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The Use of Polynucleotide RNA Probes for Detection, Identification and Cell Sorting of Microorganisms

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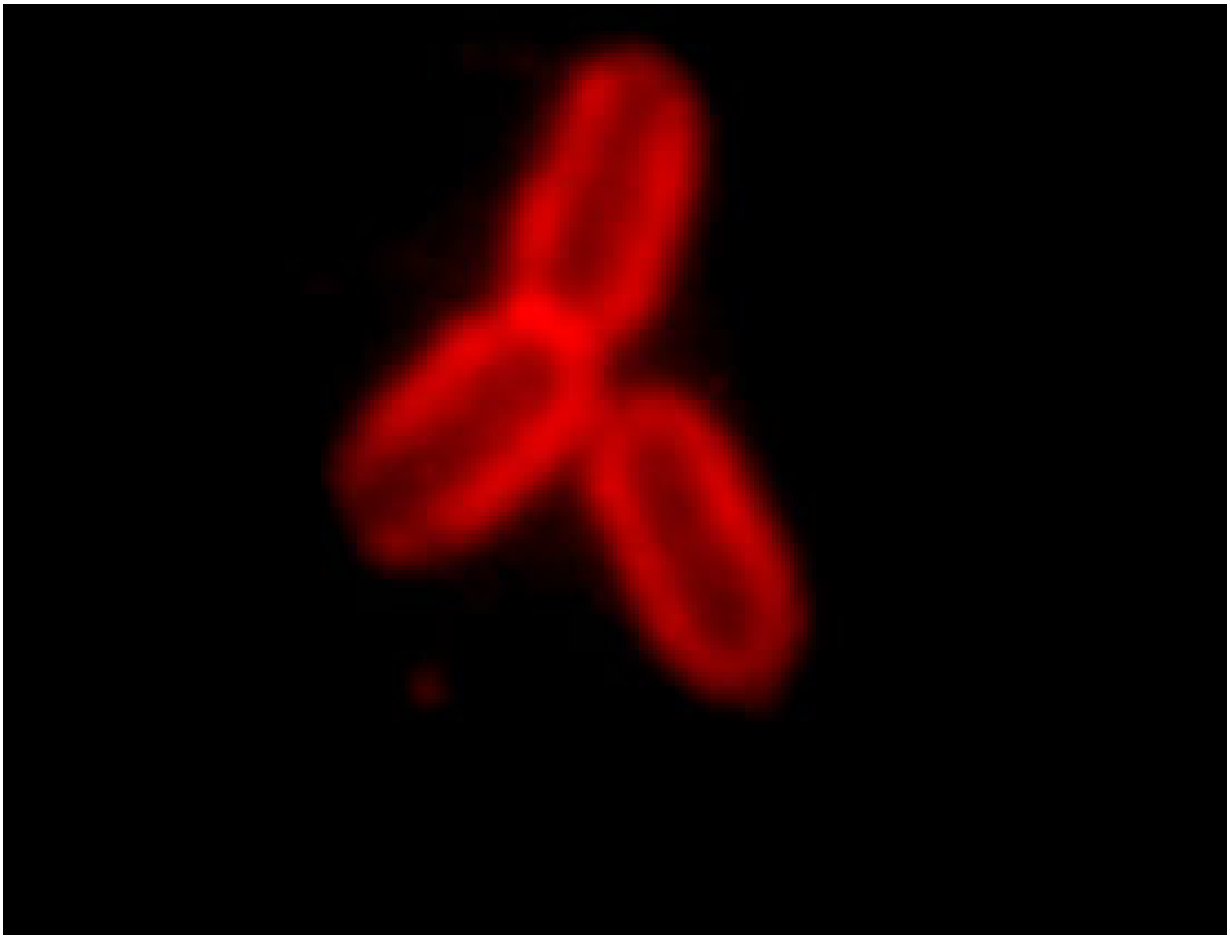
Abbreviations

A	adenine
abs.	absolute
Ac	acetate
AP	alkaline phosphatase
amp	ampicillin
APS	ammoniumperoxodisulfate
BIO	biotin
bp	base pairs
°C	degree centigrade
C	cytosine
CIA	chloroform : isoamylalcohol = 24:1 (v/v)
cf.	confer
cm	centimeter
d	day(s)
DAPI	4', 6'-Diamino-2-phenylindol-dihydrochloride
dATP	deoxyadenosinetriphosphate
dCTP	deoxycytidinetriphosphate
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
DSMZ	Deutsche Sammlung für Mikroorganismen und Zellkulturen, Braunschweig, Germany
dTTP	deoxythymidinetriphosphate
E	extinction
EDTA	ethylenediamintetraacetate
e.g.	exemplum gratiae
et al.	et alteri
etc.	et cetera
EtBr	ethidiumbromide
$EtOH_{abs.}$	100% ethanol
FA	formamide
FISH	fluorescence *in situ* hybridiszation
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

HEPES	2-[4-(2-hydroxyethyl)-piperazinyl-(1)]-ethansulfonic acid $C_8H_{18}N_2O_4S$
i.e.	id est
IPTG	isopropyl-β-D-thiogalactoside
kb	kilo bases
kDa	kilodalton
l	liter
LB	Luria-Bertani medium
LMG	Laboratorium voor Microbiologie Universiteit Gent, Belgien
M	molar
m	meter; milli(10^{-3})
min	minutes
mRNA	messenger RNA
µ	micro (10^{-6})
n	nano (10^{-9})
n.a.	not applicable
NaAc	sodium acetate
n.d.	no data, not determined
nt	nucleotides
OD	optical density
o/n	overnight
p	pico (10^{-12})
Pa	pascal
p.a.	pro analysi
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFA	paraformaldehyde
pH	negative decadic logarithm of the proton concentration
pv.	pathovar
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
ss	single stranded
T	thymine
TAE	tris-acetate-EDTA
TE	tris-EDTA
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
WS	Weihenstephan, bakteriologisches Institut des Forschungszentrums für Milch und Lebensmittel der TUM, Freising, Germany
X-Gal	5-chlor-4-brom-3-indolyl-β-D-galactoside



One Ring to rule them all
One Ring to find them
One Ring to bring them all
And in the darkness bind them

(J.R.R.Tolkien, Lord of the Rings)

A. Introduction

rRNA based taxonomy

Bacteria, as opposed to (multicellular) eukaryotic organisms, offer only limited possibilities for morphological identification. Classic microbiological methods therefore rely mainly on physiological characteristics. With the rise of molecular biology in the aftermath of the disclosure of the molecular structure of DNA (Watson and Crick, 1953) and the recognition of macromolecules as documents of evolutionary history (Zuckerkandl and Pauling, 1965) microbiologists turned to conserved structures within the microbial cell for identification and phylogenetic classification. Ribosomal RNA, as a key molecule in protein synthesis present in all living cells, soon emerged as the most promising candidate for molecular based taxonomy (reviewed by Woese, 1987). Containing both strongly conserved and highly variable regions, it can be used to describe phylogenetic relationships at different levels, from kingdoms down to species (with the threshold between two species being defined as 97% rRNA sequence identity).

The prokaryotic rDNA operon encodes three rRNA molecules, named after their respective sedimentation coefficients (measured in Svedberg units) 16S (with a length of ~1500nt), 23S (~3000nt) and 5S (~120nt). In the early days of rRNA based taxonomy, much emphasis has been laid on the 16S rRNA, leading to a vast amount of available sequence data. In recent years the 23S rRNA, which contains about twice as much sequence information as the 16S, has gained increasing interest.

Fluorescence *in situ* hybridisation as the central technique in microbial ecology

A key interest of microbial ecology is the analysis of structure and function of microbial communities. Classic methods for the identification of microorganisms rely mostly on culturing and subsequent physiological differentiation. But since only a small portion of all free living bacteria are culturable under laboratory conditions (possibly as few as 1% [Amann et al., 1995], although recently 14% have been reported [Giovannoni et al., 2002]), this approach is certainly unsatisfactory. Therefore, to evaluate the microbial diversity of a sample, a culture independent method is necessary. rRNA sequence analysis offers an elegant solution to this problem. With the information gained from rRNA sequence alignment phylogenetic relationships can be determined and it is possible to design specific

oligonucleotide probes (with a typical length of 18-24nt). Labelling these probes with a fluorescent dye and hybridising them to cells in environmental samples then allows the detection and taxonomic identification of microorganisms *in situ*, a method termed FISH (fluorescence *in situ* hybridisation) (DeLong et al., 1989). FISH is now a widely accepted standard method in microbial ecology. However, one drawback of the technique is its dependence on ribosome copy numbers. Signal intensities detectable with an epifluorescence microscope require large numbers of the (monolabelled) oligonucleotide probes to bind to their ribosomal targets. While log-phase cells can contain up to 10^5 ribosomes per cell, the numbers for slowly growing or metabolically inactive cells (which may represent a considerable fraction of any environmental sample) are much lower, thus impeding a comprehensive detection of all cells in a sample.

Amplifying the signal intensity of the probes can alleviate this problem. One approach, the so called tyramide signal amplification (TSA) is based on oligonucleotide probes labelled with horse radish peroxidase, which then catalyses the deposition of fluorescently labelled tyramine, resulting in a 10-20-fold signal amplification relative to fluorescein-monolabelled probes (Schönhuber et al., 1997). The method has been applied successfully for the *in situ* detection of stationary phase cells (Schönhuber et al., 1997) and mRNA (Bakermans et al., 2002, Wagner et al., 1998). However, comprehensive detection of all target cells is hampered, possibly due to insufficient permeability of the cell envelope for the relatively large enzyme coupled to the probe (Schönhuber, et. al, 1997).

Polynucleotide transcript probes

Another way to avoid low signal intensities is the use of multiply labelled polynucleotide probes with a size of about 100-250nt (Trebesius, 1994). The probes consist of RNA and are generated via *in vitro* transcription. The labelling is achieved through incorporation of labelled UTP (labelled with a fluorescent dye, biotin or digoxigenin) during the transcription, resulting in a labelling density of about 1 labelled nucleotide every 10-20nt. Polynucleotide probes have been reported to detect a higher percentage of total cell numbers than oligonucleotides in FISH analyses of environmental samples (Pernthaler et al., 2002). While Pernthaler et al. attributed the effect to slower bleaching dynamics of the multiply labelled probes, this work explores a different hypothesis for the origin of the phenomenon, i.e. the possibility of the probes forming a network around the target cell. This would render the signal intensity independent of the number of ribosomes, since more probe molecules than

target molecules can be involved in generating the signal, an assumption that has important implications for probe design and eventually led to the development of probes targeting plasmids and even chromosomal DNA – targets that have previously been thought to be unsuitable for FISH due to their low copy number.

Polynucleotide probes targeting plasmid and chromosomal DNA

While rRNA targeted probes allow a taxonomic identification of organisms in an environmental sample, analysis of the metabolic potential of an organism requires addressing the genetic information encoded in the chromosomal or plasmid DNA. Previous approaches to this task include *in situ* PCR (Hodson et al., 1995) and detection of mRNA (Wagner et al., 1998). Indirect evidence of genes coding for certain metabolic capabilities can be gained from monitoring the uptake of radioactively labelled substrate in combination with FISH (fluorescence *in situ* hybridisation-microautoradiography, FISH-MAR, Lee et al, 1999 and Ouverney et al, 1999). While mRNA detection and FISH-MAR are limited to metabolically active genes, *in situ* PCR can make use of the complete genome. Yet this technique failed to become widely accepted due to the problematic balance between sufficient permeability of the cell envelope to enable the polymerase to access the cell and sufficient cell integrity to prevent efflux of PCR product.

This work explores the use of polynucleotide transcript probes for detection of chromosomal or plasmid encoded genes *in situ*, a completely new approach, which opens the door to a wide range of clinical and ecological applications.

Cell sorting with polynucleotide probes

The fluorescence signal observed in hybridisations with polynucleotide probes differs markedly from that seen with oligonucleotides, a feature first described by Trebesius et al. (1994). With polynucleotides the fluorescence is concentrated in the periphery of the cell, forming a ring or halo-like structure, whereas with oligonucleotide probes the fluorescence is usually spread evenly throughout the cell.

The appearance of the halo, which has a circumference slightly larger than the cell itself, provoked the assumption, that only part of the probe actually binds to the target rRNA, while the remaining part extends into or beyond the cell envelope (Trebesius, 1994). The presence of probes or partial probes outside the cell permits capturing the thus labelled cells and separating them from unlabelled cells in a sample. This novel concept of cell sorting was first addressed by Stoffels et al. (1999), who used streptavidin-coated paramagnetic beads binding

to the biotin labelled probe, allowing a separation of labelled and unlabelled cells in a magnetic field. Magnetic cell separation has previously only been known using antibodies targeting (surface) antigens (e.g. Skjerve et al., 1990, Luk et al, 1991, Christensen et al., 1992), whereas rRNA based cell sorting (using fluorescenly labelled oligonucleotides), has been used in connection with flow cytometry, requiring expensive equipment.
In the course of this work it was possible to improve the concept of polynucleotide probe based cell sorting. The procedure was tranferred to microplates, thus permitting automation of the process. In addition to that, the cell sorting could be rendered independent of the (possibly insufficient) labelling of the probe with biotin. Instead, the microplate based technique relies on the hybridisation of the probes extending from the target cells to complementary DNA bound to the surface of the microplates.

Aims of this work

The aims of this work can be formulated as follows:

- Analysing the features of polynucleotide probes, such as general reaction parameters, stringency, probe length and the influence of secondary structures on the hybridisation signal should lead to a deeper understanding of the functional principle of these probes and facilitate future probe design.
- Due to the size of polynucleotide probes the cell envelope of an organism presents a considerable obstacle that prevents the probes from hybridising to their target inside the cell. Previously, polynucleotide probes have only been used for gram-negative bacteria. The adaptation of the method for organisms with more rigid cell envelopes (i.e. gram-positive bacteria and yeasts) requires modifications of the cell fixation procedures to permeabilise the cell envelope.
- The advantages of polynucleotide probes compared to oligonucleotides regarding signal intensity suggest investigating nucleic acid probe targets other than rRNA and present in lower copy numbers.
- Cell sorting based on the concept of partial probes protruding from the cells is to be improved.

B. Material and methods

B.1. Organisms

Organisms used in this work

Organism	Strain	Growth Medium	Growth Conditions
gram-negative bacteria			
Acinetobacter calcoaceticus	ATCC 17978 (=19606T)	LB	30°C, aerobic
Burkholderia cepacia	LMG 1222T	LB	30°C, aerobic
Enterobacter areogenes	DSM 30053	LB	37°C, aerobic
Escherichia coli	DSM 498	LB	37°C, aerobic
Escherichia coli	ATCC 1775T (=DSM30083)	LB	37°C, aerobic
Escherichia coli (carrying plasmid pCR2.1 TOPO)	Top10 Invitrogen	LB	37°C, aerobic
Escherichia coli (carrying plasmid pBBR1 MCS4)	Kovach et al. 1995	LB	37°C, aerobic
Escherichia coli (carrying plasmid pUN121)	Nilsson et al. 1983	LB	37°C, aerobic
Klebsiella pneumoniae	DSM 30104	LB	37°C, aerobic
Neisseria canis	LMG 8383T	LB	37°C, aerobic
Pseudomonas aeruginosa	DSM 6279	LB	30°C, aerobic
Xanthomonas campestris pv. campestris	LMG 568	LB	37°C, aerobic
Xanthomonas campestris pv. raphani	LMG 860	LB	37°C, aerobic
Xanthomonas campestris pv. pelargonii	LMG 817	LB	37°C, aerobic
Xanthomonas hortorum pv. pelargonii	LMG 7314	LB	37°C, aerobic
Xanthomonas campestris pv. pelargonii	LBP 672	LB	37°C, aerobic
Xanthomonas campestris pv. lobelia	LBP 687	LB	37°C, aerobic
gram-positive bacteria			
Bacillus subtilis	DSM1087	LB	37°C, aerobic
Micrococcus luteus	DSM20030T	LB	37°C, aerobic
Streptomyces divaceus griseolus	TÜ2353	SM	37°C, aerobic
Streptomyces venezuelae	TÜ2193	SM	37°C, aerobic
yeasts			
Candida cantarellii	PYCC 3073T	YM	30°C, aerobic
Candida diversa	PYCC 3408T	YM	30°C, aerobic
Candida etchellsii	PYCC 3059T	YM	30°C, aerobic
Candida intermedia	PYCC 2482T	YM	30°C, aerobic
Candida sake	PYCC 2742T	YM	30°C, aerobic
Candida tropicalis	PYCC 3097T	YM	30°C, aerobic
Candida vanderwaltii	PYCC 3671T	YM	30°C, aerobic
Candida vini	PYCC 2597T	YM	30°C, aerobic
Debaryomyces hansenii	PYCC 2968T	YM	30°C, aerobic
Dekkera anomala	PYCC 5153T	YM	30°C, aerobic
Dekkera bruxellensis	PYCC 4801T	YM	30°C, aerobic

Organism	**Strain**	**Growth Medium**	**Growth conditions**
Kluyveromyces lactis	PYCC 2538T	YM	30°C, aerobic
Kluyveromyces marxianus	PYCC 3886T	YM	30°C, aerobic
Pichia guilliermondii	PYCC 3440T	YM	30°C, aerobic
Pichia membranifaciens	PYCC 3796T	YM	30°C, aerobic
Rhodotorula mucilaginosa	PYCC 5166T	YM	30°C, aerobic
Saccharomyces cerevisiae	CBS 1171	YM	30°C, aerobic
Torulaspora delbrueckii	PYCC 3477T	YM	30°C, aerobic
Zygosaccharomyces bailii	PYCC 5167T	YM	30°C, aerobic
Zygosaccharomyces bisporus	PYCC 5335T	YM	30°C, aerobic
Zygosaccharomyces microellipsoides	PYCC 2534T	YM	30°C, aerobic
Zygosaccharomyces rouxii	PYCC 5276T	YM	30°C, aerobic
mammalian cells			
NIH-3T3 (mouse) *	DSM ACC59		
Jurkat (human) *	DSM ACC282		

Tab. B.1. Organisms
* fixed cells were kindly provided by Dr. Anke Burger-Kentischer, Fraunhofer Inst. f. Grenzflächen-und Bioverfahrenstechnik, Stuttgart)

ATCC: American Type Culture Collection, Manassas, Virginia, USA; http://www.atcc.org

CBS: Centraalbureau voor Schimmelcultures, Utrecht, Netherlands; http://www.cbs.knaw.nl/

DSM: DSMZ-Deutsche Sammlung 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

LBP: Bayerische Landesanstalt für Bodenkultur und Pflanzenanbau, Freising, Germany; http://www.LBP.bayern.de

PYCC: Portuguese Yeast Culture Collection, Lisboa, Portugal

TÜ: Universität Tübingen, Germany

B.2. Cultivation of organisms

Culture media were prepared with H_2O and sterilised in an autoclave for 20min at 121°C and an excess pressure of 1 bar. Solid media were supplemented with 12g/l agar (Difco Bacto Agar, Gibco BRL, Eggenstein, FRG).

LB – (Luria-Bertani)-medium		
tryptone (tryptic digest of casein peptone)	10.0g	
yeast extract	5.0g	
NaCl	5.0g	
H_2O	ad 1000 ml	pH 7.0 –7.2
HD – trypton	10.0g	
glucose	5.0g	
yeast extract	5.0g	
NaCl	8.0g	
H_2O	ad 1000 ml	pH 7.2-7.5
SM – Streptomyces medium		
glucose	4.0g	
yeast extract	4.0g	
malt extract	10.0g	
CaCO3 (only for solid medium!)	2.0g	
H_2O	ad 1000 ml	pH 7.2
YM – yeast medium		
yeast extract	3.0g	
malt extract	3.0g	
peptone	5.0g	
glucose	10.0g	
H_2O	ad 1000 ml	pH 7.0 –7.2

B.3. Primers

Primers for amplification of 23S rDNA domain III

Name	Sequence 5' – 3'	E. coli pos.	T_D (2+4)
1900V	(A/C)A(A/G/T) GCG TAG (G/C/T)CG A(A/T)G	1366 – 1380	50°C
317RT3	*ATA GGT ATT AAC CAC TAA AGG G* ACC (A/T)GT GTC (C/G)GT TT(A/C/T) (C/G/T)GT AC	1601 – 1621	58°C
1900V-Euk	GCG CCG TTC CGA AGG GAC	1366 – 1380	62°C
317RT3-Euk	*ATA GGT ATT AAC CAC TAA AGG G* AC CTG CTG CGG ATA TGG GTA C	1601 – 1621	60°C

Tab. B.2. Primers for amplification of domainIII, 23S rDNA. V = forward (vorwärts) primer, R = reverse primer, T3 = binding site for T3 polymerase (underlined)

Primers for probe set 1024_1-11 (23S rRNA)

Name	Sequence 5'-->3'	E. coli pos.	T_D (2+4)	size of PCR product with 1024R as reverse primer
1024R-T3	*ATA GGT ATT AAC CAC TAA AGG G* GA ACG CTC (CT)(CT)C TAC C	1186 – 1200	48°C	n.a.
1151	ACC GAA GCT GCG GCA GCG	1151 – 1168	62°C	50bp
1136	GGG GCT AAA CCA TGC ACC	1136 – 1153	58°C	65bp
1119	TGC GCG GAA GAT GTA ACG	1119 – 1136	56°C	82bp
1023	GCG TAA (CT)AG CTC ACT	1091 – 1105	45°C	110bp
324Va	GTT GGC TT(AG) GAA GCA GCC	1059 – 1076	56°C	142bp
1019VN	ATA TGC GGC CGC CTG GTT CTC (CT)CC GAA A	807 – 821	49°C	394bp
992	AGT ACC G(GT)G AGG GAA AG	457 – 473	53°C	744bp
312	AGG CTA AAT ACT C	423 – 435	36°C	778bp
939	AGT AG(CT) GGC GAG CGA A	241 – 256	51°C	960bp
940VII	GAA (GC)TG AAA CAT CT(ACT) AGT	189 – 206	48°C	1012bp
3010	AAG CGT ACA (CT)GG TGG ATG CC	13 – 32	62°C	1188bp

Tab. B.3. Primers for probe set 1024_1-11. V = forward (vorwärts) primer, R = reverse primer, T3 = binding site for T3 polymerase (underlined), N = *Not* restriction site

Other rRNA (rDNA) targeted primers

Name	Sequence 5' – 3'	E. coli pos.	T_D (2+4)
616Valt	AGA GTT TGA T(CT)(AC) TGG CTC AG	16S, pos. 8-27	58°C
630R	CA(GT) AAA GGA GGT GAT CC	16S, pos.1529-1546	50°C
985R	CCG GTC CTC TCG TAC T	23S, pos. 2654-2669	52°C
1019N	ATA TGC GGC CGC CTG GTT CTC (CT)CC GAA A	23S, pos. 806-821	49°C

Tab. B.4. Other rDNA targeted primers. V = forward (vorwärts) primer, R = reverse primer, N = *Not* restriction site

Primers targeting plasmids

Name	Sequence 5' – 3'	Target	T_D (2+4)
M13-R	CAG GAA ACA GCT ATG AC	multiple cloning site of plasmid pCR2.1 (Invitrogen, Carlsbad, CA, USA)	50°C
M13-V	GTA AAA CGA CGG CCA G	(sequencee cf. F.1.)	50°C
M13-V T3	*ATA GGT ATT AAC CAC TAA AGG G* GTA AAA CGA CGG CCA G		50°C
M13R-T3	*ATA GGT ATT AAC CAC TAA AGG G* CA GGA AAC AGC TAT GAC		50C
beta-Lact-R	ACC AAT GCT TAA TCA GTG	beta lactamase gene (sequence cf. F.1.)	50°C
beta-Lact-R-T3	*ATA GGT ATT AAC CAC TAA AGG G* ACC AAT GCT TAA TCA GTG		50°C
beta-Lact-V	GAG TAT TCA ACA TTT CCG		50°C
beta-Lact-VT3	*ATA GGT ATT AAC CAC TAA AGG G* GAG TAT TCA ACA TTT CCG		50°C

Tab.B.5. Primers targeting plasmids. V = forward (vorwärts) primer, R = reverse primer, T3 = binding site for T3 polymerase (underlined).

Primers targeting the Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)

Name	Sequence 5' – 3'	T_D (2+4)
Eco-GAPDH-RT3	*ATA GGT ATT AAC CAC TAA AGG G* CA GTT TCG TCA GTC AGG A	54°C
Eco-GAP-V-A	GTT GAC GTT GTC GCT GAA	54°C
Eco-GAP-V-B	TGA ACG TGA TCC GGC TAA	54°C
Eco-GAP-V-C	GTG AAA GAC GGT CAT CTG	54°C
Eco-GAP-V-D	TAC ATG GCA TAC ATG CTG	52°C
Eco-GAP-V-E	GCT GCT CAG AAA CGT TCT	54°C
Eco-GAP-V-F	CTA ACA AAT AGC TGG TGG	52°C
Eco-GAP-V-G	AGT CAG TCG CGT AAT GCT	54°C
Eco-GAP-V-H	TGC ACC TAA ATC GTG ATG	52°C
Eco-GAP-V-I	CTC TGT CCC ATG ATT CTG	52°C
Eco-GAP-V-J	CAA ACA GTG ATA TAC GCC	54°C
Eco-GAPDH-VT3	*ATA GGT ATT AAC CAC TAA AGG G* GC TGC TCA GAA ACG TTC T	54°C
Eco-GAPDH-V2T3	*ATA GGT ATT AAC CAC TAA AGG G* CT CTG TCC CAT GAT TCT G	52°C
Euk-GAPDH-V	CCC ATC ACC ATC TTC CAG	56°C
Euk-GAPDH-VT3	*ATA GGT ATT AAC CAC TAA AGG G* CCC ATC ACC ATC TTC CAG	56°C
Euk-GAPDH-RT3	*ATA GGT ATT AAC CAC TAA AGG G* AA GGC CAT GCC AGT GAG C	58°C
Euk-GAPDH-R	AAG GCC ATG CCA GTG AGC	58°C

Tab. B.6. Primers targeting the GAPDH gene. V = forward (vorwärts) primer, R = reverse primer, T3 = binding site for T3 polymerase (underlined).

Primers targeting *Xanthomonas campestris* genes

Primer	specific for	Sequence 5'-->3'	Target gene	T_D (2+4)
Xantho6.8.-RT3	*X. campestris pv. campestris*	*ATA GGT ATT AAC CAC TAA AGG G* CGC AGC CAG CAA CTT GAA	XpsO (PilC like protein)	56°C
Xantho6.8.- V	*X. campestris pv. campestris*	ACA ACC TCT ACA TGC CAG	XpsO (PilC like protein)	54°C
Xantho4.6b -RT3	*X. campestris pv. raphani*	*ATA GGT ATT AAC CAC TAA AGG G* CTG CCC CGT CGG AAA AGT	VirB11	58°C
Xantho4.6b – V	*X. campestris pv. raphani*	GAC AGC ACG CAT CTC GAA	VirB11	56°C
Xantho2.11. -RT3	*X. campestris pv. lobelia*	*ATA GGT ATT AAC CAC TAA AGG G* CGA ATA CCT TGC TCA TGA	n.d.	52°C
Xantho2.11. -V	*X. campestris pv. lobelia*	TGG TGG TTT CTA TCT TCC	n.d.	52°C

Tab. B.7. Primers targeting *Xanthomonas campestris* genes. V = forward (vorwärts) primer, R = reverse primer, T3 = binding site for T3 polymerase (underlined).

Primers for yeasts

Name	Sequence 5' – 3'	Binding position *(S.cerevisiae)*	T_D (2+4)
Y1-V	CGG CGA GTG AAG CGG	LSU, 247-261	52°C
Y1-R-T3	*ATA GGT ATT AAC CAC TAA AGG G* TTC CCT TTC AAC AAT TTC	LSU, 563-580	46°C

Tab. B.8. Universal primers targeting the LSU rDNA of yeasts. V = forward (vorwärts) primer, R = reverse primer, T3 = binding site for T3 polymerase (underlined).

