625857 was isolated from, also allowed the detection of RINGFISH signals as can be seen in Figs. C.52 A and B below, but no double hybridisation signals was obtained, if additionally using an oligonucleotide probe specific for *E. faecalis*.

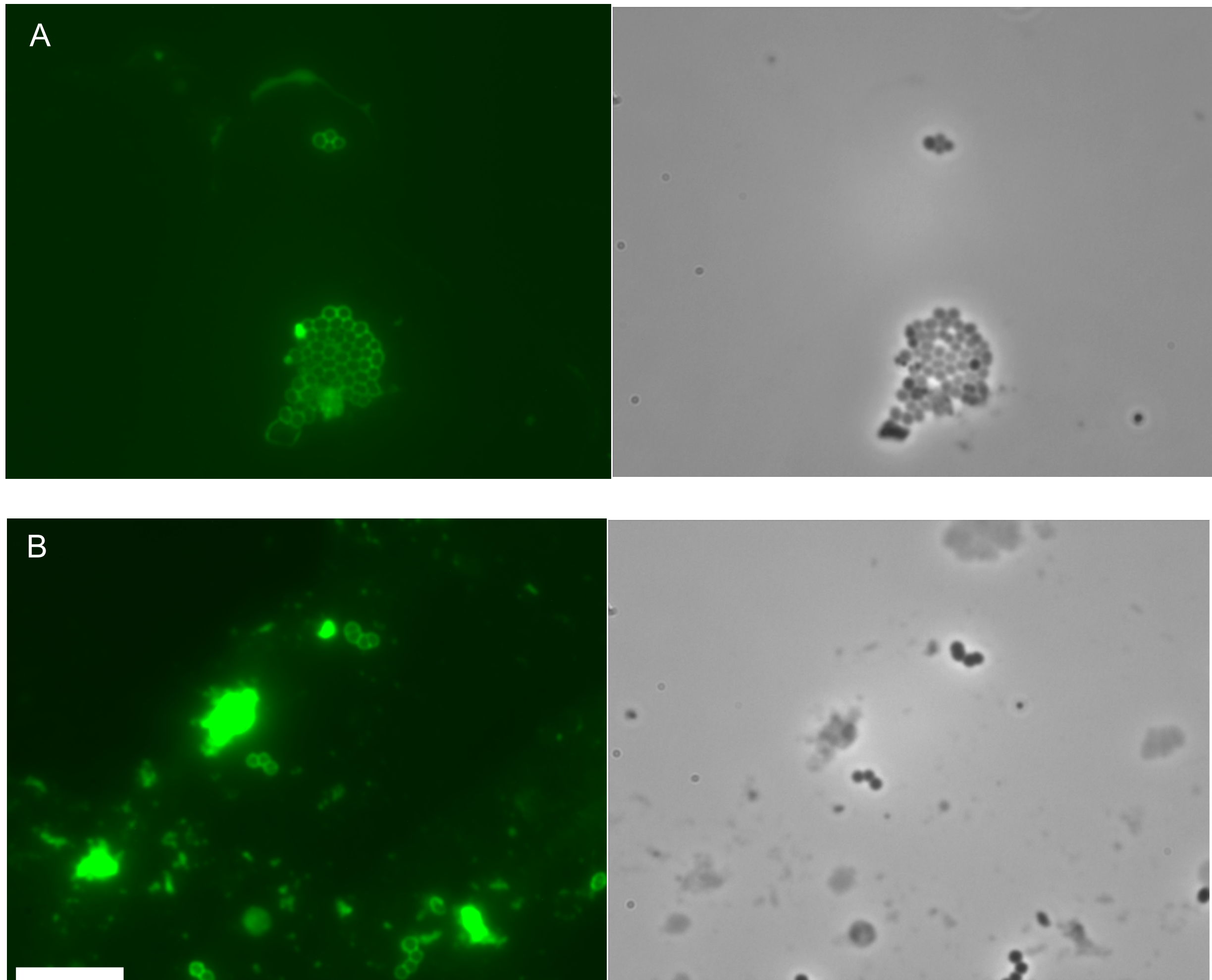


Fig.C.52. A, B: Fluorescence *in situ* hybridisation with real clinical sample number 6, containing cells of *E. faecalis,* RINGFISH probe parC 60% formamide, Fluos-12-UTP labelled (left) phase contrast image (right image); bar 10µm

4.3. Enrichment hybridisations using RINGFISH probes

After the best conditions with respect to halo signal, signal intensity and specificity had been evaluated, all RINGFISH probes were applied to enrichment hybridisations for testing their appropriateness for cell fishing.

For all given probes, it was possible to show the applicability for enrichments in general.

For examples of RINGFISH probes which were designed for organisms natively present in the available real clinical samples (samples number 1 and 2, targeted by RINGFISH probes EcoAmpCV3) enrichment hybridisations from the real sample materials were performed. Afterwards, PCR detection of enriched organism with conserved primers and subsequent sequencing of the PCR products was carried out to confirm the identity of the enriched organism.

For one example of RINGFISH probes no real clinical sample material was available to perform enrichment hybridisations (probes TetAJ) and for two examples not enough sample was left (probes ermB and parC). In these cases, enrichment hybridisations from the pure culture clinical strains have been performed to test *in situ* the enrichment capacities of the given probes *in situ* by comparing the signal intensities relying on a streptavidin peroxidase system for biotin-labelled probes.

4.3.1. Application of RINGFISH probes for enrichment of cells from real clinical samples

The clinical strains *E. coli* AF 421100 and *E. coli* AF 430319, isolated from real clinical samples numbers 1 and 2, according to the patient`s record contain resistances against several ß-lactam antibiotics, especially ampicillin. With PCR for amplification of a part of the a*mpC* gene (Table B.8), it was possible to obtain the amplicons for AmpCV3 of the DNA of both clinical strains and generate the respective probes EcoAmpCV3 #1 and EcoAmpCV3 #2. The strains *E. coli* AF 421100 and *E. coli* AF 430319 (and in case of *E. coli* AF 421100 also the sample number 1) allowed detection of positive signals after hybridisations on slides (see Fig.C.37), and therefore were used in enrichment hybridisations from real clinical samples.

4.3.1.1. Enrichment hybridisation using probe EcoAmpCV3 #1

For this enrichment, the RINGFISH probe EcoAmpCV3 was used, generated from DNA of the strain *E. coli* AF 421100 which was isolated from real clinical urine sample number 1, AF 421100.

The hybridisation in solution with the real clinical sample number 1, was performed under evaluated conditions for probe EcoAmpCV3 (Table C.14). The enrichment success was confirmed afterwards by PCR. The primers used in this case were TEM F and TEM R (Table B.12) for amplification of plasmid encoded bla_{TEM} genes.

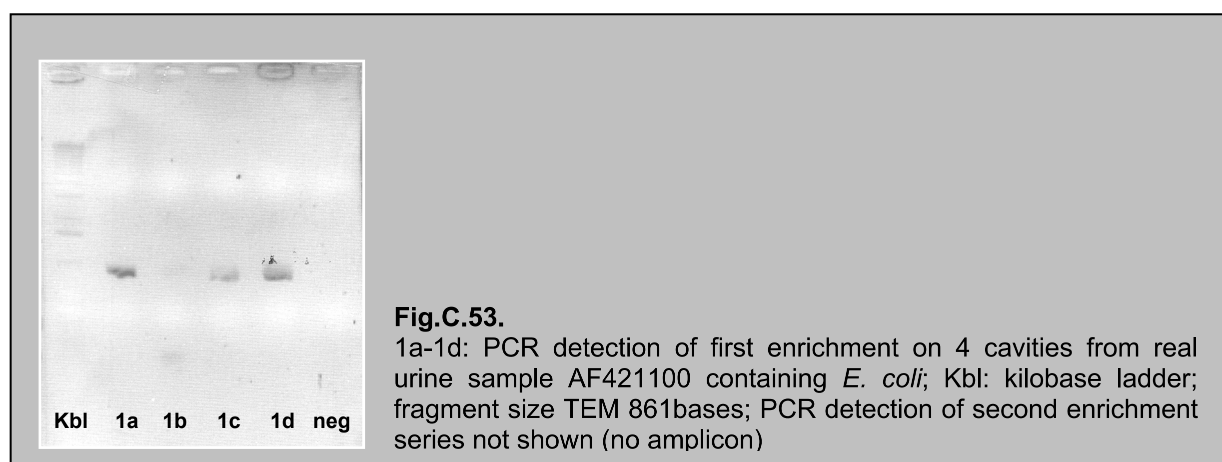


Fig.C.53.
1a-1d: PCR detection of first enrichment on 4 cavities from real urine sample AF421100 containing *E. coli*; Kbl: kilobase ladder; fragment size TEM 861bases; PCR detection of second enrichment series not shown (no amplicon)

Successful detection of immobilised cells with PCR was possible for all 4 cavities (Fig.C 53), which were used for the first enrichment hybridisation. The size of the amplicon corresponds to the expected size of 860 kb. The supernatants of the first enrichment hybridisation were transferred to fresh cavities, but PCR did not allow further detection of immobilised cells, indicating that most of the target cells were already removed during the first enrichment row (image not shown). Amplicons obtained from the first enrichment were cloned. Cloning, sequencing and analysis of sequence data was carried out as described in B.XIII. The similarity comparison of nucleic acid sequences (BLAST) was used for confirmation of the identity of the enriched organism. All sequenced clones belong to the group of the target organism, which was supposed to be enriched from the particular sample. Table C.15 given below sums up the results obtained from sequence analysis.

Table C.15. Result of sequence annotation of cloned and sequenced PCR products obtained from successful enrichments of target organism *E. coli* from real clinical sample urine list number 1, material using RINGFISH probe EcoAmpCV3 #1

Sample material	Primers used for PCR	Name of clones	Result of sequence allocation
			Blast similarity
Urine number 1, containing *E. coli*	TEM	b	99% to AY794046 *Escherichia coli* blaTEM-1 gene for TEM-1 beta-lactamase
		d	98% to AB194682 *Escherichia coli* blaTEM-1 gene for TEM-1 beta-lactamase
		e	96% to AB194682 *Escherichia coli* blaTEM-1 gene for TEM-1 beta-lactamase

As the PCR product indicates the existence of plasmid located ß-lactamase TEM, the sequences obtained showed highest similarity to known database entries for TEM ß-lactamases. The similarity of the examined clone sequence to the first named NCBI BLAST database entry, which appeared in order of the highest similarity to the request sequence, are listed in Table C.15.

All TEM ß-lactamases examined were similar in sequence and therefore had 96-99% similarity to NCBI BLAST entry accession number AB 194682 *Escherichia coli* *bla*TEM-1 gene for TEM-1 beta-lactamase and AY 794046 *Escherichia coli* beta-lactamase TEM1E (blaTEM1E) gene. This result fits well with the analysis of TEM sequences of cells of *E. coli* enriched using DIII targeted polynucleotide probes from the same sample urine AF 421100 (see further part C.I.4.2., Table C.11).

4.3.1.2. Enrichment using probe EcoAmpCV3 #2

For this enrichment, the RINGFISH probe EcoAmpCV3 was used, generated from the DNA of the strain *E. coli* AF 430319 which was isolated from real clinical aerobic blood culture sample number 2, AF 430319.

The hybridisation in solution with the real clinical sample number 2, was performed under evaluated conditions for probe EcoAmpCV3 (Table C.14). The enrichment success was confirmed afterwards by PCR. The primers used in this case were118F and 985R.

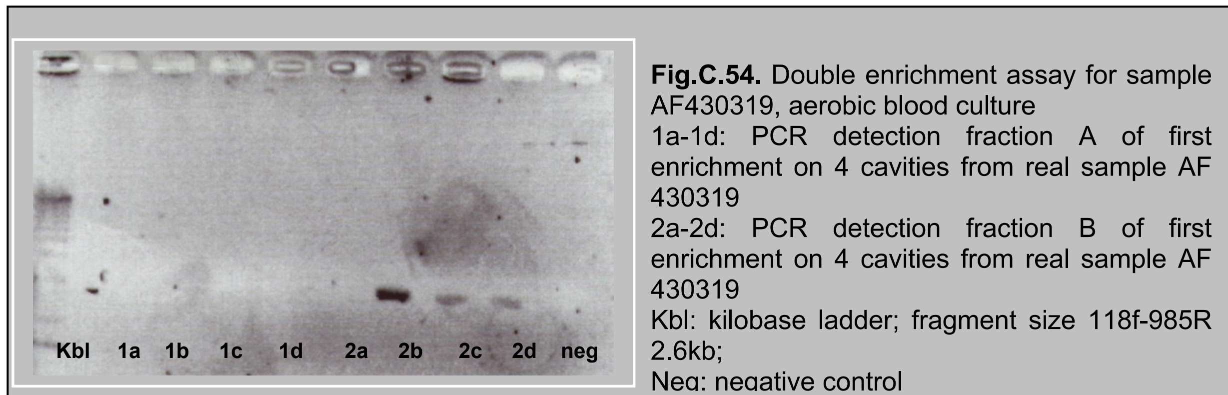


Fig.C.54. Double enrichment assay for sample AF430319, aerobic blood culture
1a-1d: PCR detection fraction A of first enrichment on 4 cavities from real sample AF 430319
2a-2d: PCR detection fraction B of first enrichment on 4 cavities from real sample AF 430319
Kbl: kilobase ladder; fragment size 118f-985R 2.6kb;
Neg: negative control

This enrichment was performed in an identical double assay, here called fraction A and B (Fig. C.54). Successful detection of immobilised cells with PCR was possible for 3 cavities of fraction B on which the first enrichment hybridisation was carried out. From fraction A, no amplicon could be retrieved. The size of the amplicon agrees with the expected size of 2.4 kb. The supernatants of fractions A and B of the first enrichment hybridisation were transferred to fresh cavities for further enrichment. Here, PCR did not allow further detection of immobilised cells, indicating, that most of the target cells had already been fished out during the first enrichment. The amplicons obtained from the first enrichment were cloned. Cloning, sequencing and analysis of sequence data was carried out as described in B.XIII. Positive clones with the correct insert size in the plasmid were sequenced, followed by sequence analysis using BLAST and an ARB database alignment. Similarity comparison of nucleic acid sequences (BLAST) showed 96-99% similarity to different *E. coli* database entries of NCBI (see Table C.16). This result was confirmed by examination of the diagnostic regions of sequences after alignment in an ARB sequence data base. All sequences could be allocated to belong to *E. coli*.

Table C.16. Result of sequence annotation of cloned and sequenced PCR products obtained from successful enrichments of target organism *E. coli* from real clinical sample aerobic blood culture list number 2, material using RINGFISH probe EcoAmpCV3

Sample material	Primers used for PCR	Name of clones	Result of sequence allocation	
			ARB*	Blast similarity
Aerobic blood culture, containing *E. coli*	118f-985R	3c	*E. coli*	99% to U00096 and V00348 (23S *E. coli*)
		3e	*E. coli*	98% to AF053967 (23S *E. coli*)
		4e	*E. coli*	96% to U00096 and V00348 (23S *E. coli*)

*ARB: confirmed by examination of the diagnostic regions in an rRNA ARB database alignment

4.3.2. Application of RINGFISH probes for enrichment of pure culture cells

Enrichments from clinical samples were not possible using RINGFISH probes ermB, parC and TetAJV1 and TetAJV2, because the amount of sample material was too small (ermB, parC) or the target strain was only available as pure culture (TetAJ). To determine whether enrichments are generally possible using these probes, qualitative enrichment hybridisations were performed using pure culture cells. After the first hybridisation with the respective biotin labelled polynucleotide probe with target and non-target organisms in pure culture, the signal intensities obtained from cavities covered with the particular probe complementary nucleic acids were measured using a streptavidin-peroxidase system. An uncovered cavity was used as a negative control.

4.3.2.1. RINGFISH probe parC for Enterococcus faecalis AF 625857

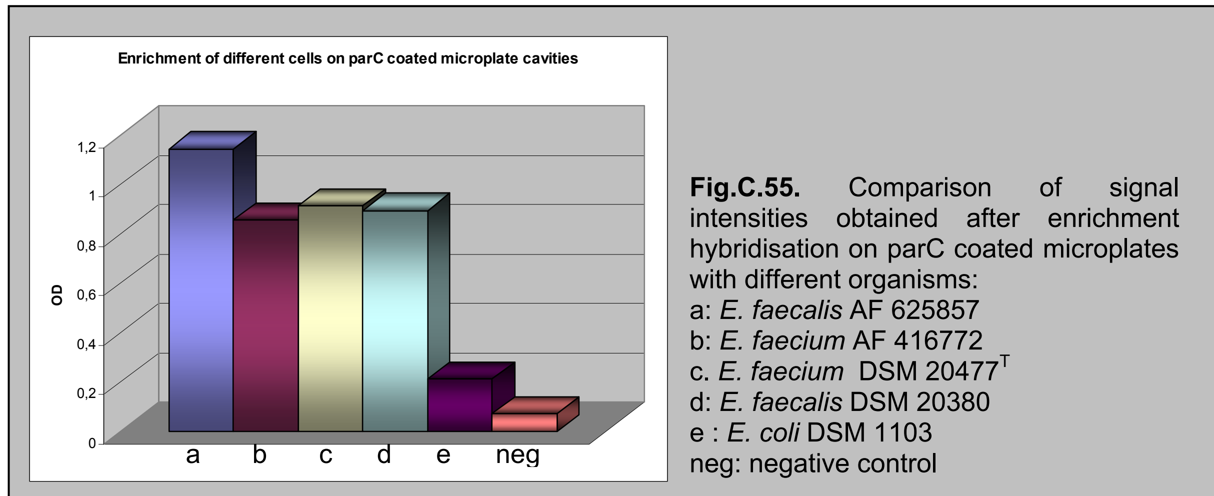


Fig.C.55. Comparison of signal intensities obtained after enrichment hybridisation on parC coated microplates with different organisms:
a: *E. faecalis* AF 625857
b: *E. faecium* AF 416772
c. *E. faecium* DSM 20477^{T}
d: *E. faecalis* DSM 20380
e : *E. coli* DSM 1103
neg: negative control

The comparison of the signal intensities obtained after enrichment hybridisation of the listed strains on microplates shows very clearly, that the binding of hybridised cells of *Enterococcus faecalis* AF 625857, the original target organism of probe parC, was achieved to the highest degree (Fig. C.55). The values obtained from *Enterococcus faecium* AF 416772, DSM 20477^{T} and *E. faecalis* DSM 20380 are comparatively high, too, indicating successful probe hybridisation, as already observed with hybridisation on slides (see C.II.2.). In contrast to that, the signal intensities obtained with *E. coli* DSM 1103 are significantly lower, as the probe does

not bind to this organism. The value is comparable to the value obtained from an uncovered cavity (negative control), indicating that no probe binding was possible.

4.3.2.2. RINGFISH probe ermB for Enterococcus faecium AF415772

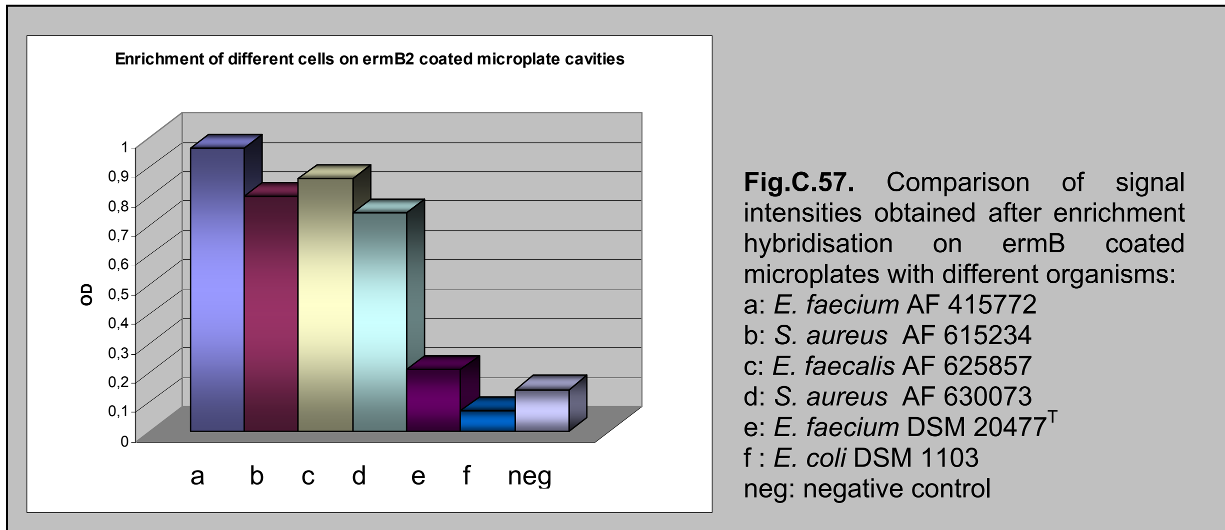


Fig.C.57. Comparison of signal intensities obtained after enrichment hybridisation on ermB coated microplates with different organisms:
a: *E. faecium* AF 415772
b: *S. aureus* AF 615234
c: *E. faecalis* AF 625857
d: *S. aureus* AF 630073
e: *E. faecium* DSM 20477^T
f : *E. coli* DSM 1103
neg: negative control

The comparison of the signal intensities obtained after enrichment hybridisation of the listed strains on microplates, that were coated with nucleic acids complementary to probe ermB after RINGFISH with the probe ermB clearly shows, that the binding of hybridised cells of *Enterococcus faecium*, the original target organism of probe ermB2, was achieved at the highest value level (Fig.C.57). The values obtained from *Enterococcus faecalis* AF 625857 *and Staphylococcus aureus* AF 615234 and AF 630073 are very high, indicating successful probe hybridisation and immobilisation of the cells in the microplate cavities. This result in turn fits with the results observed from hybridisation on slides (see C.II.2.), as these organisms allow amplification of the ermB gene with the primers designed for *E. faecium* AF 415772, too. In contrast to that, the signal intensities obtained from the cells applied as negative control, *E. faecium* DSM 20477^T, which did not allow PCR amplification using primers ermB, and cells of *E. coli* DSM 1103, are significantly lower, as the probe cannot bind to these organism, and no immobilisation in microplate cavities is possible. This result is consistent with the results obtained after hybridisations on slides (see C.II.2.). The values are comparable to the value obtained from an uncovered cavity (negative control).

4.3.2.3. RINGFISH probe TetAJ for Proteus mirabilis AF 413702

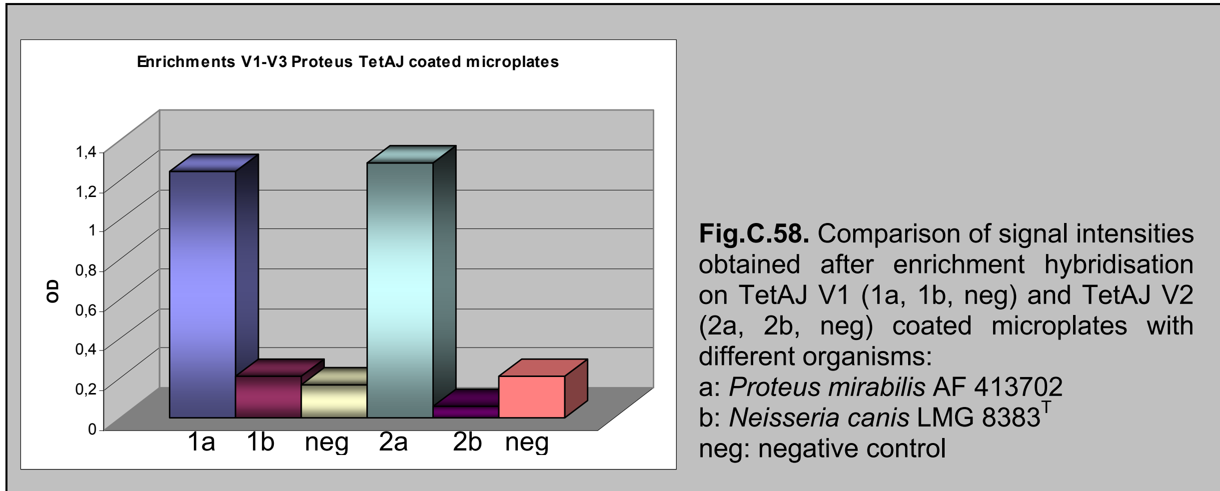


Fig.C.58. Comparison of signal intensities obtained after enrichment hybridisation on TetAJ V1 (1a, 1b, neg) and TetAJ V2 (2a, 2b, neg) coated microplates with different organisms:
a: *Proteus mirabilis* AF 413702
b: *Neisseria canis* LMG 8383^T
neg: negative control

The comparison of the signal intensities obtained after enrichment hybridisation of the listed strains on microplates, that were coated with nucleic acids complementary to probes TetAJV1 and TetAJV2 after RINGFISH with the probe TetAJV1 andV2 indicate the highest value for the cavities, where target cells of *Proteus mirabilis* were applied, for both of the probes which differ in length (Fig.C.58). The values obtained from non-target organism *Neisseria canis* LMG 8383^T are significantly decreased and in the same range as obtained from an uncovered cavity (negative control). This result confirms the findings described for hybridisations on slides (see C.II.2.).

III. Synthetic construct probes

1. Synthetic DNA polynucleotide probes

During this work, the application of synthetic DNA polynucleotide probes consisting of four-time repetition of a species-specific oligonucleotide probe was tested for several organisms (see Table B.16). Halo signals could be derived from 50 nucleotide long RNA polynucleotide probes (Zwirglmaier et al 2004), indicating network formation and utility for enrichment hybridisations. Based on this, it was now determined whether DNA polynucleotide probes with a length of 72-76 nucleotides - allow the confirmation of this result.

The DNA probes were applied to pure cultures o target cells and target cells in mixtures with non target cells to evaluate the specificity. The result was the same for most of the probes: strong whole cell fluorescence signals, no single halo signal (Fig.C.59). Various adjustments did not change the outcome. The specificity was limited. The probes PseAer_4Cy3 and PseFl_4 did not allow differentiation between *Pseudomonas aeruginosa* and *Pseudomonas fluorescens*, as was expected according to the specificity of the respective single oligonucleotide probe. Other bacterial cells could be differentiated (e.g. *E. coli, Klebsiella* spp). The probe Kpn_4, which was designed for *Klebsiella* sp, was completely unspecific, showing signals with all other tested bacterial species (e.g. *E. coli*, *Pseudomonas* spp and others).

Table C.17. Characteristics and behaviour of synthetic DNA polynucleotide probes consisting of a 4-time repetition of a species-specific oligonucleotide probe sequence; +: yes; -: no; (see Table B.16)

Synthetic DNA probe	Length of probe	Species specificity	family specificity	Formation of secondary structures	Nature of signal
PseAer_4Cy3	72	-	+	+	Strong whole cell fluorescence
PseFl_4*	76	-	+	+	Strong whole cell fluorescence
Kpn_4 *	72	-	-	+	Strong whole cell fluorescence

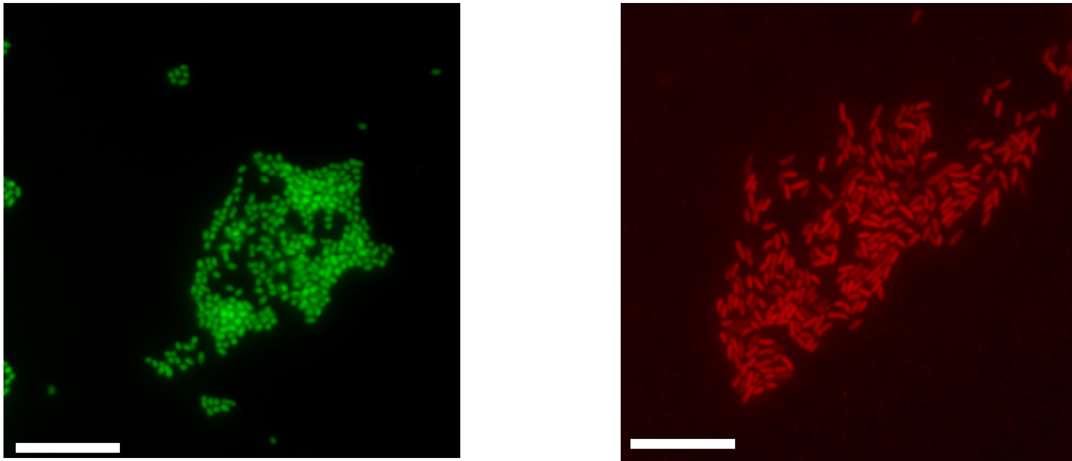


Fig.C.59. Fluorescence *in situ* hybridisation using probes Kpn_4 (left) and PseAr_4 (right image) on cells of *Klebsiella pneumoniae* (left) and *Pseudomonas aeruginosa* (right).

2. Synthetic RNA polynucleotide probes

The synthetic RNA polynucleotide probes were generated via *in vitro* transcription from a synthetic template (B.X.2.), and were multilabelled using Fluos-12-UTP during transcription. Again, the RNA polynucleotide probes consist of a repetition of a species-specific oligonucleotide probe stretch, but the individual oligonucleotide probe parts were divided by a poly A spacer of 5 nucleotides in length (Tables B.17 and C.18). This should keep the single species-specific stretches apart and prevent mismatch-binding due to shifted sequences. *In silico* analysis of the probes demonstrated the formation of secondary structures (see D.III.1.2.)

Two probes were developed:

Table C.18. Sequence of RNA polynucleotide probes generated from a synthetic ss template after *in vitro* transcription (see further Table B.17); blue marked nucleotides: poly-A spacer

Target organism	Name of probe	sequence of probe 5`-3`direction	TD [°C]	GC [%]
Pseudomonas aeruginosa	PseAer4_sp	**UCUCGGCCUUGAAACCCC***AAAAA***UCUCGGCC UUGAACCCC***AAAAA***UCUCGGCCUUGAAACCC C***AAAAA* **UCUCGGCCUUGAAACCCC**	93,8	56,5
Pseudomonas fluorescens	Psefl4_sp	**CUCGACUACUGCUCAAUAG***AAAAA***CUCGACU ACUGCUCAAUAG***AAAAA***CUCGACUACUGCUC AAUAG***AAAAA* **CUCGACUACUGCUCAAUAG**	90.6	52,9

2.1. Synthetic construct probe for *Pseudomonas aeruginosa*

2.1.1. Evaluation of hybridisation conditions

A combination of oligonucleotide probe FISH conditions necessary for specific signals with oligonucleotide probe PseAer (Table B.14) applying the denaturation step from polynucleotide hybridisations resulted in the best signals for hybridisation using the transcript synthetic construct probe PseAer_4sp. The signals were showed highly specific, as signals could not be observed with negative control species of *Pseudomonas*.

The optimal result with regard to formation of a network of probe molecules (resulting in a halo or part-halo) was achieved by using 30% formamide in the oligonucloetide hybridisation buffer (this is also the given concentration of the oligonucleotide probe), 80 °C denaturation for 20 minutes prior to a hybridisation at 46°C for three hours, with a washing step of 20 minutes at 48 °C in the according washing buffer (Table B.20).

Longer hybridisation, changes in FA concentration and omitting the washing step resulted in loss of specificity, halo signal or occurrence of signal. Table C.19 summarises the different results obtained after hybridisations.

Table C.19. Conditions of hybridisation and nature of signal after different hybridisation conditions; +: yes; -: no.

Denaturation	Fa [%]	conditions	Washing step	Species-specificity	Nature of signal
20`at 80°C	Series from 10-80%	46°C over night	-	-	weak whole cell fluorescence from all *Pseudomonas* spp tested
20`at 80°C	Series from 10-80%	46°C, 3h	-	-	weak whole cell fluorescence, some halos from *P. aeruginosa*
20`at 80°C	30%	46°C, 3h	+, 15´at 48°C	+	Halo signals, intermediate signals and whole cell fluoescence, but weaker than above

The nature of the halo signal obtained after use of the optimal hybridisation protocol varied. Halo signals and signals somewhere in between halo signals and whole cell fluorescence signals were observed, along with whole cell fluorescence signals (see Figs.C60-C.63).

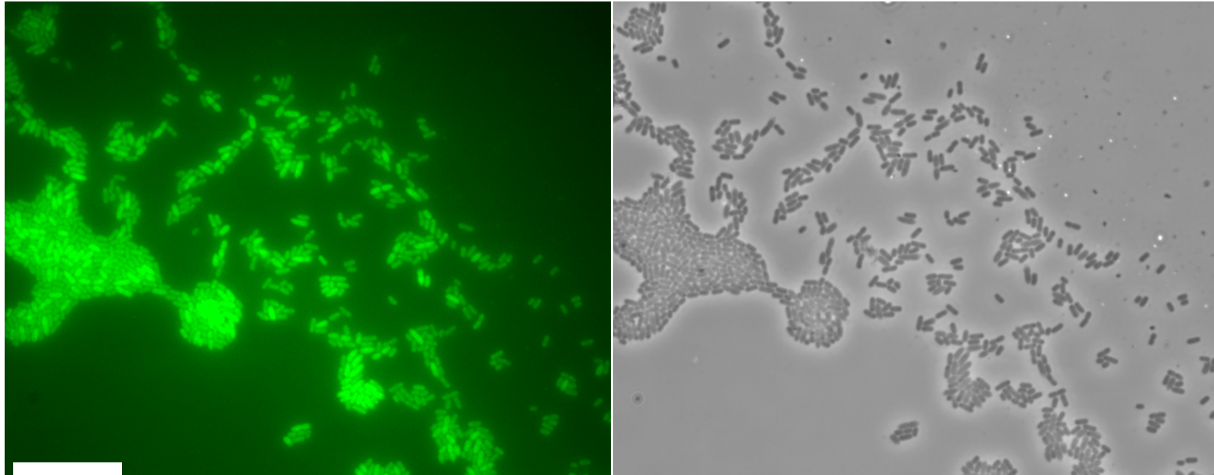


Fig.C.60. Fluorescence *in situ* hybridisation with *Pseudomonas aeruginosa* using probe PseAer_4sp, 30% formamide in the oligonucleotide probe hybridisation buffer, hybridisation over night, epifluorescence (left) and phase contrast (right image); bar 10µm

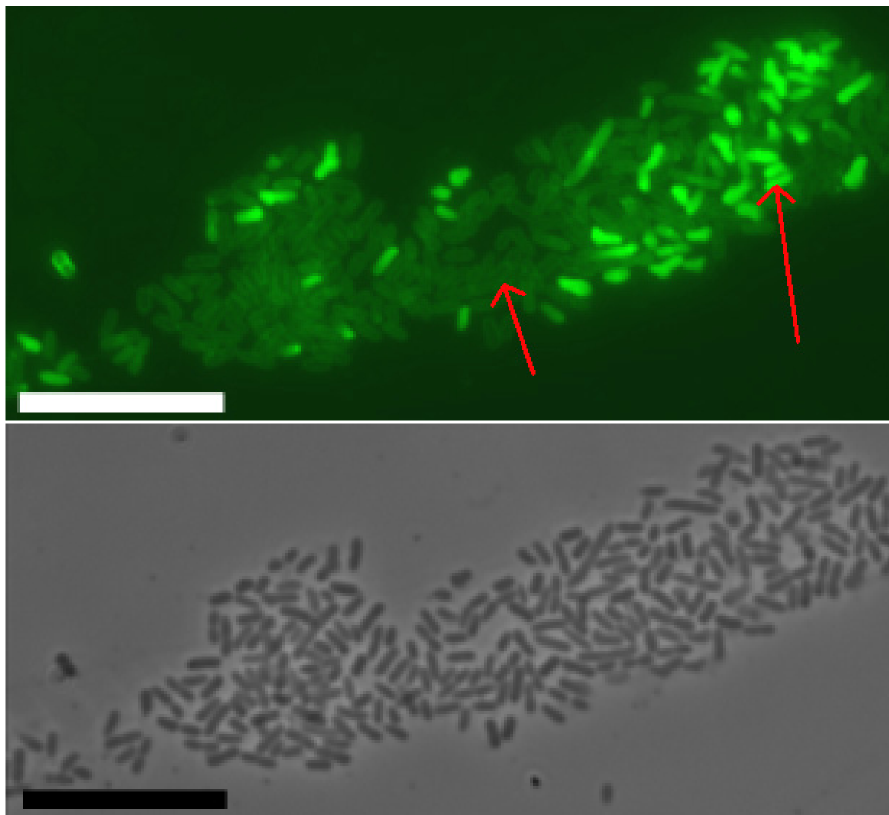


Fig.C.61. Fluorescence *in situ* hybridisation with *Pseudomonas aeruginosa* using probe PseAer_4sp, 30% formamide in the oligonucleotide probe hybridisation buffer, hybridisation 3h at 53°C, washing step in WB 15 min, epifluorescence (upper) and phase contrast (lower image); arrows indicate the presence of whole cell fluorescence showing cells and halo formation of some cells; bar 10µm

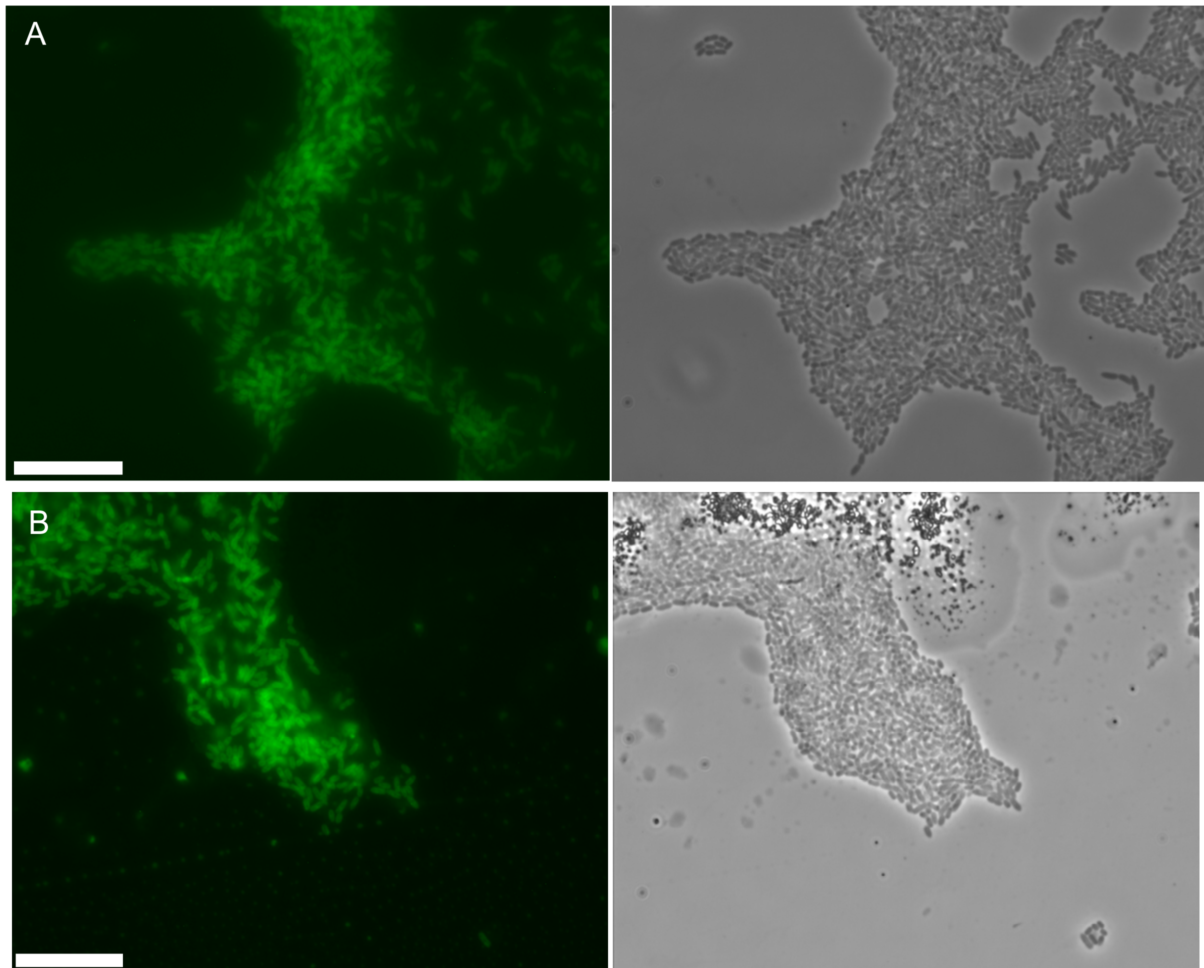


Fig.C.62. Fluorescence *in situ* hybridisation with *Pseudomonas aeruginosa* in mixture with *Pseudomonas spp* and probe PseAer_4sp, 30% formamide in the oligonucleotide probe hybridisation buffer, hybridisation 3h at 53°C, washing step in WB 15min, epifluorescence (left) and phase contrast image (middle) and phase contrast (right image); bar 10µm
A: *Pseudomonas aeruginosa* in mixture with *E. coli* and *Pseudomonas fluorescens*
B: *Pseudomonas aeruginosa* in mixture with *Pseudomonas fluorescens*

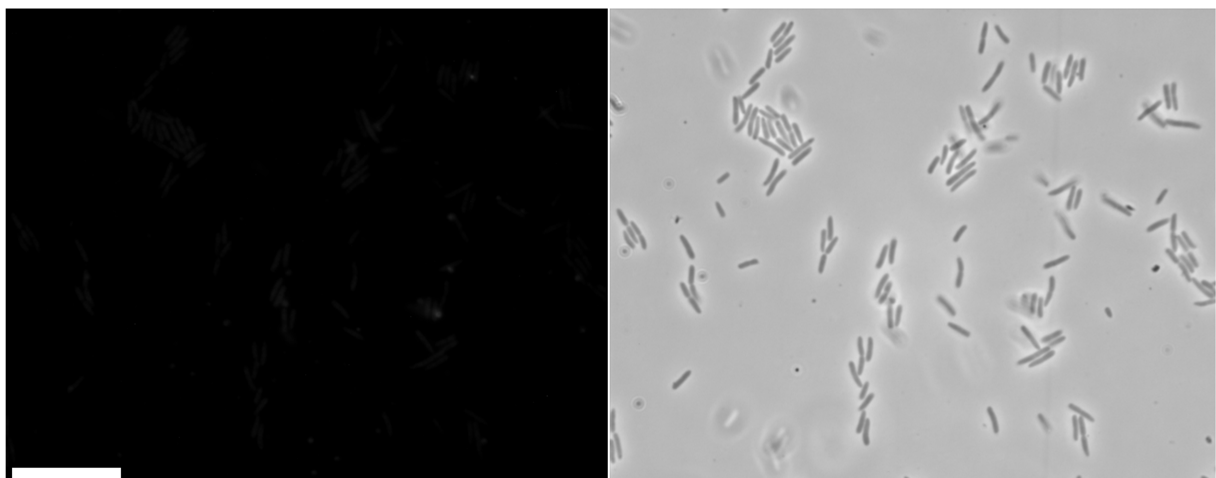


Fig.C.63. Fluorescence *in situ* hybridisation with *Pseudomonas stutzeri,* and probe PseAer_4sp, 30% formamide in the oligonucleotide probe hybridisation buffer, hybridisation 3h at 53°C, washing step in WB 15min, epifluorescence (left) and phase contrast (right image); bar 10µm

2.1.2. Enrichment hybridisations using probe PseAer4_sp

Despite the fact that the halo signal was present, but not dominant after hybridisations on slides, probe PseAer4_sp was applied in enrichments experiments with pure culture cells from *Pseudomonas aeruginosa* as well as on real clinical sample number 3, containing originally *P. aeruginosa* AF 625811, where this strain was isolated from.