B.4. Probes

rRNA targeted oligonucleotide probes

Probe	specific for	Sequence 5'-->3'	Binding position (E. coli)	T_D (2+4)
Aca23a-FL	*Acinetobacter calcoaceticus*	ATC CTC TCC CAT ACT CTA GCG AT	SSU, pos 647-669	60°C
Kpn1129-FL	*Klebsiella pneumoniae*	GTT CCC GGC CTA ACC GCT	SSU, pos. 1129-1147	60°c
Kpn74-FL	*Klebsiella pneumoniae*	CCG AGA GCA AGC TCT CTG	SSU, pos. 74-96	60°C
Pae153-FL	*Pseudomonas aeruginosa*	TAG CGC CCG TTT CCG GAC	SSU, pos. 153-171	60°C
Eco444-FL	*Escherichia coli*	CTT TAC TCC CTT CCT CCC	SSU, pos. 444-462	56°C
Eub338 I-III-Cy3	*Eubacteria*	GC(AT) GCC (AT)CC CGT AGG (AT)GT	SSU, pos 338-355	60°C
C602-Cy3	*Yeasts*	GCA TCA GAA AGA TGG ACC	SSU, pos 603-629	54°C

Tab. B.9. Oligonucleotide probes

rRNA targeted polynucleotide probes

Probe	Binding site	E. coli position	Probe length (nt)	Universal primers used to generate probe (cf. B.3.)
DIII	23S rRNA, domain III	1365-1617	236 *(E. coli)*	1900V / 317RT3
DIII-Euk	28S rRNA	1365-1617	321 *(H.sapiens)*	1900V-Euk / 317RT3-Euk
367	23S rRNA, domain I	115-370	255 *(E. coli)*	118V / 367RT3
Y1	28S rRNA	246-529	333 *(S. cerevisiae)*	Y1-V / Y1-RT3
1024-1	23S rRNA, domain II	1151-1201	50 (*E. coli)*	1151 / 1024RT3
1024-2	23S rRNA, domain II	1136-1201	65 *(E. coli)*	1136 / 1024RT3
1024-3	23S rRNA, domain II	1119-1201	82 *(E. coli)*	1119 / 1024RT3
1024-4	23S rRNA, domain II	1091-1201	110 *(E. coli)*	1023 / 1024RT3
1024-5	23S rRNA, domain II	1059-1201	142 *(E. coli)*	324Va / 1024RT3
1024-6	23S rRNA, domain II	807-1201	394 *(E. coli)*	1019N / 1024RT3
1024-7	23S rRNA, domain I-II	457-1201	744 *(E. coli)*	992 / 1024RT3
1024-8	23S rRNA, domain I-II	423-1201	778 *(E. coli)*	312 / 1024RT3
1024-9	23S rRNA, domain I-II	241-1201	960 *(E. coli)*	939 / 1024RT3
1024-10	23S rRNA, domain I-II	189-1201	1012 *(E. coli)*	940VII / 1024RT3
1024-11	23S rRNA, domain I-II	13-1201	1188 *(E. coli)*	3010 / 1024RT3

Tab. B.10. rRNA targeted polynucleotide probes

Plasmid targeted probes

Probe	Target	Probe length (nt)	Primers used to generate probe (cf. B.3.)
Eco2.1	multiple cloning site of plasmid pCR2.1	203	M13-V / M13-RT3
β-Lact	plasmid encoded beta lactamase gene	856	β-Lact-V / β-Lact RT3

Tab. B.11. Plasmid targeted polynucleotide probes

DNA targeted probes

Probe	Target	Probe length (nt)	Primers used to generate probe (cf. B.3.)
E-GAPDH-A	E. coli GAPDH gene	48	Eco-GAP-V-A / Eco-GAPDH-RT3
E-GAPDH-B	E. coli GAPDH gene	88	Eco-GAP-V-B / Eco-GAPDH-RT3
E-GAPDH-C	E. coli GAPDH gene	138	Eco-GAP-V-C / Eco-GAPDH-RT3
E-GAPDH-D	E. coli GAPDH gene	198	Eco-GAP-V-D / Eco-GAPDH-RT3
E-GAPDH-E	E. coli GAPDH gene	258	Eco-GAP-V-E / Eco-GAPDH-RT3
E-GAPDH-F	E. coli GAPDH gene	338	Eco-GAP-V-F / Eco-GAPDH-RT3
E-GAPDH-G	E. coli GAPDH gene	446	Eco-GAP-V-G / Eco-GAPDH-RT3
E-GAPDH-H	E. coli GAPDH gene	548	Eco-GAP-V-H / Eco-GAPDH-RT3
E-GAPDH-I	E. coli GAPDH gene	738	Eco-GAP-V-I / Eco-GAPDH-RT3
E-GAPDH-J	E. coli GAPDH gene	795	Eco-GAP-V-J / Eco-GAPDH-RT3
Euk-GAPDH	human/mouse GAPDH gene	472	Euk-GAPDH-V / Euk-GAPDH-RT3
Xc-ca	*Xanthomonas campestris pv. campestris*, XpsO(PilC like gene)	140	Xantho 6.8 - V / -RT3
Xc-ra	*Xanthomonas campestris pv. Raphani,* VirB11 gene	272	Xantho 4.6b – V / -RT3
Xc-lo	*Xanthomonas campestris pv. lobelia,* target region n.d.	352	Xantho 2.11. – V / RT3

Tab. B.12. DNA targeted polynucleotide probes

B.5. Isolation of nucleic acids

B.5.1. Isolation of genomic DNA

Genomic DNA was isolated as described by Wisotzkey et al. (1990). This method is suitable for a small-scale (up to 50ml bacterial culture) isolation of DNA in a relatively short period of time. The resulting DNA may be slightly fragmented and still contain some contaminations. Therefore it should not be used for assays requiring highly purified, high molecular DNA, e.g. GC determination. However, the quality is sufficient for PCR.

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

Reagents:

Saline-EDTA (SE-buffer)	0.15M NaCl 0.01M EDTA, pH 8.0
SSC, 20x (standard saline citrate)	3M NaCl 0.3M trisodiumcitrate pH 7.0
Tris/HCl	10mM Tris/HCl, pH 8.5
NaAc	5M sodiumacetate
SDS	25% (w/v) sodiumdodecylsulfate; not autoclaved
CIA	chloroform : isoamylalcohol = 24:1 (v/v)
lysozyme	10 mg/ml lysozyme in 10mM Tris/HCl, pH 8.5
proteinase K	20 mg/ml Proteinase K (Roche, Mannheim, Germany) in H_2O_{MQ}
RNase A	0.5mg/ml in 2x SSC
$EtOH_{abs}$, EtOH 70%	

- Centrifuge 4ml of an overnight culture 2min at 12 000g
- Remove medium and resuspend pellet in 500µl SE-buffer
- add 20µl lysozyme and incubate at 37°C for 30min
- add 2µl RNase A and incubate at 37°C for 30min

- add 2µl proteinase K and incubate at 37°C for 45min
- add 40µl 25% SDS and incubate at 60°C for 10min to complete cell lysis
- add 745µl CIA and 180µl NaAc to extract proteins and cell fragments; shake carefully and centrifuge 5min at 12 000g
- transfer upper phase into fresh reaction tube, discard lower phase
- precipitate DNA by adding 2vol. $EtOH_{abs}$, leave 1-2h at –20°C
- centrifuge 15min at 12 000g, wash with 70% EtOH and resuspend DNA pellet in 50-200µl H_2O
- store at –20°C

B.5.2. PurificationDNA (Birnboim and Doly, 1979, lysis by alkali)

This method is suitable for a fast purification of plasmid DNA. It is based on the different behaviour of plasmid and chromosomal DNA under alkaline conditions. A high pH disrupts the base pairing and causes the linear chromosomal DNA to denature irreversibly, whereas the supercoiled plasmid DNA stays intact and can therefore be separated from genomic DNA and cell fragments.

Reagents:

buffer 1	10mM EDTA
buffer 2	0.2M NaOH/ 1% SDS, freshly prepared
buffer 3	2.5M potassium acetate 2.5M acetic acid pH 4.7
TE-buffer	10mM tris/HCl, pH 7.0 1mM EDTA
isopropanol	
EtOH 70%	

- centrifuge 2ml of an overnight culture 1min at 12 000g
- discard medium and resuspend bacterial pellet in 200µl buffer 1
- add 400µl buffer 2
- add 300µl buffer 3
- centrifuge 15min at 12 000g and 4°C
- transfer clear supernatant to a fresh 1.5ml reaction tube
- pecipitate plasmid DNA by adding 0.7 vol isopropanol, leave 1-2h at –20°C
- centrifuge 15min at 12 000g and 4°C, wash with 70% EtOH and resuspend DNA in 20-100µl H2O
- store at –20°C

B.6. Photometric measurement of nucleic acid solutions

The aromatic ring systems of the DNA have a maximum absorption of light at a wavelength of 260nm. To determine the concentration of nucleic acid solutions as described by Clark and Swika (1977) an aliquot of nucleic acid was measured in a spectral photometer (Pharmacia, Freiburg, Germany) at 260nm. Calculation of the concentration was based on the following approximated values (Cryer et al., 1975):

double stranded (ds) DNA	1 OD_{260} is equivalent to 50µg/ml
single stranded (ss) RNA	1 OD_{260} is equivalent to 40µg/ml

For calculating the concentration of single stranded oligonucleotides the following fomula was applied in conjunction with values from table B.13.:

$$c = \frac{V_c \times OD_{260nm}}{V_{sample} \times \varepsilon_{oligo} \times d}$$

V_c: cuvette volume

V_{sample}: sample volume

d: diameter of cuvette

ε_{oligo}: oligonucleotide specific extinction coefficient, derived from the sum of the extinction coefficients of the bases (cf. tab. B.13.) of the oligonucleotide.

nucleotide	extinction coefficient ε at 260nm [$\mu mol^{-1}\ cm^2$]	molecular weight [$g\ mol^{-1}$]
dAMP	15.20	312.2
dCMP	7.05	288.2
dGMP	12.01	328.2
dTMP	8.40	303.2

Tab. B.13. Extinction coefficients of oligonucleotides

The ratio of the absorption at 260nm/280nm and 260nm/230nm can indicate the presence of proteins and other contaminants. Uncontaminated DNA solutions should approximate these values (Marmur, 1961):

$$E_{260nm}/E_{280nm} > 1.8$$

$$E_{260nm}/E_{230nm} > 2.2$$

B.7. Agarose gel electrophoresis

Nucleic acids can be analysed on agarose gels by applying an electric field. Due to their negative charge, they move towards the anode and are separated according to their molecular mass and conformation (linear, open circular or supercoiled). Intercalation of ethidiumbromide or adhesion of SYBR Green™ can render the nucleic acids visible under UV light (302nm).

Agarose gel electrophoresis was used to verify size, purity or concentration of nucleic acids (chromosomal DNA, PCR products, RNA transcript probes). For evaluation of size and concentration of the samples a standard marker was used in a given concentration.

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) boiled in 1xTAE
loading buffer 1:	10 mM	EDTA
	5% (w/v)	Ficoll (Sigma, Deisenhofen, Germany)
	0.05%	bromphenol blue
	0.05%	xylenecyanol
	0.10%	SYBR Green™ (FMC BioProducts, Rockland, ME, USA)

loading buffer 2:	10 mM 5% (w/v) 0.05% 0.05%	EDTA Ficoll (Sigma, Deisenhofen, Germany) bromphenol blue xylenecyanol
ethidiumbromide staining solution:	1 µg/ml	ethidium bromide
marker:	1 µg	1-kb-standard (Gibco/BRL, Eggenstein, Germany)
gel chamber:		Gibco/BRL, Eggenstein, Germany type H3: 11 x 14 cm, 100 ml gel volume
gel documentation:		UV-transilluminator, wave length 302nm (Bachofer, Reutlingen, Germany) Cybertech CS1 Image Documentation System (Cybertech, Berlin, Germany)

Electrophoresis with loading buffer containing SYBR Green™ :

- mix 1vol. sample (resp. marker) + 1vol. loading buffer 1 (max. final volume 20µl), load gel
- electrophoresis in 1x TAE at 90-120mA (constant current) for 45-90min
- documentation on transilluminator

Electrophoresis without SYBR Green™:

- mix 1vol. sample (resp. marker) + 1vol. loading buffer 2, load gel
- electrophoresis in 1x TAE at 90-120mA (constant current) for 45-90min
- stain gel for 20min in ethidium bromide solution, wash 5min with dH_2O
- documentation on transilluminator

B.8. Polymerase chain reaction (PCR) (Saiki et al. 1988)

The polymerase chain reaction is an enzymatic technique for specific amplification of defined DNA fragments by cyclic repetition of the following steps:

- denaturation: thermic denaturation of the DNA
- annealing: binding of the oligonucleotide primers to the target sequence
- elongation: elongation of the primers in 5‘-3‘-direction by a thermostable DNA polymerase (e.g. Taq polymerase from *Thermus aquaticus*)

DNA synthesised in one cycle can serve as template during the following cycles, thus allowing exponential amplification of the target sequence. Under optimal conditions a 10^6 fold amplification of a primer flanked target sequence can be achieved in 25-35 cycles. When

a certain concentration of PCR product has been reached, the amplification ceases to be exponential and further increase of the number of cycles will not enhance the yield significantly (Saiki et al, 1988).
The annealing temperature has to be chosen with respect to the dissociation temperature T_D of the primers, which in turn depends on their length and sequence. An approximate value for T_D can be derived from the following formula ("2+4 rule", Suggs et al., 1981):

T_D [°C] = 2 x (A+T) + 4 x (G+C)
(A,C,G,T : number of nucleotides)

The optimal annealing temperature usually lies at T_D +/- 2°C, but should be determined empirically for each pair of primers.
The elongation period depends on the length of the amplificate. Standard polymerases can synthesise ca. 1-1.5kb per minute.

PCR reactions were performed in a Primus 96plus Thermal Cycler (MWG, Ebersberg, Germany) using the TaKaRa ExTaq™ system (TaKaRa Shuzo Co., Otsu, Japan).

Reaction mixture (per 100µl PCR reaction):

buffer (10x)	10	µl
dNTP-mix (2.5 mM each)	8	µl
primer 1 (50 µM)	0.5	µl
primer 2 (50 µM)	0.5	µl
DNA	ca. 100	ng
Ex Taq™	0.5	µl
H_2O_{MQ}	ad 100	µl

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

Reaction	**Temperature**	**Time**	**Number of cycles**
initial denaturation	94 °C	3 min	1
denaturation	94 °C	1 min	30
annealing	x °C	1 min	
elongation	72 °C	y min	
final elongation	72 °C	5 min	1

x: T_D of primer
y: ca. 1 min per 1 kb fragment length

B.9. Labelling of nucleic acids with biotin, digoxigenin or fluorescent dyes

B.9.1. Oligonucleotide probes

Oligonucleotides labelled with either biotin, digoxigenin, fluorescein or Cy-3 at the 5‘-terminus were obtained from MWG Biotech (Ebersberg, Germany).

B.9.2. Polynucleotide RNA probes – *in vitro* transcription

Polynucleotide RNA probes were generated via *in vitro* transcription. A variable region of the rDNA was amplified via PCR using universal primers (i.e. primers that are complementary to a highly conserved region of the rRNA (rDNA) flanking the variable region[cf. B.3.]). Therefore one set of primers could be used for all tested bacterial species while the resulting PCR product (and eventually the transcript probe) would be specific only for the species whose DNA was used to generate the probe (cf. table B.2.-B.8. for list of primers).

The reverse primer contained the promotor for the T3 RNA polymerase at the 5'-end, which is necessary for the subsequent *in vitro* transcription. During the transcription the probe was labelled with either biotin, digoxigenin or fluorescein by using labelled UTP in a ratio of 0.65/0.35 labelled/unlabelled UTP. (Higher amounts of labelled UTP decrease the yield, while lower amounts result in insufficient labelling (Stoffels et al. 1999).)

Up to 20µg RNA transcript probe can be expected per µg DNA template.

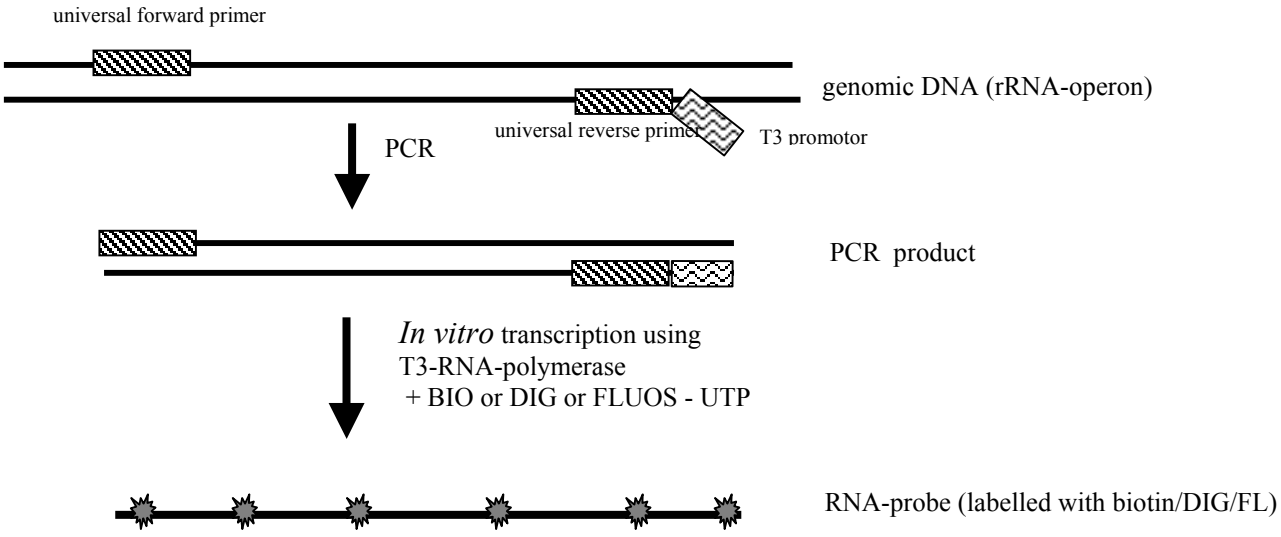


Fig. B.1. Schematic representation of the generation of labelled RNA polynucleotide probes via *in vitro* transcription

- *PCR reaction:*

for parameters of PCR reaction cf. B.9.

- *In vitro transcription:*

Nucleotides and enzymes were obtained from Roche (Mannheim, Germany).

NTP-Mix:	
ATP [100mM]	3.9µl
CTP [100mM]	3.9µl
GTP [100mM]	3.9µl
UTP [100mM]	1.4µl
BIO-16-UTP or DIG-11-UTP or FLUOS-12-UTP [10mM]	25µl

Transcription mix (per reaction):	
NTP-Mix	3µl
10x transcription buffer	3µl
T3-RNA polymerase	3µl
RNase-inhibitor	1.5µl
PCR product	1 – 2µg
H_2O_{MQ}	ad 30µl

further Reagents:

NH4-Ac	Ammoniumacetate, 10 M
EDTA	Ethylenediaminetetraacetate, 0.2M, pH 8.0

EtOH abs., 70% EtOH

- incubate transcription mix 3h at 37°C
- add 3µl DNaseI (RNase free) to degrade template DNA, incubate 15min at 37°C
- add 3µl EDTA to stop enzymatic reaction
- precipitate RNA at least 1h at –20°C by adding 16µl NH4-Ac and 156µl EtOH abs
- centrifuge 15min at 14 000rpm
- wash pellet with 70% EtOH
- resuspend in 30µl H_2O_{MQ} + 1µl RNase inhibitor
- run agarose gel to check integrity of transcript probe (2µl probe + 5µl formamide + 5µl loading buffer)
- store at –20°C

B.10. Cell fixation

Cell fixation is necessary to make cells walls permeable for oligo- or polynucleotide probes. In addition to that it helps to preserve the status quo of the cells by denaturing proteins and enzymes and thereby preventing degradation of the target RNA or DNA. For standard fixation a 4% PFA solution was used as described below. For gram-positive bacteria and yeasts other fixatives and/or additional pre-/postfixational procedures were tested. (cf. C.1)

B.10.1. Cell fixation with PFA

Reagents

PBS (phosphate buffered saline):	130mM NaCl 1.5mM K_2HPO_4 8.0mM Na_2HPO_4 2.7mM KCl
PFA: (paraformaldehyde)	4% paraformaldehyde (w/v) in PBS, pH 7.0 (heat PBS to ca. 60°C, add PFA, add NaOH until PFA dissolves, titrate to pH 7.0)
$EtOH_{abs}$	

- harvest cells during the exponential growth phase (OD_{600} 0.4-0.8), centrifuge 10min at 5000rpm
- resuspend cell pellet in 1-2ml PBS
- add 3 vol. 4% PFA solution
- incubate at 4°C for 12-16h
- centrifuge 10min at 5000rpm
- wash with 5ml PBS, centrifuge again
- resuspend in 1ml PBS
- add 1ml $EtOH_{abs}$.
- store at –20°C up to 1 year

B.10.2. Cell fixation with ethanol

- harvest cells during the exponential growth phase (OD_{600} 0.4-0.8), centrifuge 10min at 5000rpm
- resuspend cell pellet in 1-2ml PBS

- add 1 vol. EtOH abs.
- incubate at 4°C for 12-16h
- centrifuge 10min at 5000rpm
- wash with 5ml PBS, centrifuge again
- resuspend in 1ml PBS
- add 1ml $EtOH_{abs}$.
- store at –20°C

B.10.3.Cell fixation with formaldehyde / acetic acid

- harvest cells during the exponential growth phase (OD_{600} 0.4-0.8), centrifuge 10min at 5000rpm
- resuspend cell pellet in 1-2ml 20% formaldehyde / 50% acetic acid
- incubate at room temperature for 15min
- centrifuge 10min at 5000rpm
- wash with 5ml PBS, centrifuge again
- resuspend in 1ml PBS
- add 1ml $EtOH_{abs}$.
- store at –20°C

B.10.4. Cell fixation with methanol

- harvest cells during the exponential growth phase (OD_{600} 0.4-0.8), centrifuge 10min at 5000rpm
- resuspend cell pellet in 1-2ml 90% methanol / 3.7% formaldehyde
- incubate at room temperature for 15min
- centrifuge 10min at 5000rpm
- wash with 5ml PBS, centrifuge again
- resuspend in 1ml PBS
- add 1ml $EtOH_{abs}$.
- store at –20°C

B.10.5. Postfixational treatments

B.10.5.1. Heat fixation

For heat fixation either fresh or cells fixed with any of the above methods can be used.

- apply cells on microscope slides, dry at 60°C
- move slide 3x through flame of Bunsen burner
- dehydrate cells with 50%, 80%, 100% ethanol, 2min each
- hybridise with nucleic acid probe (cf. B.11.)

B.10.5.2. Lysozyme (gram-positive bacteria)

Lysozyme specifically hydrolyses the □(1→4) glycosidic bond between the N-acetylmuramic acid and the N-acetylglucosamine of the peptidoglycane layer of the bacterial cell wall. While this treatment is usually not necessary for gram-negative bacteria, it can help to decrease the rigidity of the thick peptidoglyane layer of gram-positive bacteria and therefore facilitate the passage of nucleic acid probes through the cell wall.

Cells on microscope slides:

- apply 2-5µl fixed cells on microscope slide, dry at 60°C
- put 10µl PBS on each field of slide
- add 2µl lysozyme [10mg/ml in 50mM Tris/HCl pH 8.0]
- incubate 5min at 37°C
- rinse slide with dH_2O
- dehydrate cells with 50%, 80%, 100% ethanol, 2min each
- hybridise with nucleic acid probe (cf. B.11.)

Cells in reaction tube

- put 2-5µl fixed cells in 0.5ml reaction tube
- add 200µl PBS, centrifuge 3min at 12000rpm to remove ethanol
- resuspend in 10µl PBS
- add 2µl lysozyme [10mg/ml in 50mM Tris/HCl pH 8.0]
- incubate 5min at 37°C
- add 200µl PBS, centrifuge 3min at 12000rpm
- resupend in hybridisation buffer (cf. B.11.3.). Formamide in hybridisation buffer will deactivate residual lysozyme
- hybridise with nucleic acid probe (cf. B.11.)

B.10.5.3. Proteinase K (gram-positive bacteria and yeasts)

Proteinase K is a non-specific, highly active protease obtained from *Tritirachium album*. In the pretreatment of cells prior to hybridisation it should 1) degrade proteins embedded in the cell envelope and thereby permeabilise the cell envelope and 2) degrade ribosomal proteins and thus possibly facilitate access of the probes to the ribosomal target sites.

Cells on microscope slides:

- apply 2-5µl fixed cells on microscope slide, dry at 60°C
- put 10µl PBS on each field of slide
- add 2µl proteinase K [5mg/ml in H_2O_{MQ}]
- incubate 30min at 37°C
- rinse slide with dH_2O
- dehydrate cells with 50%, 80%, 100% ethanol, 2min each
- hybridise with nucleic acid probe (cf. B.11.)

Cells in reaction tube

- put 2-5µl fixed cells in 0.5ml reaction tube
- add 200µl PBS, centrifuge 3min at 12000rpm to remove ethanol
- resuspend in 10µl PBS
- add 2µl proteinase K [5mg/ml in H_2O_{MQ}]
- incubate 30min at 37°C
- add 200µl PBS, centrifuge 3min at 12000rpm
- resupend in hybridisation buffer (cf. B.11.3.). Formamide in hybridisation buffer will deactivate residual proteinase
- hybridise with nucleic acid probe (cf. B.11.)

B.11. Hybridisation techniques

B.11.1. Evaluation of hybridisation conditions

For evaluation of the hybridisation conditions the following formulas were employed to calculate T_m. The hybridisation temperature should be about 20°C below T_m. However, to maximise signal specificity and intensity optimal hybridisation conditions had to be determined experimentally for each individual probe.

i) DNA-DNA-hybridisations

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

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

Polynucleotides (Howley et al., 1979):

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

ii) RNA-RNA-hybridisations (max. 500nt) (Wetmur et al., 1991, Wahl et al., 1987):

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

iii) RNA-DNA-hybridisations

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

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

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

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

T_m:	melting temperature in °C (polynucleotides)
T_D:	dissociation temperature in °C (oligonucleotides)
M:	molar concentration of sodium ions in hybridisation buffer
%GC:	percent guanine plus cytosine
D, n:	length of duplex
%FA:	percent formamide in hybridisation buffer
P:	percent of mismatches

B.11.2. Hybridisation with oligonucleotide probes

If hybridisations with both poly- and oligonucleotide probes are to be performed, the oligonucleotide hybridisation has to be done after the polynucleotide hybridisation, since polynucleotide probes require considerably higher stringency conditions.

Hybridisation buffer:	900 mM	NaCl
	20 mM	Tris-HCl, pH 8.0
	0.01 %	SDS
	x %	formamide
Washing buffer:	x mM	NaCl (cf. table B.14)
	20mM	Tris-HCl
	0.01 %	SDS

- use 2-5µl of a suspension of PFA fixed cells per field of microscope slide
- dry at 60°C, 5min
- dehydrate cells in 50%, 80%, 100% ethanol, 2min each
- put 9µl of hybridisation buffer on each field of slide
- add 1µl oligonucleotide probe (50-100ng/µl)
- put tissue paper in 50ml tube, moisten with 1-2ml hybridisation buffer
- insert slide into 50ml tube and close tube
- incubate 1.5-2 h at 46°C
- rinse slide with dH_2O
- put slide into 50ml tube filled with washing buffer (preheated to 46°C)
- wash 15 min at 48°C
- rinse with dH_2O, air dry

The NaCl concentration of the washing buffer can be derived from table B.14. To avoid the use of large amounts of (carcinogenic) formamide the stringency in the washing buffer is adjusted via the salt concentration. The stringency of the washing buffers as listed in table B.14. is equivalent to the stringency of the corresponding hybridisation buffers. (cf. B11.1.)

% formamide in hybridisation buffer	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*	-*

* for >20% formamide add 5mM Na_2EDTA to washing buffer

Tab. B.14. In the washing buffer formamide is omitted and equivalent stringency is achieved by decreasing the salt concentration.

B.11.3. Hybridisation with RNA polynucleotide probes

B.11.3.1. Probes targeting rRNA

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

Hybridisation with RNA polynucleotide probes was carried out either on microscope slides or in 0.5ml reaction tubes.

Hybridisation on slides:

- use 2-5µl of a suspension of PFA fixed cells per field of slide
- dry at 60°C, 5min
- dehydrate cells in 50%, 80%, 100% ethanol, 2min each
- put 12µl hybridisation buffer on each field of slide
- add 4µl transcript probe (~ 3-4µg) [cf. B.9.2.]
- put tissue paper in 50ml tube, moisten with 1-2ml hybridisation buffer
- insert slide into 50ml tube and close tube
- denature 20min at 80°C

- hybridise 5-16h at 53°C
- rinse slide with dH_2O
- detection cf. B.13.
- (if detection is not done immediately after hybridisation, dry slide and store at –20°C)

Hybridisation in 0.5ml reaction tubes:

- use 5-10µl PFA fixed cells per tube
- add 200µl PBS and centrifuge 3min at 12000rpm to remove residual ethanol
- resuspend cell pellet in 30µl hybridisation buffer
- add 5µl transcript probe (~4-5µg)
- denature 20min at 80°C
- hybridise 5-16h at 53°C
- detection cf. B.14.
- (if detection is not done immediately after hybridisation, store at –20°C)

B.11.3.2. Probes targeting plasmids

The procedure is essentially the same as described above, but due to the thermally less stable DNA-RNA-hybrid (as compared to the RNA-RNA-hybrid of B.11.3.1.) and the much lower number of target molecules, the formamide concentration in the hybridisation buffer and the hybridisation time have to be adjusted.