2.1.2.1. Enrichment hybridisation from pure culture cells of Pseudomonas aeruginosa DSM 50071

To elucidate whether enrichment hybridisations are generally possible using probe PseAer4_sp, either signal intensities were measured or PCR detection was applied after an enrichment hybridisation.

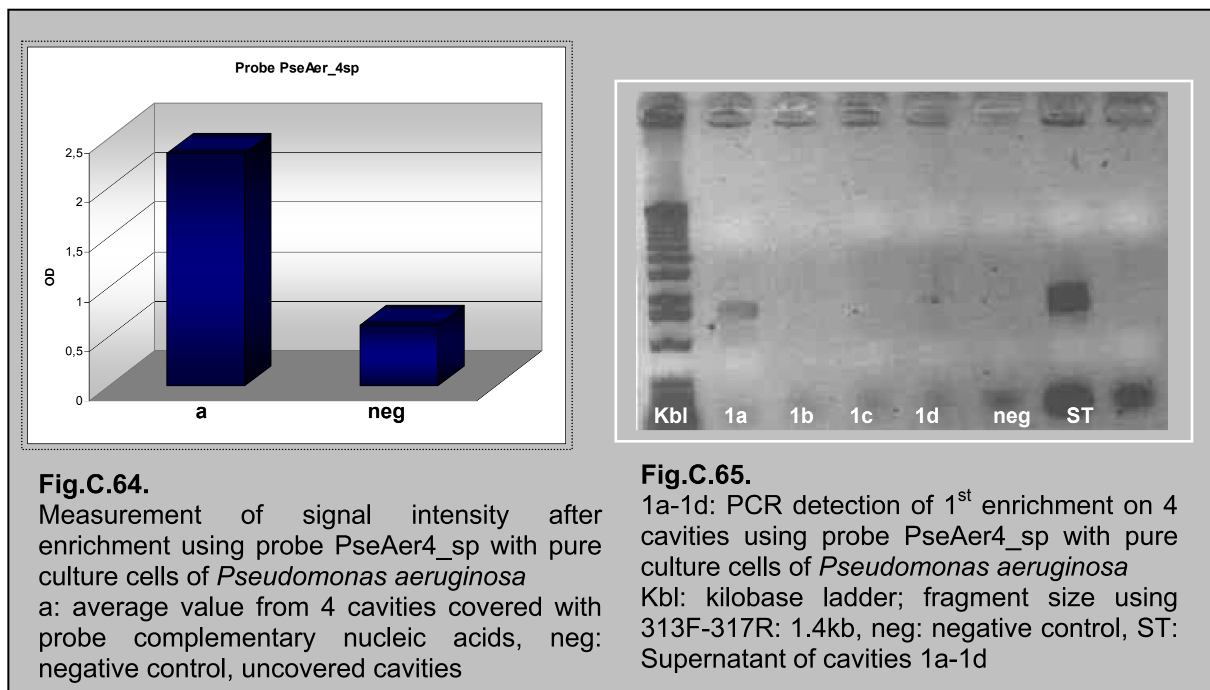


Fig.C.64.
Measurement of signal intensity after enrichment using probe PseAer4_sp with pure culture cells of *Pseudomonas aeruginosa*
a: average value from 4 cavities covered with probe complementary nucleic acids, neg: negative control, uncovered cavities

Fig.C.65.
1a-1d: PCR detection of 1st enrichment on 4 cavities using probe PseAer4_sp with pure culture cells of *Pseudomonas aeruginosa*
Kbl: kilobase ladder; fragment size using 313F-317R: 1.4kb, neg: negative control, ST: Supernatant of cavities 1a-1d

The comparison of average signal intensities obtained from the cavities covered with complementary nucleic acids to those cavities that were not covered is highly increased, indicating the successful immobilisation of hybridised target cells (Fig.C.64).

The detection of immobilised cells with PCR after enrichment on covered microplate cavities resulted in one amplicon for cavitiy 1a (Fig.C.65). The supernatant was analysed by the same PCR and also showed amplification of 23S rDNA of *Pseudomonas aeruginosa* (Fig.C.65). Though not all cavities allowed PCR detection of immobilised cells afterwards, general applicability of this probe can be assumed. The outcome of only one possible PCR detection in cavity 1a (Fig.C.65) could be an indication, that not all target cells exhibit the halo signals, thus not offering enough parts of the network of probe molecules for being linked to the immobilised nucleic acids during second hybridisation for enrichment of the cells. This can also be concluded from the ambiguous nature of signals obtained after hybridisation on slides, see Fig.C.61. Finally, the supernatant that was applied for PCR analysis, too, gives a strong amplicon, indicating the presence of many unbound target cells still present in the liquid.

2.1.2.2. Enrichment hybridisation from real clinical sample wound smear number 3 AF 625811

For enrichment of *Pseudomonas aeruginosa* target cells from real clinical sample number 3, 25 µl of the PFA fixed wound smear sample were used for hybridisation with probe PseAer4_sp under conditions mentioned in Table C.19, before being applied further in the enrichment hybridisation. The hybridised sample material was transferred into 4 cavities covered with nucleic acids complementary to probe PseAer4_sp for the first enrichment, before the supernatant was applied to fresh cavities for repeated hybridisations. Afterwards, PCR detection was performed using primers 313F-317R.

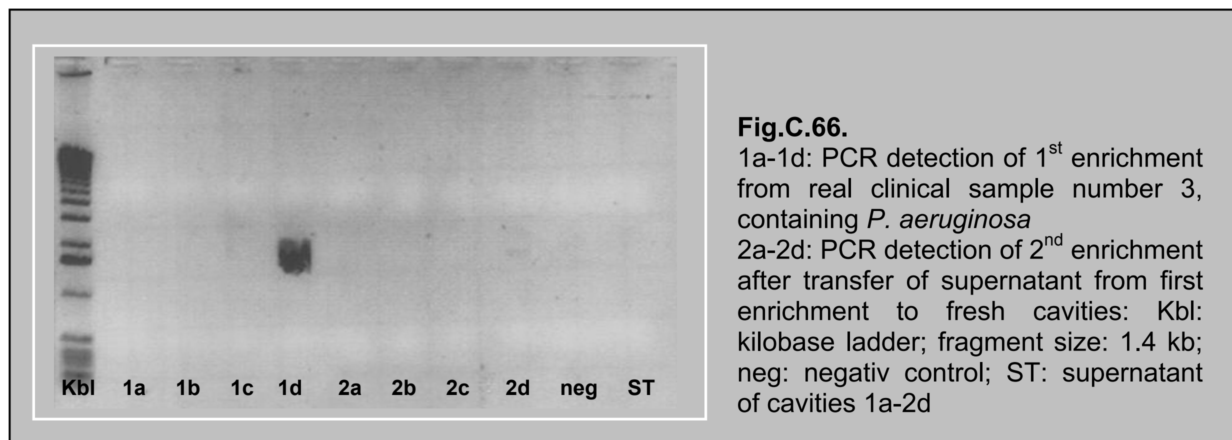


Fig.C.66.
1a-1d: PCR detection of 1st enrichment from real clinical sample number 3, containing *P. aeruginosa*
2a-2d: PCR detection of 2nd enrichment after transfer of supernatant from first enrichment to fresh cavities: Kbl: kilobase ladder; fragment size: 1.4 kb; neg: negativ control; ST: supernatant of cavities 1a-2d

Only one amplicon was present in the fourth cavity of the first enrichment. In the second enrichment nothing could be detected with PCR, and also the analysis of the pooled supernatant did not allow further amplification (Fig.C.66). The amount of sample which was used for the enrichment was very low; the distribution of the cells within the sample material has to be assumed to be variabel, resulting in unequal distribution in the cavities.

The obtained PCR product was cloned (B.XIII). Clones were picked arbitrarily and in numbers of 5-10 per cloning plate. Positive clones with the correct size of insert in the plasmid were sequenced, followed by sequence analysis using BLAST and ARB database alignment.

Table C.20. Result of sequence annotation of cloned and sequenced PCR products obtained from successful enrichments of the target organism *Pseudomonas aeruginosa* from real clinical sample wound smear list number 3, using synthetic construct probe PseAer4_sp

Sample material	Name of clones	Sequencing Primers	Result of sequence allocation	
			ARB*	Blast similarity
Wound smear number 3 containing *Pseudomonas aeruginosa*	PsAerDa	M13F-R 992V-1020R 1019V-335R	*P. aeruginosa*	99% to AE004949PAO1
	PsAerDb		*P. aeruginosa*	99% to AE004949PAO1
	PsAerDf		*P. aeruginosa*	98% to AE004949PAO1
	PseAerPPa		*P. aeruginosa*	99% to AE004949PAO1
	PseAerPPb		*P. aeruginosa*	98% to AE004949PAO1

*ARB: confirmed by examination of the diagnostic regions in an rRNA ARB database alignment

All cloned and analysed sequences belonged to the group of the target organism, which was supposed to be enriched from the particular sample. The similarity of the sequences compared to BLAST database entries ranged from 98-99% for the first named database entry, *Pseudomonas aeruginosa* PAO 1, accession number AE 004949. This result was confirmed by examination of the diagnostic regions of sequences after alignment in ARB sequence data base. All sequences could be allocated to belong to *Pseudomonas aeruginosa*.

2.2. Synthetic construct probe for *Pseudomonas fluorescens*

In the case of probe PseFl4_sp for *Pseudomonas fluorescens*, the result was different after hybridisation with pure culture cells of DSM 50124. The combination of the specific oligonucleotide probe PsAG1 hybridisation conditions (Table B. 14), with the denaturation step from polynucleotide hybridisations resulted in specific whole cell fluorescence signals.

Alterations of the hybridisation conditions such as extended hybridisation time, changes in FA concentration and omitting the washing step did not change the nature of the signal, but resulted in loss of specificity. The possible role of the particular secondary structures which might be causative for halo signals is discussed in D. III.

D. Discussion

I. Application of DIII 23S rRNA targeted polynucleotide probes

1. DIII 23S rRNA as target

16S rRNA, intergenic spacer-rRNA and 23S rRNA targeted fluorescence *in situ* hybridisation (FISH) using fluorescently labelled, synthetic DNA oligonucleotide probes with a length of 16-25 nucleotides are nowadays a standard method for the identification of microorganisms in different kinds of habitats, e.g. in environmental and clinical samples (Kempf et al, 2000; Hogardt et al, 2000). In contrast to oligonucleotide probes, rRNA targeted polynucleotide probes offer a length of 250 to 350 nucleotides or longer and are generated by *in vitro* transcription from a double stranded DNA template. Especially 23S rRNA DIII targeted polynucleotide probes have been used for a variety of applications since their first use in 1994 (Trebesius et al 1994). The target sequence of the probes, *E. coli* position 1366 -1601, is the longest coherent, variable part of bacterial 23SrRNA (Ludwig et al, 1994). Insertions of a length up to 100 nucleotides and deletions of up to 80 nucleotides have been reported to be stable for different bacterial groups within in the target site of the probes (Roller et al, 1992, Ludwig et al 1994, Höpfl et al, 1989, Larsen, 1992). Several studies revealed the special applicability of this region for polynucleotide probe based hybridisations.
With the first use of 23S rRNA DIII targeted polynucleotide probes for whole cell hybridisations of *Pseudomonas putida* and *Acinetobacter* spp (Trebesius et al, 1994), the special nature of the hybridisation signal, the halo, was observed for the first time. Trebesius et al (1994) and Stoffels et al (1999) proposed as an explanation that the polynucleotide probes, multi-labelled during transcriptional generation, bind only partially to their target structures inside of the cell, whereas the rest of the probe remain outside, spanning the cell envelope. Zwirglmaier et al (2003) extended this theory to the “Network hypothesis”. The polynucleotide probes do not simply span the cell envelope, but are in addition to target binding involved in the formation of secondary structures and inter-probe hybridisations, resulting in a three dimensional network around the whole cell. Based on these theories, it was possible to use the halo phenomenon for further applications, i.e. for the enrichment of microbial cells,

using the projecting parts of the polynucleotide probes for binding (of biotin-labelled probes) to streptavidin-covered magnetic beads for application in a MACS system (magnetic activated cell sorting, Stoffels et al, 1999) or for hybridisation to probe-complementary nucleic acids in microplates (Zwirglmaier et al, 2004). Fig.D.1. displays a secondary structure model of *E. coli* 23S rRNA, with the nucleotides of the probe binding part of the DIII sequence in red.

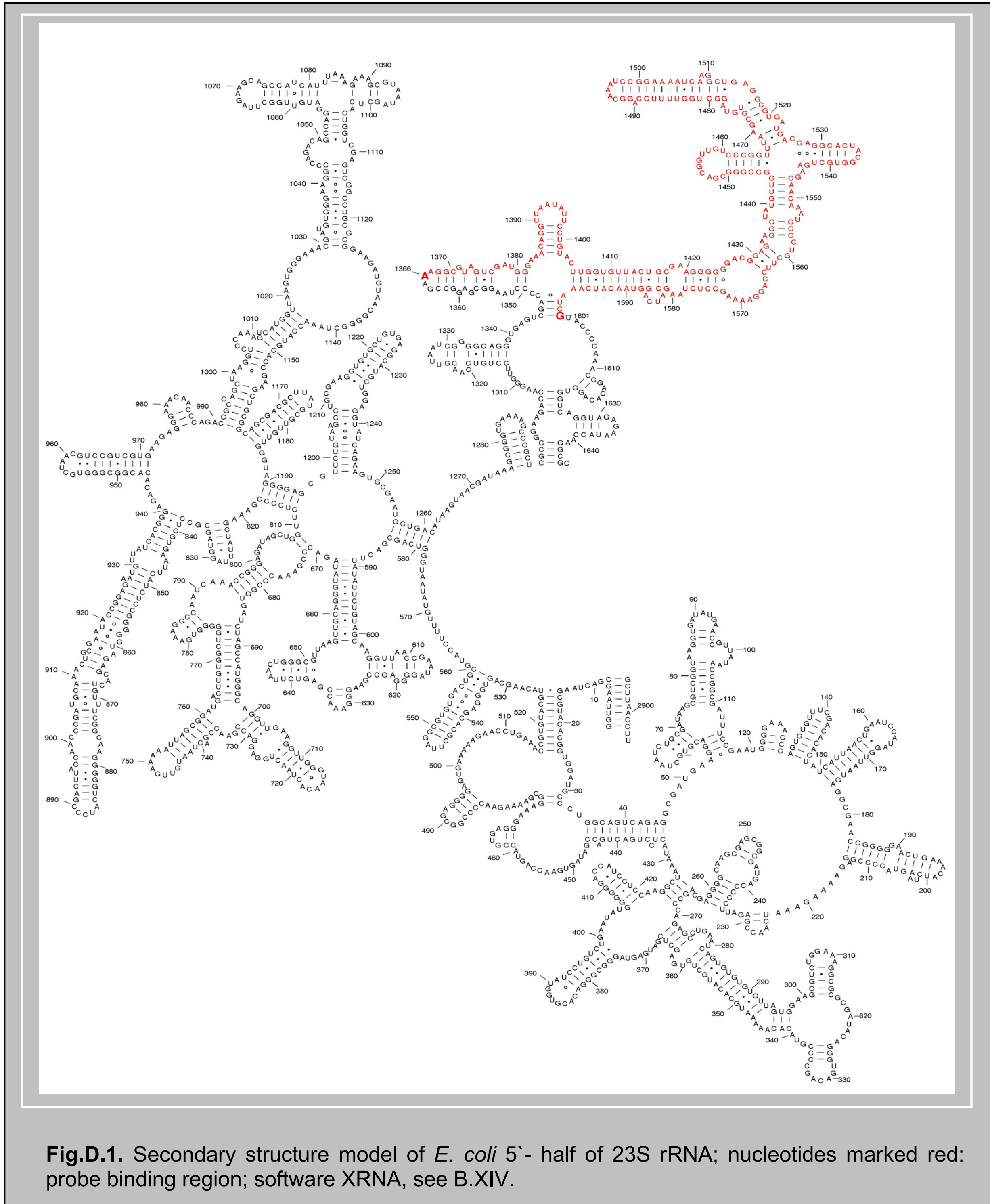


Fig.D.1. Secondary structure model of *E. coli* 5`- half of 23S rRNA; nucleotides marked red: probe binding region; software XRNA, see B.XIV.

The design of DNA polynucleotide probes targeting the same region as the RNA transcript polynucleotide probes turned out to be an alternative for some applications, offering possible advantages in comparison to RNA probes: higher stability, faster generation, and seemingly less unspecific binding to non-target components, especially when applied to environmental samples (Zimmermann et al, 2001). The disadvantages of DNA polynucleotide probes lie in an increased cost for generation caused by the cost intensive labelling during PCR-based generation and the low yield of probes obtained. Moreover, the hybridisations with DNA polynucleotide probes did not always yield halo signals, but rather whole cell fluorescence when applied in environmental samples (Zimmermann et al, 2002), which lessens their application in enrichment hybridisations, because efficient binding to complementary nucleic acids may be reduced.

In contrast, RNA polynucleotide probes allowed solid halo detection of target bacteria in real clinical samples examined in this work (see part C.I.4.). The yield obtained after generation of RNA polynucleotide probes is very high in contrast to DNA polynucleotide probes, (~20µg from a template of 2-4µg, this work, Stoffels et al, 1999; Zwirglmaier et al, 2004) and the generation less expensive. The stability of RNA polynucleotide probes is high enough, to be applied to a broad range of samples.

Further advantages of RNA polynucleotide probes over DNA polynucleotide probes are evident, e.g. the stable formation of a network during hybridisation, resulting in the typical halo signal, even for Gram-positive bacteria. The RNA probes can generally be used over a range of formamide concentration in the hybridisation buffer from 50-80% which makes them quite flexible in use, e.g. if successive double hybridisations are necessary for some projects.

The RNA polynucleotide probes, obtained via *in vitro* transcription of a double stranded template DNA are single stranded with the main target site being located on the ribosomes (and on the chromosomal DNA). Thus, target binding might be more efficient in comparison to DNA polynucleotide probes, where both labelled strands are present during hybridisation. This could result in preferred renaturation of both strands, rather than in binding of one strand to the target on the ribosome. The nature of the halo signal after DNA polynucleotide probe hybridisations probably confirms this theory: it has been reported to be significantly weaker in intensity,

broader and granier compared to the signal obtained with RNA polynucleotide probes (Zimmermann et al, 2002).

1.1. Spectrum of specificity

In former studies, DIII targeted polyribonucleotide probes were shown to be group specific (Ludwig et al 1994). The evaluation of the specificity of all DIII targeted RNA polynucleotide probes used in this study could not always confirm this. As mentioned in C.I.1.1.2., four different similarity matrices were calculated over the target sequences, to obtain information about differences in the four sections (see Fig.D.2).

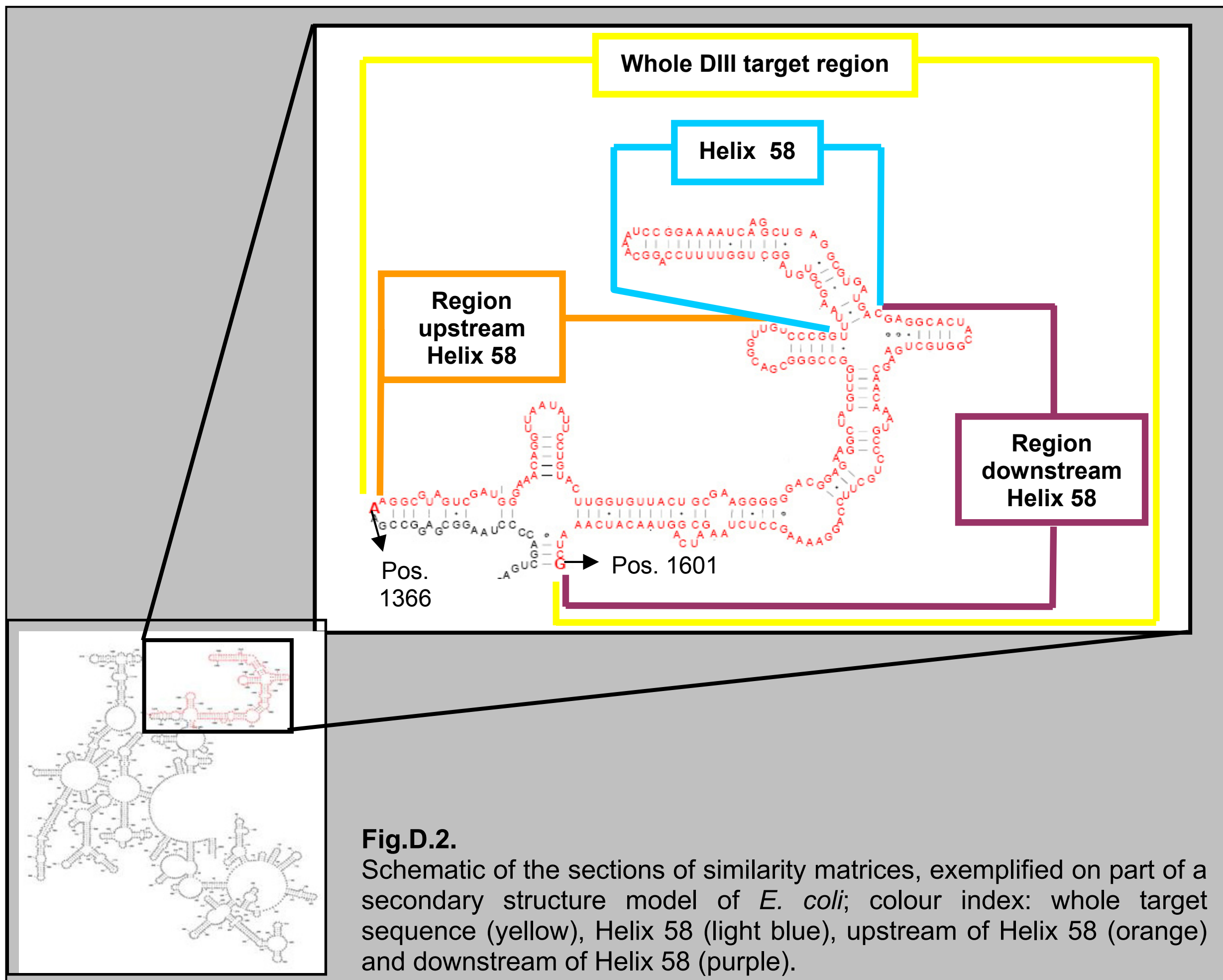


Fig.D.2.
Schematic of the sections of similarity matrices, exemplified on part of a secondary structure model of *E. coli*; colour index: whole target sequence (yellow), Helix 58 (light blue), upstream of Helix 58 (orange) and downstream of Helix 58 (purple).