The optimal formamide concentration for probes targeting plasmids ranges between 5-20% FA, the hybridisation time between 18-24h.

B.11.3.3. Probes targeting genomic DNA

The procedure is essentially the same as described above, but due to the thermally less stable DNA-DNA-hybrid (as compared to the RNA-RNA-hybrid of B.11.3.1.) and the much lower number of target molecules, the formamide concentration in the hybridisation buffer and the hybridisation time have to be adjusted.

The optimal formamide concentration for probes targeting genomic DNA ranges between 5-15% FA, the hybridisation time between 24-30h.

B.12. Cell sorting

The cell sorting procedure can be divided into two distinct steps – a hybridisation step using polynucleotide probes to label the target cells (cf. B.11.) and and the actual cell sorting step where labelled cells are separated from unlabelled cells. This separation can either be based on a second hybridisation (using DNA-coated microplates [B.12.2.2.]) or can be mediated by biotin-streptavidin binding (using biotin labelled polynucleotide probes in the first step and streptavidin coated paramagnetic beads [B.12.1.] or streptavidin coated microplates [B.12.2.1.] for the second step).

B.12.1. Magnetic cell sorting (MACS) (Stoffels et al, 1999)

After hybridisation in 0.5ml tubes (cf. B.11.3.1.) using biotin labelled polynucleotide probes:

- add 60µl binding buffer (PBS/0.5M EDTA) + 10µl streptavidin coated microbeads (Miltenyi, Germany)
- incubate o/n at 4°C
- retain 10µl for later microscopic analysis (fraction A)
- put MACS column (Miltenyi, Germany) into magnetic field and equilibrate with 1ml binding buffer
- apply sample onto MACS column (Milteny, Germany)
- wash 2x with 500µl binding buffer, keep flow through (fraction B)
- remove column from magnetic field
- eluate with 2x 500µl PBS (fraction C)
- wash and centrifuge fractions A, B and C, resuspend in 10-20µl PBS
- quantify cell sorting efficiency (cf. B.14.):
  fraction A represents the original distribution of target and non-target cells
  fraction B should be depleted in target cells
  fraction C should be highly enriched in target cells

B.12.2. Cell sorting in microplates

B.12.2.1. Streptavidin coated microplates

After hybridisation in 0.5ml tubes (cf. B.11.3.) using biotin labelled polynucleotide probes:

- retain 5µl for later microscopic analysis (fraction A)
- add 1ml PBS

- centifuge 5min at 12000 rpm, resuspend in 50µl PBS/2mM EDTA per µl PFA fixed cells used for hybridisation (i.e. if 5µl cells were used for hybridisation [B.11.3.1.], resupend in 250µl PBS/2mM EDTA)
- put solution into streptavidin coated microwells (Advanced Biotechnologies Ltd., Surrey, UK), 50µl per microwell
- incubate first 1h on ice, then 30min at room temperature
- carefully remove the solution without disturbing the walls of the microwells
- if several microwells were used, pool contents, centifuge and resupend in 10-20µl PBS (fraction B)
- quantify cell sorting efficiency (cf. B.14.):
  fraction A represents the original distribution of target and non-target cells
  fraction B should be depleted in target cells
- target cells immobilised in streptavidin coated microwells can be directly used for PCR analysis

B.12.2.2. DNA coated microplates

B.12.2.2.1. Coating of microplates with DNA

Microplates (either Maxisorp or Nucleolink [both Nalge Nunc, Roskilde, Denmark]) were coated with PCR product corresponding to the probe sequence.

In Maxisorp plates the binding of DNA is due to hydrophobic/hydrophilic interactions, whereas in Nucleolink plates DNA is bound covalently via carbodiimide condensation. Both types of microplates proved to be equally suitable for the cell sorting process. Nucleolink plates, however, have the advantage to allow both photometric detection and PCR, while the shape of Maxisorp plates is not suitable for PCR.

- amplify the probe sequence by PCR using the standard protocol (cf. B.8.) and probe specific primers (cf. B.4.); use 16 100µl PCR reactions to get sufficient product for a complete 96 well plate (~ 1µg DNA per well)
- purify the PCR product by precipitation with 2.5 vol EtOH abs., 2h at –20°C, centrifugation at 12 000 rpm for 15min; resuspend in 100µl H_2O_{MQ}
- denature DNA for 10min at 94°C, place tube on ice immediately afterwards to avoid renaturing
- add ice cold PBS/0.1M $MgCl_2$, freshly prepared, 50µl per well
- dispense solution over microplate, 50µl per well, while microplate is on ice

- incubate 45min at 37°C
- discard supenatant
- dry 1h at 60°C
- coated plates can be stored at room temperature for several weeks in a dark and dry place
- prior to use, plates should be washed with 100µl PBS to remove unbound DNA

B.12.2.2.2. Cell sorting (Hybridisation in coated microplates – HYCOMP)

Reagents:
MP buffer: 5x SSC
0,02% SDS
2% blocking
0,1% N-laurysarcosin
33% formamide

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

After *in situ* hybridisation in 0.5ml tubes (cf. B.11.3.) using labelled (biotin, digoxigenin, fluoresceine, Cy3) or unlabelled polynucleotide probes:

- add 1ml PBS
- centifuge 5min at 12000 rpm, resuspend in 100µl MP buffer per µl PFA fixed cells used for hybridisation (i.e. if 5µl cells were used for hybridisation [B.11.3.], resupend in 500µl MP buffer)
- wash microwells with 100µl PBS to remove unbound DNA
- prehybridise microwells with 50µl MP buffer, 5min at room temperature
- put one half of solution into DNA coated microwells (cf. B.12.2.2.1.), 50µl per microwell (B)
- put the other half into uncoated microwells, 50µl per microwell (= negative control) (A)
- cover microplate with adhesive tape
- incubate 90min at 53°C (for rRNA targeted probes) / 37°C (for plasmid and chromosomal DNA targeted probes)
- carefully remove the solution without disturbing the walls of the microwells
- if several microwells were used, pool contents, centrifuge and resupend in 10-20µl PBS

- target cells immobilised in microwells can either be directly used for PCR analysis or the cells can be recovered by adding 100µl H_2O per microwell, incubation at 94°C for 5min and thorough rinsing to wash the cells off the walls of the microplate (C)
- quantify cell sorting efficiency (cf. B.14.):
  fraction A represents the original distribution of target and non-target cells
  fraction B should be depleted in target cells
  fraction C should be enriched in target cells

B.13. Detection of cells after cell sorting

B.13.1. Detection of biotin or digoxigenin labelled cells in microplates

During the following procedure it is crucial to gently pipette the solutions in order to avoid disturbing the cells bound to the microplate.

- remove supernatant from microplates; keep supernatant for microscope analysis (cf.B.13.2.)
- wash with 100µl PBS
- for biotin labelled probes: add 50µl streptavidin-peroxidase conjugate, diluted 1:1000 in PBS/1% blocking per microwell
  or:
- for digoxigenin labelled probes: add 50µl anti-digoxigenin-peroxidase conjugate, diluted 1:1000 in PBS/1% blocking per microwell
- incubate 30 min at room temperature
- discard supernatant
- wash 3x with 100µl PBS
- add 100µl BM blue (POD substrate [Roche, Germany])
- incubate 5-15min at room temperature; colour change to blue indicates substrate reaction
- add 100µl 1M H_2SO_4 to stop enzyme reaction; colour will change to yellow
- measure absorption in microplate reader at 450nm against 650nm reference

B.13.2. Detection for microscope analysis

Since the fluorescent dyes are unstable at light, the detection has to be done in the dark.

- recover supernatant from microplates after cell sorting (cf. B.12.2.2.2.); if the original sample was dispersed over several wells, pool supernatants
- centrifuge 5min at 12000 rpm
- wash 2x with 500µl H_2O
- resuspend in 10µl H_2O
- apply up to 5µl on microscope slide, dry at 60°C
- for biotin labelled probes: add 30µl streptavidin-fluorescein or streptavidin-Cy3 1:200 in DPBS (137mM NaCl, 8mM Na_2HPO_4, 2.7mM KCl) to each field of the slide

  or
- for digoxigenin labelled probes: add 30µl anti-digoxigenin-fluorescein 1:5 in DPBS
- incubate 45min at room temperature
- rinse slide with 2ml DPBS
- put slide in 50ml tube filled with DPBS
- incubate 20min
- air dry slide
- use anti-fading mounting medium (e.g. Vectashield, www.vectorlabs.com) to reduce bleaching of fluorescent dyes

When kept in the dark, the slides can be stored at room temperature for several weeks.

B.14. Quantification of cell sorting efficiency

To quantify the enrichment or depletion of target cells an aliquot of the sample after the cell sorting was compared to an aliquot of the same sample that had not undergone the cell sorting process.

B.14.1. Semiquantitative PCR

The idea of a semiquantitative PCR is to dilute a sample in several steps until no more template is present or the amount of template is too low to yield a detectable PCR product. The highest dilution where a detectable (visualised on an agarose gel) amount of PCR product is generated allows estimates about the relative amount of template in the original sample. Comparing dilution series before and after cell sorting, while using two sets of PCR primers -

I) specific for target cells and II) specific for non-target cells (whose number should remain constant) it is possible to assess the enrichment/depletion of target cells in the cell sorting process.

A difference of one dilution step – when using a 1:1 dilution series - is equivalent to 50% depletion of target cells, two dilution steps – 75% (=50+25), three dilutions steps – 87.5% (=50+25+12.5), etc. PCR with primers specific for non-target cells is necessary to adjust dilution steps to account for differences in the initial amount of sample before and after cell sorting.

For the amplification a standard PCR programm was used (cf. B8.).

Example:

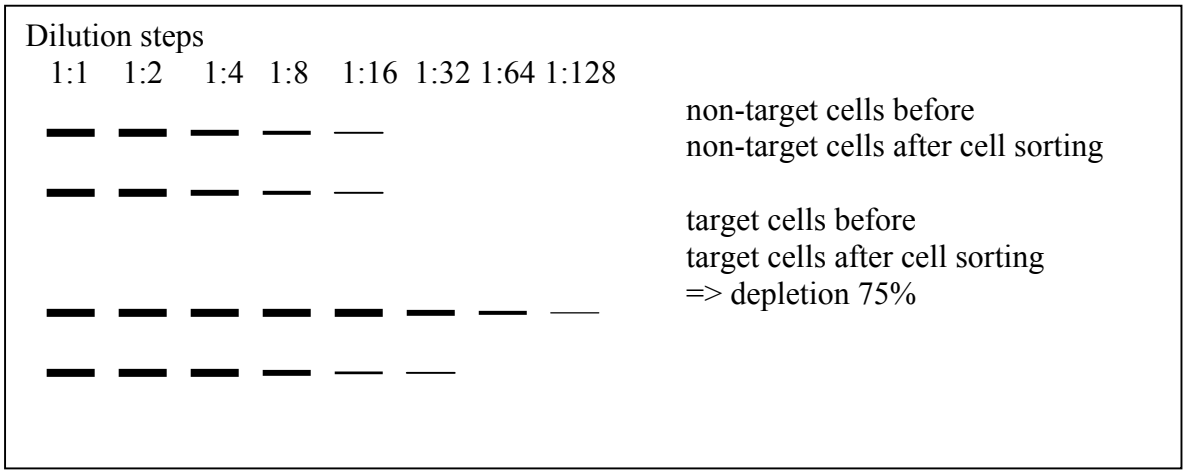


Fig.B.2. Semiquantitative PCR. Dilution series of target cells before and after cell sorting are amplified with target cell specific PCR primers. Comparing the number of dilution steps before and after cell sorting with a detectable PCR product (detected as bands on an agarose gel) allows conclusions about the cell sorting efficiency. A difference of one dilution step (in a 1:1 dilution series) is equivalent to 50% depletion of target cells, two dilution steps – 75% (50+25), etc. Amplification of a dilution series of non-target cells before and after cell sorting is necessary as a reference to adjust differences in the initial amount of sample before and after cell sorting.

B.14.2. Cell counts

Target and non-target cells of 10 randomly chosen microscopc fields were counted manually. Comparison of percentages before and after cell sorting allows calculation of depletion values.

B.14.3. LSM / KS400 software

For quantification with the LSM in connection with the KS400 software (Zeiss, Germany) samples have to be stained with fluorescently labelled oligonucleotide probes (cf. B.11.2.) using probe EUB338 (Amann et al., 1990) to stain all (bacterial) cells and a target cell specific probe, labelled with a different fluorescent dye.

The LSM was used to take pictures of A) the EUB signal and B) the target cell signal of 10-20 randomly chosen microscope fields. The files were then exported in .tif format. The subsequent software based quantification (macro within the KS400 software [Brühl, 1999]) relies on the comparison of the over-all area (pixels) of the EUB signal and the signal of the specific probe and is expressed in percent, i.e. "x % of all cells (area of EUB signal) are target cells (area of specific probe signal)". Comparison of percentages before and after cell sorting allows calculation of depletion values.

The depletion value is defined as:

$$\frac{(\%\ \text{target cells before} - \%\ \text{target cells after cell sorting})}{\%\ \text{target cells before cell sorting}} * 100$$

e.g. target cells before cell sorting: 40%
target cells after cell sorting: 20%
=> depletion value = 50%

B.15. Software and equipment

The following software and equipment was used for data analysis and processing :

- *rRNA sequence analysis and probe design:*

ARB software package (Ludwig, Strunk, 1996, http://www.arb-home.de)

- *Analysis of secondary structure of polynucleotide probes:*

Wisconsin Sequence Analysis Package, GCG (Genetics Computer Group), version 8.0, Madison, Wisconsin, USA

- *Epifluorescence microscopy imaging*

Zeiss Axioplan microscope

CCD camera, Princeton Instruments CCD 1035x1317 (model RTE-CCD 1317-K/2)

WinView software version 2.1.7.6., Princeton Instruments

Image processing: Adobe Photo Deluxe BE 1.0

- *Confocal laser scanning microscopy imaging and quantification*

confocal laser scanning microscope: Zeiss LSM 510 Ver. 2.01 SP2

quantification: KS400 ver. 3.0 software

Microsoft Office 2000

C. Results

The work presented here can be divided into two distinct parts –1. the exploration of the nature and functional principle of polynucleotide transcript probes, leading to an extended applicability regarding target organisms and molecules and 2. the implementation of this increased understanding to develop an improved method for cell sorting.

Part I – Fluorescence *In situ* Hybridisation (FISH) with Polynucleotide Transcript Probes

Fluorescence *in situ* hybridisation (FISH) is a widely-used method for identification of microorganisms in environmental samples. Typically, the probes used for conventional FISH are synthetic (DNA) oligonucleotides of no more than 15-25 nt in length.

The probes studied in this work differ from the tradtional FISH probes in that they are 1) polynucleotides (typically 200-300nt, but probes ranging from 50-1200nt have been tested successfully) and 2) they consist of RNA, generated via *in vitro* transcription. These features entail certain differences in the hybridisation procedure and detection, which required reevaluation and adjustment of existing FISH protocols.

C.1. Groups of organisms targeted with polynucleotide probes

Due to the length of polynucleotide probes experiences gained with oligonucleotide probes cannot necessarily be transferred. The applicability of these probes for groups of organisms differing in their cell envelope, i.e. gram-negative and gram-positive bacteria, yeasts and mammalian cells, had to be tested individually. Figure C.1 shows the position of the target region of the probes within the secondary structure of the bacterial 23S rRNA. Probe DIII, targeting the domain III, was the first polynucleotide probe analysed (Trebesius et al, 1994) and therefore served as a positive control in many experiments. Probes 367 and 1024R-1_11 were created to test the general applicability of polynucleotide probes and to verify that the special characteristics observed with polynucleotide probes are not due to certain features (e.g. secondary structure) inherent to the the domain III.

Probe DIII comprises ca. 250nt, as does probe 367, which targets domain I of the 23S rRNA. Probe 1024R-1_11 is a set of probes varying in length between 50-1200 nt. It was used to evaluate the influence of probe length on the quality of the signal and is discussed in more detail under C.2.1.2.

Figures C.2 - C.8. show examples of hybridisiations with polynucleotide probes with various gram-negative organisms.

The probes were labelled with biotin during the *in vitro* transcription and later detected with streptavidin-fluorescein as described in Material and Methods (cf. B.13.2.) and analysed using an epifluorecence microscope. Mixtures of morphologically distinguishable bacteria were used to verifiy the specificity of the probes. Each figure shows a phase contrast image (left) where all cells can be seen and an epifluorescence image (right) where only the cells targeted by the probe are visible. The fluorescence signal has the shape of a halo, which is characteristic for hybridisations with polynucleotide probes (Trebesius et al, 1994).

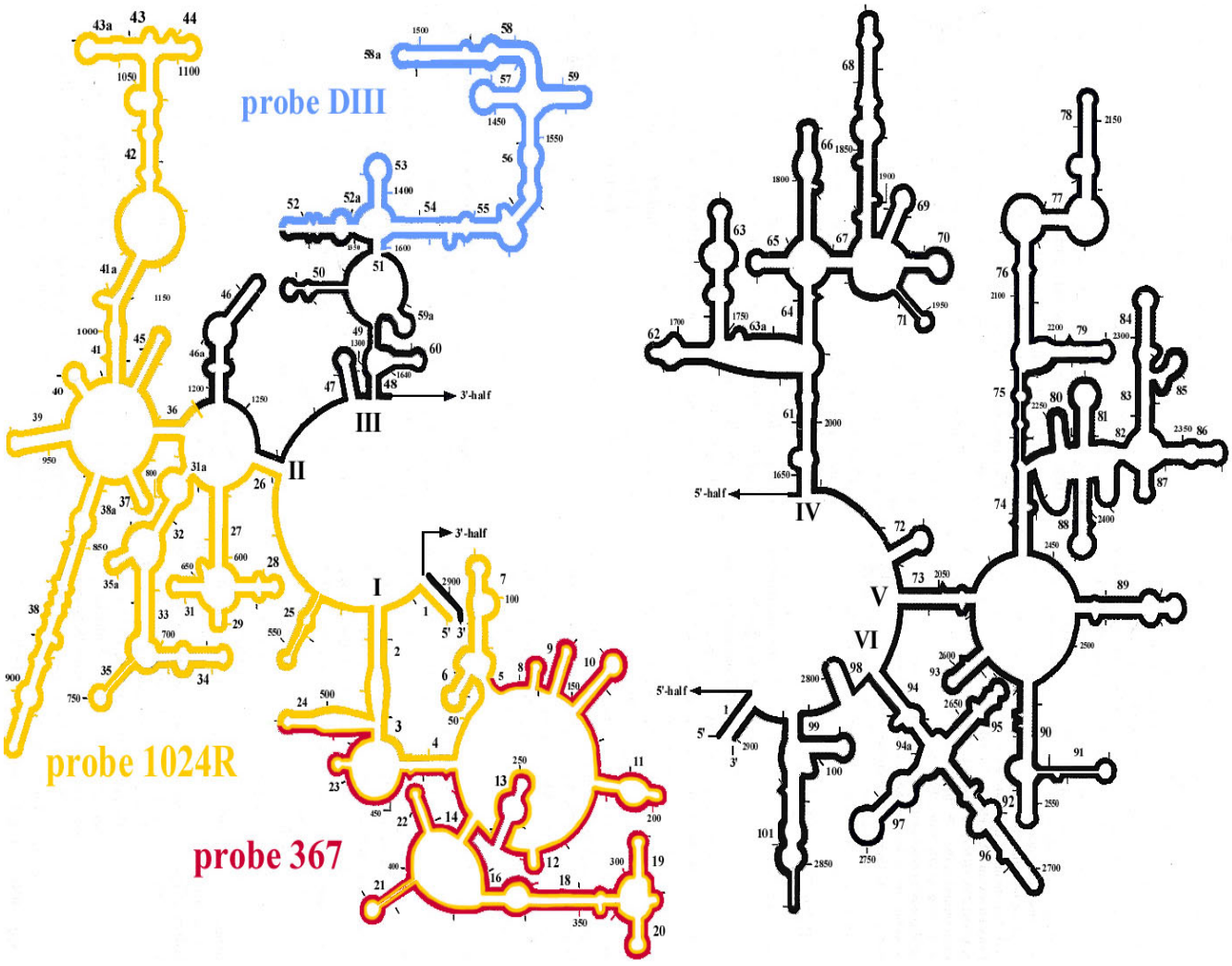


Fig. C.1. Secondary structure model of the 23S rRNA. The target positions of probes used in this study are marked in colour. Probe DIII (blue), probe 1024R (yelllow), probe 367 (red).

C.1.1. Gram-negative bacteria

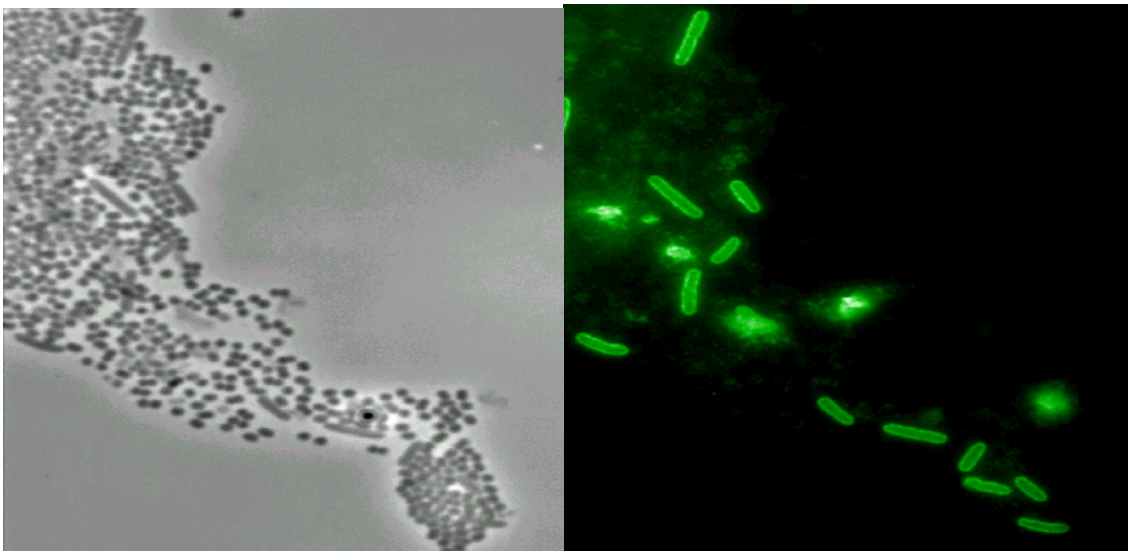


Fig. C.2. Fluorescence in situ hybridisation using probe DIII-Eco, specific for *E. coli* and a mixture of (roc shaped) *E. coli* and (coccoid) *N. canis* cells. Epifluorescence image (right), phase contrast image (left)

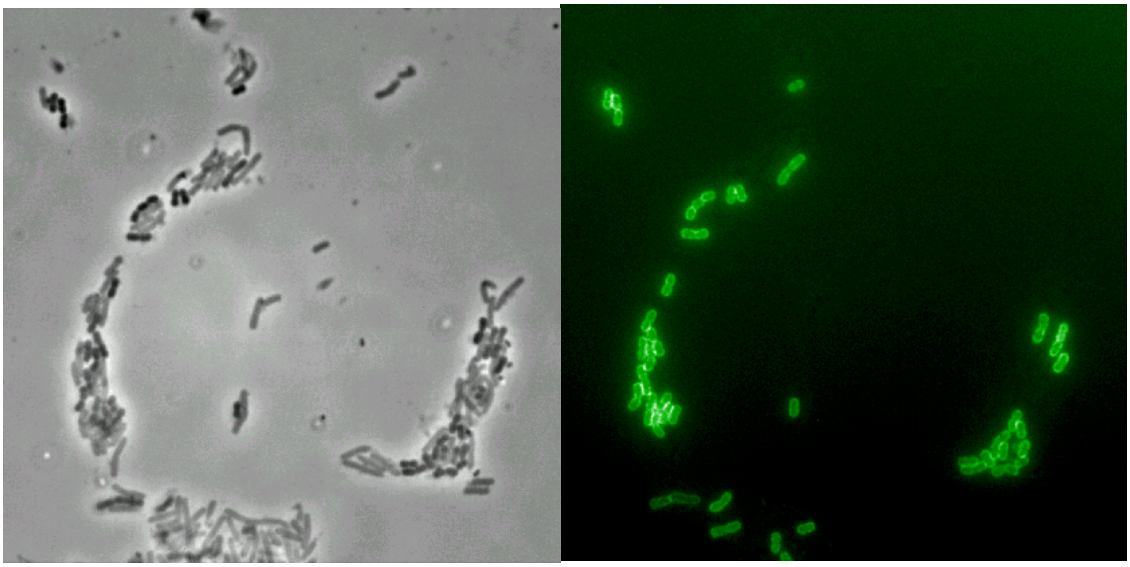


Fig. C.3. Fluorescence in situ hybridisation using probe DIII-Aca, specific for *E. coli* and a mixture of *E. coli* and *A. calcoaceticus* cells. Epifluorescence image (right), phase contrast image (left)

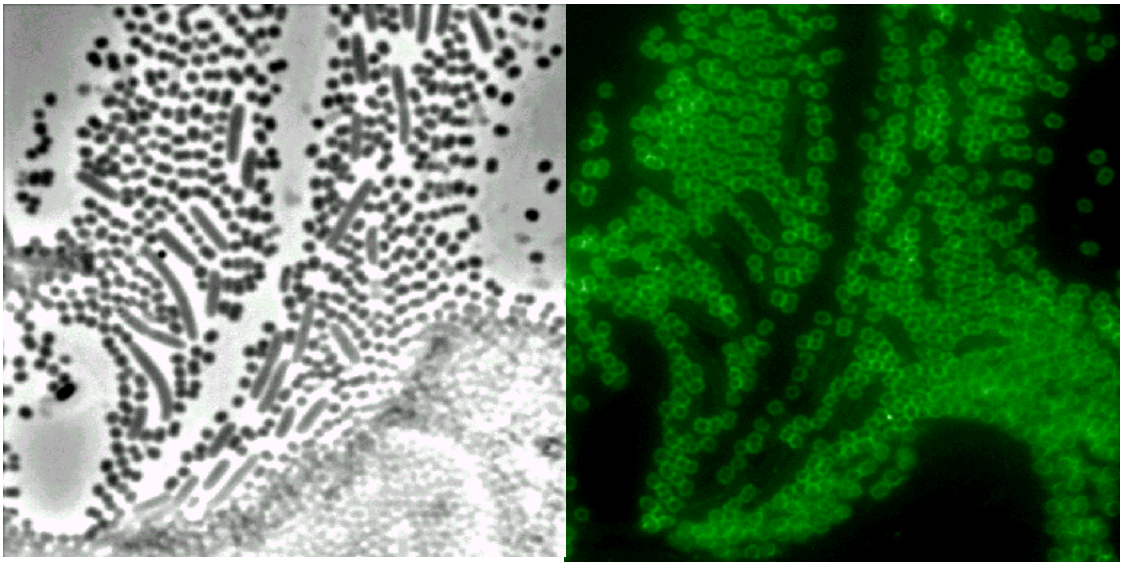


Fig. C.4. Fluorescence in situ hybridisation using probe DIII-Nca, specific for *N. canis* and a mixture of *E. coli* and *N. canis* cells. Epifluorescence image (right), phase contrast image (left)

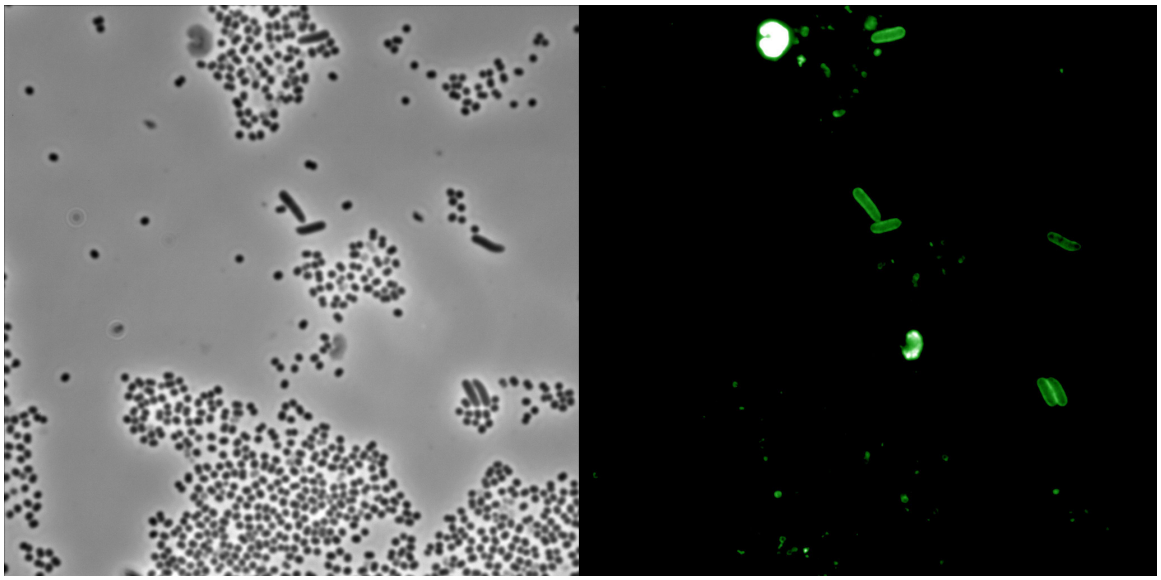


Fig. C.5. Fluorescence *in situ* hybridisation using probe DIII-Eae, specific for *E.aerogenes* and a mixture of *E.aerogenes* (rods) and *N. canis* (cocci) cells. Epifluorescence image (right), phase contrast image (left)

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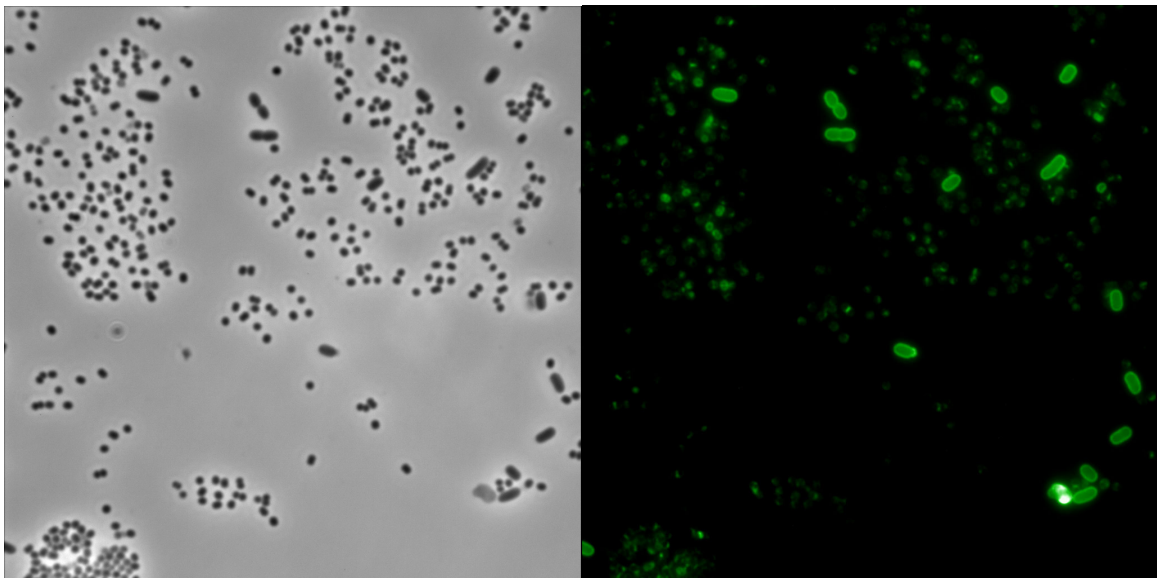


Fig. C.6. Fluorescence ir situ hybridisation using probe DIII-Kpn, specific for *K. pneumoniae* and a mixture of *K.pneumoniae* and *N. canis* cells. Epifluorescence image (right), phase contrast image (left)

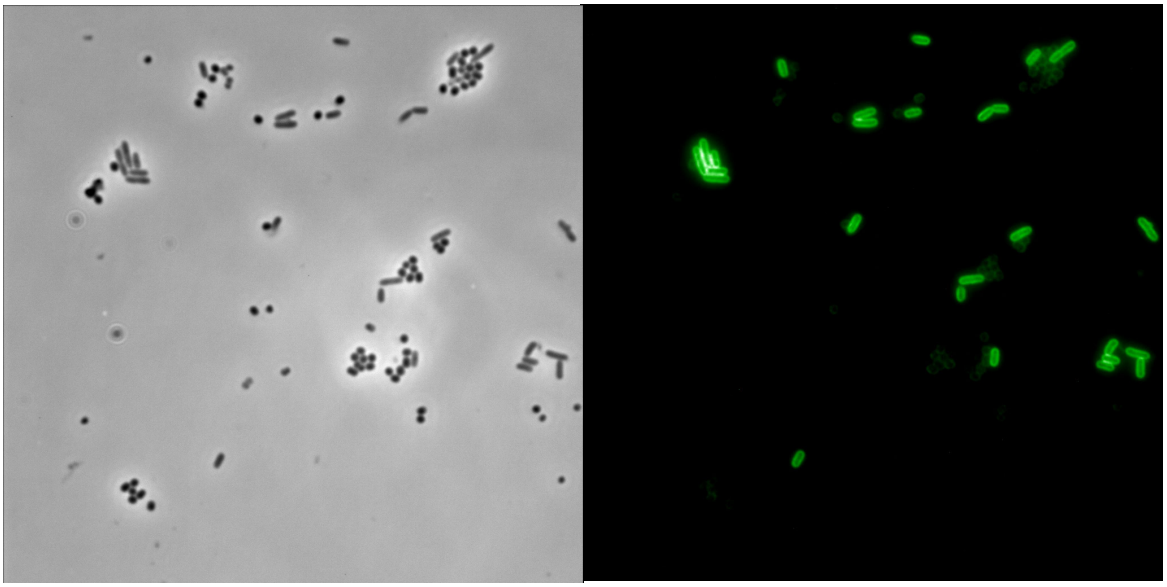


Fig. C.7. Fluorescence in situ hybridisation using probe DIII-Pae, specific for *P.aeruginosa* and a mixture of *P.aeruginosa* and *N. canis* cells. Epifluorescence image (right), phase contrast image (left)

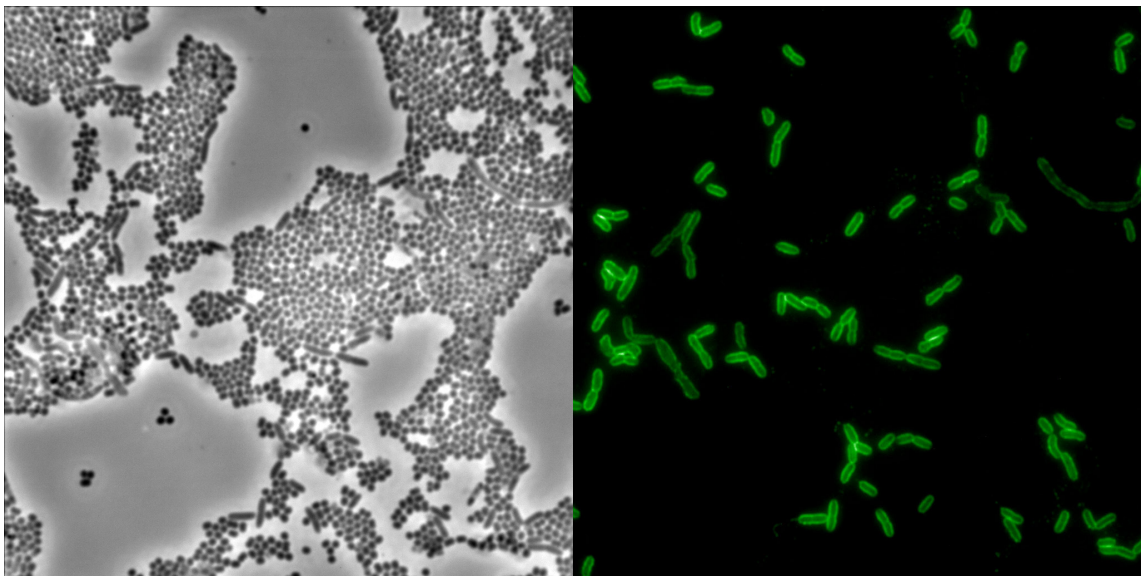


Fig. C.8. Fluorescence in situ hybridisation using probe 367-Bce, specific for *B.cepacia* and a mixture of *B.cepacia* and *N. canis* cells. Epifluorescence image (right), phase contrast image (left)

C.1.2. Gram-positive bacteria

The thick and rigid peptidoglycan layer of gram-positive bacteria can impede the passage of the probes through the cell wall. Treating the cells with lysozyme prior to hybridisation (cf. B.10.5.) can improve the permeability, but the incubation period has to be carefully balanced to avoid complete cell lysis (see Fig. C.9-10). Increased permeability may lead to a cellwide fluorescence signal instead of the characteristic halo (observed in *B. subtilis* and *Mc. luteus*, after 2min lysozyme incubation, Fig. C.9-10). *Streptomyces venezuelae* and *S. divaceus griseolus* showed a strong halo signal and no sign of cell lysis after 5min of lysozyme treatment (Fig. C.11-12).

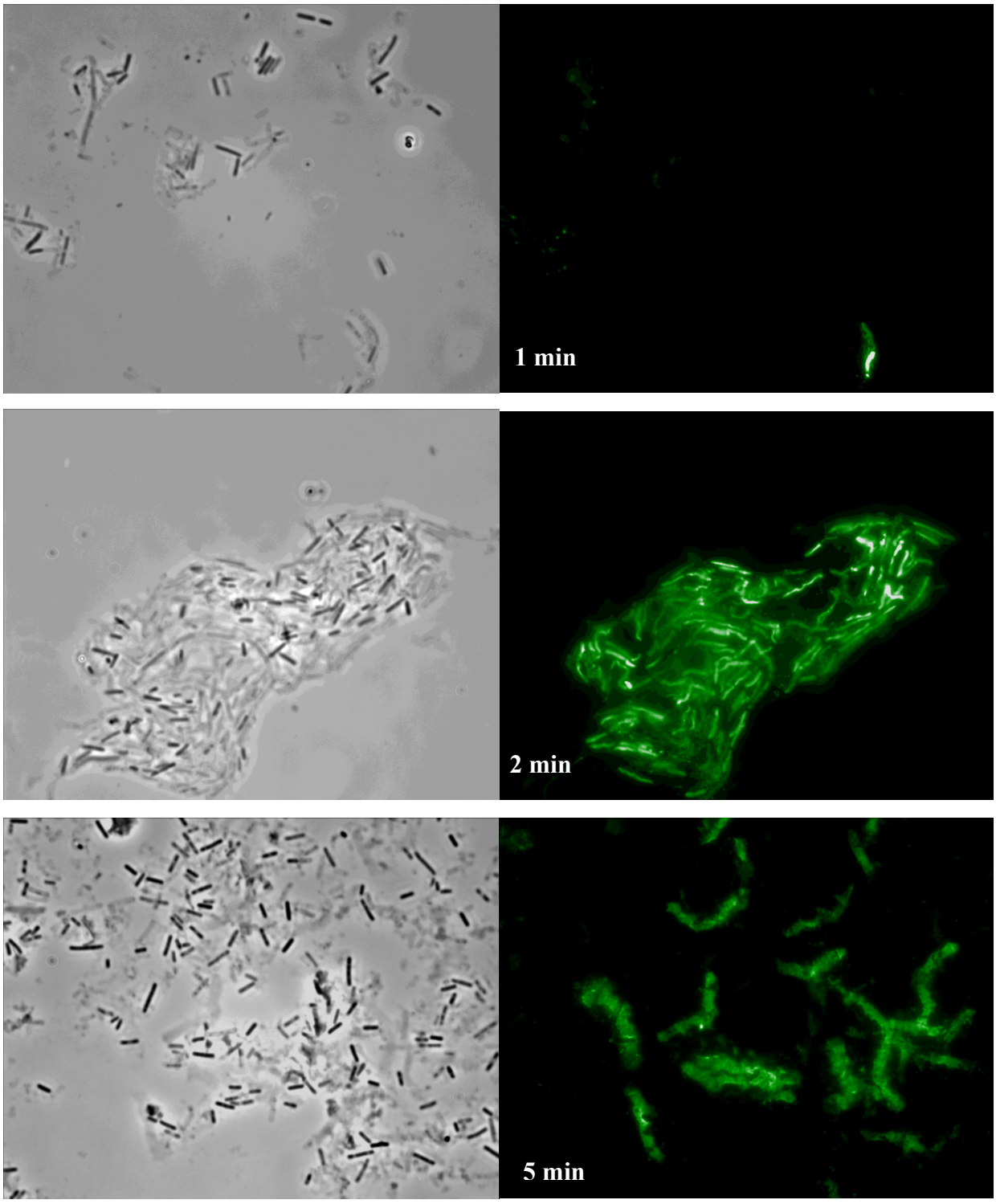


Fig.C.9. Influence of lysozyme on hybridisation signal in *Bacillus subtilis*. Upper images: hybridisation after 1min lysozyme treatment, middle images: hybridisation after 2min lysozyme treatment, lower images: hybridisation after 5min lysozyme treatment

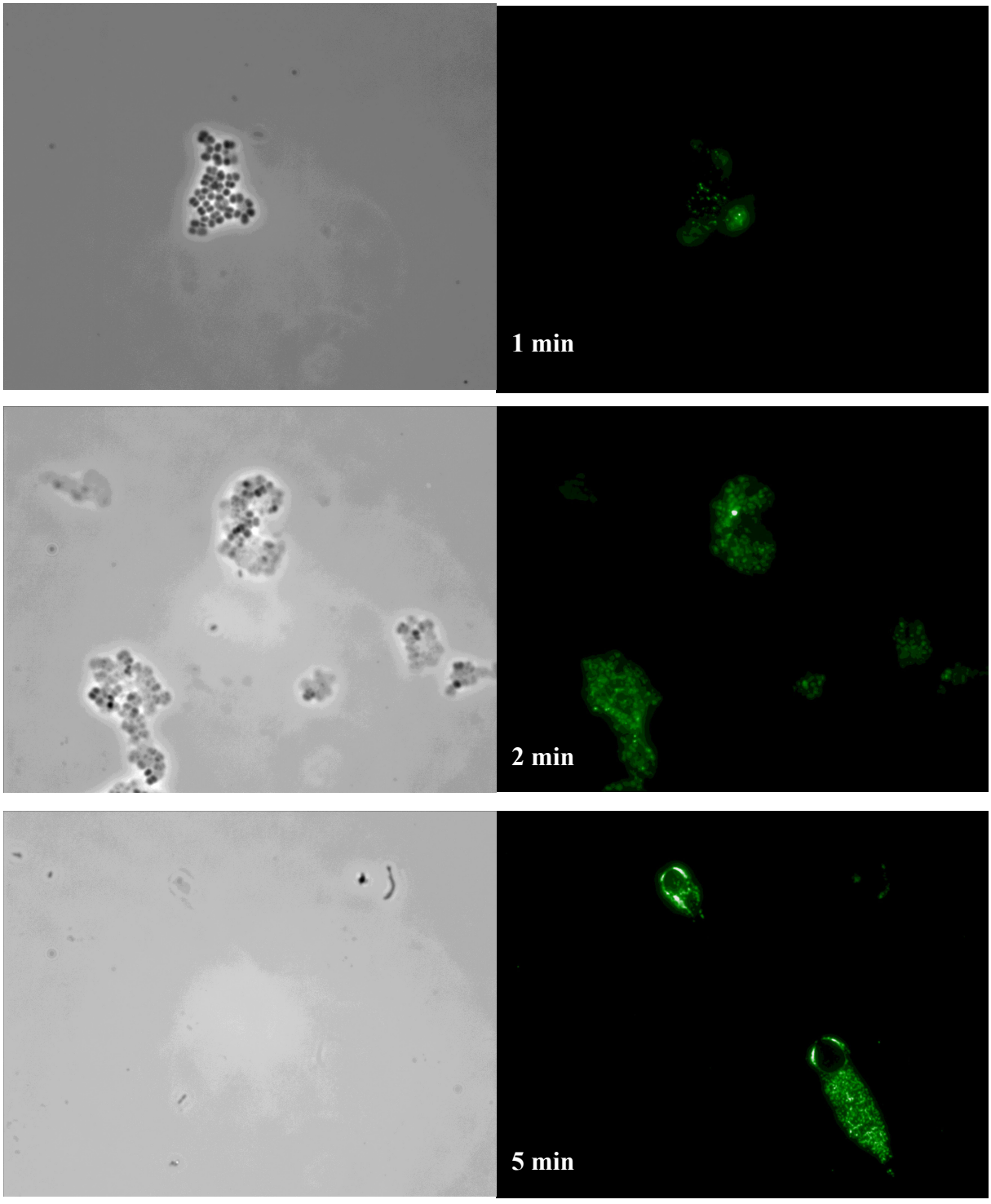


Fig.C.10. Influence of lysozyme on hybridisation signal in *Micrococcus luteus*. Upper images: hybridisation after 1min lysozyme treatment, middle images: hybridisation after 2min lysozyme treatment, lower images: hybridisation after 5min lysozyme treatment

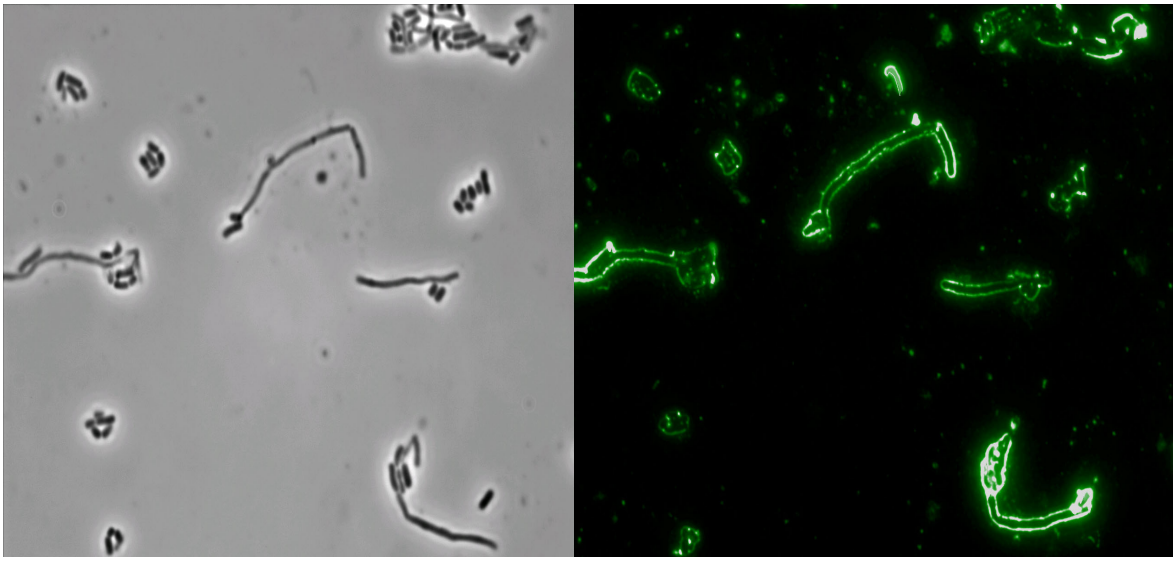


Fig. C.11. Fluorescence *in situ* hybridisation with *Streptomyces venezuelae* cells using probe DIII-Sve. Epifluorescence image (right) , phase contrast image (left)

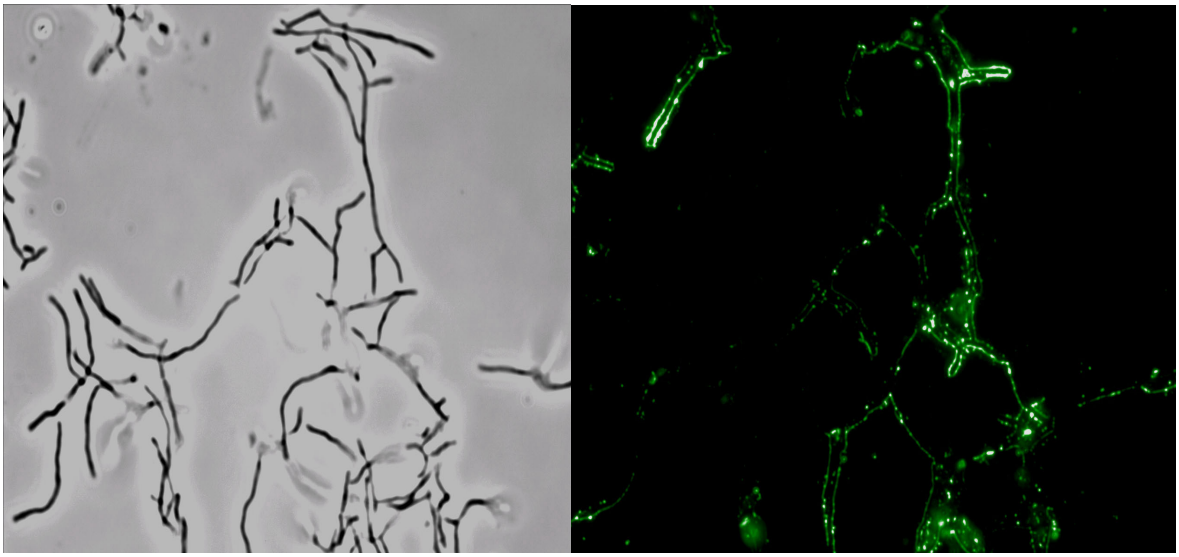


Fig. C12. Fluorescence *in situ* hybridisation with *Streptomyces divaceus griseolus* cells using probe DIII-Sdg. Epifluorescence image (right) , phase contrast image (left)

C.1.3. Yeasts

rRNA targeted polynucleotide probes have so far been used exclusively for prokaryotic cells. By applying these probes to yeasts, it was possible to extend their applicability to eukaryotes. Yeasts pose a certain challenge for this method, because they tend to have very thick cell envelopes, consisting chiefly of glucans, mannans and proteins in various proportions. In most cases modified cell fixation and/or pretreatment of the cells with proteinase was necessary to enable the probes to pass through the cell envelope. Examplary results of different cell fixation methods and protease treatment are shown in Fig. C.13 – C.14.

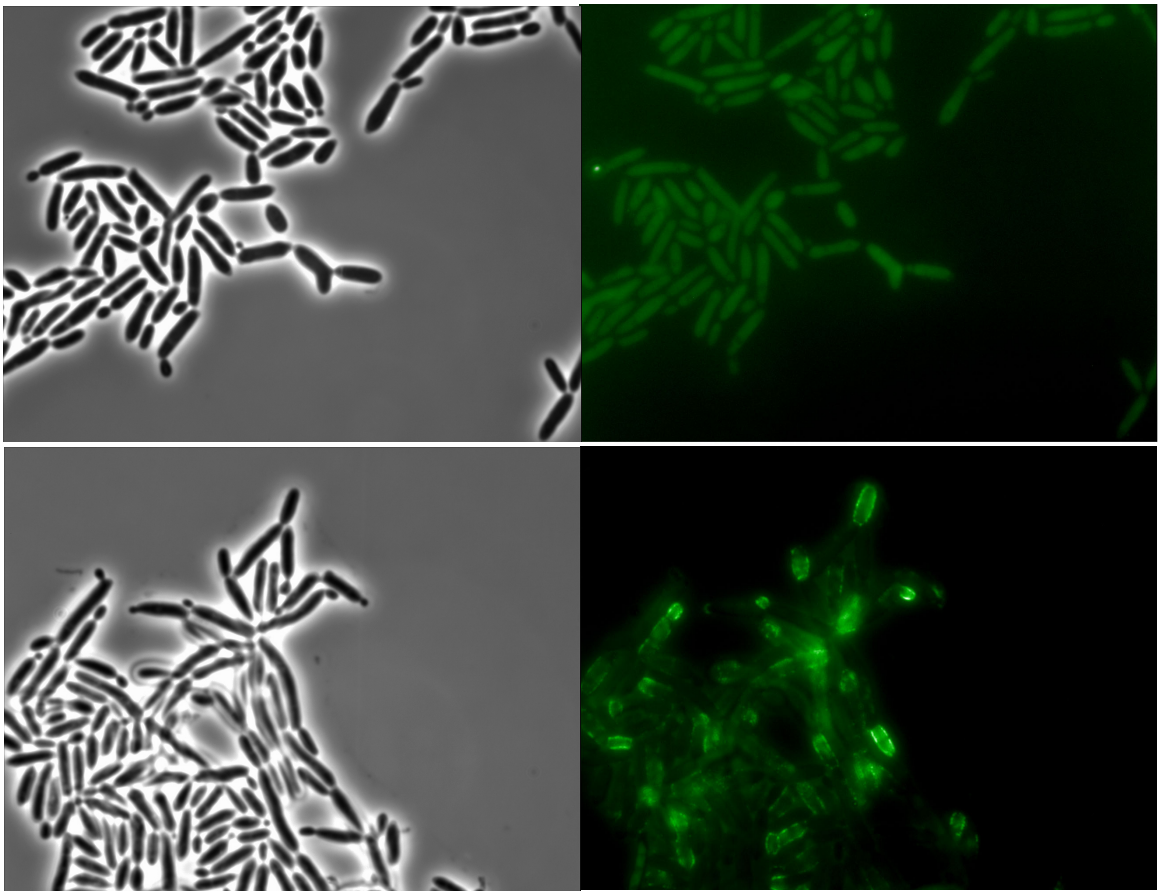


Fig. C.13. Fluorescence *in situ* hybridisation of *Dekkera anomala* cells using probe Y1-Dan. Upper images: Ethanol fixed cells. Lower images: PFA fixed cells. Right panel: epifluorescence image - the biotin labelled probes were detected with streptavidin-fluorescein. Left panel: phase contrast image of the same microscopic field.

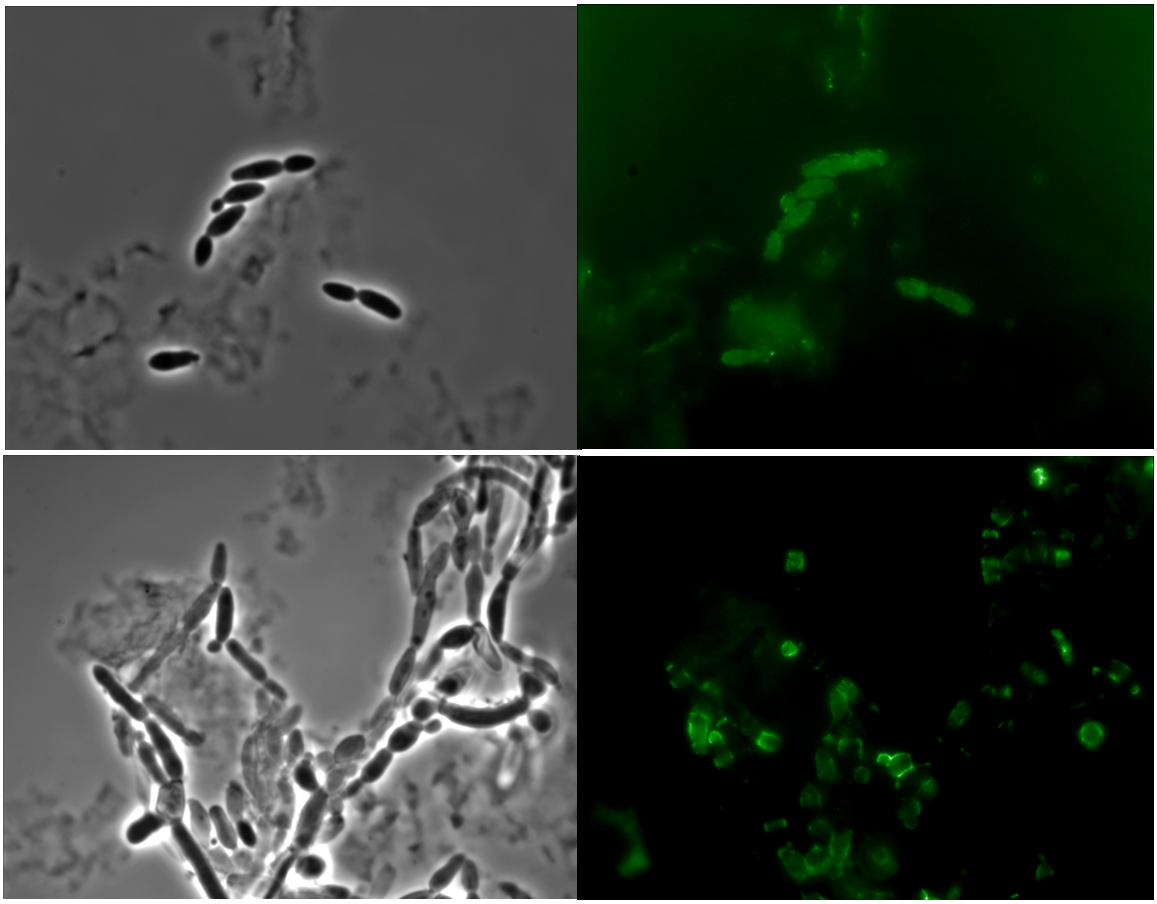


Fig. C.14. Fluorescence *in situ* hybridisation of *Kluyveromyces marxianus* cells using probe Y1-Kma..
Upper images: hybridisation with PFA fixed cells. Lower images: PFA fixed cells treated with proteinase K prior to hybridisation. Right panel: epifluorescence image - the biotin labelled probes were detected with streptavidin-fluorescein. Left panel: phase contrast image of the same microscopic field.