Regarding the values calculated over the whole target sequence in combination with the results from direct *in situ* hybridisations with the probes from all bacterial strains relevant for the project a similarity of 70-76% of the target sequence has to be regarded as the cut off for obtaining positive or negative hybridisation signals (see Table D1, copy of Table C.2). This means that below 70% of target sequence

similarity of two compared bacterial species, the probe from one species will not bind to the other target sequence and thus allow differentiation. In contrast, a similarity above 76% of the target sequence will enable oppositional probe binding and allow detection of a positive hybridisation signal. The highest value of similarity, which allowed differentiation in FISH, was 76%.

Table D.1. Bacterial group-combined values from matrices calculated over whole target sequence, Helix 58,region upstream and downstream of Helix 58; colour index: **green**: + FISH result; **red**: - FISH result; **blue**: + and – results in repeated FISH experiments; vs.: versus, in meaning of compared to each other

Groups of bacteria	Range of similarity values for			
	Whole DIII target region	Upstream Helix 58	Helix 58	Downstream Helix 58
Length of sequence in the different matrix sections*	233	100	58	75
Enterobacteriaceae (without 10*)	84-99.6%	99-100%	78-96.6%	94-100%
***Pseudomonas* spp**	88-94%	87-91%	89-93%	87-92%
***Acinetobacter* spp**	93-96%	96-100%	86-95%	96-100%
Enterobacteriaceae **vs.** *Pseudomonas* spp	73-78%	82-86%	65-72%	69-73%
Enterobacteriaceae **vs.** *Acinetobacter* spp	68-70%	79-81%	50-65%	61-67%
Pseudomonas spp **vs.** *Acinetobacter* spp	75-80%	81-84%	70-75%	73-77%
***Enterococcus* spp**	85-92-100%	88-95%	72-83%	92-95%
***Streptococcus* spp**	100%	100%	100%	100%
***Staphylococcus* spp**	92-100%	92-100	93.1-100%	92-100%
Enterococcus spp **vs.** Streptococcus spp	70-76%	72-80%	65-74%	73-79%
Enterococcus spp **vs.** *Staphylococcus*	69-73%	70-82%	60-74%	69-74%
Streptococcus spp **vs.** *Staphylococcus* spp	67-68%	69-72%	65-69%	63-66%
Enterobacteriaceae **vs.** *Enterococcus* spp	60-65%	69-72%	50-61%	54-63%
Enterobacteriaceae **vs.** *Streptococcus* spp	57-60%	66-68%	54-58%	51-54%
Enterobacteriaceae **vs.** *Staphylococcus* spp	61-63%	66-69%	71-79%	49-53%
Pseudomonas spp **vs.** *Enterococcus* spp	60-67%	68-74%	49-57%	61-70%
Pseudomonas spp **vs.** *Streptococcus* spp	60-61%	65-68%	67-73%	61-66%
Pseudomonas spp **vs.** *Staphylococcus* spp	65-68%	66-74%	50-54%	60-65%
Acinetobacter spp **vs.** *Enterococcus* spp	53-63%	61-64%	40-52%	50-56%
Acinetobacter spp **vs.** *Streptococcus* spp	59-61%	65-66%	47-52%	57-60%
Acinetobacter spp **vs.** *Staphylococcus* spp	57-60%	64-66%	57-63%	54-61%

* without 10: Similarity values from organism 10, *Stenotrophomonas maltophilia*, Table C.1, not included

Gram-positive bacteria and Gram-negative bacteria did not allow detection of signals after hybridisations using the opposite probes. The value of similarity over the whole sequence ranges from 53-68%.

1.1.1. Focus on Gram-positive bacteria

Real group specificity can only be assigned to staphylococci, as they could not be detected with probes from any other group, including enterococci and streptococci.

Table D.2. Bacterial group-combined values from matrices calculated over whole target sequence, Helix 58, the region upstream and downstream of Helix 58; colour index: **green**: + FISH result; **red**: - FISH result; vs.: versus, compared to each other

Groups of bacteria	Differentiation possible	Range of similarity values for			
		Whole DIII target region	Upstream Helix 58	Helix 58	Downstream Helix 58
***Staphylococcus* spp**	**-**	92-100%	92-100	93.1-100%	92-100%
Enterococcus spp **vs.** *Staphylococcus* spp	+	67-68%	69-72%	65-69%	63-66%
Streptococcus spp **vs.** *Staphylococcus* spp	+	69-73%	70-82%	60-74%	69-74%

Compared to enterococci and streptococci, the similarity over the whole probe binding region is 67-73% (Tables D1 and D.2).

Regarding the matrices calculated over the single sections, the similarity of Helix 58 offers a moderate similarity (65-69% in comparison to enterococci, and 60-74% to streptococci (only one member in matrix). The parts upstream of Helix 58 and especially the parts downstream of Helix 58 in comparison to enterococci yet show very low similarity, too. The overall similarity is in the case of staphylococci compared to enterococci low enough, to enable the differentiation.

Between the closely related enterococci and streptococci differentiation failed in about 50% of attempts after identical, repetitive hybridisations.

Table D.3. Bacterial group-combined values from matrices calculated over whole target sequence, Helix 58, region upstream and downstream of Helix 58; colour index: **green**: + FISH result; **red**: - FISH result; **blue**: + and – results in repeated FISH experiments; vs.: versus, compared to each other

Groups of bacteria	Differentiation possible	Range of similarity values for			
		Whole DIII target region	Upstream Helix 58	Helix 58	Downstream Helix 58
***Enterococcus* spp**	**-**	85-92-100%	88-95%	72-83%	92-95%
***Streptococcus* spp**	-	100%	100%	100%	100%
Enterococcus spp **vs.** *Streptococcus* spp	+ and -	70-76%	72-80%	65-74%	73-79%

These varying results in repetitive hybridisations could be based in variabel target binding which results in differing stability of the binding and might also be influenced by physical aspects (e.g. temperature of hybridisation ovens etc.) and small changes in hybridisation procedure, such as slight changes of salt/buffer concentration changes. The similarity value over the whole sequence ranges from 70-76%. Regarding the part upstream of Helix 58, the value is increased to 72-76%, as is the value from the part downstream of Helix 58, 73-79%. Helix 58 reveals a low similarity between 65-74%.

According to the values mentioned above, probe binding might be possible to regions upstream and downstream of Helix 58, but not directly to Helix 58. The length of the region upstream of Helix 58 is 100 nucleotides (regarding *E. coli* positions), and downstream of Helix 58 it is 75 nucleotides, whereas within Helix 58, only 58 nucleotides are provided. Suggested, that the length of the parts upstream (100 nucleotides) and downstream of Helix 58 (75 nucleotides) allows successful binding of the probes under the right conditions, this binding could be sufficient for establishing a stable network of probe molecules, and the discrimination power of Helix 58 in this case might not be able to affect this probe binding. Non-binding to Helix 58 could be possible while binding of one or more likely several polynucleotide probes to the parts upstream and downstream of Helix 59 can take place. The non-binding parts of the probe molecules otherwise could be involved in inter-probe molecular binding and thus enable and strengthen the formation of a network.

This random binding or non-binding could be influenced by physical aspects as mentioned above.

1.1.2. Focus on Gram-negative bacteria

In the case of Gram-negative bacteria, differentiation is only possible between *Acinetobacter* spp from *Enterobacteriaceae* by *in situ* hybridisation and vice versa, but not for *Acinetobacter* spp compared to *Pseudomonas* spp and *Enterobacteriaceae* in comparison to *Pseudomonas* spp.

Table D.4. Bacterial group-combined values from matrices calculated over whole target sequence, Helix 58,region upstream and downstream of Helix 58; colour index: **green**: + FISH result; **red**: - FISH result; vs.: versus, compared to each other

Groups of bacteria	Differentiation possible	Range of similarity values for			
		Whole DIII target region	Upstream Helix 58	Helix 58	Downstream Helix 58
Enterobacteriaceae (without 10*)	**-**	84-99.6%	99-100%	78-96.6%	94-100%
***Pseudomonas* spp**	**-**	88-94%	87-91%	89-93%	87-92%
***Acinetobacter* spp**	**-**	93-96%	96-100%	86-95%	96-100%
Enterobacteriaceae **vs.** *Pseudomonas* spp	**-**	73-78%	82-86%	65-72%	69-73%
Enterobacteriaceae **vs.** *Acinetobacter* spp	+	68-70%	79-81%	50-65%	61-67%
Pseudomonas spp **vs.** *Acinetobacter* spp	-	75-80%	81-84%	70-75%	73-77%

* without 10: Similarity values from organism 10, *Stenotrophomonas maltophilia*, Table C.1, not included

As already mentioned in C. I. 1.1., the group of *Pseudomonas* spp also showed signals with probes derived from members of *Enterobacteriaceae* and *Acinetobacter* spp, whereas differentiation between *Acinetobacter* spp and *Enterobacteriaceae* was possible.

Pseudomonas spp offer, similarly to the examined staphylococci, a relative equally distributed similarity over the three matrices Helix 58, the region upstream and downstream of Helix 58 of 87-93%.

This could be the reason why the differentiation of *Pseudomonas* spp with DIII probes from *Enterobacteriaceae* is complicated and not possible, though especially the value of similarity of a comparison of Helix 58 between *Enterobacteriaceae* and *Pseudomonas* spp is quite low with a range from 65-72%.

However, the region upstream of Helix 58 obviously offers an increased value of 82-86% in similarity. This high similarity could again be enough for successful binding of the probe in the region upstream of Helix 58 and downstream of Helix 58, especially in combination with the relative high similarity of the region downstream of Helix 58, 69-73%.

As mentioned above, Helix 58 only comprises 58 nucleotides and a non-binding to this region could be possible while at the same time binding of one or, more likely, different polynucleotide probes to the regions upstream and downstream of Helix 59 can take place. The non-binding parts of these probe molecules otherwise could be implicated in inter-probe molecular binding and thus enable and strengthen the formation of a network. The positive results from hybridisations could be a support for this theory.

Table D.5. Bacterial group-combined values from matrices calculated over whole target sequence, Helix 58, region upstream and downstream of Helix 58; colour index: **green**: + FISH result; **red**: - FISH result; vs.: versus, compared to each other

Groups of bacteria	Differentiation possible	Range of similarity values for			
		Whole DIII target region	Upstream Helix 58	Helix 58	After Helix 58
***Pseudomonas* spp**	-	88-94%	87-91%	89-93%	87-92%
***Acinetobacter* spp**	-	93-96%	96-100%	86-95%	96-100%
Pseudomonas spp **vs.** *Acinetobacter* spp	-	75-80%	81-84%	70-75%	73-77%

On the other hand *Pseudomonas* spp compared to *Acinetobacter* spp also show the typical low similarity in Helix 58 with a range of 70-75%, but again the similarity of the region upstream of Helix 58 is increased to 81-84%, what could enable binding due to the same reason as mentioned above.

Table D.6. Bacterial group-combined values from matrices calculated over whole target sequence, Helix 58, region upstream and downstream of Helix 58; colour index: **green**: + FISH result; **red**: - FISH result; vs.: versus, compared to each other

Groups of bacteria	Differentiation possible	Range of similarity values for			
		Whole DIII target region	Upstream Helix 58	Helix 58	Downstre m Helix 58
Enterobacteriaceae	-	84-99.6%	99-100%	78-96.6%	94-100%
***Acinetobacter* spp**	-	93-96%	96-100%	86-95%	96-100%
Enterobacteriaceae **vs.** *Acinetobacter* spp	-	68-70%	79-81%	50-65%	61-67%

Contrarily, the comparison of *Acinetobacter* spp with *Enterobacteriaceae* displays the low value of 68-70% over the whole probe binding region, and especially the region of Helix 58 is highly diverse with a similarity of only 50-65%, comparable to the values of Gram-positive bacteria. The similarity upstream of Helix 58 indeed ranges from 79 to 81%, but is significantly lower than the 81-84% of the comparison *Acinetobacter* spp to *Pseudomonas* spp. Together with the similarity downstream of Helix 58 of only 61-67%, obviously the overall dissimilarity is too high under hybridisation conditions and thus enables differentiation.

In summary, the specificity of DIII targeted polynucleotide probes does not allow a high resolution differentiation of bacteria. The probes are appropriate for applications, where **order or family** specificity is desired. For application to clinical specimen the differentiation scope of the RNA polynucleotide probes tested in this study is high enough to be applied for subsequent research, as occurrence of bacterial groups in real clinical sample is dependent on the specimen. Additionally, prior known information about the specimen, origin of specimen and related numbers of target organism in the specimen than non-target organisms can be expected.

1.2. The Cut Off as a benchmark for future polynucleotide probe design

Based on the results of the similarity matrix, future rRNA directed polynucleotide probe design with the aim to increase the specificity to real family- and perhaps species-specificity could be facilitated: the cut off value of 70-76% in target sequence similarity, in other words, 24-30% dissimilarity, can be used as a benchmark necessary for differentiation when new rRNA targeted polynucleotide probes are developed.

Thus, polynucleotide probe design targeting not only DIII of the 23S rRNA can be considered, but also domain I and II, (already performed exemplarily from Zwirglmaier et al, 2003) as well as domains IV, V, VI of 23S rRNA, 16 S rRNA and spacer rRNA.

Demonstrated on an example, the use of a polynucleotide probe targeting only Helix 58, (with a proposed length from 50 to 60 nucleotides) applied for differentiation between the families of *Enterobacteriaceae* and *Pseudomonaceae* (similarity 65-72%) will possibly result in the desired specificity.

The construction of a special software (possibly implemented in already existing alignment and probe design program packages such as ARB), with the aim to enable the design of polynucleotide probes by using filters that account the cut off value of 70-76% for the species to be differentiated, could make the process of probe design easy. The minimum probe length of this future polynucleotide probe design targeting rRNA is proposed to be 50 nucleotides, when formation of halo is desired, based on the results of Zwirglmaier et al (2003).
Moreover, the development of the software mentioned above could be used not only for facilitating the polynucleotide probe design for rRNA targeted probes, but also for the design of RINGFISH probes, if the validity of the benchmark can be confirmed in this area, too.

4. The cell wall of Gram-positive bacteria: the great barrier

Solutions for applicability of polynucleotide probes to Gram-positive bacteria

The chemical structure of the cell wall of Gram-positive bacteria is highly variable among different genera and species and has been used for chemotaxonomic differentiation of these organisms (Schleifer, K. H., Kandler, O., 1972). With the development of FISH, this interstrain variability rendered several genera difficult for *in situ* hybridisation. The rigid peptidoglycan layer of Gram-positive bacteria has a thickness of 30-80 nm, which is considerably higher compared to 10 nm of the cell walls of Gram-negative bacteria. Most of the cell walls of Gram-positive bacteria generally require a step of permeabilisation of the cell walls before any kind of *in situ* hybridisation, especially for polynucleotide probe hybridisations. Exceptions from this rule are e.g. some Gram-positive bacilli, where halo signals were obtained without any pretreament (Fichtl, unpublished).
For Gram-positive bacterial FISH using oligonucleotide probes, a standard pretreatment with lysozyme was applied, which acts as muramidase by hydrolysing specifically the ß-1-4-glycosodic bond between N-acetylmuramic acid and N-acetylglucosamin. In combination with two ethanol series (before and after), this enzymatic treatment is generally enough to allow access for the 15-20 nucleotide long oligonucleotide probes. Protocols developed for rapid detection of Gram-positive bacterial cells using FISH in clinical samples also suggest an incubation of fixed

sample material with different lysis buffers, containing e.g. lysostaphin (a glycyl-glycine hydrolysing endopeptidase) for permeabilisation of *Staphylococcus* spp (Krimmer et al, 1999, Jansen et al, 2000).
This standard method is insufficient for polynucleotide probe hybridisations applied on Gram-positive bacteria. Former studies revealed, that even in between the different genera of Gram-positive bacteria, the conditions of accessibility of target structures vary considerably (Fichtl, unpublished, Sandjong, unpublished, Ludyga unpublished). Often, the species- and strain-specific characteristics in the cell wall composition necessitated different pretreatment protocols even between two closely related strains, e.g. decreased incubation with enzymes or heat for two species of streptococci (Lydiga, unpublished). In this work and two diploma related theses (Sandjong 2003, Ludyga 2004), several protocols could be established for different kinds of pretreatments for the three project relevant groups of Gram-positive bacteria: enterococci, staphylococci and streptococci, listed in Table B.2 and B.3.
The protocols proposed in Table C.3 allow controlled and moderate pretreatment for permeabilisation of the different Gram-positive cell walls (Figs.C.4 and C.5). A stable formation of the network is assured, which is necessary for subsequent enrichment hybridisations and results in a homogeneous halo signal similar to that observed for Gram-negative bacteria. The vast majority of the cells still showed cellular integrity, without losing shape or bursting.

The efficiency of the pretreatment protocols could also be confirmed after successful application to clinical samples. All polynucleotide probe FISH based detections of Gram-positive bacteria in this work were performed after application of the special protocols developed during this work. In addition, the protocols were used before hybridisations with RINGFISH probes to Gram-positive bacteria, e.g. for RINGFISH probes targeting virulence factors of *Streptococcus pyogenes* and *Streptococcus agalactiae* (Ludyga, unpublished) or the antibiotic resistance gene *ermB* of *Enterococcus faecium,* as well as *parC* gene of *Enterococus faecalis* in this work, see further C.II.4.

5. Time is money: reduction of time

Time reduction assays

The time required for the whole approach of cell or sample fixation for hybridisation in solution, enrichment hybridisation and subsequent examination of the enrichment hybridisation ranges from 11-23 h, if the standard protocols for single steps are used. To make the whole procedure more time efficient (especially with regard to clinical applicability) a time reduction was focussed.
In this work, it was examined whether shortening of single steps within the whole method still allows an equivalent enrichment success without losing cell conservation and integrity, signal intensity of the halo or binding capacities of hybridised bacterial cells to complementary nucleic acids in microplate cavities during the enrichment hybridisation itself.
Two steps in the whole procedure could be regarded as potentially amenable for time reduction: the duration of the fixation time and the time of the first hybridisation for binding of the polynucleotide probes to their ribosomal target structure.

The comparison of different fixatives for the application of polynucleotide probes on real clinical samples spiked with target bacteria revealed that PFA is the fixative, which best fulfils the special demands on polynucleotide probe hybridisations. The demands consist of the formation of a stable network of probe molecules, which afterwards allowed the probe based enrichment hybridisation in microplates and. Though not as fast as ethanol fixation, but moderate in effort, it proved superior to Bouin`s fixation, which required more time and effort, and formalin, which also could not be performed in a fast way.
In a series of hybridisations with cells that had been fixed for 0.5-5 h, it could be shown, that the halo formation was not affected by a fixation time of only 0.5 h, neither with Gram-negative nor with Gram-positive bacteria.
Application in enrichment hybridisations did not lead to loss of amplicon intensity when detected via PCR or loss of signal intensity after photometrical detection (Figs.C.16 and C.17).
For shortening the hybridisation time, the step of microwaving the cells before subjecting them to hybridisation was very effective. As reported by Franks et al (1998) and Lan et al (1996), microwaving microbial cells or sample material before

FISH is a widely used method in all fields of FISH applications, which can be applied to enhance the accessibility of the target structure, permeabilise the cell walls and trigger the specificity of probes. Representatives of Gram-positive and Gram-negative bacteria, fixed for standard times were hybridised with polynucleotide probes for 20, 40 and 60 minutes. No difference in comparison to standard time hybridised cells with regard to halo signal, cellular integrity or specificity was observed (Fig.C.18).

In the course of this study, it was possible to show qualitatively and quantitatively that the combination of both time reduction methods is possible. The quantification of the signal intensities using the DAIME software package, (Daims, unpublished) compared the signal intensities from microbial cells that were fixed and hybridised according to the minimum of the standard protocol (with a fixation of 5h and hybridisation of 9h), to the signal intensities of cells, that were subjected to highly reduced (0.5, 1, 2, 3, 4, and 5h) fixation times and hybridisation times (20, 40, 60min), see Figs.C.20 and C.21. There were no significant differences.

Possible reasons for low signal intensities in comparison to the standard could also be based in image acquisition from randomly chosen microscopic visual fields. Often, cell layers or agglomerates of cells have to be taken into account for calculation, thus changing slightly the overall result.

Especially microscopic analysis did not reveal differences in the nature or intensity of the halo signal. In comparison to the standard procedure (11-23h), the combined time reduced protocol resulted in 1.5-3.5h for the whole hybridisation based approach (Table C.5).

Furthermore, the time reduced hybridisation protocol could successfully been used for all enrichment hybridisations performed from spiked clinical samples and for one example of enrichment hybridisation from real clinical samples, showing the transferability to real sample material under natural conditions.

6. Application of DIII 23S rRNA targeted polynucleotide probes to clinical samples

6.1. Role of natural compartments and possible reasons for enrichment failure

6.1.1. Characteristics of EDTA blood, plasma and urine

EDTA blood

EDTA blood consists of erythrocytes, leukocytes and other cellular structures. The pH ranges from 7.38 to 7.42. Numerous electrolytes are dissolved: Na^{+} (135-145 mmol/l) and additionally K^{+}, Ca^{2+}, Mg^{2+}, Cl^{-}. EDTA is added as an anti-coagulans. It acts by inhibiting Ca^{2+} ions. During hybridisation, this could cause a shift in the conditions of the hybridisation buffer, resulting in decreased probe binding to bacterial target cells. Red blood cells have a size 10-20 fold larger than bacteria, and additionally, bacterial cells often stick close to these cells, so that successful binding of probe molecules to the target organisms could be physically inhibited, too. Especially the binding of the three-dimensional network to complementary nucleic acids during the second hybridisation step for the enrichment of microbial target cells in microplate cavities could be inhibited to a high degree.

Plasma

Plasma is the liquid blood component and contains proteins, lipids, amino acids, salts and water. This results in a viscous structure, which can embed bacterial cells in a matrix and hamper access of the polynucleotide probes to the ribosomal target structure during first hybridisation and contact to complementary nucleic acids in microplate cavities during the second hybridisation (see further 4.1.2.).

Urine

Urine can contain erythrocytes, leukocytes, different epithelial cells, several cristalline components such as calciumoxalate, uric acid, triphosphate and other salts, but the accessibility of bacteria is generally not physically impeded.

6.1.2. Reasons for enrichment failure

On the one hand, successful hybridisation in solution (enabling the first binding of the polynucleotide probes to their ribosomal target structures) is dependent on the fixation method of the sample material, which could have an influence, as some fixation methods might lead to better signals than others (see Table C.6).
On the other hand, this hybridisation could also be negatively affected by components of the sample, as mentioned for EDTA blood, or physical inhibition of hybridisation due to growth of bacteria in agglomerates, sticking to organic matter or embedding in an impermeable matrix (e.g. tracheal swabs later discussed).
Nevertheless, the polynucleotide probes are “mobile” during the first hybridisation, agitated by Brownian molecular movement, and failure is quite more unlikely than in the following steps.

The second hybridisation for enrichment of bacterial target cells does not allow “mobility” of the bacterial cells and is highly dependent on direct contact of the hybridised bacterial cells to the complementary nucleic acids on the bottom of the microplate cavities. If the contact is inhibited, e.g. when the hybridised bacteria simply can not contact the complementary nucleic acids because they are embedded in a matrix, no second hybridisation for immobilisation of the cells can take place.
Another reason is the possibility of PCR biases, e.g. if the PCR reaction is disturbed by residues of hybridisation buffer ingredients, or if the number of immobilised target cells is simply not high enough to allow detection (depending on the particular PCR sensitivity and successful break of the examined cells).

6.2. Spiked clinical samples

Spiked clinical samples were used to examine the applicability of 23S DIII targeted polynucleotide probes and the polynucleotide probe based enrichment technique. After evaluation of the best fixation with respect to the most important parameter, the characteristic halo signal, the results obtained from spiked clinical samples provided the basis for all following hybridisations with real clinical samples. Furthermore, it was possible to estimate the detection limit by using spiked clinical samples, which was in the particular cases estimated $8x10^3$ cells. Real clinical samples of EDTA blood, urine and plasma were spiked in different dilutions (10^{-1}-10^{-4}) with pure culture organisms as target bacteria.

Detection of halo signals after application of 23S rRNA DIII targeted polynucleotide probes on slides turned out to be possible for all organisms tested and in all dilutions of the particular sample material. A precondition was to find the optimum fixative for the samples with respect to different important parameters: time, effort and halo signal. Table C.6 exemplifies, that PFA fixation leads to the best results when all three parameters are considered, especially the most important feature, the halo signal, as this is the preconditions for enrichment hybridisations.

Polynucleotide probe based enrichment hybridisation for specific cell fishing from spiked environmental samples has already been successfully applied by Zimmermann (2002) using DNA polynucleotide probes targeting the same sequence.

The results obtained after enrichment hybridisations of spiked clinical samples (see Table D.7, copy of C.8) in this work revealed different facts. Generally, it is easier to enrich Gram-negative bacteria, as pretreatment is not necessary and the halo formation is relatively stable even in clinical samples. The pretreatment protocols work well for Gram-positive bacteria, but nevertheless can lead to increased loss of target cells due to several additional washing steps.

The efficiency of the pretreatment protocols may be hampered or aided by natural components of the sample material or the growth state of the organism. The impact of pretreatments can be different in spiked bacteria and pure cultures, according to the point mentioned before. The success of enrichments depends on the sample material especially for Gram-positive bacteria: Gram-positive bacteria could not be enriched from spiked blood samples and plasma samples.

Table D.7. Successful enrichments from spiked clinical sample material; n.r.: no result; n.t.: not tested; * Enrichment success confirmed by PCR, but not by cloning and sequencing.

Spiked organism / sample material	Enrichment successful in dilution		
	EDTA blood	urine	plasma
E. coli	10^{-1} - 10^{-2}	10^{-1}	10^{-1} - 10^{-3}
Pseudomonas aeruginosa	10^{-1} - 10^{-4}	10^{-1} - 10^{-4}	10^{-1} - 10^{-4}
Enterobacter aerogenes	10^{-1} - 10^{-2}	n.r.	10^{-1} - 10^{-4}
Stenotrophomonas maltophilia*	10^{-2} - 10^{-4}	10^{-1}	n.r.
Haemophilus influenzae*	n.t.	10^{-1} - 10^{-2}	n.t.
Klebsiella oxytoca	n.t.	10^{-1} - 10^{-2}	n.t.
Enterococcus faecium	n.r.	10^{-1} - 10^{-4}	n.r.
Enterocccus faecalis	n.r.	10^{-1} - 10^{-4}	n.r.

In summary, all enriched organisms that were detected with conservative 23S PCR, could be allocated to the organism the samples were spiked with, though many other bacteria may have been present in the natural sample as well.
The application of the 23S DIII targeted polynucleotide probe based enrichment technique could successfully be applied for the specific cell fishing from the spiked clinical sample within the scope of the mentioned probe specificity and the potential restrictions due to inhibiting ingredients and conditions.

6.3. Real clinical samples

Detection of microorganisms in clinical samples using olignucleotide FISH is a standard and highly valuable method for specific and rapid screening and identification of pathogenic microorganisms in clinical specimen without cultivation (e.g. Kempf et al, 2002; Hogardt et al, 2000).

Polynucleotide probes have successfully been applied to environmental samples (DeLong et al, 1999, Zimmermann et al, 2002, Pernthaler et al, 2002, Zwirglmaier et al, 2003) but their applicability and behaviour in clinical samples has not been tested up to now. The applicability to clinical specimen was successfully shown for spiked clinical samples in this work. The successful transfer of the technique to real clinical samples was the next challenge.

The success of DIII targeted polynucleotide probe hybridisations on slides demonstrated the applicability of the probes for the detection of bacteria in clinical samples. The same reasons which hamper successful polynucleotide hybridisations and especially enrichment hybridisations in spiked clinical samples (see D.4.1.) are also true for polynucleotide FISH on real samples, too. But additionally, more challenges emerge, because several facts are unknown and / or can not be influenced or estimated:

1. **Amount of sample material:** varying with sample and patient.
2. **Number of target organism**: depending on the sample material, the point in time of sampling during infection and the consistency of sample, the number of the target bacterium can vary greatly.
3. **Growth of target bacterium in real sample**: during infection, different states of microbial growth have great influence; possibly (supplied) antibiotic medication may have slowed or stopped growth or bacteria could be embedded in a polymatrix (e.g. agglomerated or stuck to matter in the sample).
4. **Natural components of sample material**: role as possible inhibitors of hybridisations, see D.4.1.2.

The applicability of the polynucleotide probe based enrichment hybridisation on real clinical samples was demonstrated for 60% of all available sample material tested (see Table C.10). From three urine samples with Gram-negative and Gram-positive bacteria as targets (*Klebsiella* sp., *E. coli* and *Enterococcus faecium*), two different blood culture samples (*E. coli, Staphylococcus aureus*) and one wound smear (*Pseudomonas aeruginosa*), the target bacterium could be successfully detected using PCR with universal primers, the PCR product cloned and sequenced for confirmation of the identity (see Table C.11).
Two further target organisms could be successfully enriched from their real clinical habitat, *Staphylococcus aureus* from a wound smear and *Klebsiella pneumoniae* from a tracheal swab, but the PCR products that were cloned did not lead to clones with the correct size of insert in the plasmid, so the identity of the bacterial cells enriched could not be checked by sequencing.

The samples from which enrichment hybridisations failed completely were available in a too small amount to perform several parallel attempts and/or the occurrence of the target organisms was stated in the patients' record to be very sporadic.
For sample number 4 (Table C.10), a tracheal swab containing *Pseudomonas aeruginosa*, though available in sufficient quantity, no enrichment was successful, though detection of *Pseudomonas aeruginosa* was possible on slides with DIII targeted polynucleotide probes as well as with PseAer oligonucleotide probe. As demonstrated in image Fig.C.23 B, the bacteria are embedded in a thick and highly viscous matrix. Obviously, it is possible for the oligo- and polynucleotide probes to enter the matrix and bind to the target structures, but the bacteria are nevertheless surrounded by the matrix. For successful enrichment, it is necessary to allow hybridised cells to bind to the complementary nucleic acids on the carrier material, as already discussed in point D.4.1.2. Thus, the matrix could have inhibited this second hybridisation simply by preventing direct contact of the bacteria to the cavities.

The polynucleotide probe based technique used for the enrichment of whole bacterial cells from real clinical samples could already be successfully applied in the context of the project this work was involved in. In cooperation with project partner ITB (Institute of Technical Biochemistry, Stuttgart), it was possible to use the genetic information of immobilised cells from one real clinical sample after an enrichment hybridisation for the microarray based analysis of antibiotic resistance genes.

7. Possible applications

The application of 23S DIII targeted polynucleotide probe based enrichment on clinical samples could allow large scale enrichments from real clinical samples within the given scope of specificity, as long as the sample material is available in amounts that allow performing the procedure in several parallel attempts and the numerous occurrence of the target organism is ensured. Combined afterwards with further methods, e.g. a subsequent screening for antibiotic resistance genes in the immobilised microorganisms, it could be used for special specific diagnostics in hospitals.
To overcome the limited specificity of DIII targeted polynucleotide probes, a development of shorter probes targeting parts of the same region (in consideration of

the results obtained from analysis of the similarity matrices), but still offering the advantage of suitability for enrichment hybridisation could increase specificity, possibly to species-specificity.
Nevertheless, oligo- and also polynucleotide probe FISH presents a fixed-end methodology and detects only the target organisms at which it is aimed, whereas classical subculturing presents an open-end methodology with the possibility to detect a broader range of possible pathogens (Jansen et al, 2000). On the other hand, FISH allows detection of all targeted species present in the sample at the point of sampling (within the limits of the oligo- or polynucleotide probes used) without the danger of losing pathogens which require special culture conditions not provided in clinical routine subculturing.

II. RINGFISH probes - the quest for the Holy Grail

The development of RINGFISH (*R*ecognition of *In*dividual *G*enes, Zwirglmaier et al, 2004) and the extension of the enrichment technique to RINGFISH probes opened the door to an up to that time point seemingly impossible goal: to have the possibility not only to visualise the existence of single genes in individual bacterial cells, but also to use the special nature of the signal, the halo, for the enrichment of target cells via binding to complementary nucleic acids on different types of carrier surfaces.

1. Role of secondary structures

The formation of secondary structures and the ability to allow hybridisations between several probe molecules are very important features in the context of hybridisations with polynucleotide probes. On the one hand, the more or less complete denaturation of the probe molecules is required for a successful binding of the probe molecules to the target sequence, which is, compared to ribosomal targets (10^4- 10^5per cell) only available in very limited numbers (below 10 per cell, dependent on cellular growth state). On the other hand, the steps of renaturation, of formation of secondary structures and inter-probe hybridisations, resulting in the network of probe molecules, are necessary to visualise the successful binding of the probe molecules to the target sequence.

The ability to form secondary structures is, apart from other factors, one particular precondition for formation of a network, and thus a resulting halo signal. The construction of different synthetic polynucleotide probes showing reduced or lacking ability to form secondary structures led to the observation of whole cell fluorescence (Zwirglmaier et al, 2004; this work).

2. Application of RINGFISH probes: antibiotic resistance genes as target genes

The bacterial strains and real clinical samples chosen for application of RINGFISH methodology were received from Robert Bosch hospital and contained several resistances against antibiotics which were revealed using standard clinical screening methods. Clinical reports documented the findings for the bacterial isolates after routine screening. The genes that were chosen as RINGFISH targets belong to different groups of antibiotic resistance genes and encode ß-lactamases (*ampC*), efflux pumps (*tetAJ*), methylases (*ermB*) and topoisomerase IV (*parC*). The modes of action of the respective antibiotics differ:

- **ß-lactam antibiotics** are competitors for PBP (penicilline binding proteins) during cell wall synthesis; PBP bind preferably to ß-lactam antibiotics than catalysing the transpeptidation reaction during cell wall synthesis; crosslinking of two glycan linked peptide chains fails, therefore the cell wall weakens. In addition to that, an increased release of autolysins is stimulated by the PBP-antibiotic complex; the cells lyse because of osmotic pressure differences between inside and outside of the cell.
- **Tetracyclines** inhibit the binding of aminoacyl-t-RNA to the ribosomal A-site during translation at the 30S subunit of the ribosomes (Lengeler et al, 1999).
- **Macrolide antibiotics** like erythromycin act during early protein synthesis: the drug blocks the growth of the nascent peptide chain by inhibiting the movement of the peptidyl-t-RNA from the A-site to the P-site of the ribosome (translocation); the assembly of a new large ribosomal subunits is impeded, and a gradual depletion of functional ribosomes in the cell follows (Vester et al, 2001).
- **Fluoroquinolone antibiotics** act during nucleic acid synthesis: binding to subunits of DNA Gyrase subunit GyrB and topoisomerase IV subunit ParC

leads to inhibition of DNA replication but can also inhibit subunits of DNA-dependent RNA polymerases (RpoB).

The mode of resistance against these antibiotics also differs:

In case of ß-lactam antibiotics, genes encoding ß-lactamases cleave the ß-lactam ring of the antibiotic, after it has entered the cell. Enterobacterial *ampC* is a chromosomal encoded resistance gene that codes for a ß-lactamase that is able to hydrolyse ß-lactam antibiotics (Volkmann et al 2003).

The mechanism of resistance to tetracycline antibiotics is either based on genes encoding efflux pumps, or based on genes encoding ribosomal protection proteins (Chopra et al, 2001).

Resistance against macrolide antibiotics confers to methylases that methylate an adenosine-2058 residue of bacterial 23S rRNA, encoded by the *erm* (erythromycine ribosome methylation) gene.

Fluoroquinolone resistance is caused by point mutations of the binding site at the topoisomerase I (DNA gyrase B subunit) and IV (Petersen, 2003).

3. Evaluation of RINGFISH conditions for clinical strains

The application of the standard protocol (proposing the usage of 5-15% of formamide in the hybridisation buffer, Zwirglmaier et al 2003) led to positive hybridisation signal for polynucleotide probes TetAJV1 and V2 designed for a clinical strain of *Proteus mirabilis*.

Hybridisation under standard conditions with RINGFISH probes EcoAmpCV3, designed for two clinical strains of *E. coli*, RINGFISH probes ermB1 and ermB2, designed for one clinical strain of *Enterococcus faecium*, and RINGFISH probe parC, designed for one strain of *Enterococcus faecalis* , did not lead to positive hybridisation signals.

Therefore, some changes of hybridisation conditions were necessary: the concentration of formamide was increased, while the temperature was not changed.

A reduction of hybridisation time to 20 hours was effective in some cases. Table D.8 summarises the different conditions that are necessary to obtain halo signals or halo like signals with the respective probes.

The question to be discussed here is, whether there is a relation between all different parameters like GC content of probe and target, denaturation of secondary structures of the probe molecules, denaturation of the target and examined conditions that are necessary for particularly positive hybridisations.

Table D.8. Characteristics of all developed RINGFISH probes and conditions required for hybridisation and DIII 23S rRNA targeted poylnucleotide probe DIII as comparative

Target / probe	Length of probe	Probe / target GC content [%]	Hybridisation conditions FA [%]	Complete denaturation of probe molecule *in silico*	Real temperature [°C] denaturation (left) and hybridisation (right)		Artificial temperature* [°C] denaturation (left) and hybridisation (right)	
ermB1	402	37%	0%	86°C	80°C	53°C	80°C	53°C
ermB2	302	37 %	0%	86°C	80°C	53°C	80°C	53°C
parC	182	42,3%	20-60%	98°C	80°C	53°C	91.6-114.8°C	64.4-87.8°C
TetAJV1	278	40%	10%	91°C	80°C	53°C	85.8°C	58.8°C
TetAJV2	420	39%	10%	95°C	80°C	53°C	85.8°C	58.8°C
EcoAmpCV3	990	50%	80%	120°C	80°C	53°C	126.4°C	99.4°C
DIII general	225-300	50-57%	50-80%	94°C	80°C	53°C	109-126.4°C	82-99.4°C

* artificial temperature: physical temperature at denaturation 80°C and hybridisation 53°C plus Formamide rule of increase of 0.58°C per 1 % formamide; values calculated using RNAdraw (B.XIV.2)

4. Relation between GC content of probe, denaturation of secondary structure of the probe molecules, the target and the GC content of the target

In silico analysis of denaturation of secondary structures, melting temperature and GC content of the probe molecules and a comparison with the particular hybridisation conditions, especially concerning temperature and formamide concentration, revealed that there might be a correlation between these characteristics for each single target and probe which has to be considered when successful binding of the RINGFISH probe to target structures is desired.
In fact, the comparison of the optimal hybridisation conditions for all different designed probes in his work demonstrates the correlation and gives a hint that the target has to be considered as well, when searching for the best conditions with respect to specificity, intensity and most of all occurrence of a (halo-) signal.

Polynucleotide hybridisations consist of two steps, differing in temperature: at the beginning, a step of 20-30 minutes at 80°C is necessary to resolve secondary structures of the probe molecules and allow them to get access to the target cells. The chromosomal or plasmid single or oligo copy target should be denaturated and made accessible during that step as well. After this denaturation step the hybridisation takes place at a lower temperature to ensure the reformation of secondary structures, to enable probe-probe hybridisations, resulting in a network formation, and of course stringent probe hybridisation.

The real physical temperature conditions are equal for all probes (80°C at the denaturation step and 53°C during hybridisation). For estimation of the artificial temperature, the rule of an increase of 0.58°C per 1% formamide in the hybridisation buffer (Schmid et al 2001) was assumed. The following conclusions can be drawn from table D.8:

1. The higher the GC content of the probe (and therefore the target) the more percent of formamide in the hybridisation buffer has to be used, to enable the denaturation of probe molecules as well as the accessibility of the target structures during the denaturation step, as illustrated on Figs. D.3 –D.4.

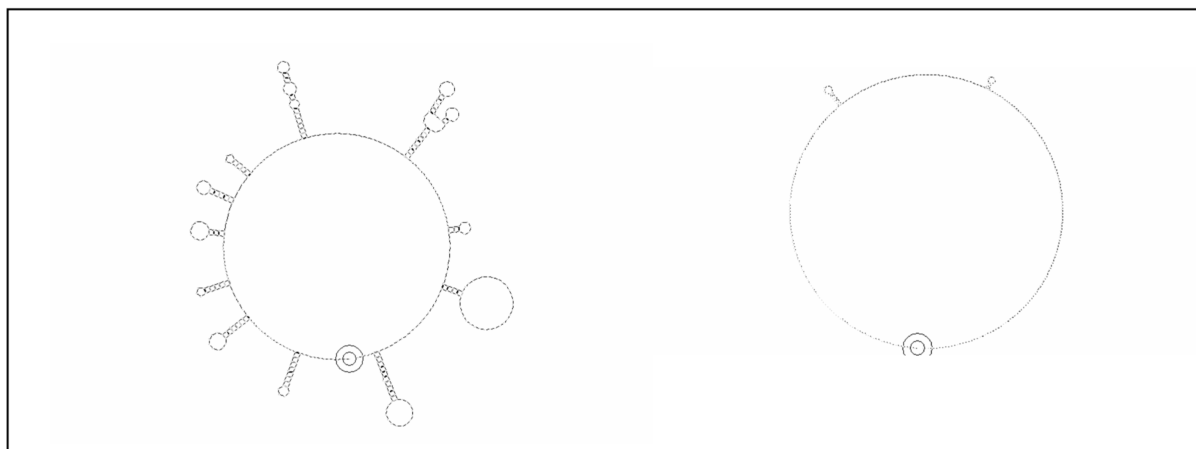


Fig.D.3. Secondary structure model of RINGFISH probe ermB2, GC content 37%, 0% formamide in the hybridisation buffer; software RNAdraw (see B.XIII.2); the circle marks the first nucleotide
Left: under hybridisation conditions 53°C; free energy value -27.01 kcal
Right: under denaturation conditions 80°C; free energy value -0.73 kcal;

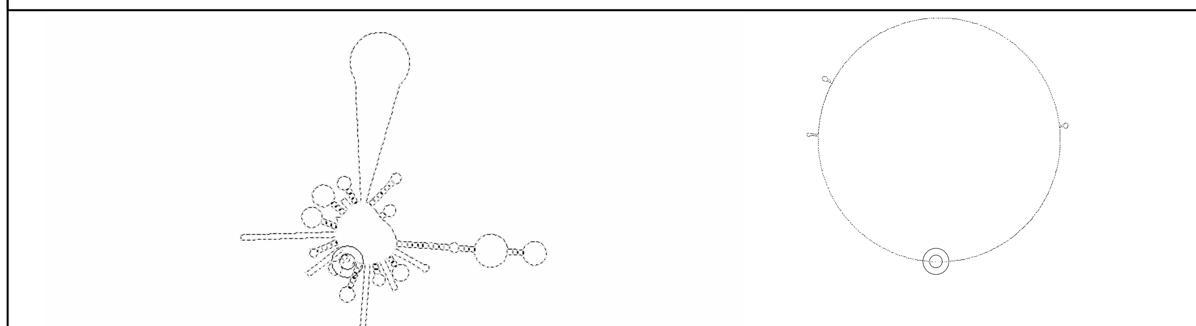


Fig.D.4. Secondary structure model of RINGFISH probe TetAJ V2, GC content 39%; 10% formamide in the hybridisation buffer; software RNAdraw (see B.XIII.2); circle: see Fig.D.3.
Left: under hybridisation conditions 53°C (58.8°C); free energy value –22.73 kcal
Right: under denaturation conditions 80°C (85.8°C); free energy value –1.1 kcal

2. The more formamide is used, the more increased is the denaturation of secondary structures also under hybridisation conditions at 53°C; sometimes, the secondary structures of the probe molecules are still more or less denaturated under hybridisation conditions (see Table D.8. for probe parC and EcoAmpCV3), but halo signals or halo-like signals are obtained. For these cases, this could be a hint, that the cellular environment influences the target accessibility as well as possible network formation.