However, even with modified cell treatment not all targeted cells showed fluorescence signals. In contrast to that, no problems with cell wall permeability and probe uptake were encountered in experiments with oligonucleotide probes.

Often the signal is concentrated on one pole of the yeast cell where a bud has been or is about to be formed, possibly indicating a) high metabolic activity reflected in a high ribosome content and b) a thinner or incomplete cell envelope in this region (see Fig. C.15).

Of the 22 yeast species tested (cf. B.1.), positive hybridisation results could be achieved with 7 species (see figures C.18a-g). The probe used here (Y1) targets domain I of the LSU rRNA and has a length of about 330nt. Figure C.16 shows the target position of probe Y1 within the secondary structure model of the LSU rRNA.The probe was generated using the universal primers Y1-V and Y1-RT3 (cf.B.3.) for PCR and *in vitro* transription of the amplificate (cf. B.9.2.). In those cases, where the polynucleotide probes were able to penetrate the cell envelope and bind to their targets, the nature of the signal was identical to that observed with procaryotes, namely a halo that has a diameter slightly larger than that of the cell (Fig. C.17).

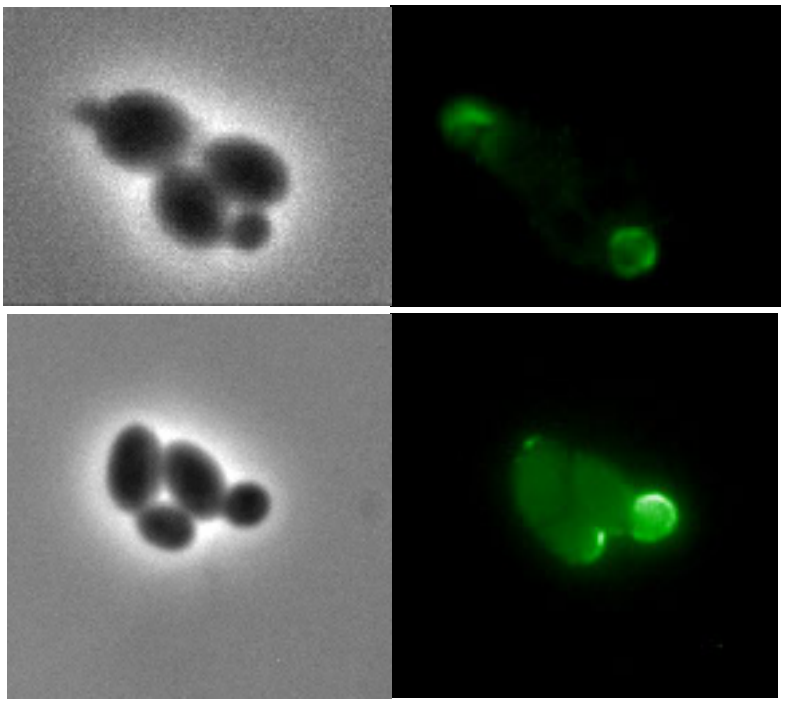


Fig. C.15. Localisation of fluorescence in regions of active cell growth. *Rhodotorula mucilaginosa* (upper images) and *Saccharomyces cerevisiae* (lower images) were hybridised with probes Y1-Rmu and Y1-Sce, respectively. Display of epifluorescence (right) and phase contrast (left) images.

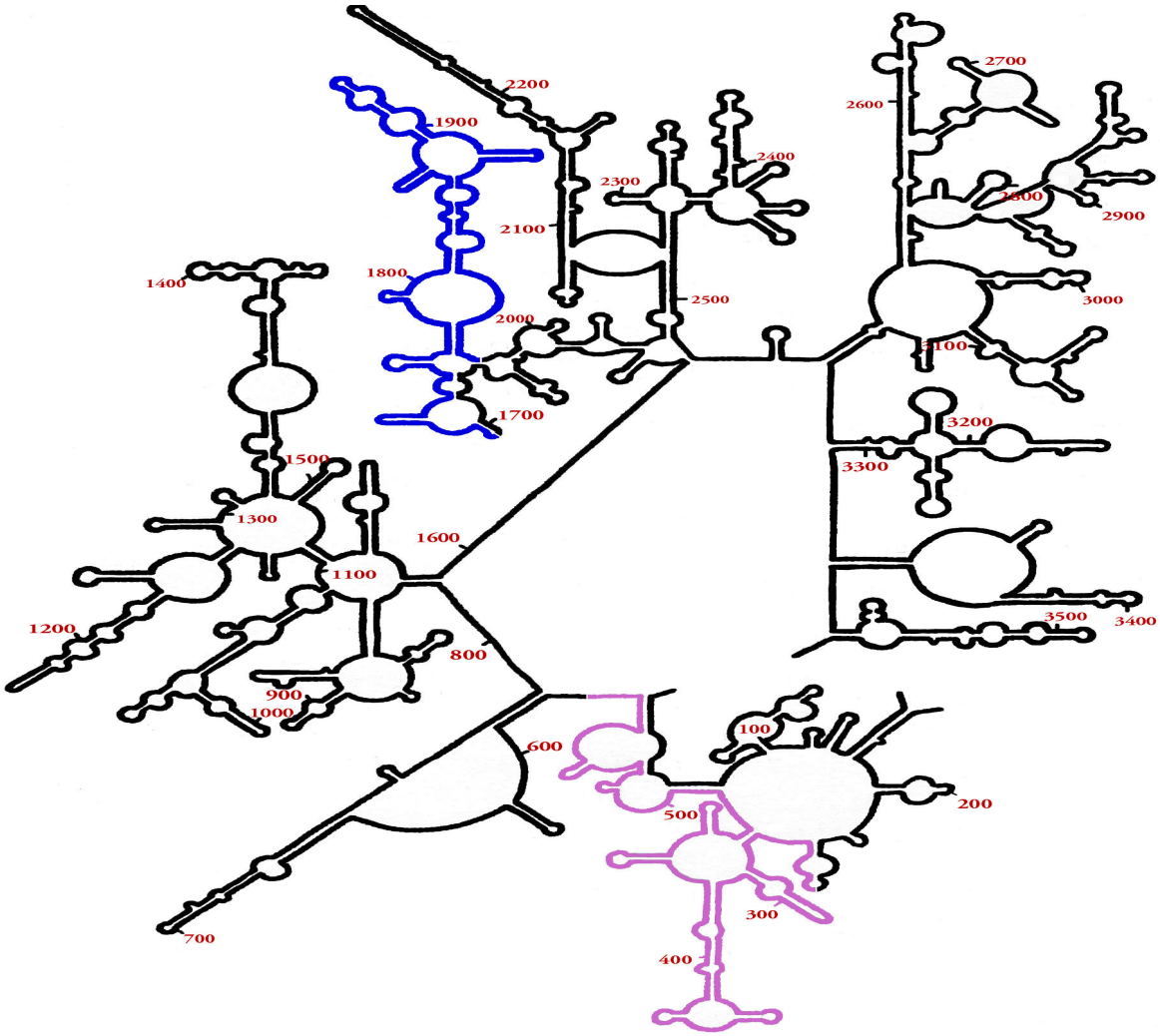


Fig. C.16. Secondary structure model of the LSU rRNA of *S. cerevisiae*. Position of the probes Y1 (pink) and DIII-Euk (blue) within this structure.

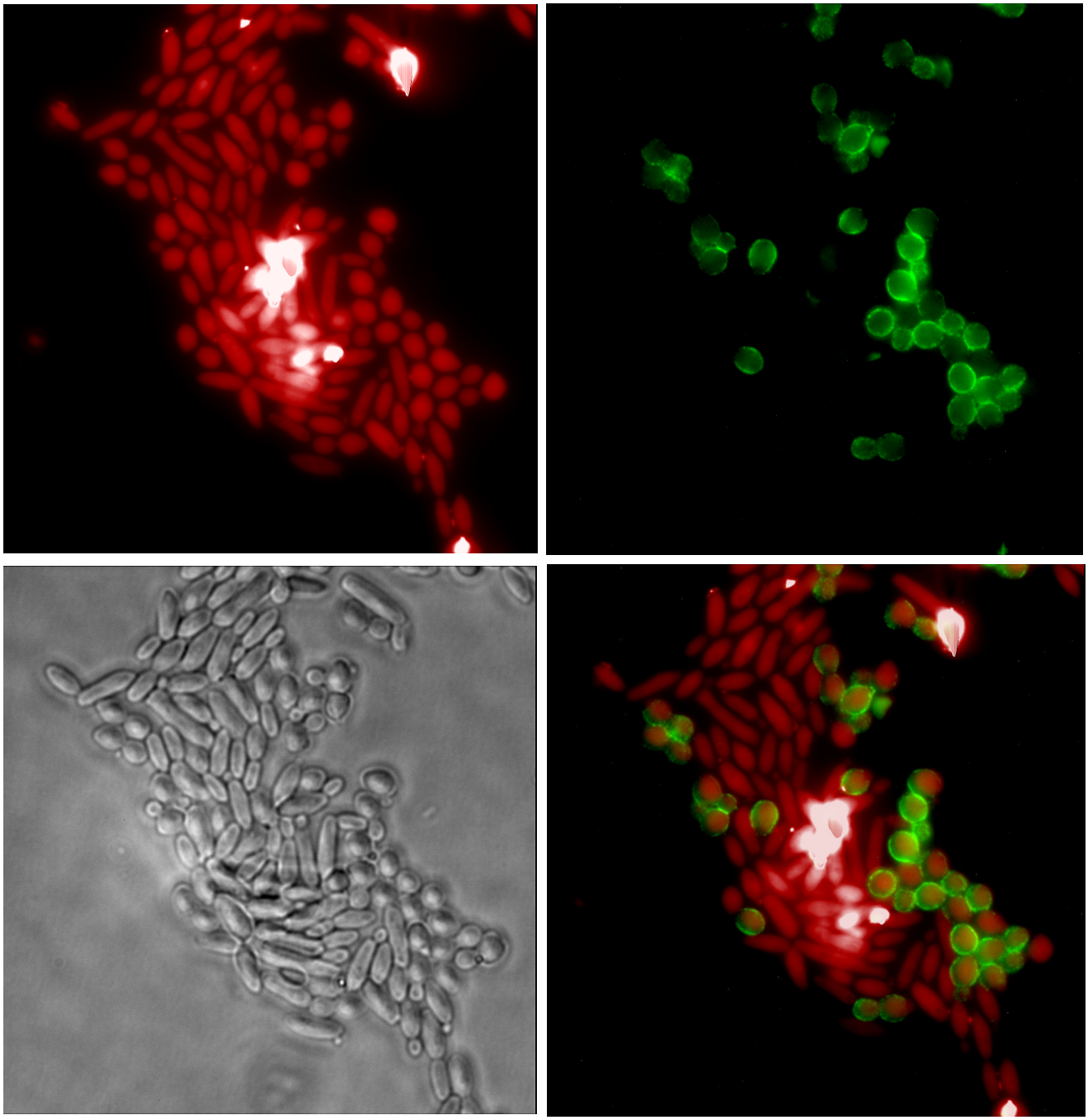


Fig. C.17. Mixture of *Torulaspora delbrueckii* and *Candida tropicalis* hybridised with Cy3 labelled oligonucleotide probe C602 (red, upper left image), which stains all cells in this image and fluorescein labelled polynucleotide probe Y1-Tde (green, upper right image), specific for *T. delbrueckii*. The signal of the oligonucleotide probe is distributed all over the cell, whereas that of the polynucleotide probe is concentrated on the outside of the cell, forming a halo that has a slightly larger circumference than the cell (lower right image). Lower left image: phase contrast.

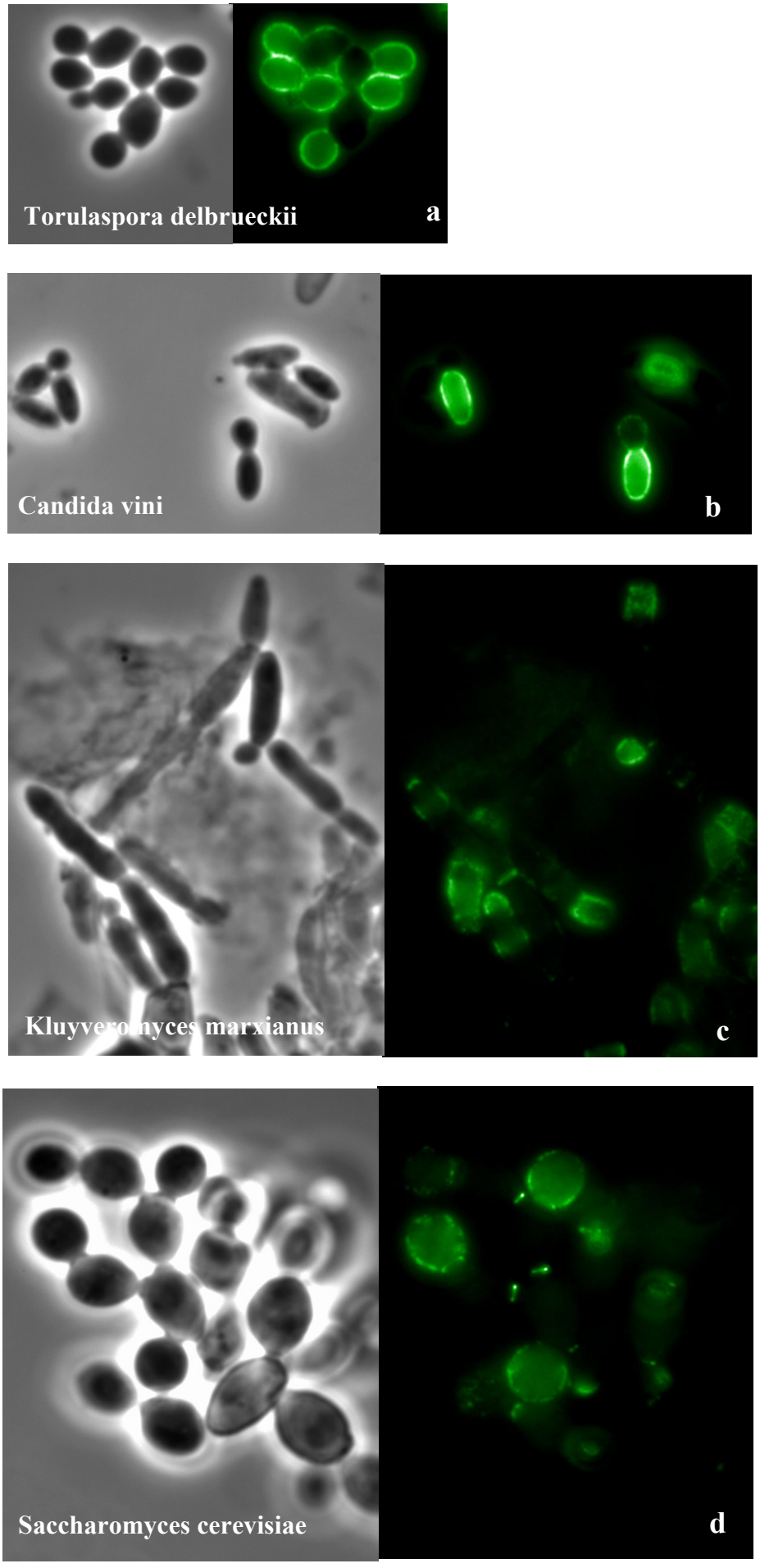
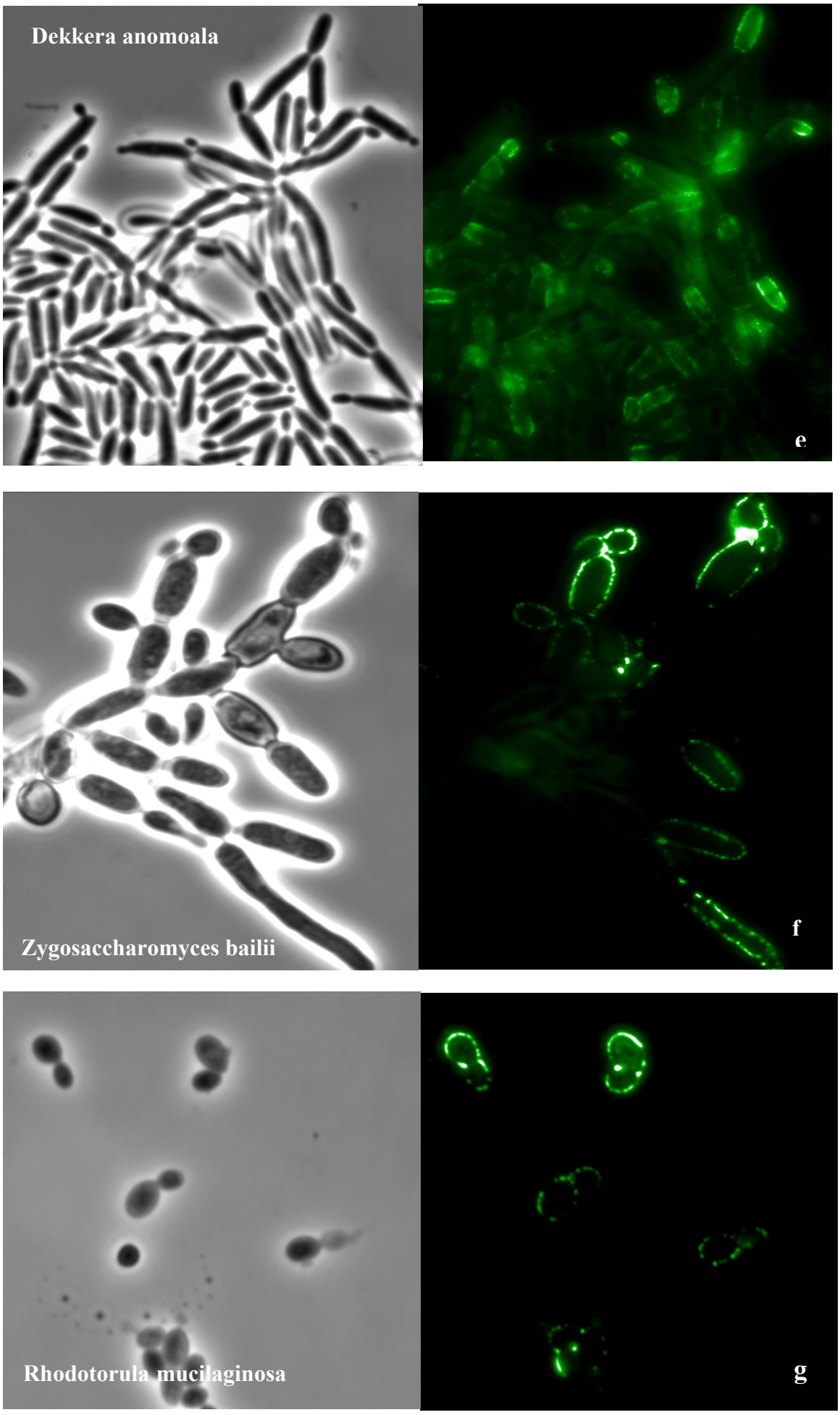


Fig. C.18. Fluorescence *in situ* hybridisation with probe Y1 specific for a) *Torulaspora delbrueckii*, b) *Candida vini*, c) *Kluyveromyces marxianus*, d) *Sacharomyces cerevisiae,* e) *Dekkera anomala*, f) *Zygosaccharomyces bailii*, g) *Rhodotorula mucilaginosa*


Dekkera anomoala
e
Zygosaccharomyces bailii
f
Rhodotorula mucilaginosa
g

C.1.4. Mammalian cells

Finally, polynucleotide probes were tested with mammalian cells. Since mammalian cells do not have a cell wall or other rigid cell structures, access of the probes to their targets is not hindered. In fact, the cell fixation could be shortened to 10 min with either 4% PFA or 100% ethanol (as opposed to 16h with 4% PFA for bacteria) and positive hybridisation signals could already be observed after 2h hybridisation (as opposed to a minimum of 5h for bacteria). The nature of the signal resembles that seen with bacteria and yeasts, with a concentration of probes on the outside of the cell, although there is also some fluorescence inside the cell.

To establish the general applicability of polynucleotide probes for mammalian cells probe DIII-Euk, which targets positions 1301-1621 (analogous to bacterial domainIII) of the mammalian LSU rRNA, was generated (target position in secondary structure cf. Fig. C.16., primer sequences cf. B.3.) and used for hybridisation of a mouse (NIH 3T3) and a human (Jurkat) cell line (see Fig. C.19).

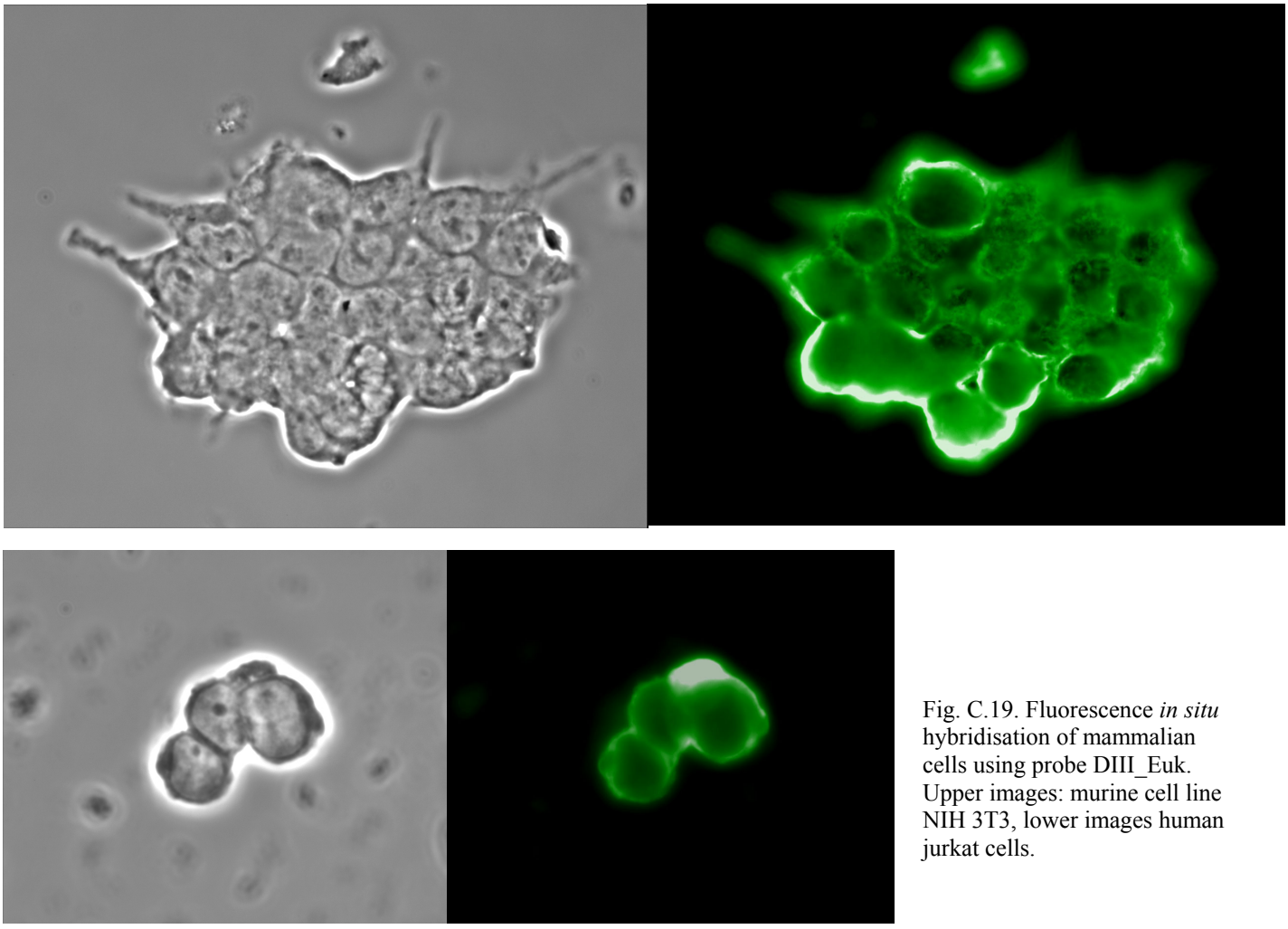


Fig. C.19. Fluorescence *in situ* hybridisation of mammalian cells using probe DIII_Euk. Upper images: murine cell line NIH 3T3, lower images human jurkat cells.

Regarding mammalian cells, however, the value of polynucleotide probes lies rather with probes targeting the chromosomal DNA, since taxonomic differentiation of mammals – in contrast to bacteria – generally does not rely on rRNA analysis.

Accordingly, a probe targeting the glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) (length 481nt) was designed to test the general applicability of DNA targeted polynucleotide probes for mammalian cells. Hybridisations with this probe resulted in a concentration of the fluorescence signal in the region of the nucleus (Fig. C.20), indicating the binding of the probe to the chromosomal DNA, although in some cases fluorescence was dispersed across the whole cell.

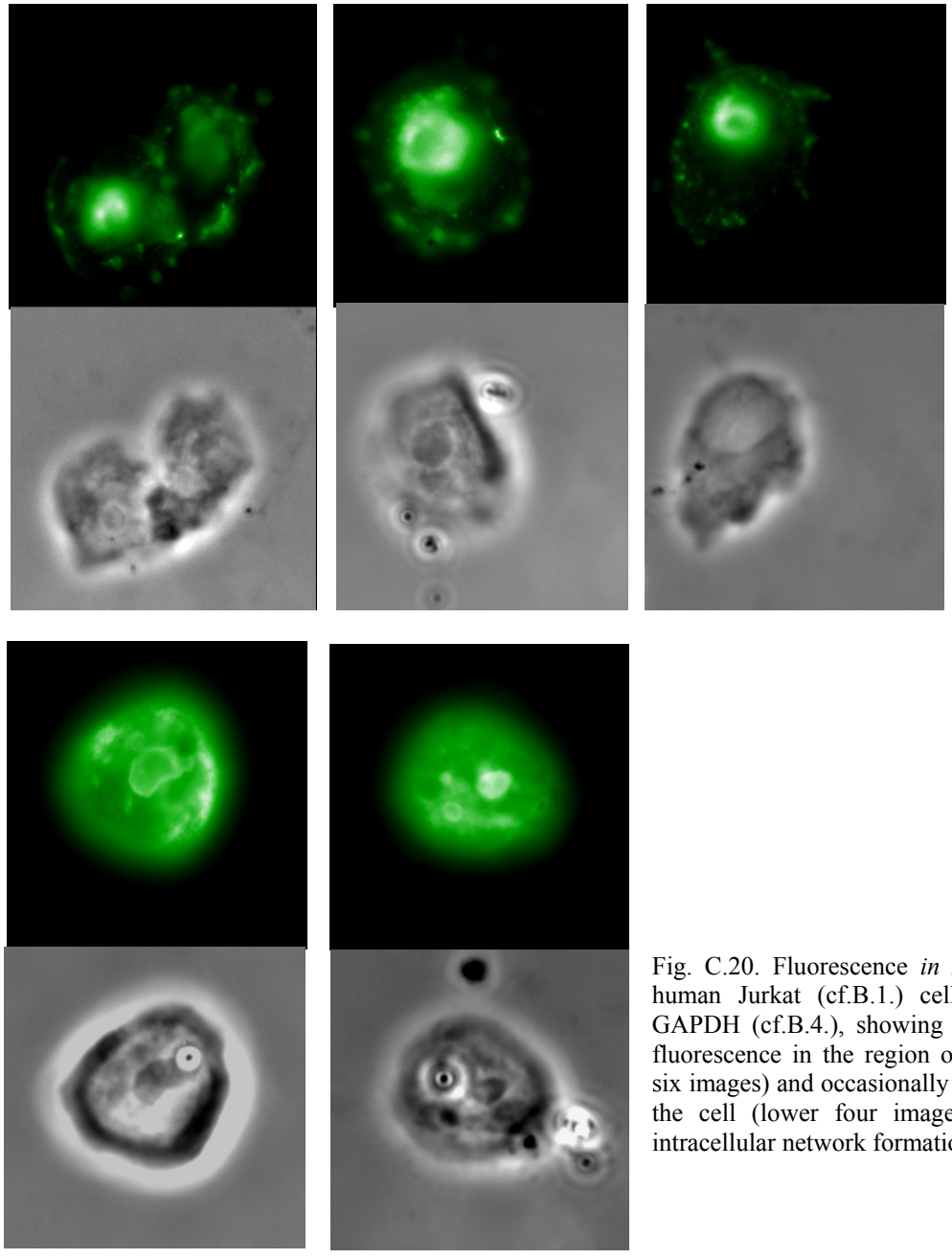


Fig. C.20. Fluorescence *in situ* hybridisation of human Jurkat (cf.B.1.) cells with probe Euk-GAPDH (cf.B.4.), showing concentration of the fluorescence in the region of the nucleus (upper six images) and occasionally fluorescence all over the cell (lower four images), possibly due to intracellular network formation

C.2. Classes of target molecules for polynucleotide probes

In addition to adapting the fluorescence *in situ* hybridisation with polynucleotide probes for different groups of organisms, another aim of this work was to gain a deeper understanding of the functional principle of these probes and the minimum requirements regarding probe length and the number of target molecules.

C.2.1. Polynucleotide probes targeting rRNA

C.2.1.1. Standard polynucleotide probes

rRNA targeted polynucleotide probes with a size of ca. 250nt (i.e. probes DIII and 367) were the starting point for this work. Examples of hybridisations with these probes are shown in Fig. C.2-8.

C.2.1.2. Evaluation of probe length – probe set 1024_1-11

One characteristic feature of hybridisations with polynucleotide probes is the halo-like signal, i.e. a ring of fluorescence around the cells hybridised with these probes, which is in sharp contrast to oligonucleotide probes that usually show fluorescence throughout the cell.

To determine the minimum – or maximum – probe length necessary to generate this specific signal, a set of probes with a size of 50 – 1200nt was tested. The universal primer 1024R-T3 (23S rRNA, Eco pos.1186-1200), containing the binding site for the T3 RNA polymerase, served as a common starting point for the *in vitro* transcription to generate the probes. Primer 1024R-T3 in combination with different forward primers binding at increasing distances upstream of 1024R (cf. Tab. B.10.) was used to generate PCR products between 50 and 1200bp in length that served as templates for the *in vitro* transcription.

In situ hybridisations with these probes resulted in halo signals for all probes (see Fig. 21). No significant difference in either the nature or the intensity of the signal was observed for the whole set of probes.

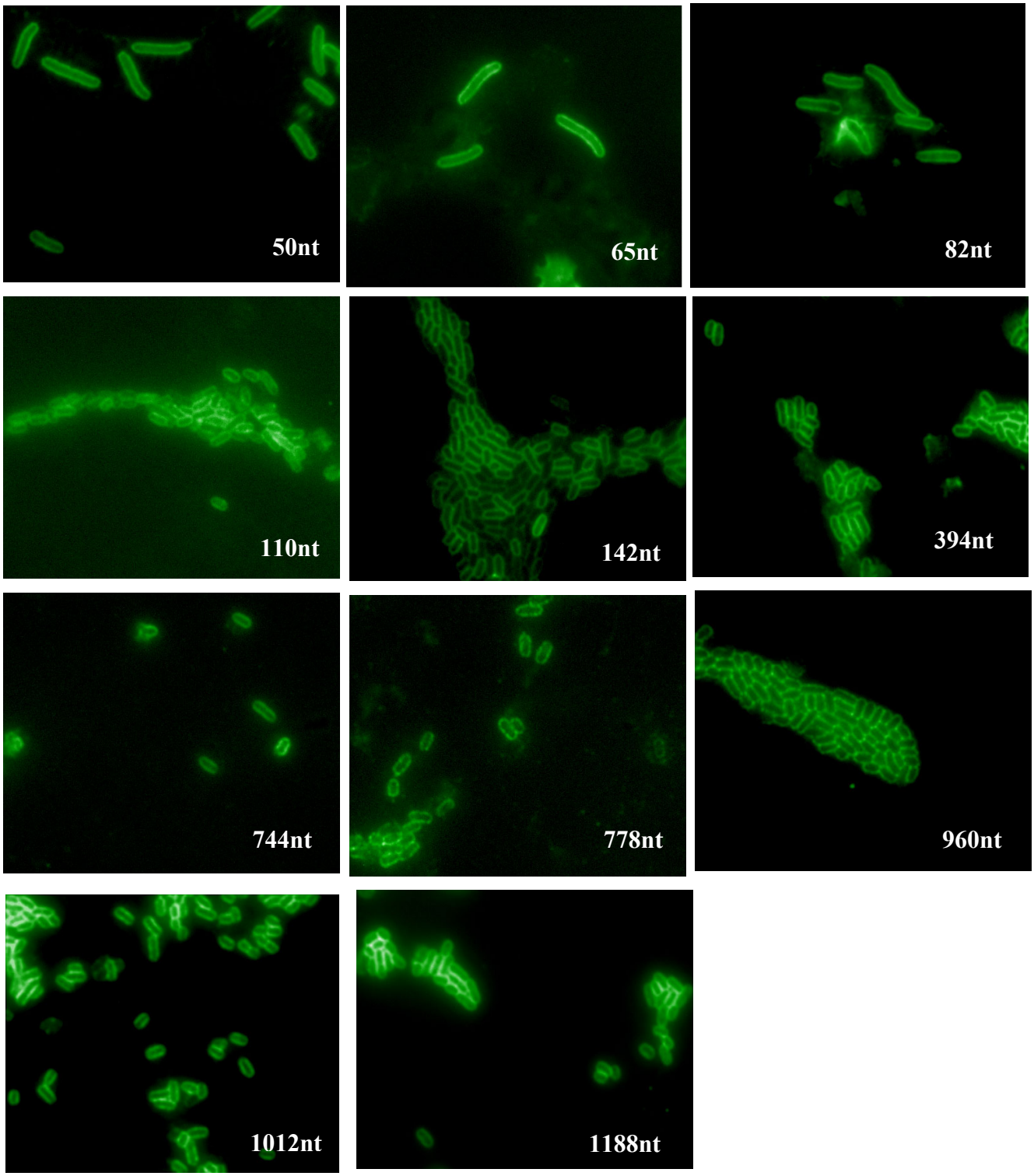


Fig. C.21. Probes 1024_1-11. Fluorescence *in situ* hybridisations with probes varying in length between 50-1188nt targeting *E. coli* cells. A halo signal can be seen in all cases.

C.2.1.3. Influence of secondary structure of the probe on the hybridisation signal – probe EcoX

The results with probe set 1024_1-11 (see previous chapter, C.2.1.2.) imply that the length of the probe is not the decisive factor responsible for the halo signal. Therefore, the possibility of the secondary structure of the probes influencing the nature of the hybridisation signal was explored. Three polynucleotide probes, designed to be able to form secondary structures with different degrees of stability and an oligonucleotide probe were compared with respect to their hybridisation signal.

Oligonucleotide probe Eco444, an 18mer, specific for *Escherichia coli*, targeting position 444-462 of the 16S rRNA, that was shown not to be able to form any secondary structure *in silico* (using the GCG software package, Wisconsin, USA), but worked well as an oligo FISH probe, was used as a basic building block for three artificial polynucleotide probes that are supposed to be able to form a) no, b) moderate and c) strong secondary structures. The three probes have a length of 90, 84 and 84 nt respectively, which – according to the results with probe set 1024_1-11 (cf. C.2.1.2.) – should be sufficient to result in a halo signal.

- Probe Eco444-5X consists of a direct repeat of 5 times oligomer Eco444 (= "X") and cannot form any reasonable secondary structure (formation of a weak structure can be forced under energy consumption [$\Delta G = +3.9$kcal/mole], whereas the other probes have negative values for ΔG). (cf. Tab. C1. and Fig. C.22)
- Probe Eco444-cX consists of the building block X, alternating with cX, the complementary sequence of X, separated by a short spacer to facilitate hairpin formation. It is able to form a moderately stable secondary structure ($\Delta G = -26$kcal/mole). (cf. Tab. C1. and Fig. C.22)
- Probe Eco444-rcX consists of the building block X, alternating with rcX, the reverse complementary sequence of X, again separated by a short spacer. It is able to form a strong secondary structure ($\Delta G = -62$kcal/mole). (cf. Tab. C1. and Fig. C.22)

Table C.1 illustrates the composition of the three probes, the secondary structures are shown in figure C.22 (right column).

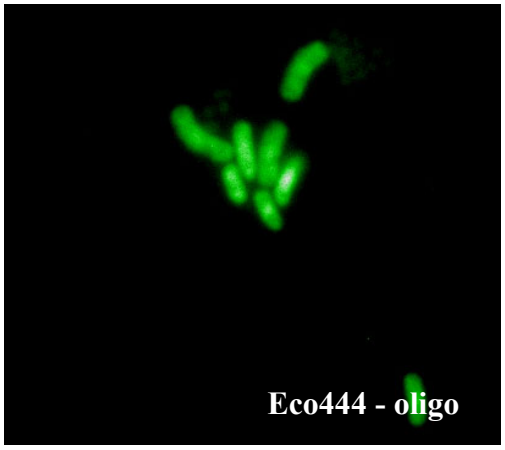
Hybridisations with these probes resulted in a very weak signal (no halo) for probe Eco444-5X, a stronger signal (but still no halo) for probe Eco444-cX and a strong signal with some halos for probe Eco444-rcX. (see Fig. C.22)

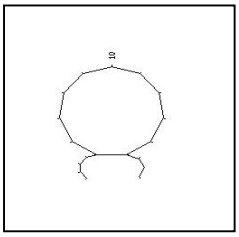
		Sequence 5‘ – 3‘
X	Eco444	5‘ - CTT TAC TCC CTT CCT CCC – 3‘
cX	complementary	5‘ - GAA ATG AGG GAA GGA GGG – 3‘
rcX	reverse complementary	5‘ - GGG AGG AAG GGA GTA AAG – 3‘

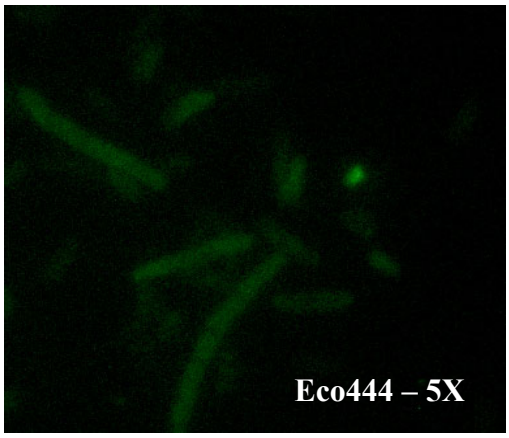
probe Eco444-5X (90nt)	5‘ – CTT TAC TCC CTT CCT CCC CTT TAC TCC CTT CCT CCC CTT TAC TCC CTT CCT CCC CTT TAC TCC CTT CCT CCC CTT TAC TCC CTT CCT CCC – 3‘
probe Eco444-cX (84nt)	5‘ – CTT TAC TCC CTT CCT CCC AAAA GAA ATG AGG GAA GGA GGG AAAA CTT TAC TCC CTT CCT CCC AAAA GAA ATG AGG GAA GGA GGG – 3‘
probe Eco444-rcX (84nt)	5‘ – CTT TAC TCC CTT CCT CCC AAAA GGG AGG AAG GGA GTA AAG AAAA CTT TAC TCC CTT CCT CCC AAAA GGG AGG AAG GGA GTA AAG – 3‘

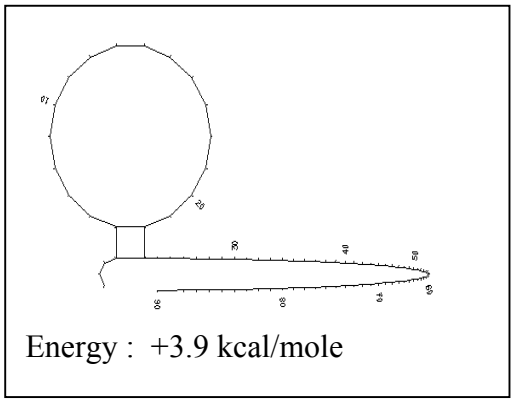
Table C.1. Structure of the EcoX probes

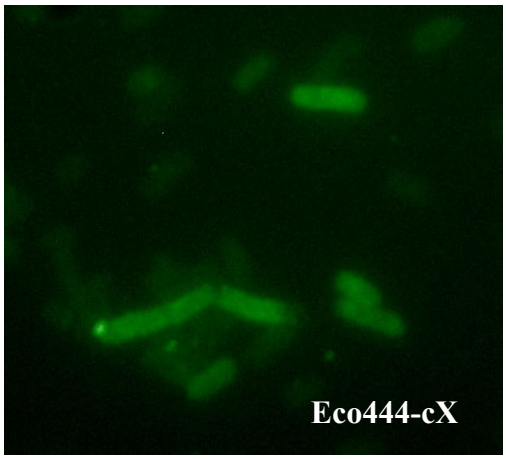
Next page:
Fig. C.22. Fluorescence *in situ* hybridisation with oligonucleotide probe Eco444 and artificial polynucluetide probes Eco-5X, Eco-cX and Eco rcX (left column) with corresponding secondary structures of the probes as determined with GCG (right column).

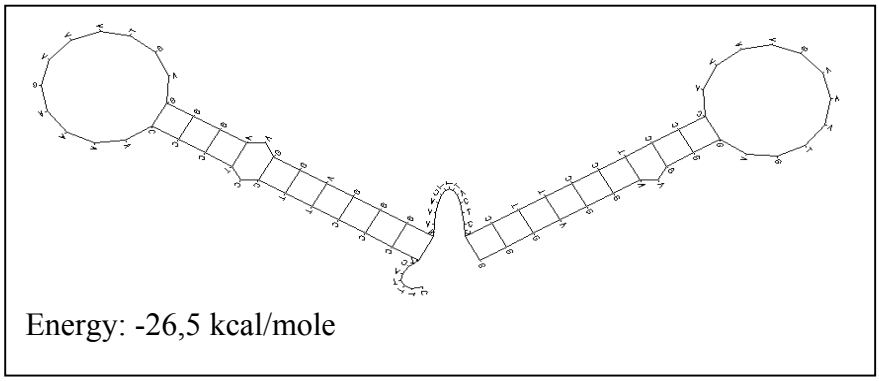


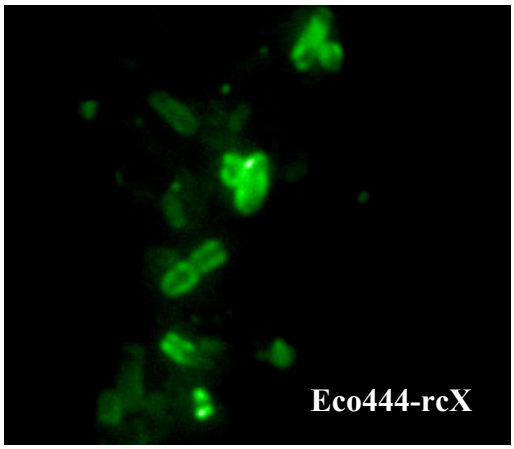


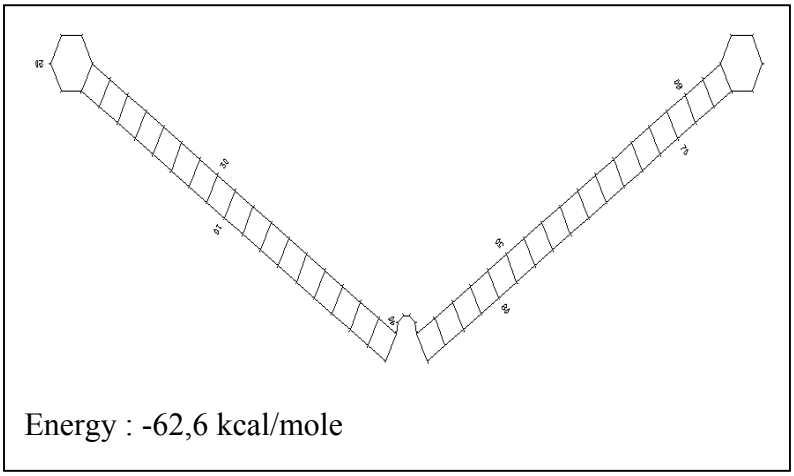



Energy : +3.9 kcal/mole




Energy: -26,5 kcal/mole




Energy : -62,6 kcal/mole

The experiments with plasmid and genomic DNA targeted probes as described in the following chapters C.2.2. and C.2.3. were carried out under the assumption, that the probes are able to interact with each other, forming a network around the cell, which results in a strong amplification of the fluorescence signal. This hypothesis is explained and discussed in detail in D.3.

C.2.2. Polynucleotide probes targeting plasmids

With the hypothesis of an amplification of the signal due to a network formation, it is possible to deviate from the concept of using rRNA as the target for the probes. Ribosomal RNA – apart from its value as a phylogenetic marker - has previously been regarded as an ideal target for *in situ* hybridisations because of its abundance in the cell, with copy numbers ranging between 10^4-10^5, depending on the growth state of the cell. Low signal intensities with oligonucleotide probes have been reported for slowly growing organisms with a low ribosome content (Amann et al., 1995). With rRNA targeted polynucleotide probes, no significant change in signal intensity was observed with hybridisations of cells fixed at different growth points (reflecting different ribosome contents).

The concept of a network formation abrogates the problem of detection limits due to low copy numbers of the target molecule, since, theoretically, a few target molecules within the cell could be sufficient to serve as an anchor and starting point for the network.

To test this hypothesis, probes targeting several plasmids were generated using the same strategy as for rRNA targeted probes, i.e. PCR amplification of the probe sequence with one of the primers containing the recognition site for the T3 RNA polymerase and subsequent *in vitro* transcription of the PCR product.

The initial plasmid to be tested was the high copy (200-500 copies per cell, according to manufacturer) cloning vector pCR2.1 (Invitrogen). The first probe targets the multiple cloning site of the vector and has a size of 203nt (Fig. C.23, for complete sequence of the vector cf. F.1.). The possible formation of secondary structures could be shown *in silico* using the GCG software package (GCG, Wisconsin) (Fig. C.24).

The parameters for the hybridisation had to be adjusted, taking into account a) the lower stability of DNA:RNA (plasmid:probe) hybrids as compared to the RNA:RNA (rRNA:probe) hybrids of the conventional FISH and b) the substantially reduced number of target molecules. The optimal conditions for the hybridisation were shown to be 5-20% formamide in the hybridisation buffer (cf. B.11.3.) and a hybridisation time of 18-24h.

Under these conditions halo-like, cell specific signals, comparable to those seen with rRNA targeted probes could be observed (see Fig. C.25), although the signal intensity was slightly lower. Occasionally the appearance of the halo was less smooth than with rRNA targeted probes and with rather frayed edges (Fig. C.26). In some cases the fluorescent signal was distributed all over the cell (Fig.C.27).

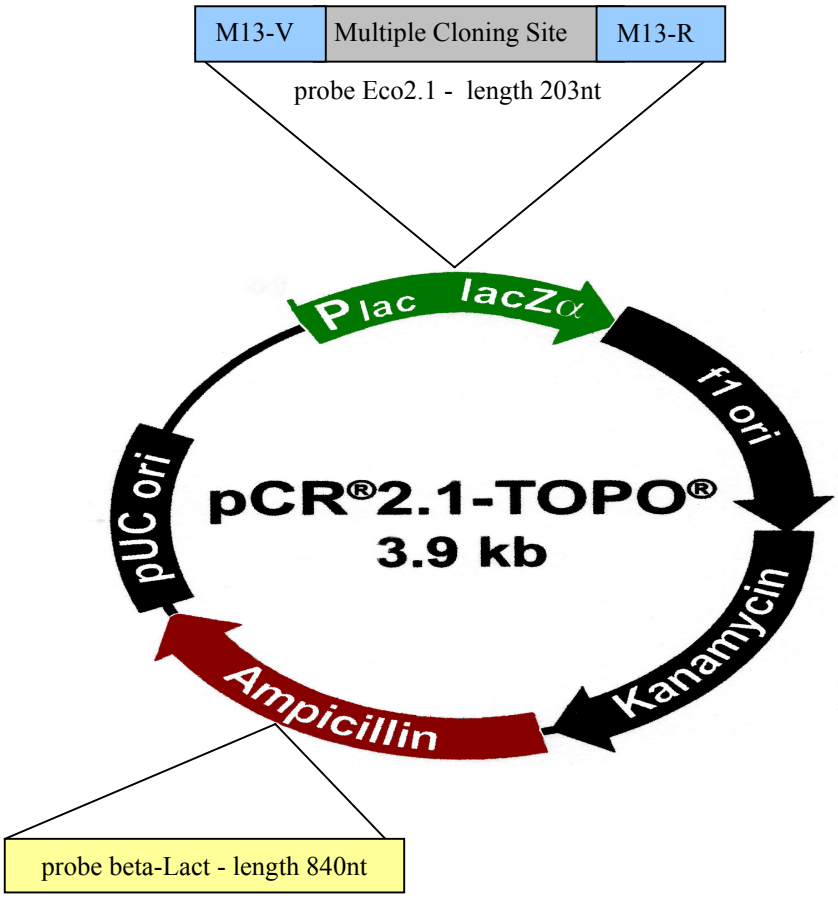


Fig. C.23. Map of the plasmid pCR2.1TOPO (Invitrogen) indicating the target positions of the probes Eco2.1 and betaLact.

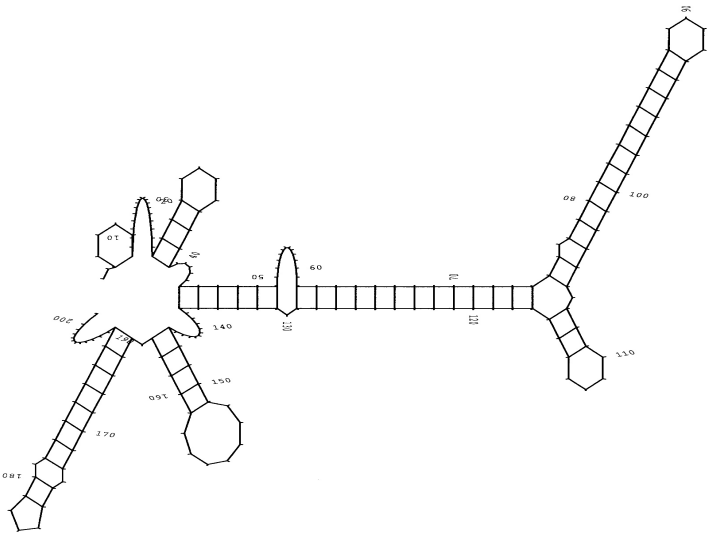


Fig.C.24. Putative secondary structure of probe Eco2.1 as calculated with the GCG software package

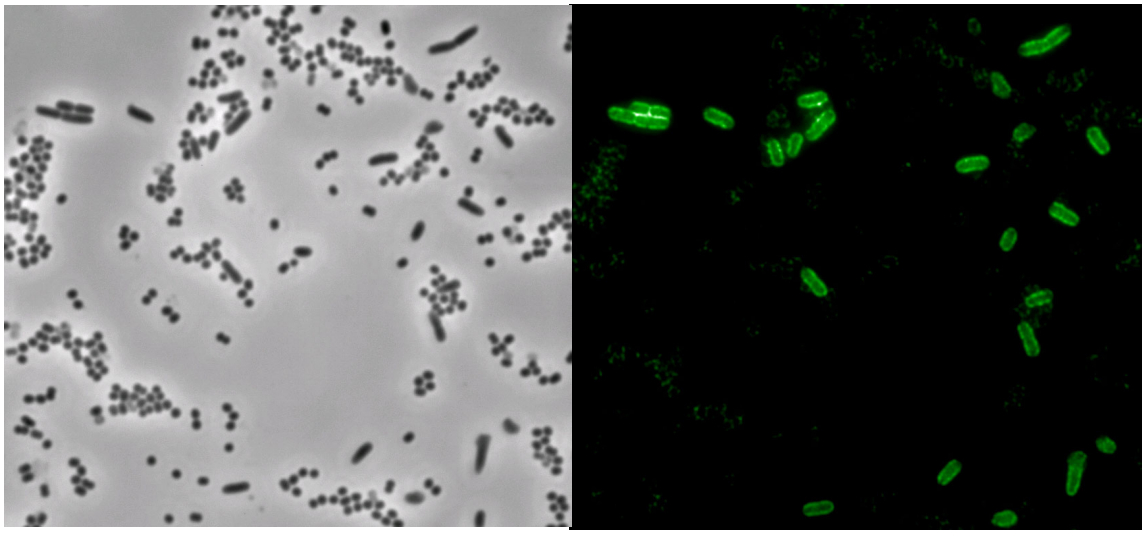


Fig.C.25. Fluorescence *in situ* hybridisation with probe Eco2.1 using a mixture of *E. coli* cells (rods) carrying the plasmid pCR2.1 and *N. canis* (, cocci, negative control). Epifluorescence image (right) and phase contrast image (left).

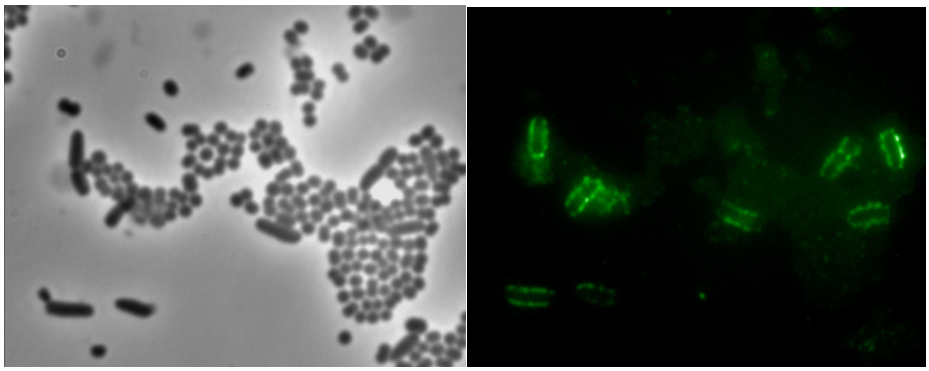


Fig. C.26. Slightly irregular shape of the halo for plasmid targeted probes.

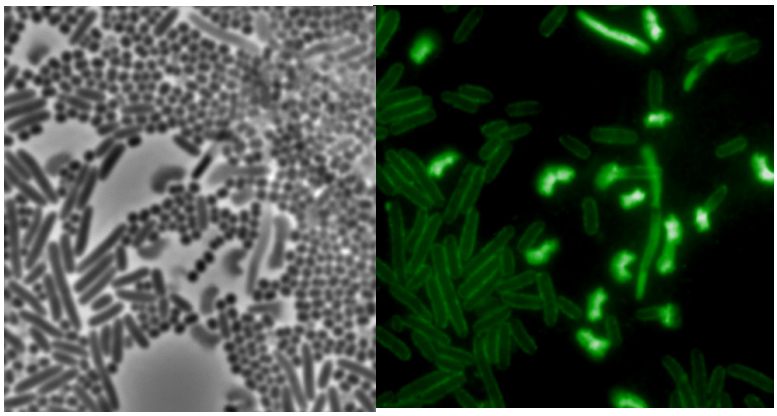


Fig. C.27. Different types of fluorescence (halo and overall) signals with plasmid targeted probes

After the general applicability of plasmids as targets for polynucleotide probes was established, the way was paved for a further reduction of the number of target molecules, i.e. by addressing plasmids with lower copy numbers.