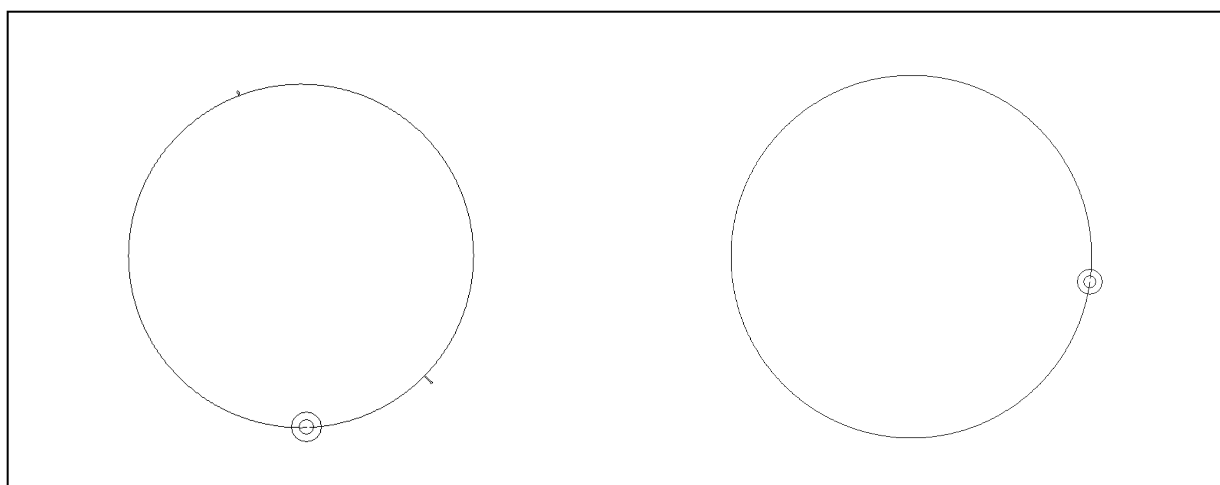


Fig.D.5. Secondary structure model of RINGFISH probe EcoAmpCV3, GC content 50%; 80% formamide in the hybridisation buffer, software RNAdraw (see B.XIV.2); circle: see Fig.D.3.
Left: under hybridisation conditions 53°C (99.4°C); free energy value –0.1 kcal
Right: under denaturation conditions 80°C (126.4°C); free energy value 0 kcal

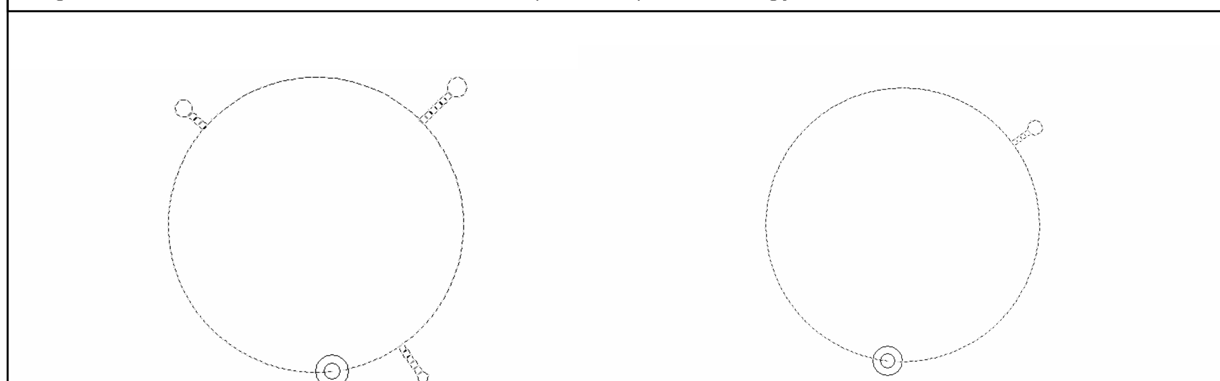


Fig.D.6. Secondary structure model of RINGFISH probe parC, GC content 42%; 20% formamide in the hybridisation buffer, software RNAdraw (see B.XIV.2); circle: see Fig.D.3.
Left: under hybridisation conditions 53°C (65°C); free energy value –7.89kcal
Right: under denaturation conditions 80°C (93°C); free energy value -0.76 kcal

3. If the GC content and therefore the melting point of probe and target is lower and allows melting next to the artificial temperature (see Table D.8.), which is apparent in the denaturation step, the target should be denaturated as well or even better accessible for the probe molecules.

4. The length of the probes does not seem to play a major role for obtaining positive hybridisation signals; as long as the target is accessible for binding of probe molecules (e.g. probe parC counts 182 nucleotides, whereas probe EcoAmpCV3 has a length of 990 nucleotides).

The examined RINGFISH probes show great consistency with these proposals, whereas the DIII targeted polynucleotide probes (here given as comparative example) are similar to proposal 2: under the given conditions for hybridisations with 50-80% of formamide in the hybridisation buffer, the probe molecules show only small loops or no secondary structures, similar to RINGFISH probes EcoAmpC and ParC. However, rRNA targeted polynucleotide FISH can not be compared to RINGFISH, because the accessibility of the ribosomal target is often unproblematic, whereas the accessibility of the chromosomal target is completely unknown. The probe molecule denaturation and renaturation are not solely crucial for successful binding to the target and thus detection of halo signals, but the target has an influence that can not be estimated in the case of RINGFISH probes.

The composition of the probe molecules and perhaps to an even higher degree, of the target for the particular RINGFISH probe, has to be considered in future applications.

5. Application of RINGFISH in real clinical samples

The application of RINGFISH to real clinical samples for detection and enrichment of bacteria is hampered by the same factors as in DIII targeted polynucleotide hybridisation. The factors can even play a more important role, as the number of potential targets in the cells is highly reduced and can, as a consequence, often lead to failure.

The RINGFISH based detection of the target organisms in the particular real clinical sample was successful for probes ermB1 and ermB2, EcoAMpCV3 and parC (C.II.4.2.).

The real clinical samples that were available for testing the developed RINGFISH probes for their suitability in enrichment hybridisations allowed the specific cell fishing of the targeted organisms (*E. coli* from real clinical samples number 1 and 2 using the respective EcoAmpC RINGFISH probes).

In both cases, PCR detection of the immobilised bacterial cells after the enrichment based on the respective EcoAmpC RINGFISH probes, confirmed the identity.

6. Possible applications

Possible application for RINGFISH on clinical samples could be the detection of antibiotic resistance genes and specific cell fishing in one step. Depending on the different genes coding for individual antibiotic resistances, it could be possible to achieve specific detection of resistance genes in combination with specific oligonucleotide probes for providing phylogenetic identification in one hybridisation step. If further applied on enrichment hybridisation, the entire genetic information of the specifically fished microbial cell can be provided for additional genetic examinations. Especially in the hospital environment, the transfer of genetic material i.e. antibiotic resistance genes among endogenous nosocomial pathogens could be monitored. Application to environmental samples is conceivable.

III. Synthetic construct probes

1. Development of synthetic construct probes: DNA probes versus RNA probes

1.1. Synthetic DNA polynucleotide probes

Application of synthetic DNA polynucleotide probes with the aim to combine the advantages of species-specific oligonucleotide probes with the network forming polynucleotide probes turned out to be ineffective (see C.III.1.). The most important drawback of these probes was the absence of halo signals. Modifications of the hybridisation conditions (increase or decrease of formamide concentration or temperature, omitting or elongating of the washing step, alterations in hybridisation time) did not lead to changes in the nature and appearance of the signals: complete whole cell fluorescence was obtained, though *in silico* analyses indicated possible secondary structure formation for every single probe (Figs.D.7 and D.8).

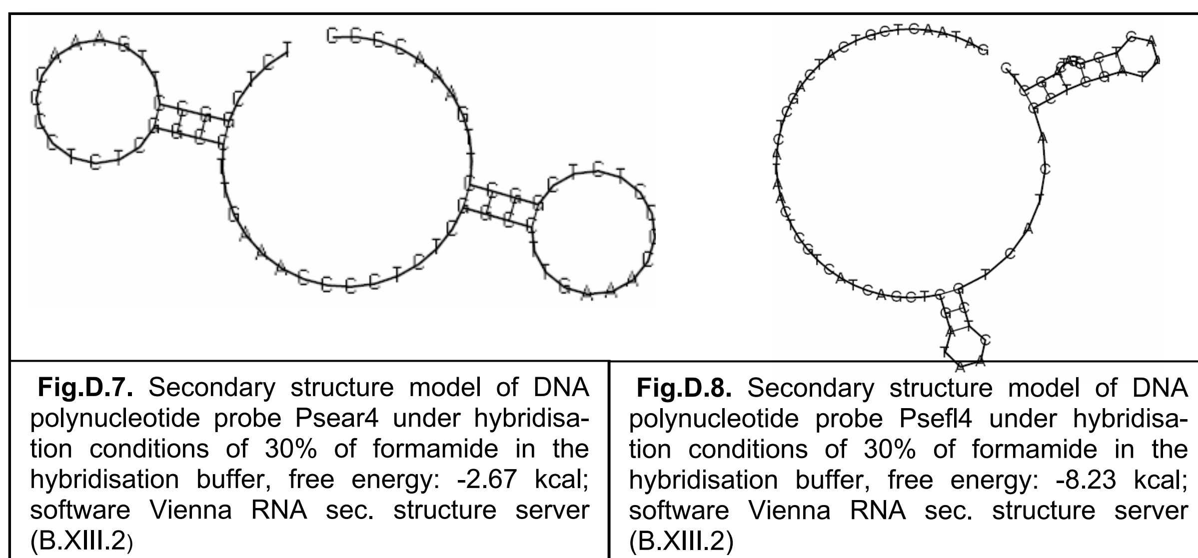


Fig.D.7. Secondary structure model of DNA polynucleotide probe Psear4 under hybridisation conditions of 30% of formamide in the hybridisation buffer, free energy: -2.67 kcal; software Vienna RNA sec. structure server (B.XIII.2)

Fig.D.8. Secondary structure model of DNA polynucleotide probe Psefl4 under hybridisation conditions of 30% of formamide in the hybridisation buffer, free energy: -8.23 kcal; software Vienna RNA sec. structure server (B.XIII.2)

No specificity was given except for probe PseAer4, consisting of a four time repetition of the oligonucleotide probe PseAer (see Table B.16), which allowed differentiation. As other studies demonstrated halo signals derived from RNA polynucleotide probes of only 50 nucleotides in length (Zwirglmaier et al, 2003), indicating network formation and suitability for enrichment hybridisations, a length of 72 to 76 nucleotides for the synthetic probes was considered to be sufficient. Besides, specific signals of similar constructs were reported by Macian (unpublished) and Zimmermann et al (2002). The DNA polynucleotide probes developed in this work could not confirm these prospects.

It has to be assumed, that not only the length of a probe and the *in silico* ability to form secondary structures and perform inter-probe hybridisations play a role for halo formation. Other possible factors are the reduced binding energies of DNA-DNA and DNA-RNA hybrids in comparison to RNA-RNA bindings and the way of labelling.

The transcriptional generation of rRNA targeted polynucleotide probes of different lengths leads to multilabelling with the use of e.g. Fluorescein-12-UTP or Biotin-16-UTP to unlabelled UTP in a ratio of 3:2. These fluorochromes are large in size and lead to a shift of up to 200 or more nucleotides (depending on the labelling rate) if loaded on an agarose gel in comparison to the size of their PCR template or an unlabelled polyribonucleotide probe.

The synthetic DNA polynucleotide probes used in this study consist of a length of 72 to 76 nucleotides and are mono-labelled at the 5´end (probe Stemal_4 and PseAer_4) or oligo-labelled using BiotinChemLink (Roche, Mannheim, Germany), with approximately every tenth base labelled, which could result in app. 7 labels per probe.

Thus, the three dimensional space filled by these synthetic polynucleotide probes could be significantly smaller than that of multilabelled RNA polynucleotide probes even of short lengths. A complete entry into the target cells during the denaturation step and behaviour similar to that of oligonucleotide probes during hybridisation is possible.

Though the labelling itself should not affect hybridisation, it might have a sterical effect, which is more pronounced with increasing labelling rate; this sterical effect could benefit network formation, as there might be a preferential formation of probe-probe hybridisation instead of formation of intra-probe secondary structures, thus facilitating and strengthening network formation.

The restricted specificity could be based in the acceptance of single mismatch binding due to the length of the probe stretches with repetitive possibilities to bind to the target.

1.2. Synthetic RNA polynucleotide probes

1.2.1. Synthetic construct probe for *Pseudomonas aeruginosa*

The synthetic RNA polynucleotide probes that were generated (Table B.17) differed in composition from the synthetic DNA polynucleotide probes. Multilabelled during transcription, the resulting RNA polynucleotide probes again consist of a 4 time repetition of a species-specific oligonucleotide probe, with the single probe parts divided by a poly A spacer of 5 nucleotides in length (Table B.17). This should keep the single species-specific probes apart and thus enable oligonucleotide probe specificity. In contrast to the DNA polynucleotide probes, which showed unspecific signals, the RNA probe for *Pseudomonas aeruginosa* was species-specific.
In silico analysis of the probes confirmed the formation of secondary structures under hybridisation conditions (Fig.D.9). The probe is completely linearised during the denaturation step (Fig.D.9). The combination of the conditions for specific oligonucleotide probe hybridisation with the denaturation step from polynucleotide hybridisations resulted in specific signals, for the developed probe PseAer4_sp and target organisms *Pseudomonas aeruginosa* (see Table C.19).

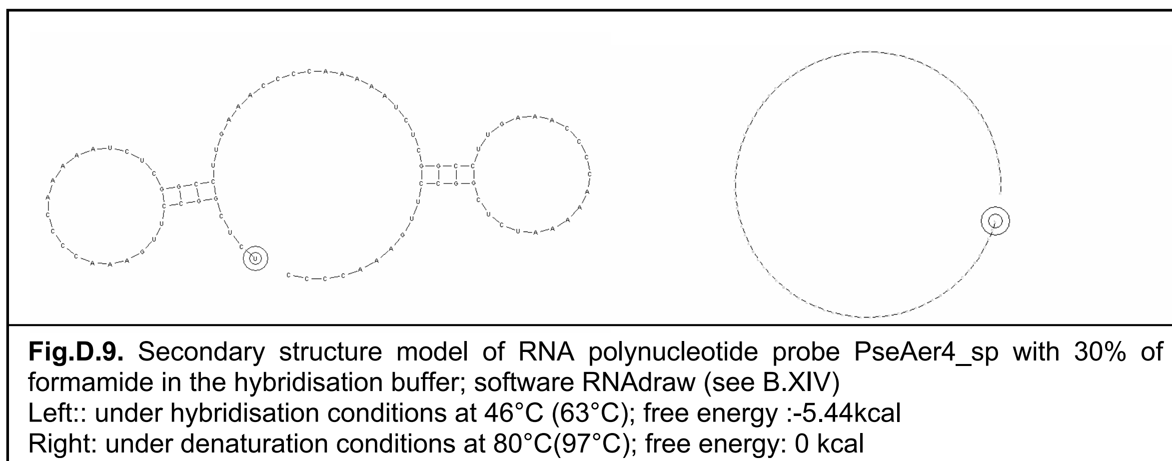


Fig.D.9. Secondary structure model of RNA polynucleotide probe PseAer4_sp with 30% of formamide in the hybridisation buffer; software RNAdraw (see B.XIV)
Left:: under hybridisation conditions at 46°C (63°C); free energy :-5.44kcal
Right: under denaturation conditions at 80°C(97°C); free energy: 0 kcal

The nature of the signal obtained after hybridisation with target cells of *Pseudomonas aeruginosa* was variable, showing signals ranging from whole cell fluorescence and halo signals (see Figs.C.60-62). As already mentioned, a bright halo signal similar to those of a standard 250 nucleotide long rRNA targeted transcript probes described by Zwirglmaier (2003) for a transcript probe counted only 50 nucleotides in length.

Although the developed probe Pseaer4_sp is slightly longer, counting 78 nucleotides in length, the occurrence of whole cell fluorescence was dominant in comparison to halo signals and intermediate signals.
One particular reason for this could be based on the composition of probe PseAer4_sp, as the repetitive character of the sequence of this probe might not be able to form those strong secondary structures during hybridisations as the probe described by Zwirglmaier (2003). Considering the secondary structure of probe PseAer4_sp during the conditions of hybridisation (Fig.D.9), a moderate low energy value is found, which might result in preferred whole cell fluorescence signal and reduced network formation. However, alterations in hybridisation conditions, namely reduced temperature or formamide concentration, did not change the character of the signal; in this case, specificity was lost due to decreased stringency, while the halo signals did not rise.

Though fewer cells allowed the detection of halo signals in comparison to whole cell fluorescence, it was possible to use probe PseAer4_sp for an enrichment hybridisation from real clinical sample urine number 3, containing *Pseudomonas aeruginosa* with subsequent successful PCR detection, cloning and sequencing for confirmation of the identity (see Table C.20).

1.2.2. Synthetic construct probe for *Pseudomonas fluorescens*

The combination of the denaturation step from polynucleotide probe hybridisation with the hybridisation conditions of the oligonucleotide probe was successful for probe PseAer4_sp, but the result for probe PseFl4_sp for *Pseudomonas fluorescens* was different: specific whole cell fluorescence. Alterations of the hybridisation conditions such as longer hybridisation, changes in FA concentration and omitting the washing step did not change the nature of the signal, but resulted in loss of specificity.
The secondary structure analysis of probe PseFl4_sp revealed that the probe is linearised not only during the denaturation step, but also during hybridisation itself, as no more possible loops or structures can be observed above 48°C (see Fig.D.11), which is a hint that network formation could be hampered significantly while specific

binding to the target structure can still take place, thus leading to whole cell fluorescence.

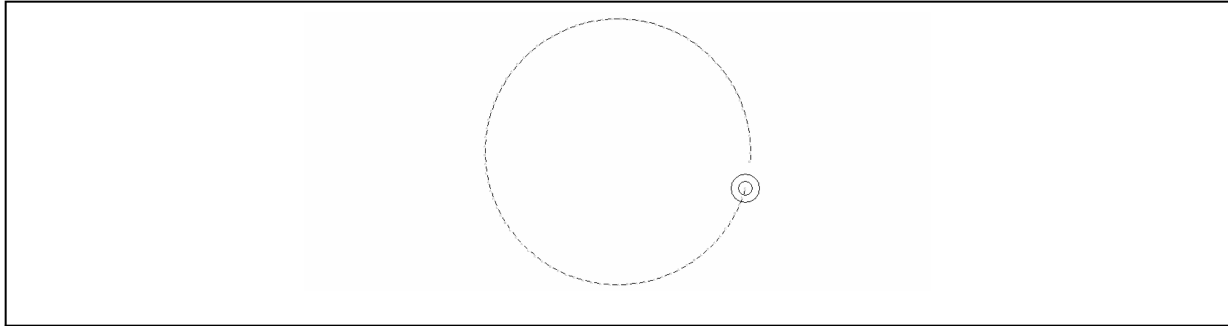


Fig.D.10. Secondary structure model of RNA polynucleotide probe Psefl4_sp with 30% of formamide in the hybridisation buffer under hybridisation conditions at 46°C (63°C); free energy : 0 kcal ; software RNAdraw (see B.XIV.2)

This result confirms the crucial role of the ability to form secondary structures under hybridisation conditions, if a halo is desired.

1.3. DNA probes versus RNA probes: the confrontation

The question why halo signals and specificity are given in the case of synthetic RNA polynucleotide probe PseAer_4sp, but not for synthetic DNA polynucleotide probes will be discussed using the example of RNA probe PseAer4_sp DNA probe PseAer4:

probe	DNA PseAer4	RNA PseAer4_sp
Composition	4 time repetition of oligonucleotide probe	4 time repetition of oligonucleotide probe with Poly A spacers in between
Labelling	5` Cy3 mono-label	Fluos-12-UTP multi-label
ΔG at hybridisation	-2.67kcal	-5.44 kcal
Specificity	-	+
Signal	whole cell fluorescence	Halo signal and whole cell fluorescence

Table D.9. Comparison of synthetic DNA and RNA polynucleotide probes with regard to composition, labelling, ΔG (= free energy), specificity and nature of signal; +: yes; -: no.