For this purpose, a second probe was generated, targeting the beta lactamase gene, a common component of many cloning vectors, which confers ampicillin resistance to the organism and serves as a selection marker. Thus, it was possible to use the same probe for several vectors with different copy numbers containing the beta lactamaes gene.

Probe betaLact comprises 840nt. Possible formation of secondary structures was again confirmed *in silico* using the GCG software.

Hybridisations with *E. coli* cells carrying the high copy plasmid pCR2.1 showed cell specific halo signals with a signal intensity slightly better than with probe E2.1.(Fig.C.28).

Subsequent hybridisations with *E. coli* cells harbouring the medium copy number plasmid pBBR1MCS4 (30-50 copies per cell, Kovach et al, 1995) and the low copy number plasmid pUN121 (15-20 copies per cell, Nilsson et al, 1983) resulted in specific halo signals for all types of plasmids (Fig. C.29). There was no significant difference in signal intensity for these three vectors.

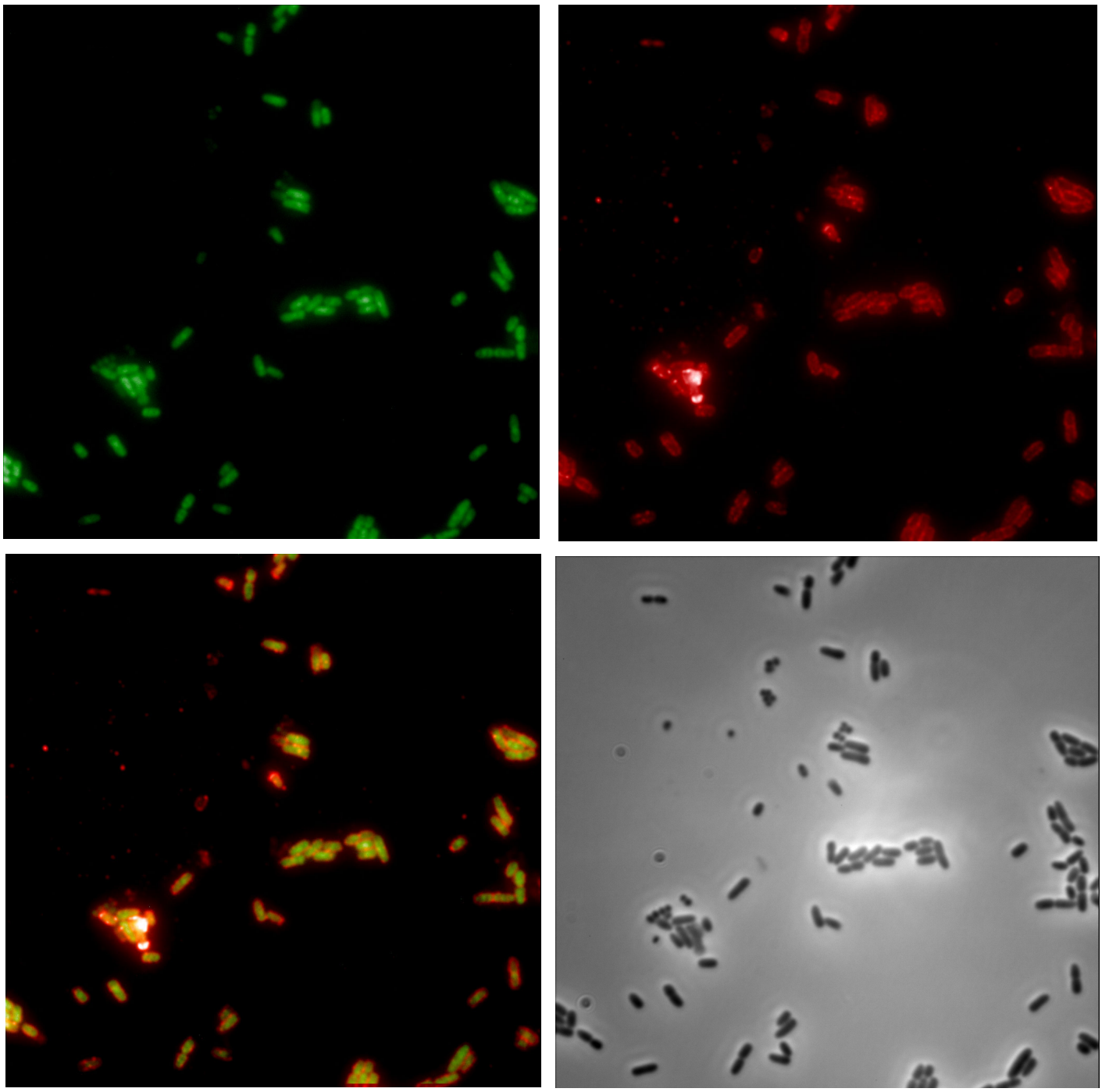


Fig. C.28. Fluorescence *in situ* hybridisation with probes betaLact (polynucleotide probe targeting the plasmid-encoded beta lactamase gene, Cy3 labelled [red], upper right image) and Eco444 (oligonucleotide probe specific for *E. coli*, FLUOS labelled [green], upper left image). The halo signal of the polynucleotide probe has a slightly larger circumference than the cell, which is evenly stained by the oligonucleotide probe (lower left image). Lower right image: phase contrast.

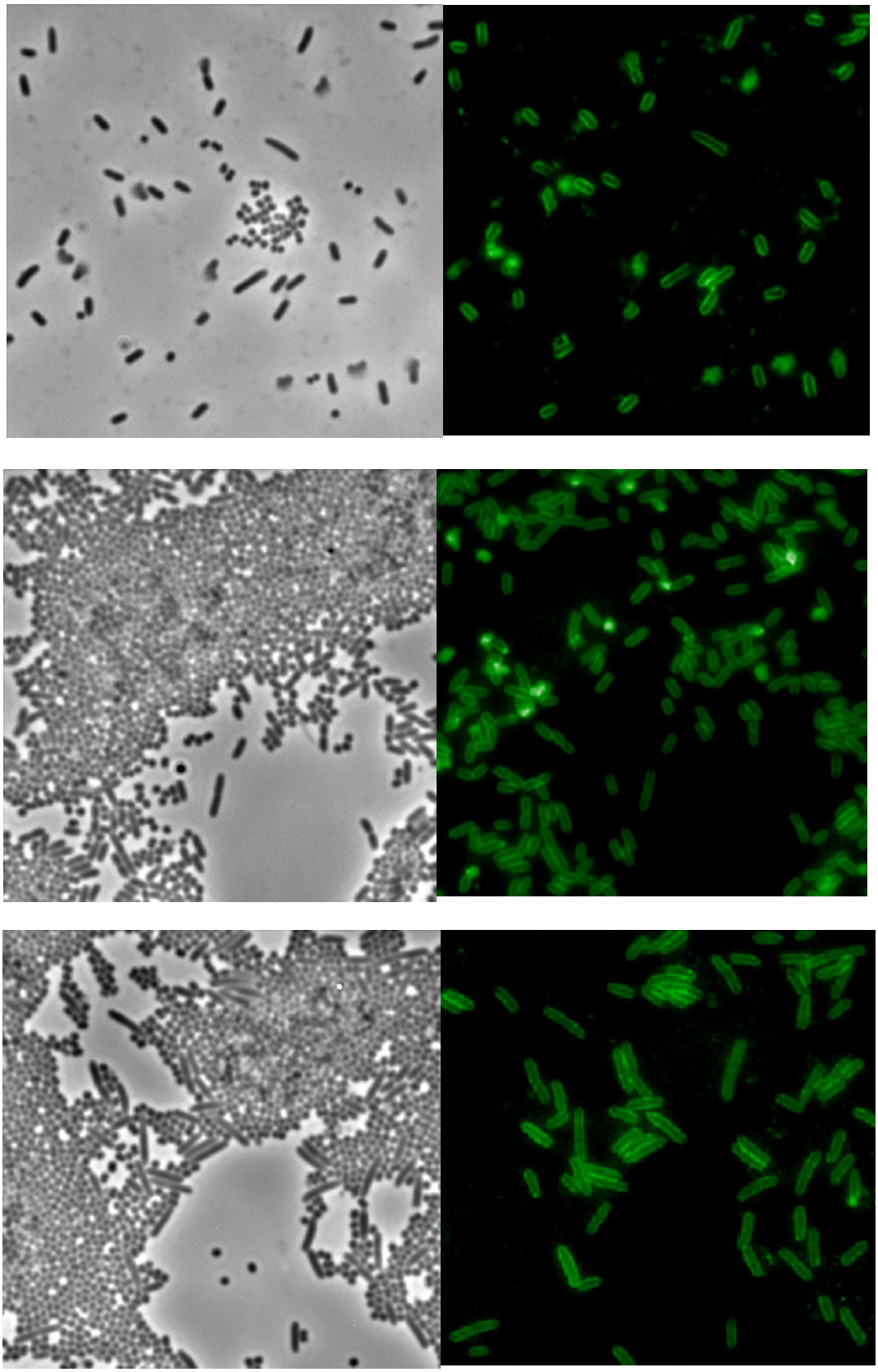


Fig.C.29. Fluorescence *in situ* hybridisation with probe betaLact using a mixture of *N.canis* (cocci, negative control) and *E. coli* (rods) carrying the high copy plasmid pCR2.1 (upper images), medium copy plasmid pBBR1MCS4 (middle) and low copy plasmid pUN121 (lower).

Control experiments

To validate the findings with plasmid targeted polynucleotide probes several controls were introduced.

1. All hybridisations were carried out with a mixture of (rod-shaped) *E. coli* and (coccoid) *N.canis* cells to verify the specificity of the probe (see previous figures).

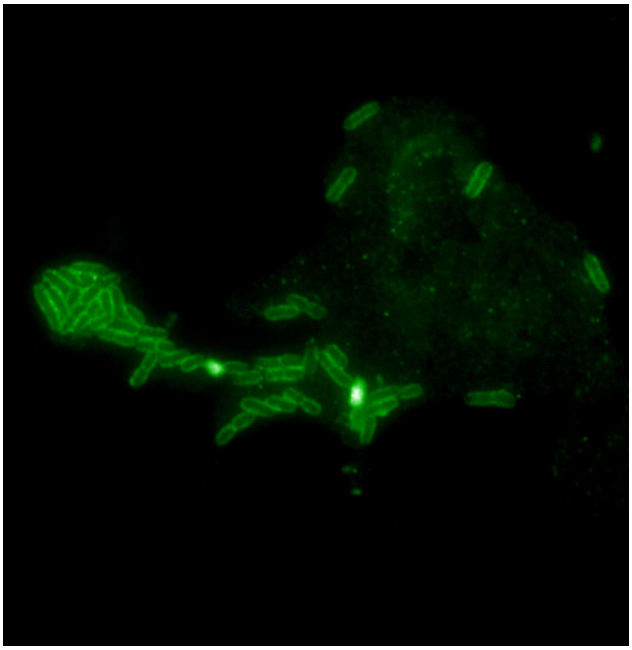
2. To exclude the possibility that the signals are due to unspecific binding of the fluorescein-tagged streptavidin to naturally occurring biotin instead of the biotin-labelled probe, the experiments were repeated with digoxigenin-labelled probes (and detection via fluorescein- or Cy3-tagged anti-digoxigenin) and directly fluorescein labelled probes, which do not require any secondary detection. The results with all types of labelling were identical.

3. To exclude the possibility of the probe binding to cell structures in *E. coli* other than the target DNA, hybridisations with the same *E. coli* strain that carried plasmid pCR2.1TOPO but this time without the plasmid were performed. No fluorescence signal was observed.

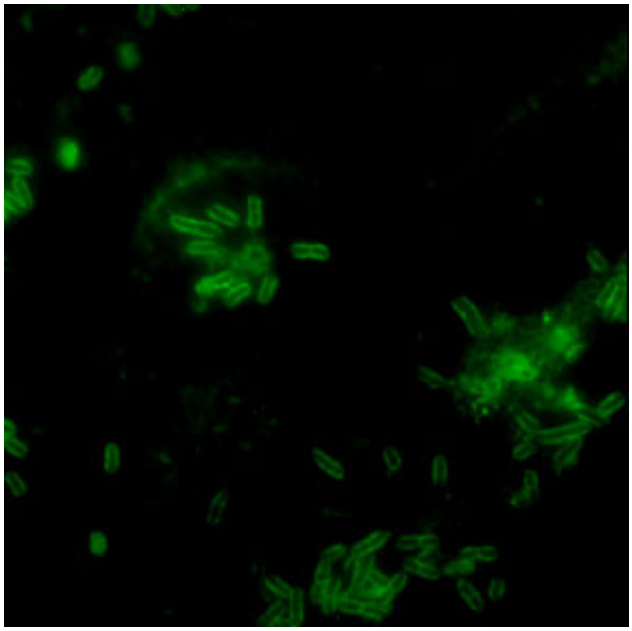
4. To exclude the possibility, that the signal resulted from the probe binding to mRNA, a sense probe was generated, which has the same sequence as the mRNA. Using only the sense probe should obviate a positive hybridisation signal if the previous findings had been based on the binding to mRNA, but should give a normal signal, if the probe binds to the plasmid DNA, since in this case it is of no importance, whether the positive or the negative strand of the plasmid DNA is targeted.

Hybridisations with a) the antisense probe, b) the sense probe and c) a mixture of antisense and sense probes resulted in positive hybridisation signals in all three cases (Fig. C.30). No significant difference in signal intensity was observed. Therefore it can be stated that the signal is not exclusively based on binding to mRNA, although it cannot be excluded that binding to mRNA contributes to the signal. An attempt to clarify this by destroying the mRNA prior to the hybridisation with RNase treatment failed, because it was not possible to completely remove the RNase afterwards, which then resulted in a degradation of the RNA polynucleotide probe.

probe betaLact - antisense



probe betaLact - sense



probe betaLact – sense+antisense

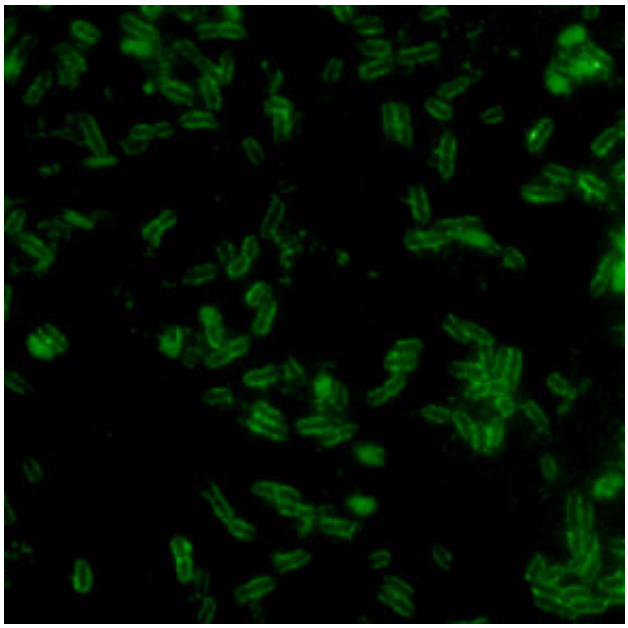


Fig. C.30. Fluorescence *in situ* hybridisation with probes betaLact – antisense (upper), sense (middle) and sense + antisense (lower image) to test possible binding to mRNA. Target was plasmid pCR2.1.

C.2.3. Polynucleotide probes targeting chromosomal DNA

The final step in the reduction of the number of target molecules was the development of probes targeting the chromosomal DNA. Using a single copy gene presumably less than 5 (with the copy number of the bacterial chromosome depending on the growth state) target sequences are available as starting point for a network formation.

C.2.3.1. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

GAPDH is a key enzyme in the glycolytic pathway, catalysing the oxidative phosphorylation of glyceraldehyde-3-phosphate to 1,3,-biphosphoglycerate. It is a well studied housekeeping enzyme and its amino and nucleic acid sequence has previously been used for phylogenetic analysis (Brown et al., 2000, Liaud et al, 1994, Lawrence et al., 1991).

Primers specific for the *E. coli* GAPDH gene were designed and PCR products were used for *in vitro* transcription to produce the RNA probes. The first probe, GAP-1 (later renamed GAP-E), had a size of 258nt (target position of probe see Fig. C.31). After adjusting the hybridisation conditions (considering the low number of target sequences and the lower stability of RNA:DNA hybrids [as compared to RNA:RNA hybrids of rRNA targeted hybridisations]) to 5-15% formamide and 24-30h hybridisation (constituting a further reduction of stringency and increase of hybridisation time compared to plasmid targeted probes) a cell specific (with *N.canis* as negative control) fluorescence signal was observed (Fig. C.32). The signal often, but not always, had the shape of a halo. Signal intensity was considerably weaker than with rRNA targeted probes, but only slightly below that of plasmid targeted probes.

To elucidate the influence of probe length on the nature of the hybridisation signal, ten probes ranging in length between 48-795nt were developed (GAP-A – GAP-J, binding positions cf. Fig. C.31). Hybridisations with these probes resulted in strong background and unspecific binding for the very short probes (GAP-A-D). With increasing probe length the signal became more specific, but with the very long probes not all target cells were detected (GAP-H – J). Optimal signal specificity and intensity could be achieved with probes GAP –E and –F (length 258 and 338, resp.) (see Fig. C.33).

A sense probe of GAP-E was generated to exclude the possibility of the signal being due to binding to mRNA (analogous to the procedure described for plasmid targeted probes, cf. chapter C.2.2.). Hybridisations with this probe showed signals equivalent to those seen with the antisense probe, thus excluding mRNA as the sole origin of the positive hybridisation signal (cf. Fig. C.34).

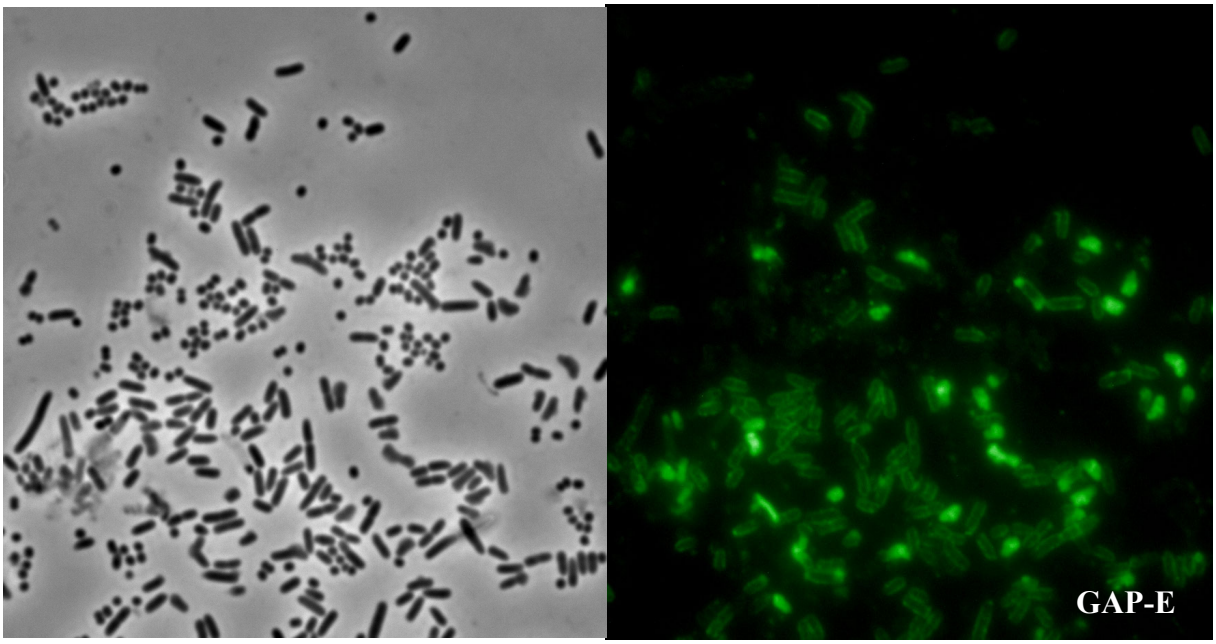
Fig.C.31. Sequence of *E. coli gap* gene for GAPDH (glyceraldehyde-3-phosphate dehydrogenase) [NCBI accession number X02662] indicating the positions of primers GAP A-J.

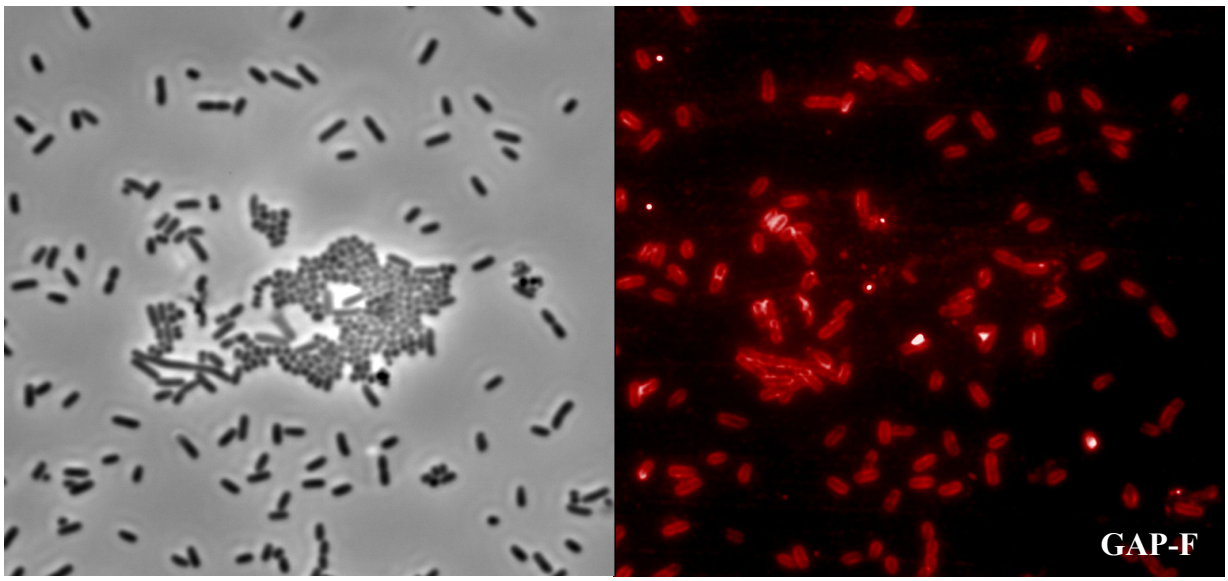
Next page:

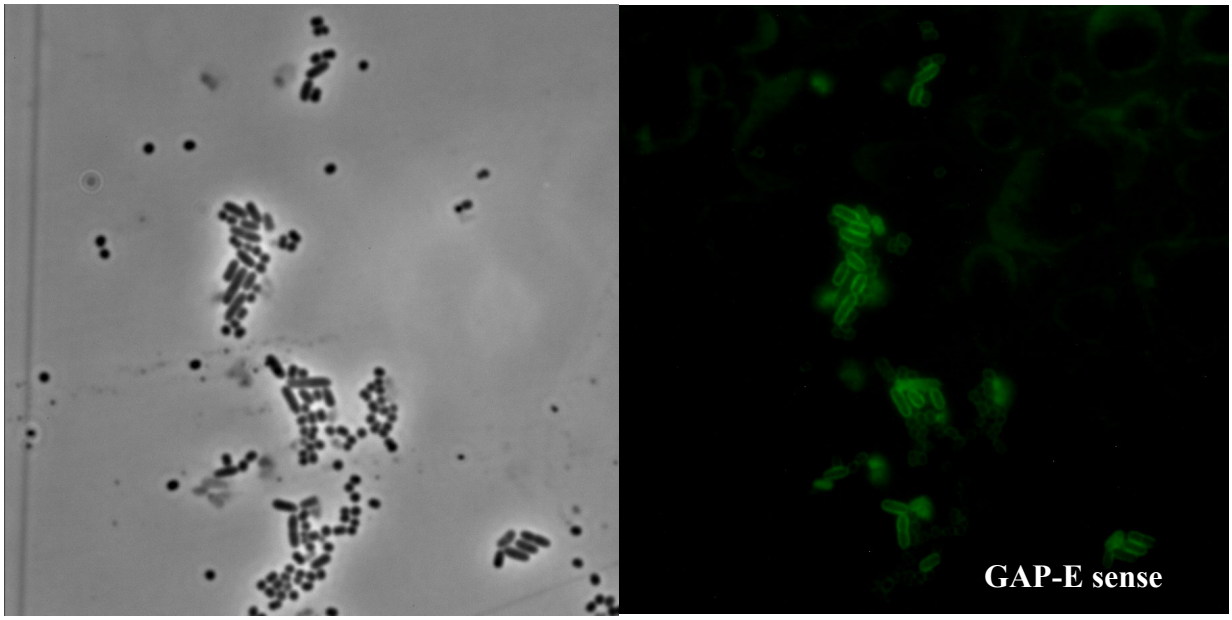
Upper images:
Fig. C.32. Fluoerscent *in situ* hybridisation with probe GAP-E, (length 258nt) targeting the *E. coli* GAPDH gene. The biotin labelled probe was detected with fluorescein-tagged streptavidin. Artificial mixture of *E. coli* (rods) and *N. canis* (cocci) to verifiy specificity of the probe. Phase contrast (left) and epifluorescence image (right).

Middle images
Fig. C.33. Fluoerscent *in situ* hybridisation with probe GAP-F, (length 338nt) targeting the *E. coli* GAPDH gene. The biotin labelled probe was detected with Cy3-tagged streptavidin. Artificial mixture of *E. coli* and *N. canis* to verifiy specificity of the probe. Phase contrast (left) and epifluorescence image (right).

Lower images
Fig C.34. Hybridisation with GAP-E sense probe, which is identical to the mRNA sequence, to rule out the possibility of positive hybridisation signals due to binding to mRNA. Phase contrast (left) and epifluorescence image (right).


GAP-E


GAP-F


GAP-E sense

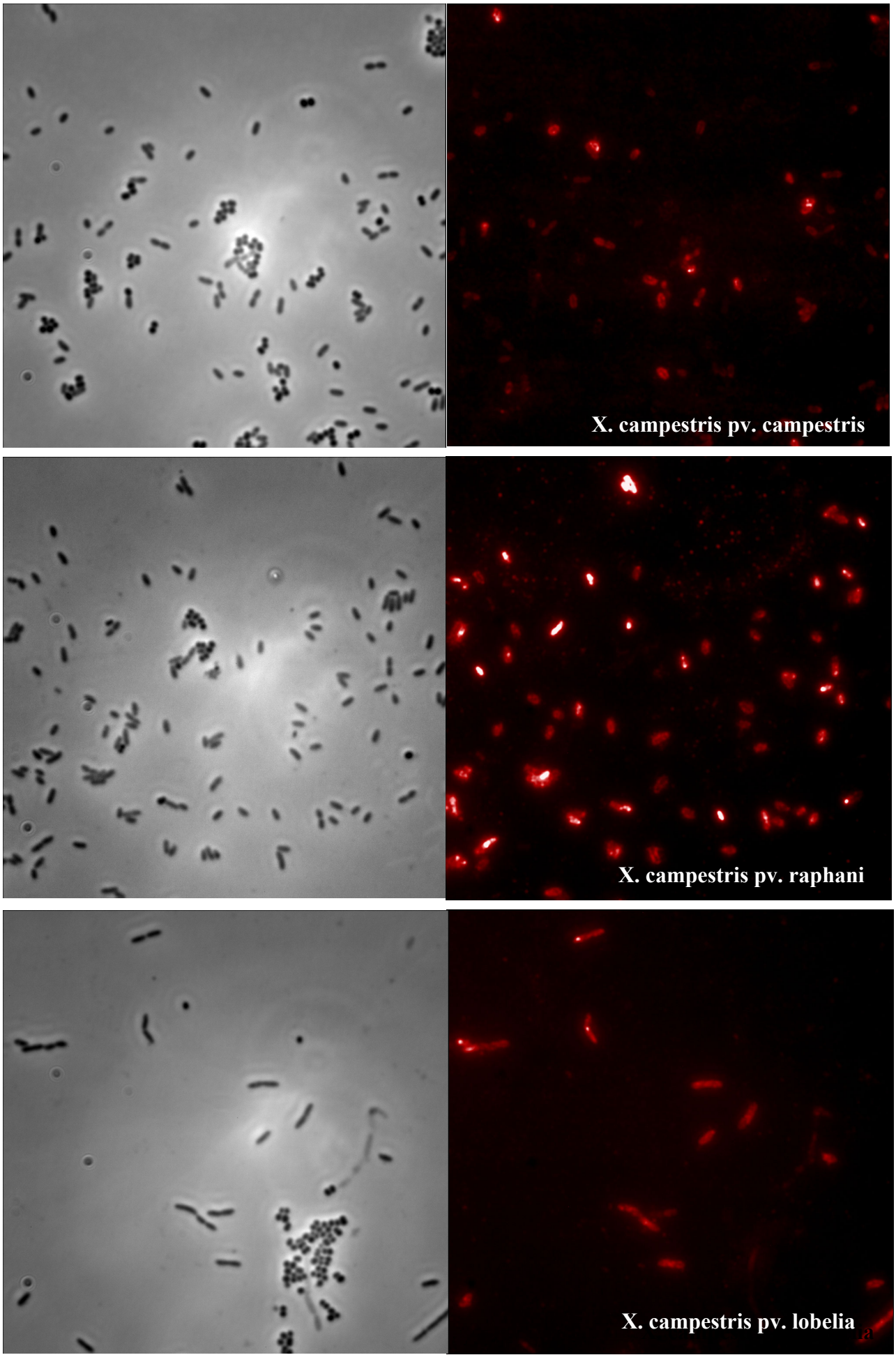
C.2.3.2. *Xanthomonas campestris* pathovars

As a second example for chromosomal DNA targeted polynucleotide probes pathovar specific regions of several *Xanthomonas campestris* pathovars were chosen. *X. campestris* is a plant pathogen that can be subdivided in "pathovars" with different host specificities. The pathovars are too closely related for discrimination by rRNA analysis, but using genomic subtractive hybridisation pathovar specific PCR primers can be developed (Mehlen, 2003). The PCR products obtained with the pathovar specific primers were used for *in vitro* transcription to generate RNA polynucleotide probes.