As already mentioned in part D 1.1, labelling could play a role with regard to network formation, as the sterical effect of the multi-labels in the RNA probe could result in preferred inter- rather than in intramolecular probe binding. In addition, the energy value under hybridisation conditions is significantly stronger for the RNA probe than for the DNA probe, which also should stabilise secondary structures and network

formation. Another point is that RNA-RNA binding energies have a higher value than RNA-DNA or DNA-DNA bindings, which might also result in more stable target structure binding and intra- and inter-probe molecular binding for probe RNA PseAer4_sp, thus facilitating network and halo signal detection. Last but not least, the spacer of poly A keeps the single oligonucleotide probe stretches apart, and might be essential under the hybridisation conditions for specificity and the additional 15 nucleotides resulting from the spacers in between the different oligonucleotide stretches could also support the hybridisation process.

4. Possible applications

The overall goal of application of synthetic construct probes is the combination of all advantages provided by polynucleotide probes with the specificity of rRNA targeted oligonucleotide probes.

Especially the high copy number of the target, the ribosomes, has to be considered as very advantageous, as the probe binding is facilitated and, due to the signal amplification obtained by multilabelling of even one probe molecule and the possible network formation, can be used for the detection and subsequent enrichment of bacteria, even with low ribosomal content. As discussed above, for further design of such synthetic DNA and RNA polynucleotide probes the length, composition, ability to form strong secondary structures and possibly the labelling have to be considered carefully. Possible applications of synthetic construct probes could be the species-specific enrichment of microbial target or non target cells from environmental or clinical samples, to have the possibility of improved cell fishing, perhaps in combination with the detection of special genes of interest using RINGFISH probes.

E. Summary

In this study, the application of FISH (Fluorescence *in situ* hybridisation) using different types of polynucleotide probes (classic and synthetic polynucleotide probes targeting rRNA and RINGFISH polynucleotide probes targeting DNA) and the related polynucleotide probe based enrichment technique were tested for their suitability for clinically relevant bacteria, artificially spiked clinical sample material and real clinical samples.

The polynucleotide enrichment technique is based on the special hybridisation feature of polynucleotide probes, visualised after hybridisations using fluorescently labelled probes as a ring-shaped fluorescence surrounding the cell envelope. The current theory assumes that the polynucleotide probe molecules form a three dimensional network around the cells by probe-probe hybridisation and formation of secondary structures. Thereby, the probe molecules are anchored by partial binding to the target structure inside of the cell, whereas the remaining probe molecule(s) is/are involved in the formation of secondary structures and hybridisations between several probe molecules. This network of protruding polynucleotide probes can be used in a second hybridisation step to bind to complementary nucleic acids which are immobilised in microplate cavities. Thus, a specific enrichment of cells with the applied polynucleotide probe is possible.

In this context, numerous further developments and evaluations of existing protocols had to be designed and tested. Especially the achievement of an adequate permeabilisation of the cell envelopes of Gram-positive bacteria, which are innately impenetrable for polynucleotide probes, was necessary to enable probe binding and application of the probe based enrichment technique. Protocols for moderate and controlled permeabilisation of the different cell envelopes could be generated, which are also applicable for real clinical sample material. Another important development concerned the time consumption of the whole hybridisation and enrichment procedure. The development of methods which allow shortening of different steps during the whole procedure led to quite a saving of time.

The new developed or modified protocols and the application of the polynucleotide probe enrichment technique were primarily tested and evaluated using classic rRNA targeted polynucleotide probes on pure culture bacteria and clinical samples, which had been spiked with different target organisms.

The successful application in this area allowed the further application of the protocols and the transfer of the technique to real clinical samples without artificial spiking, where it was possible to enrich successfully target bacteria from the respective real clinical samples.
Another step in the course of the work was the development of RINGFISH probes targeting different antibiotic resistance genes of Gram-positive and Gram-negative bacteria. For the first time, this technique could be applied to real clinical samples. The developed RINGFISH probes could also be successfully applied to enrichment hybridisations of target bacteria from real clinical samples. In this connection, the developed protocols for permeabilisation of the cell envelopes of Gram-positive bacteria were successfully integrated.
The last type of polynucleotide probes developed, synthetic polymers of specific oligonucleotide probes, could also be used successfully for detection and enrichment of bacteria from real clinical samples.

E. Zusammenfassung

Im Rahmen dieser Arbeit wurden die Anwendung von FISH (Fluorescence *in situ* hybridisation) unter Verwendung verschiedener Arten von Polynukleotidsonden (klassischen und synthetischen Polynukleotidsonden mit den Zielmolekülen rRNA und RINGFISH Polynukleotidsonden mit chromosomaler DNA als Zielmolekül) im Zusammenhang mit der Polynukleotidsonden-gestützten Anreicherungstechnik auf ihre Eignung für klinisch relevante Bakterien, künstlich mit Zielbakterien versetztem, klinischen Probenmaterial als auch realem, klinischen Probenmaterial getestet .

Die Polynukleotidsonden-gestützte Anreicherungstechnik basiert auf dem speziellen Merkmal, das bei Hybridisierungen mit fluoreszenzmarkierten Polynukleotidsonden offenbar wird: eine ringförmige Fluoreszenz im Bereich der Zellwand der hybridisierten Zellen. Aktuelle Hypothesen sehen das Phänomen darin begründet, dass die Polynukleotidsonden ein dreidimensionales Netzwerk bilden, welches durch Hybridisierungen von Sondenmolekülen untereinander als auch durch Sekundär-strukturausbildung der einzelnen Sonden hervorgerufen wird. Ein Teil der Polynukleotidsonden ist dabei am Bindungsort innerhalb der Zelle verankert, während der übrige Teil der Sonde(n) an der Netzwerkbildung beteiligt ist (sind). Dieses Netzwerk aus herauragenden Polynukleotidsonden kann in einem zweiten Hybridisierungsschritt an sondenkomplementäre Nukleinsäuren, die auf Trägermaterial immobilisiert sind, genutzt werden und erlaubt somit eine Anreicherung der hybridisierten Zielzellen im Spezifitätsrahmen der verwendeten Polynukleotidsonden.

Im Zuge der Anpassung der Polynukleotidsondentechnik an Gram-positive Bakterien und klinisches Probenmaterial mussten eine Reihe von neuen Protokollen als auch Adaptionen bereits existierender Protokolle entwickelt und evaluiert werden. Besonders eine angemessene Permeabilisierung der Zellhülle Gram-positiver Bakterien, die für Polynukleotidsonden unter Standardbedingungen undurchlässig ist, war erforderlich, um erfolgreich Sondenbindung an das jeweilige Zielmolekül zu ermöglichen als auch die Anwendung der Polynukleotidsonden-gestützten Anreicherungstechnik zu erlauben. Es gelang hierbei, Protokolle für eine kontrollierte Permeabilisierung der Zellhüllen verschiedener Gram-positiver Bakterien zu entwickeln, die auch eine Übertragung auf reales klinisches Probenmaterial erlaubten. Eine weitere wichtige Entwicklung stellte die Verkürzung des Zeitaufwandes dar, der bisher für die gesamte Hybridisierungs- und

Anreicherungstechnik erforderlich war. Die Abwandlung einzelner Schritte innerhalb der gesamten Prozedur führte zu einem enorm zeitverkürzten Protokoll.

Die neu entstandenen Protokolle als auch die Anwendbarkeit und der Transfer der Technik wurden beginnend mit klassischen rRNA zielgerichteten Polynukleotid-sonden an Reinkulturen von Bakterien und künstlich mit Zielbakterien versetztem klinischem Probenmaterial getestet und evaluiert. Die erfolgreiche Anwendung in diesem Bereich erlaubte die Ausweitung und Übertragung der Technik auf reales klinisches Probematerial, das nicht künstlich mit Zielbakterien versetzt war. Hier gelang es, spezifisch Bakterien aus den realen klinischen Probenmaterialien anzureichern.

Ein weiterer Schritt im Zuge der Arbeit war die Entwicklung von RINGFISH Polynukleotidsonden, die auf verschiedene Antibiotika-Resistenzgene Gram-positiver und Gram-negativer Bakterien zielgerichtet waren. Dabei konnte die RINGFISH Technik als auch die Polynukleotidsonden- gestützte Anreicherungstechnik zum ersten Mal auf reales, nicht künstlich mit Zielbakterien versetztes klinisches Probematerial oder Reinkulturen von Bakterien angewendet werden. Die Anwendung der entwickelten Protokolle zur Permeabilisierung der Zellhüllen Gram-positiver Bakterien wurde dabei erfolgreich integriert.

Die Entwicklung von synthetischen Polynukleotidsonden, die aus repetetiven Sequenzen spezifischer Oligonukleotidesonden bestanden, konnte ebenfalls exemplarisch die Anwendbarkeit im Rahmen der Anreicherung von Zielzellen aus realem, nicht künstlich mit Zielbakterien versetztem Probenmaterial zeigen.

F. Appendix

I. Similarity matrices

Similarity matrix analysis of the region upstream of Helix 58 (*E. coli* position 1366-1466)*

Table F.1. Similarity matrix of DIII 23S rDNA sequences of project relevant organisms calculated for the region upstream of Helix 58; * Brosius et al 1981

	Organism / probe DIII	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
1	*E. coli*	100	99	99	99	99	99	95	99	98	79,8	84	83	82	79	79	79	69,7	68,7	69,7	72,7	67,3	64,9
2	*Klebsiella pneumoniae*	99	100	100	100	100	100	96	100	99	79,8	85	82	83	80	80	80	70,7	69,7	70,7	71,7	68,4	66
3	*Klebsiella ozaenae*	99	100	100	100	100	100	96	100	99	79,8	85	82	83	80	80	80	70,7	69,7	70,7	71,7	68,4	66
4	*Klebsiella terrigena*	99	100	100	100	100	100	96	100	99	79,8	85	82	83	80	80	80	70,7	69,7	70,7	71,7	68,4	66
5	*Citrobacter freundii*	99	100	100	100	100	100	96	100	99	79,8	85	82	83	80	80	80	70,7	69,7	70,7	71,7	68,4	66
6	*Citrobacter youngae*	99	100	100	100	100	100	96	100	99	79,8	85	82	83	80	80	80	70,7	69,7	70,7	71,7	68,4	66
7	*Hafnia alvei*	95	96	96	96	96	96	100	96	95	78,8	85	82	83	77	77	77	70,7	68,7	68,7	71,7	69,4	67
8	*Enterobacter aerogenes*	99	100	100	100	100	100	96	100	99	79,8	85	82	83	80	80	80	70,7	69,7	70,7	71,7	68,4	66
9	*Enterobacter cloacae*	98	99	99	99	99	99	95	99	100	79,8	86	82	84	81	81	81	70,7	69,7	70,7	71,7	68,4	66
10	*Stenotrophomonas maltophilia*	79,8	79,8	79,8	79,8	79,8	79,8	78,8	79,8	79,8	100	80,8	79,8	78,8	73,7	75,8	75,8	61,2	64,3	62,2	63,3	68	62,5
11	*Pseudomonas aeruginosa*	84	85	85	85	85	85	85	85	86	80,8	100	96	91	84	84	84	68,7	73,7	71,7	69,7	73,5	69,1
12	*Pseudomonas fluorescens*	83	82	82	82	82	82	82	82	82	79,8	96	100	87	81	81	81	70,7	75,8	73,7	73,7	74,5	70,1
13	*Pseudomonas stutzeri*	82	83	83	83	83	83	83	83	84	78,8	91	87	100	81	81	81	66,7	69,7	69,7	67,7	69,4	66
14	*Acinetobacter baumanii*	79	80	80	80	80	80	77	80	81	73,7	84	81	81	100	96	96	60,6	65,7	63,6	61,6	65,3	62,9
15	*Acinetobacter junii*	79	80	80	80	80	80	77	80	81	75,8	84	81	81	96	100	100	60,6	65,7	63,6	61,6	66,3	63,9
16	*Acinetobacter lwoffii*	79	80	77	80	80	81	60,6	80	80	80	84	81	81	96	100	100	100	65,7	63,6	61,6	66,3	64,6
17	*Enterococcus faecium*	69,7	70,7	70,7	70,7	70,7	70,7	70,7	70,7	70,7	61,2	68,7	70,7	66,7	60,6	60,6	60,6	100	91,6	95,3	92,5	76,4	74,3
18	*Enterococcus faecalis*	68,7	69,7	69,7	69,7	69,7	69,7	68,7	69,7	69,7	64,3	73,7	75,8	69,7	65,7	65,7	65,7	91,6	100	91,6	87,9	82,1	78,1
19	*Enterococcus durans*	69,7	70,7	70,7	70,7	70,7	70,7	68,7	70,7	70,7	62,2	71,7	73,7	69,7	63,6	63,6	63,6	95,3	91,6	100	91,6	75,5	72,4
20	*Enterococcus gallinarum*	72,7	71,7	71,7	71,7	71,7	71,7	71,7	71,7	71,7	63,3	69,7	73,7	67,7	61,6	61,6	61,6	92,5	87,9	91,6	100	72,6	70,5
21	*Staphylococcus aureus*	67,3	68,4	68,4	68,4	68,4	68,4	69,4	68,4	68,4	68	73,5	74,5	69,4	65,3	66,3	66,3	76,4	82,1	75,5	72,6	100	91,5
22	*Staphylococcus epidermidis*	64,9	66	66	66	66	66	67	66	66	62,5	69,1	70,1	66	62,9	63,9	63,9	74,3	78,1	72,4	70,5	91,5	100
23	*Streptococcus pyogenes*	66,7	67,7	67,7	67,7	67,7	67,7	65,7	67,7	67,7	59,2	67,7	67,7	64,6	65,7	64,6	64,6	75,2	80,2	77,2	72,3	72	66,7

Similarity matrix analysis of Helix 58 (*E. coli* position 1467-1525)*

Table F.2. Similarity matrix of DIII 23S rDNA sequences of project relevant organisms calculated for the region of Helix 58; * Brosius et al 1981

	Organism / probe DIII	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
1	*E. coli*	100	93,1	77,6	93,1	79,3	79,3	69	79,3	79,3	60	69	69	65,5	63,8	56,9	63,8	50,9	50,9	52,6	56,1	73,2	71,4
2	*Klebsiella pneumoniae*	93,1	100	77,6	98,3	79,3	79,3	69	79,3	79,3	54,5	67,2	65,5	62,1	65,5	55,2	62,1	57,9	56,1	54,4	56,1	73,2	71,4
3	*Klebsiella ozaenae*	77,6	77,6	100	77,6	94,8	94,8	67,8	94,9	94,8	54,5	67,2	67,2	70,7	63,8	56,9	60,3	50,9	52,6	52,6	61,4	75	71,4
4	*Klebsiella terrigena*	93,1	98,3	77,6	100	79,3	79,3	67,2	79,3	79,3	54,5	67,2	65,5	62,1	65,5	55,2	62,1	56,1	54,4	54,4	56,1	73,2	71,4
5	*Citrobacter freundii*	79,3	79,3	94,8	79,3	100	98,4	72,4	96,6	100	58,2	67,2	67,2	74,1	61	54,2	57,6	50,9	56,1	56,1	61,4	78,9	75,4
6	*Citrobacter youngae*	79,3	79,3	94,8	79,3	98,4	100	72,4	96,6	100	58,2	67,2	67,2	74,1	61	54,2	57,6	50,9	56,1	56,1	61,4	78,9	75,4
7	*Hafnia alvei*	69	69	67,8	67,2	72,4	72,4	100	71,7	72,4	56,4	69	67,2	69	60,3	55,2	60,3	50,9	49,1	45,6	47,4	60,7	58,9
8	*Enterobacter aerogenes*	79,3	79,3	94,9	79,3	96,6	96,6	71,7	100	96,6	58,2	70,7	70,7	74,1	63,8	56,9	60,3	54,4	56,1	56,1	61,4	76,8	73,2
9	*Enterobacter cloacae*	79,3	79,3	94,8	79,3	100	100	72,4	96,6	100	58,2	67,2	67,2	74,1	61	54,2	57,6	50,9	56,1	56,1	61,4	78,9	75,4
10	*Stenotrophomonas maltophilia*	60	54,5	54,5	54,5	58,2	58,2	56,4	58,2	58,2	100	65,5	72,7	69,1	61,8	61,8	60	45,5	45,5	40	41,8	59,3	58,5
11	*Pseudomonas aeruginosa*	69	67,2	67,2	67,2	67,2	67,2	69	70,7	67,2	65,5	100	93,1	89,7	70,7	67,2	72,4	54,4	50,9	52,6	56,1	71,4	69,6
12	*Pseudomonas fluorescens*	69	65,5	67,2	65,5	67,2	67,2	67,2	70,7	67,2	72,7	93,1	100	89,7	74,1	70,7	75,9	59,6	50,9	49,1	57,9	69,6	67,9
13	*Pseudomonas stutzeri*	65,5	62,1	70,7	62,1	74,1	74,1	69	74,1	74,1	69,1	89,7	89,7	100	74,1	70,7	75,9	49,1	52,6	50,9	52,6	73,2	71,4
14	*Acinetobacter baumanii*	63,8	65,5	63,8	65,5	61	61	60,3	63,8	61	61,8	70,7	74,1	74,1	100	84,7	94,9	52,6	45,6	50,9	45,6	63,2	63,2
15	*Acinetobacter junii*	56,9	55,2	56,9	55,2	54,2	54,2	55,2	56,9	54,2	61,8	67,2	70,7	70,7	84,7	100	86,4	47,4	40,4	45,6	40,4	57,9	57,9
16	*Acinetobacter lwoffii*	63,8	62,1	60,3	62,1	57,6	57,6	60,3	60,3	57,6	60	72,4	75,9	75,9	94,9	86,4	100	49,1	42,1	47,4	42,1	59,6	59,6
17	*Enterococcus faecium*	50,9	57,9	50,9	56,1	50,9	50,9	50,9	54,4	50,9	45,5	54,4	59,6	49,1	52,6	47,4	49,1	100	76,3	83,1	83,1	67,9	65,5
18	*Enterococcus faecalis*	50,9	56,1	52,6	54,4	56,1	56,1	49,1	56,1	56,1	45,5	50,9	50,9	52,6	45,6	40,4	42,1	76,3	100	74,6	72,9	60,7	61,8
19	*Enterococcus durans*	52,6	54,4	52,6	54,4	56,1	56,1	45,6	56,1	56,1	40	52,6	49,1	50,9	50,9	45,6	47,4	83,1	74,6	100	81,4	73,2	74,5
20	*Enterococcus gallinarum*	56,1	56,1	61,4	56,1	61,4	61,4	47,4	61,4	61,4	41,8	56,1	57,9	52,6	45,6	40,4	42,1	83,1	72,9	81,4	100	66,1	69,1
21	*Staphylococcus aureus*	73,2	73,2	75	73,2	78,9	78,9	60,7	76,8	78,9	59,3	71,4	69,6	73,2	63,2	57,9	59,6	67,9	60,7	73,2	66,1	100	93,1
22	*Staphylococcus epidermidis*	71,4	71,4	71,4	71,4	75,4	75,4	58,9	73,2	75,4	58,5	69,6	67,9	71,4	63,2	57,9	59,6	65,5	61,8	74,5	69,1	93,1	100
23	*Streptococcus pyogenes*	50,9	56,1	56,1	54,4	57,9	57,9	57,9	61,4	57,9	45,5	54,4	52,6	50,9	52,6	47,4	49,1	74,1	65,5	70,7	67,2	69,6	65,5

Similarity matrix analysis downstream of Helix 58 (*E. coli* position 1526-1525)*

Table F.3. Similarity matrix of DIII 23S rDNA sequences of project relevant organisms calculated for the region downstream of Helix 58;
* Brosius et al 1981

	Organism / probe DIII	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
1	*E. coli*	100	97,4	98,7	98,7	97,3	96,1	81,6	97,4	93,4	65,7	67,1	68	67,1	61,8	63,2	63,2	57,5	56,2	58,9	58,9	47,9	50,7
2	*Klebsiella pneumoniae*	97,4	100	98,7	98,7	97,3	96,1	81,6	97,4	93,4	65,7	67,1	68	67,1	61,8	63,2	63,2	57,5	56,2	58,9	56,2	47,9	50,7
3	*Klebsiella ozaenae*	98,7	98,7	100	100	98,7	97,4	84,2	100	96,1	67,1	69,7	70,7	69,7	64,5	65,8	65,8	58,9	57,5	61,6	58,9	50,7	53,5
4	*Klebsiella terrigena*	98,7	98,7	100	100	98,7	97,4	82,9	98,7	94,7	67,1	68,4	69,3	68,4	63,2	64,5	64,5	58,9	57,5	60,3	57,5	49,3	52,1
5	*Citrobacter freundii*	97,3	97,3	98,7	98,7	100	100	84	97,3	94,7	66,7	70,7	71,6	70,7	65,3	66,7	66,7	61,1	58,3	62,5	59,7	50	52,9
6	*Citrobacter youngae*	96,1	96,1	97,4	97,4	100	100	82,9	96,1	94,7	67,1	69,7	70,7	69,7	64,5	65,8	65,8	60,3	57,5	61,6	58,9	49,3	52,1
7	*Hafnia alvei*	81,6	81,6	84,2	82,9	84	82,9	100	84,2	81,6	71,4	76,3	77,3	73,7	68,4	67,1	67,1	58,9	58,9	63	60,3	56,3	59,2
8	*Enterobacter aerogenes*	97,4	97,4	100	98,7	97,3	96,1	84,2	100	94,7	67,1	68,4	69,3	68,4	63,2	64,5	64,5	57,5	56,2	61,6	58,9	49,3	52,1
9	*Enterobacter cloacae*	93,4	93,4	96,1	94,7	94,7	94,7	81,6	94,7	100	67,1	72,4	73,3	72,4	67,1	65,8	65,8	56,2	54,8	58,9	56,2	52,1	54,9
10	*Stenotrophomonas maltophilia*	65,7	65,7	67,1	67,1	66,7	67,1	71,4	67,1	67,1	100	67,1	66,7	68,6	60	62,9	62,9	58,2	59,7	58,2	58,2	56,9	61,5
11	*Pseudomonas aeruginosa*	67,1	67,1	69,7	68,4	70,7	69,7	76,3	68,4	72,4	67,1	100	92,2	87,3	76,9	73,1	73,1	61,6	63	65,8	63	60,8	60,8
12	*Pseudomonas fluorescens*	68	68	70,7	69,3	71,6	70,7	77,3	69,3	73,3	66,7	92,2	100	88,3	77,9	74	74	66,7	68,1	70,8	68,1	65,3	65,3
13	*Pseudomonas stutzeri*	67,1	67,1	69,7	68,4	70,7	69,7	73,7	68,4	72,4	68,6	87,3	88,3	100	83,3	79,5	79,5	60,3	64,4	64,4	61,6	60,8	60,8
14	*Acinetobacter baumanii*	61,8	61,8	64,5	63,2	65,3	64,5	68,4	63,2	67,1	60	76,9	77,9	83,3	100	96,2	96,2	52,1	56,2	56,2	53,4	57,5	61,6
15	*Acinetobacter junii*	63,2	63,2	65,8	64,5	66,7	65,8	67,1	64,5	65,8	62,9	73,1	74	79,5	96,2	100	100	50,7	54,8	54,8	52,1	54,8	58,9
16	*Acinetobacter lwoffii*	63,2	63,2	65,8	64,5	66,7	65,8	67,1	64,5	65,8	62,9	73,1	74	79,5	96,2	100	100	50,7	54,8	54,8	52,1	54,8	58,9
17	*Enterococcus faecium*	57,5	57,5	58,9	58,9	61,1	60,3	58,9	57,5	56,2	58,2	61,6	66,7	60,3	52,1	50,7	50,7	100	93,3	94,7	93,3	73,2	74,6
18	*Enterococcus faecalis*	56,2	56,2	57,5	57,5	58,3	57,5	58,9	56,2	54,8	59,7	63	68,1	64,4	56,2	54,8	54,8	93,3	100	93,3	92	71,8	73,2
19	*Enterococcus durans*	58,9	58,9	61,6	60,3	62,5	61,6	63	61,6	58,9	58,2	65,8	70,8	64,4	56,2	54,8	54,8	94,7	93,3	100	96	69	70,4
20	*Enterococcus gallinarum*	58,9	56,2	58,9	57,5	59,7	58,9	60,3	58,9	56,2	58,2	63	68,1	61,6	53,4	52,1	52,1	93,3	92	96	100	69	70,4
21	*Staphylococcus aureus*	47,9	47,9	50,7	49,3	50	49,3	56,3	49,3	52,1	56,9	60,8	65,3	60,8	57,5	54,8	54,8	73,2	71,8	69	69	100	92
22	*Staphylococcus epidermidis*	50,7	50,7	53,5	52,1	52,9	52,1	59,2	52,1	54,9	61,5	60,8	65,3	60,8	61,6	58,9	58,9	74,6	73,2	70,4	70,4	92	100
23	*Streptococcus pyogenes*	52,6	52,6	53,9	53,9	53,3	52,6	56,6	52,6	51,3	54,3	61,8	66,7	61,8	60,5	57,9	57,9	74,3	79,7	75,7	73	63,4	66,2

II. Primer binding sites and gene sequences of RINGFISH probe targeted genes

1. *ampC* gene sequence

ampC gene sequence of NCBI 124204 and *E. coli* AF 421100 (EcoAmpC#1) and *E. coli* AF 430319 (Eco AmpC#2, see also Table B.4)

```
                 1          11         21         31         41         51         61         71         81         91        100
   EscCol91   1  CGATCTGAAA ATTCACGTGC CTGCGGGCAA ATGGGTTTTC TACGGTCTGG CTGCTATCTT GACAGTTGTC ACGCTGATTG GTATCGTTAC AATCTAACGT  100    EscCol91
 #1_EcoAmpC   1  CGATCTGAAA ATTCACGTGC CTGCGGGCAA ATGGGTTTTC TACGGTCTGG CTGCTATCCT GACAGTTGTC ACACTGATTG GTGTCGTTAC AATCTAACGC  100 #1_EcoAmpC
 #2_EcoAmpC   1  CGATCTGAAA ATTCACGTGC CTGCGGGCAA ATGGGTTTTC TACGTTCTGG CTGCTATCCT GACAGTTGTC ACGCTGATTG GTGTCGTTAC AATCTAACGC  100 #2_EcoAmpC
                 EcoAmpC V

                                       121        131        141        151        161        171        181        191        200
   EscCol91 101  ATCGCCAATG TAAATCCGGC CCGCCTATGG CGGGCCGTTT TGTATGGAAA CCAGACCTTA TGTTCAAAAC GACGCTCTGC GCCTTATTAA TTACCGCCTC  200    EscCol91
 #1_EcoAmpC 101  ATCGCCAATG TAAATCTGGC CCGCCTATGG CGGGCCGTTT TGTATGGAAA CCAGACCCTA TGTTCAAAAC GACGCTCTGC ACCTTATTAA TTACCGCCTC  200 #1_EcoAmpC
 #2_EcoAmpC 101  ATCGCCAATG TAAATCCGGC CCGCCTATGG CGGGCCGTTT TGTATGGAAA CCAGACCCTA TGTTCAAAAC GACGGTCTGC GCCTTATTAA TTACCGCCTC  200 #2_EcoAmpC

                 201        211        221        231        241        251        261        271        281        291        300
   EscCol91 201  TTGCTCCACA TTTGCTGCCC CTCAACAAAT CAACGATATT GTGCATCGCA CAATTACCCC GCTTATAGAG CAACAAAAGA TCCCCGGTAT GGCGGTGGCG  300    EscCol91
 #1_EcoAmpC 201  TTGCTCCACA TTTGCCGCCC CTCAACAAAT CAACG-TATT GTGCATCGCA CAATTACCCC GCTTATAGAG CAACAAAAGA TCCCCGGTAT GGCGGTGGCG  299 #1_EcoAmpC
 #2_EcoAmpC 201  TTGCTCCACA TTTGCTGCCC CTCAACAAAT CAACGATATT GTGCATCTCA CAATTACCCC GCTTATAGAG CAACAAAAGA TCCCGGGTAT GGCGGTGGCG  300 #2_EcoAmpC

                 301        311        321        331        341        351        361        371        381        391        400
   EscCol91 301  GTAATTTATC AGGGTAAACC TTATTACTTT ACCTGGGGCT ATGCGGACAT CGCCAAAAAG CAGCCCGTCA CACAGCAAAC GTTGTTTGAG TTAGGTTCGG  400    EscCol91
 #1_EcoAmpC 300  GTAATTTATC AGGGTAAACC TTATTACTTT ACCTGGGGCT ATGCGGACAT CGCCAAAAAG CAGCCCGTCA CACAGCAAAC GTTGTTTGAG TTTGGTTCGG  399 #1_EcoAmpC
 #2_EcoAmpC 301  GTAATTTATC AGGGTAAACC TTATTACTTT ACCTGGGGCT ATGCGGACAT CGCCAAAAAG CAGCCCGTCA CACAGCAAAC GTTGTTTGAG TTAGGTTCGG  400 #2_EcoAmpC

                 401        411        421        431        441        451        461        471        481        491        500
   EscCol91 401  TCAGCAAAAC ATTTACGGGC GTGCTTGGTG GCGACGCTAT TGCTCGAGGG GAAATCAAGT TAAGCGATCC CGCAACAAAA TACTGGCCTG AACTTACCGC  500    EscCol91
 #1_EcoAmpC 400  TCAGCAAAAC ATTTACGGGC GTGCTTGGTG GCGACGCTAT TGCTCGAGGG GAAATCAAGT TAAGCGATCC CACAACAAAA TACTGGCCTG AACTTACCGC  499 #1_EcoAmpC
 #2_EcoAmpC 401  TCAGCAAAAC ATTTACTGGC GTGCTTGGTG GCGACGTGAT TGCTCGAGGG GAAATCAAGT TAAGCGATCC CACAACAAAA TACTGGCCTG AACTTACCGC  500 #2_EcoAmpC

                 501        511        521        531        541        551        561        571        581        591        600
   EscCol91 501  TAAACAGTGG AATGGGATCA CACTATTACA TCTTGCGACC TACACCGCTG GCGGCCTGCC ATTGCAGGTG CCGGATGAAG TGAAATCCTC AAGCGACTTG  600    EscCol91
 #1_EcoAmpC 500  TAAACAGTGG AAAGGGATCA CACTATTACA TCTCGCAACC TACACCGCTG GCGGCCTGCC ATTGCAGGTG CCGGATGAGG CGAAATCCTC AAGCGACTTG  599 #1_EcoAmpC
 #2_EcoAmpC 501  TAAACAGTGG AATGGGATCA CACTATTACA TCTCGCAACC TACACTGCTG GCGGCCTGCC ATTGCAGGTG CCGGATGAGG TGAAATCCTC AAGCGACTTG  600 #2_EcoAmpC

                 601        611        621        631        641        651        661        671        681        691        700
   EscCol91 601  CTGCGCTTCT ATCAAAACTG GCAGCCTGCA TGGGCTCCAG GAACACAACG TCTGTATGCC AACTCCAGTA TCGGTTTGTT CGGCGCACTG GCTGTGAAGC  700    EscCol91
 #1_EcoAmpC 600  CTGCGCTTCT ATCAAAACTG GCAGCCTGCA TGGGCTCCAG GAACACAACG TCTGTATGCC AACTCCAGTA TCGGTTTGTT CGGCGCACTG GCTGTGAAGC  699 #1_EcoAmpC
 #2_EcoAmpC 601  CTGCGCTTCT ATCAAAACTG GCAGCCTGCA TGGGCTCCAG GAACACAACG TCTGTATGCC AACTCCAGTA TCGGTTTGTT CGGCGCACTG GCTGTGAAGC  700 #2_EcoAmpC

                 701        711        721        731        741        751        761        771        781        791        800
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 #2_EcoAmpC 701  CGTCTGGTTT GAGTTTTGAG CAGGCGATGC AAACTCGTGT CTTCCAGCCA CTCAAACTCA AACATACGTG AATTAATGTA CCGCCCGCAG AAGAAAAGAA  800 #2_EcoAmpC

                 801        811        821        831        841        851        861        871        881        891        900
   EscCol91 801  TTACGCCTGG GGATATCGCG AAGGTAAGGC AGTGCATGTT TCGCCAGGGG CGTTAGATGC TGAAGCTTAT GGTGTGAAGT CGACCATTGA AGATATGGCC  900    EscCol91
 #1_EcoAmpC 800  CTACGCCTGG GGATATCGCG AAGGTAAGGC AGTACATGTT TCGCCAGGGC CGTTAGATGC TGAAGCTTAT GGTGTGAAGT CGACCATTGA AGATATGGCC  899 #1_EcoAmpC
 #2_EcoAmpC 801  TTACGCCTGG GGATATCGCG AAGGTAAGGC AGTGCATGTT TCGCCTGGGA CGTTAGATGC TGAAGCTTAT GGTGTGAAGT CGACCATTGA AGATATGGCC  900 #2_EcoAmpC

                 901        911        921        931        941        951        961        971        981
   EscCol91 901  CGCTGGGTAC GAAGCAATAT GAATCCCCGT GATATCAACG ACAAAACACT TCAGCAAGGG ATACAACTGG CACAATCTCG CTACTGGCAA             990    EscCol91
 #1_EcoAmpC 900  CGCTGGGTGC AAAGCAATTT AAAACCCCTT GATATCAATG AGAAAACACT TCAACAAGGG ATACAACTGG CACAATCTCG CTACTGGCAA             989 #1_EcoAmpC
 #2_EcoAmpC 901  CGCTGGGTGC AAAGCAATTT AAAACCCCTT GATATCAATG AGAAAACACT TCAACAAGGG ATACAACTGG CACAATCTCG CTACTGGCAA             990 #2_EcoAmpC
                                                                                                 EcoAmpC R (rev.compl.)
```

Fig.F1. *ampC* gene sequence of NCBI AF 124204 and *E. coli* AF 421100 (EcoAmpC#1) and *E. coli* AF 430319 (EcoAmpC#2); red boxes: forward primer binding site, blue boxes: reverse primer binding site

2. *tetAJ* gene sequence

tetAJ gene sequence of NCBI AF 038993 and *Proteus mirabilis* AF 413702 (see also Table B.4)

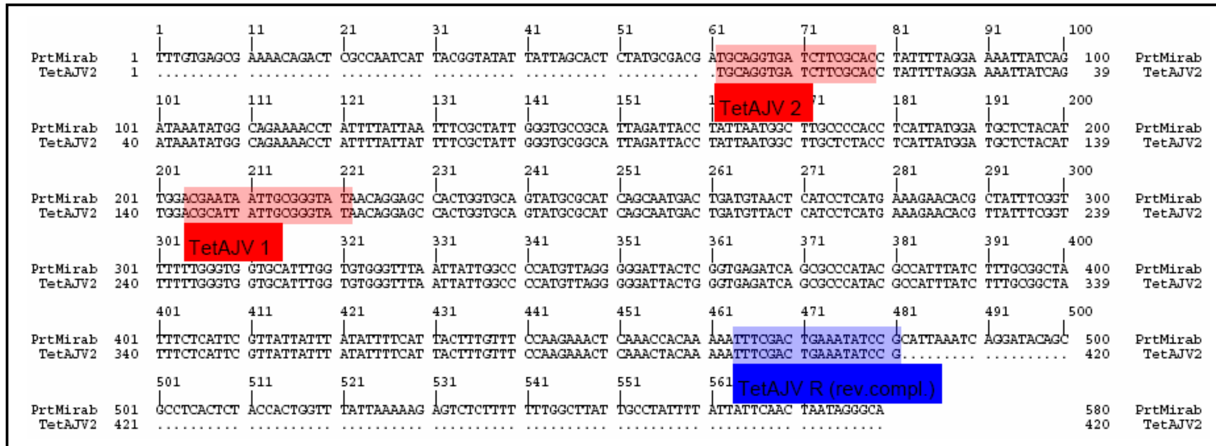

Fig.F2. *tetAJ* gene sequence of NCBI AF 038993 and *Proteus mirabilis* AF 413702; red boxes: forward primer binding sites, blue boxes: reverse primer binding site

3. *ermB* gene sequence

ermB gene sequence of NCBI AF 229200 and *Enterococcus faecium* AF 415772 (see also Table B.4)

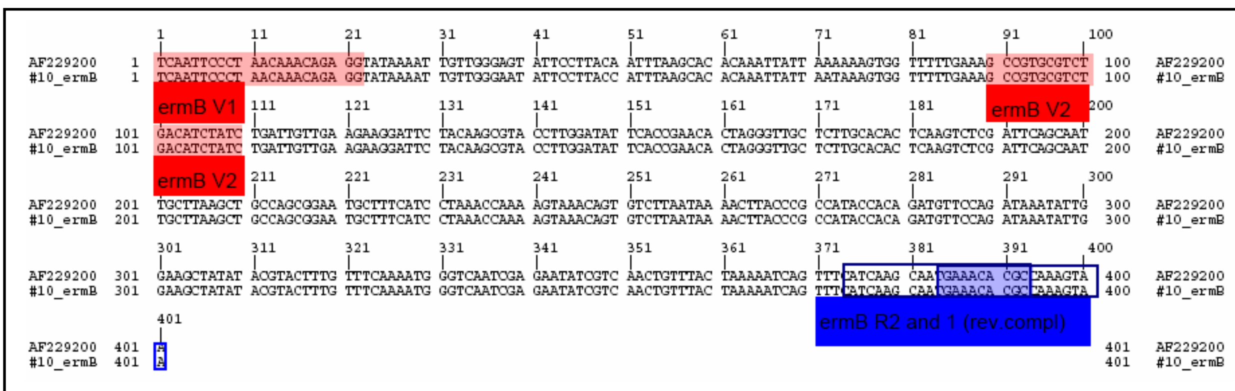

Fig.F3. *erm*B gene sequence of NCBI AF 038993 and *Enterococcus faecium* AF 415772; red boxes: forward primer binding sites (ermB V1 and V2), blue boxes: reverse primer binding sites (ermB R1 and R2); light blue area: overlapping primer binding region of primers ermB R1 and ermB R2; (binding sites ermB R1: the first 5 binding nucleotides not included in sequence)

4. *parC* gene sequence

parC gene sequence of NCBI AB 005036 and clinical strain *Enterococcus faecalis* AF 625857 (see also Table B.4)

```
                 1          11         21         31         41         51         61         71         81         91        100
                 |          |          |          |          |          |          |          |          |          |        |
AB005036     1   GACGGCAATA CCTTTGATAA AGGCTTTCGT AAATCAGCAA AATCTGTCGG AAACATTATG GGGAATTATC ATCCCCATGG CGACAGTAGT ATTTATGAAG  100   AB005036
#_6_parC     1   GACGGCAATA CCTTTGATAA AGGCTTTCGT AAATCAGCAA AATCTGTCGG AAACATTATG GGGAATTATC ATCCCCATGG CGACAGTAGT ATTTATGAAG  100   #_6_parC
                 parC V
                            111        121        131        141        151        161        171        181
                            |          |          |          |          |          |          |          |
AB005036   101   CAATGGTCCG TCTAAGTCAA GACTGGAAAT TACGGGAAGT ACTAATTGAA ATGCACGGAA ACAACGGAAG TATGGATGGC G                    181   AB005036
#_6_parC   101   CGATGGTCCG TCTAAGTCAA GACTGGAAAT TACGGGAAGT ACTAATTGAA ATGCACGGAA ACAACGGAAG TATGGATGGC G                    181   #_6_parC
                                                                                   parC R (rev.compl.)
```

Fig.F4. *parC* gene sequence of NCBI AB 005036 *Enterococcus faecalis* AF 625857; red boxes: forward primer binding site, blue boxes: reverse primer binding site

III. Secondary structure models of all RINGFISH probes under hybridisation and denaturation conditions

1. RINGFISH probe TetAJ V1

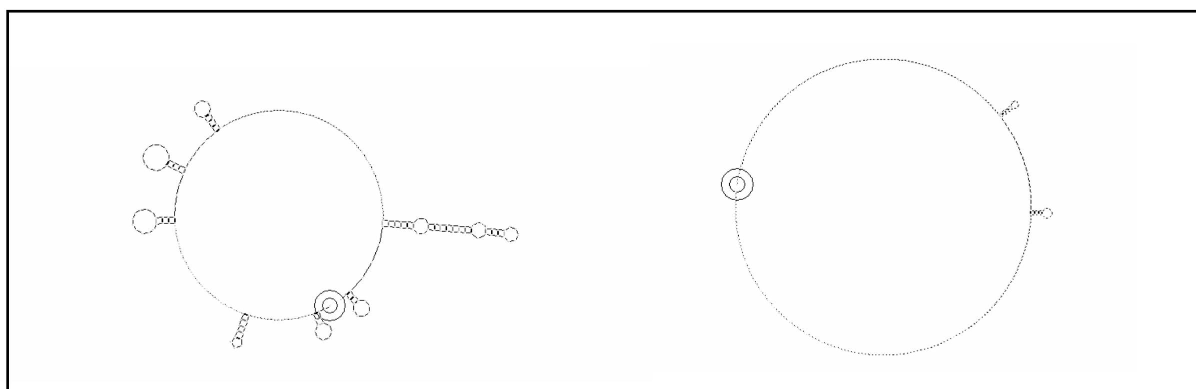

Fig.F.5. Secondary structure model of RINGFISH probe TetAJ V1, GC content40%; 10% formamide in the hybridisation buffer; circle: see Fig.D.3.
Left: under hybridisation conditions 53°C (58.8°C); free energy value –14.04 kcal
Right: under denaturation conditions 80°C (85.8°C); free energy value –1.01 kcal; software RNAdraw (see B.XIV.2)

2. RINGFISH probe ermBV1

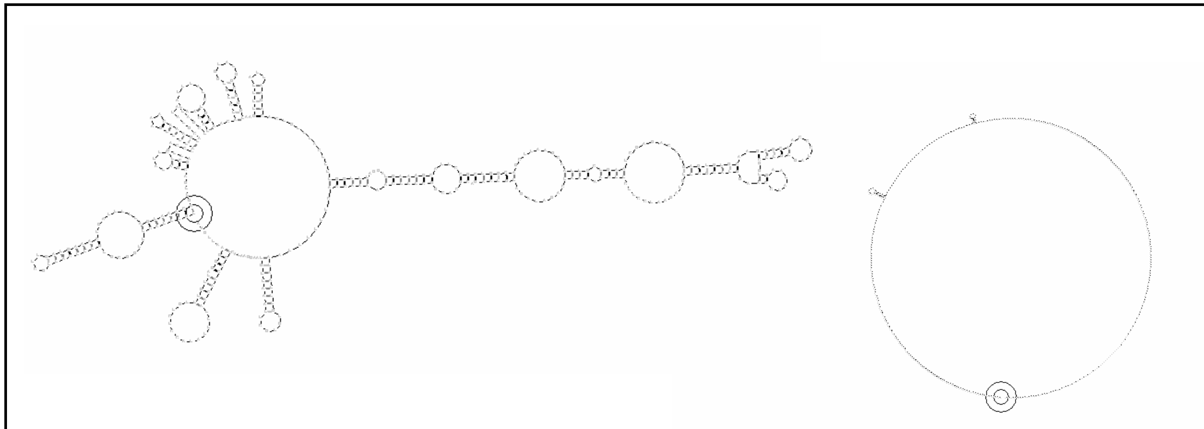

Fig.F.6. Secondary structure model of RINGFISH probe ermB V1, GC content 37%; 0% formamide in the hybridisation buffer; circle: see Fig.D.3.
Left: under hybridisation conditions 53°C (58.8°C); free energy value –26.73 kcal
Right: under denaturation conditions 80°C (85.8°C); free energy value –0.73 kcal; software RNAdraw (see B.XIV.2)

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