In situ hybridisation with probes Xc-ca (targeting the xpsO gene of *X. campestris pv. campestris*, length 140nt), Xc-ra (targeting the virB11 gene of *X. campestris pv. raphani*, length 272nt) and Xc-lo (targeting an unspecified region of *X. campestris pv. l*obelia, length 352nt) showed species specific signals (using *N. canis* as negative control) either in the shape of a halo or as cellwide fluorescence (Fig. C.35). However, the probes were not suitable for distinguishing different pathovars of *X. campestris*.

Next page:

Fig. C.35. Fluorescence *in situ* hybridisation with probes targeting chromosomal DNA of several *Xanthomonas campestris* pathovars. Biotin labelled probes targeting the xpsO gene of *X. campestris pv. campestris* (upper images), virB11 gene of *X. campestris pv. raphani* (middle images) and an unspecified region specific for *X. campestris pv. lobelia* (lower images) were detected with Cy3-tagged streptavidin. Artificial mixtures of *Xanthomonas* and *N. canis* were used to verify specificity of the probes.


X. campestris pv. campestris
X. campestris pv. raphani
X. campestris pv. lobelia

Part II – Cell sorting with polynucleotide probes

C.3. Cell sorting methods

In the course of this work several cell sorting methods were compared and improved. Starting point was a technique described by Stoffels et al (1999), which is based on the usage of paramagnetic beads (method A, Fig. C.36.). The next step was the transfer of the process to microplates, which are easier to handle and allow to work with many samples at the same time (method B, Fig.C.37.). In the final stage, which is still based on microplates, it was possible to render the cell sorting independent of labelling the probes with biotin, thereby avoiding possible artefacts due to insufficient labelling efficiency or unspecific binding to biotin (method C, Fig. C.38.).

All three methods have the first step in common, which consists of hybridising the sample with an RNA polynucleotide probe. For methods A and B this polynucleotide probe has to be labelled with biotin. This is achieved by incorporating biotin labelled UTP during the *in vitro* transcription, resulting in a labelling density of about 10-20 biotin-UTP for a 200nt probe. Using method C it is not necessary for the cell sorting to label the probes. Labelling may, however, be desirable for a later detection of the cells. In this case not only biotin, but also digoxigenin or fluorescent dyes (Fluorescein, Cy3, Cy5 etc.) attached to UTP are available, allowing the simultaneous use of differently labelled probes during one hybridisation.

The different principles underlying methods A, B and C are schematically depicted in figures C.36 – C.38.

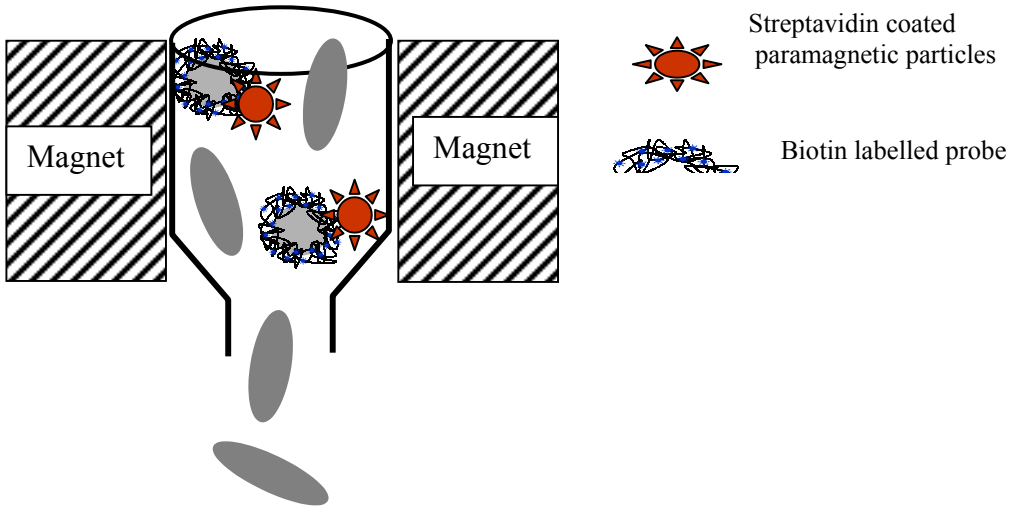


Fig. C.36. Method A – Cell sorting with streptavidin coated paramagnetic particles
After hybridisation with a biotin labelled polynucleotide probe streptavidin coated paramagnetic beads are added to the sample, which bind to probes located on the outside of the cell, forming a stable biotin-streptavidin bond. The sample is then applied onto a column filled with steel wool, which is placed in a magnetic field. The cells attached to the paramagnetic beads will be held back in the magnetic field, while unlabelled cells will pass through the column. Removing the column from the magnetic field, it is possible to obtain the labelled cells. Both labelled and unlabelled fractions can then be used for further analysis. By comparing these fractions with a fraction of the sample before the cell sorting, it is possible to determine the cell sorting efficiency (cf. chapter C.5.).

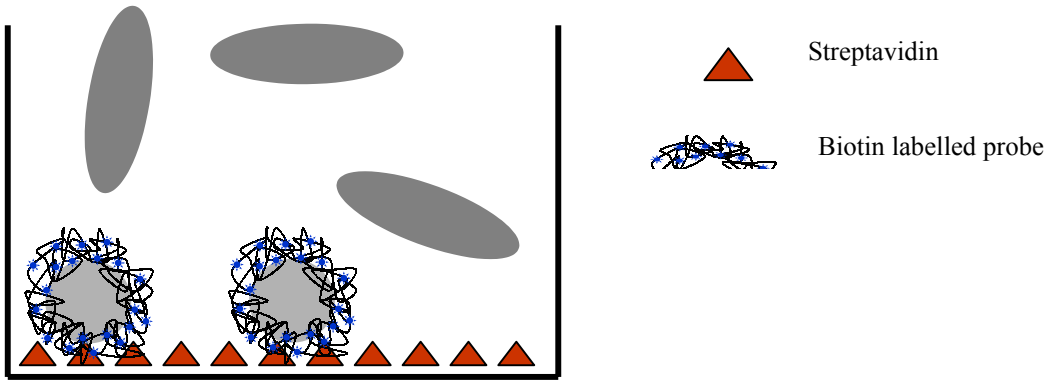


Fig. C.37. Method B – Cell sorting in streptavidin coated microplates
After hybridisation with biotin labelled polynucletide probes, the sample is transferred into streptavidin coated microwell plates. As in method A, the separation of labelled (i.e. cells that have taken up the probe) and unlabelled cells is achieved via a biotin-streptavidin bond. Considering the fact that in this case the separation is done on a two-dimensional area (the surface of the microwell plate), as opposed to the three-dimensional space inside the column in method A, it is advisable to spread the sample over several microwells to circumvent insufficient cell sorting efficiency due to saturation of the surface of the microplate.
Unlabelled cells can easily be recovered from the supenatant and used for further microscopic or molecular analysis. It is not possible, however, to retrieve the cells that are bound to the microplate for microscopic analysis, although, using specially (conically) shaped microplates, they are still available for molecular analysis (PCR, sequencing etc.).

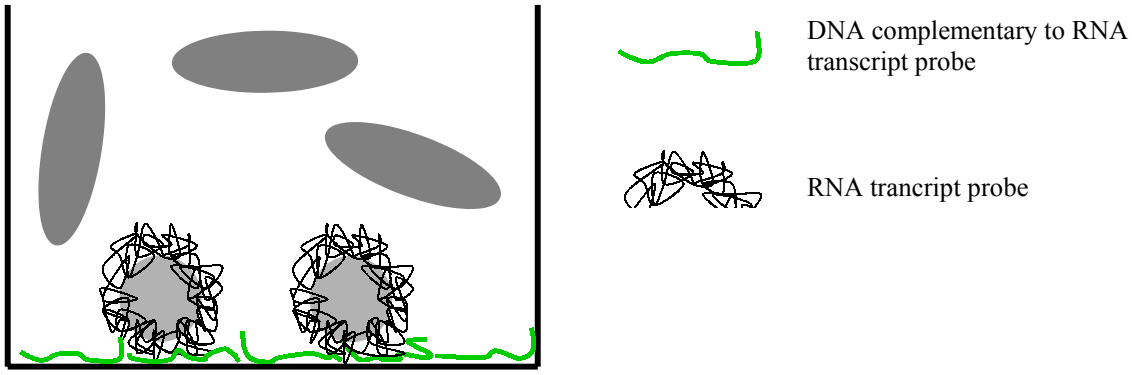


Fig. C.38. Method C – Cell sorting with DNA coated microplates
After hybridisation with polynucleotide probes the sample is transferred into microwell plates that are coated with DNA complementary to the probe sequence. Here, the separation is based on a second hybridisation step between the probes or part of the probes that are presumably located outside the cells and the DNA on the surface of the microwells. As in method B it is again advisable to spread the sample over several microwells to avoid saturation of the surface. Unlabelled cells can be recovered from the supernatant and in contrast to method B it is also possible to retrieve the cells bound to the surface by heating the plates and thereby denaturing the hybrids. Using specially (conically) shaped microplates it is also possible to directly use the immobilised cells for molecular analysis.

Method A as the starting point and method B as an intermediate step were only used during the early stages of development. Later all efforts were concentrated on method C, which is discussed in greater detail in the following chapers and D.6. The results of cell sorting experiments as described under C.5 solely refer to method C.

C.4. Optimisation of the reaction parameters

A successful hybridisation and subsequent cell sorting depends on numerous parameters that can be divided into three groups: treatment of the sample before the first hybridisation, during the hybridisation and during the cell sorting (second hybridisation). To maximise the cell sorting efficiency parameters at all three steps had to be optimised.

C.4.1. Treatment of the sample before the first hybridisation

The effects of different cell fixation protocols and lysozyme or proteinase treatment on the hybridisation signal are described in chapters C.1.2 and C.1.3.

C.4.2. Parameters for 1st hybridisation

C.4.2.1. Stringency

The first hybridisation should be carried out under rather stringent conditions in order to ensure a specific binding of the probe to the target cells. The second hybridisation, on the other hand, requires less stringent conditions to enable all labelled cells, respectively the probes protruding from these cells, to bind to the DNA coated surface of the microplates.

The standard temperature of the first hybridisation was 53°C, with the stringency being adjusted through the formamide concentration in the buffer. A series of different formamide concentrations was tested using the rRNA targeted probe DIII (domain III of the 23S rRNA) and an artificial mixture of *E. coli* and *A. calcoaceticus* cells. Microscopic analysis revealed unspecific binding of the probe at <60% formamide and a slightly decreasing signal intensity > 90% formamide. Using these cells for cell sorting in microplates resulted in no significant differences in signal intensity when the (biotin labelled) cells (i.e. probes attached to cells) were detected with streptavidin-peroxidase in the microplates (Fig. C.39.). However, it is important to state that at formamide concentration lower than 60% the signal measured in the microplate is partially due to non-target cells that bound unspecifically to the probe during the first hybridisation. Since the second hybridisation can only differentiate between labelled and unlabelled cells, it is vital for a successful cell sorting to ensure specific and comprehensive binding of the probes during the first hybridisation.

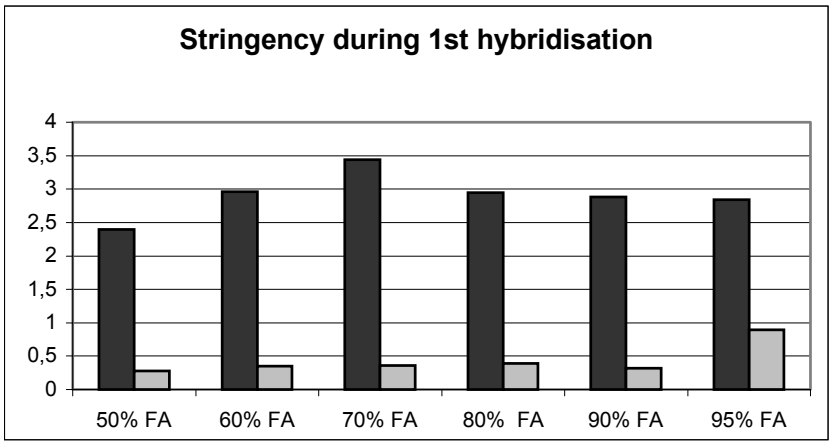


Fig. C.39. Detection of immobilised cells in microplates after hybridisation at different formamide concentrations. Dark grey columns: signal intensity in microwells that were coated with DNA complementary to the probe. Light grey columns: negative control/background – microwells were coated with DNA other than the probe sequence.

C.4.2.2. Hybridisation time

With regard to speeding up the whole cell sorting procedure, different incubation times for the first hybridisation were tested, followed by cell sorting in microplates and subsequent comparison of the signal intensities. A 4-5 hour hybridisation yielded the highest signal intensities in the microplates, with the values being ~3 times as high as with 2 hours hybridisation. After an overnight hybridisation the values decrease again, which may be due to a gradual degradation of the RNA probe.

These findings are in accordance with microscopic analysis, where the highest signal intensities were also observed after 4-5 hours hybridisation.

This comparison of hybridisation times was done using the rRNA targeted DIII probe. For subsequent experiments with this probe a standard hybridisation time of 5 hours was used. Plasmid and chromosomal DNA targeted probes require significantly longer incubation periods, but nevertheless the gradual degradation of the RNA probe (which will also lead to unspecific binding) has to be considered. The optimal hybridisation times for plasmid and chromosomal DNA targeted probes was shown to be 18-24h and 24-30h, respectively.

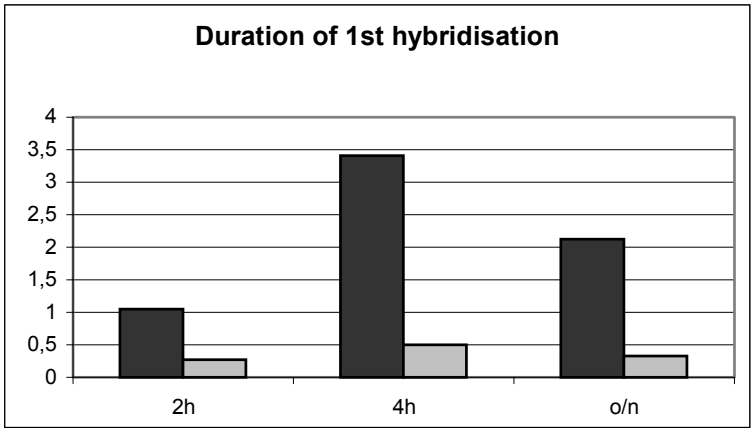


Fig. C.40. Incubation period of first hybridisation. Dark grey columns: signal intensity in microwells that were coated with DNA complementary to the probe. Light grey columns: negative control/background – microwells were coated with DNA different from the probe sequence.

C.4.2.3. Amount of probe

To determine the correlation between the halo signal and the amount of probe (i.e. does the halo only appear, when the probe is used in large excess, what happens at low probe concentrations [possibly a cellwide fluorescence]?) a series of different probe concentrations was tested.

Hybridisations were carried out with 0.1 - 4µl (appr. 0.1 – 4µg) of transcript probe (23S DIII) + 12µl hybridisation buffer containing 80% formamide. Adding varying amounts of probe to the buffer leads to varying dilutions of the buffer, resulting in a lower final concentration of formamide (e.g. 4µl probe + 12µl buffer[80% FA] results in 60% FA f.c., whereas 1µl probe

+ 12µl buffer results in 73.8% FA f.c.). To compensate this, a second series of hybridisations was carried out in parallel, where the probe was complemented with H_2O to a volume of 4µl + 12µl buffer, resulting in a constant formamide concentration of 60%.

The signal intensity was measured with the confocal laser scanning microscope in combination with the KS400 software (Zeiss, Germany).

The strongest signals were observed with 4µl of probe, decreasing rapidly with decreasing probe concentrations. This effect was even more pronounced when the probe was not complemented with H_2O to 4µl, resulting in increasing formamide concentrations with decreasing probe concentrations (c.f. Fig. C.41.). Below 0.5µl the signals were too weak for quantification of signal intensity. All visible signals had the typical halo shape.

This experiment was carried out with an rRNA targeted probe. In tests with plasmid and DNA targeted probes, the minimum amount of probe necessary to yield a detectable fluorescence signal was shown to be 2µl (approximately 2µg).
For subsequent hybridisations 4µl probe + 12µl buffer for hybridisations on microscope slides and 6µl probe + 30µl buffer for hybridisations in solutions were used as standard concentrations.

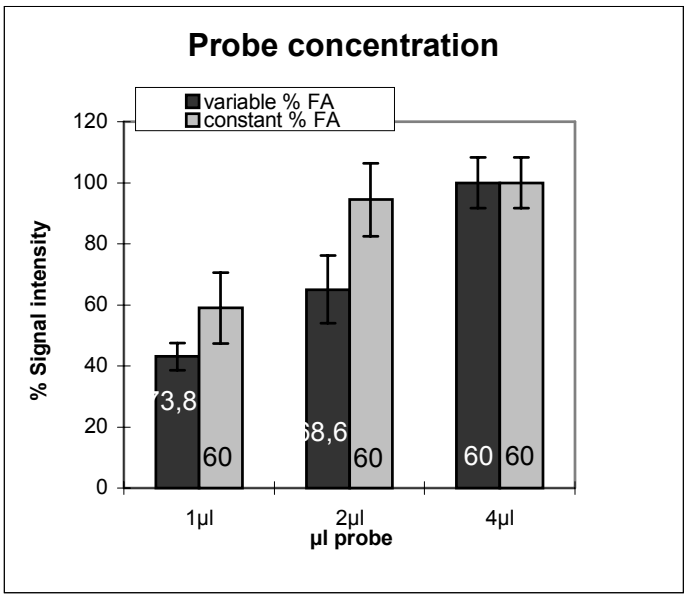


Fig. C.41. Probe concentration. The signal intensity was measured for different probe concentrations. Grey columns: % signal intensity with constant formamide concentration in the hybridisation buffer, black columns: % signal intensity for increasing formamide concentrations with decreasing probe concentrations. Numbers on columns indicate the formamide concentrations.

C.4.3. Parameters for 2nd hybridisation

C.4.3.1. Stringency

The second hybridisation (i.e. the cell sorting in microplates) should be carried out at a considerably lower stringency than the first hybridisation, since in this case it is desirable to let all labelled cells bind to the microwells via the probes attached to them. It is, however, not advisable to use absolutely unstringent conditions, since this will result in slightly lower signal intensity and higher background (when detecting the cells in microplates in order to monitor the cell sorting efficiency) as can be seen in Fig. C.42.

For subsequent hybridisations with rRNA targeted probes a standard temperature of 53°C with a buffer containing 33% formamide was used. For plasmid and DNA targeted probes, which require a much lower stringency during the first hybridisation, the temperature during the second hybidisation was lowered to 37°C (with 33% formamide in the buffer).

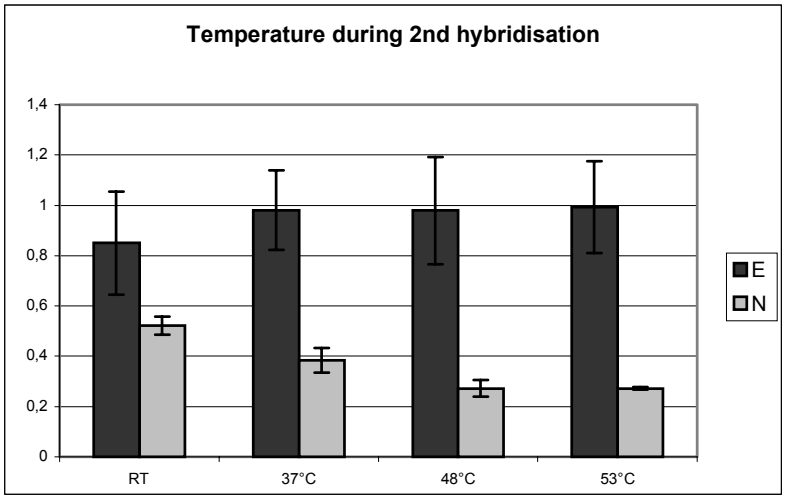


Fig. C.42. Stringency during the second hybridisation. Black columns: signal intensity in microwells that were coated with DNA complementary to the probe. Grey columns: negative control/background – microwells were coated with DNA different from the probe.

C.4.3.2. Hybridisation time

Testing different incubation times for the second hybridisation showed no significant variation in signal intensity between 1 and 4 hours incubation. Therfore a standard incubation for 60-90min was used for subsequent experiments.

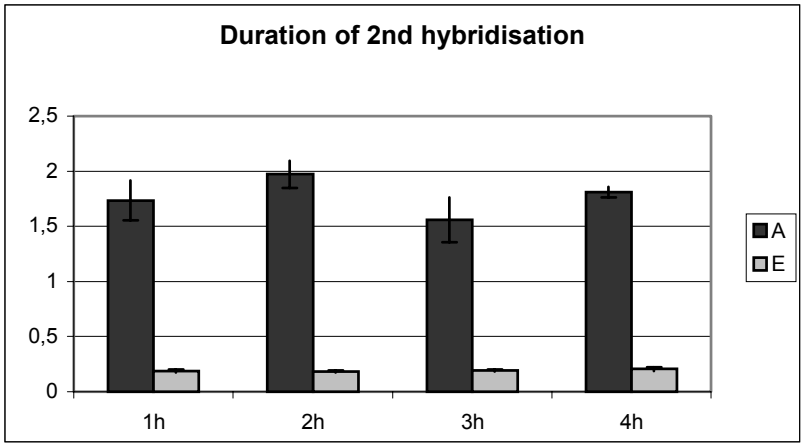


Fig. C.43. Incubation period of second hybridisation. Black columns: signal intensity in microwells that were coated with DNA complementary to the probe. Grey columns: negative control/background – microwells were coated with DNA different from the probe.

C.5. Cell sorting efficiency

C.5.1. Evaluation via semiquantitative PCR

Semiquantitative PCR was carried out as described in Material and Methods (cf. B.14.2.). Figure C.44. shows a typical example. The sample contained both *E. coli* and *A. calcoaceticus* cells and was hybridised with the rRNA targeted probe 23S-DIII-Aca, specific for *A. caloaceticus*. Cell sorting was carried out in DNA-coated (23S-DIII-Aca) microplates (cf. B.12.2.) and aliquots before and after the cell sorting were used for PCR.

For *E. coli* (the non-target cells in this experiment) the number of dilution steps with a detectable PCR product remains constant before and after cell sorting, whereas for *A. calcoaceticus* (the target cells) there is a reduction after the cell sorting, indicating a depletion of *A. calcoaceticus* cells.

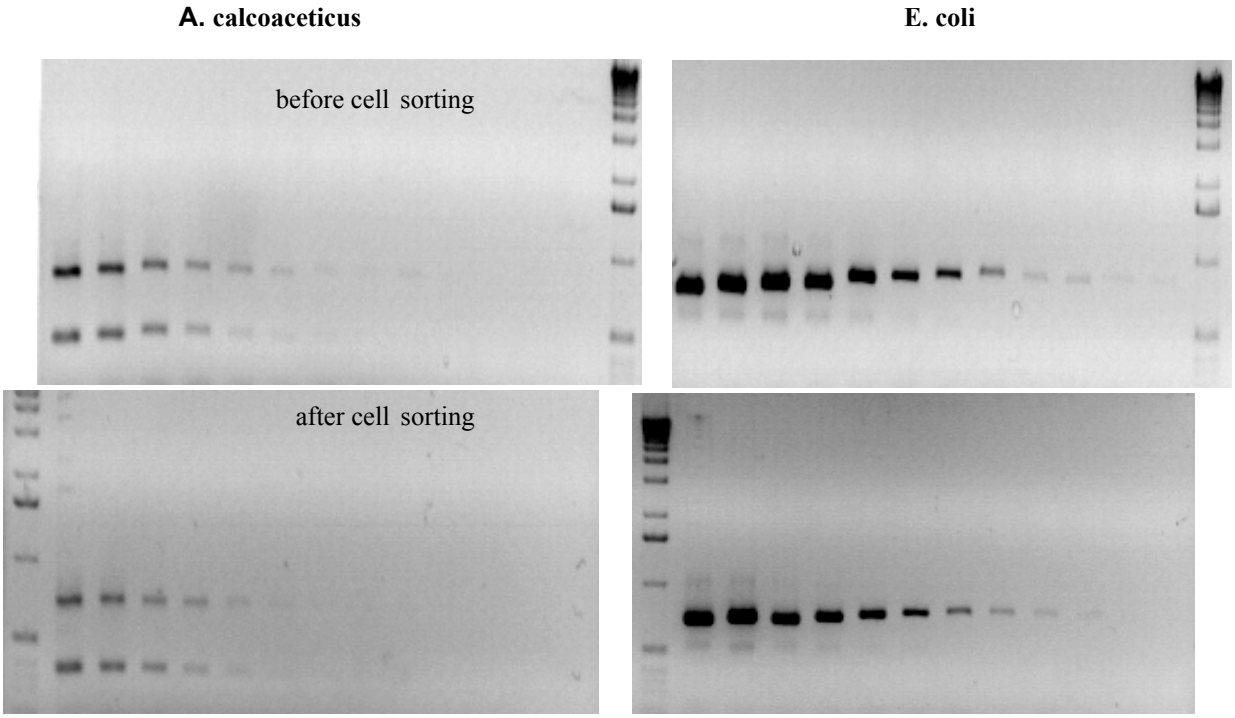


Fig. C.44. Semiquantitative PCR with primers specific for *E. coli* (right) and *A.calcoaceticus*(left) before (top) and after (bottom) the cell sorting. The number of dilution steps with detectable PCR products remains constant for *E. coli* (non-target cells) and decreases for *A. calcoaceticus* (target cells) after the cell sorting, indicating a depletion of *A. calcoaceticus* cells during the cell sorting.

The semiquantitative PCR certainly allows a general evaluation whether the cell sorting process has been successful, but does not permit an exact measurement of the enrichment/depletion of target cells, since – using a 1:1 dilution series - a difference of one dilution step before and after cell sorting is equivalent to 50% depletion, 2 steps difference are equivalent to 75% (50+25) depletion etc. Due to this lack of accuracy and the disproportional amount of time required for this method, it was soon abandoned and replaced first by manual cell counts and eventually by computer based quantification (cf. B.14.3., C.5.3.).

C.5.2. Quantification via manual cell counts

Manual cell counts were only used in the early stages of the study for quantification cell sorting efficiency using artificial mixtures of morphologically dicriminable bacterial species. Due to the inaccuracy of this method it was later replaced by computer based quantification (cf. B.14.3., C.5.3.). In the summary of the cell sorting experiments (Fig. C.46) results obtained by manual cell counts are marked with an asterisk.

C.5.3. Computer based quantification

Computer based quantification was carried out as described in Material and Methods (cf. B.14.3.).

Figure C.45. shows an example a cell sorting experiment using the rRNA targeted probe DIII-Eco to remove *E. coli* cells from an artificial mixture of *E. coli* and *N. canis*. The values for target cells (*E. coli*) before and after cell sorting are depicted. The depletion value (calculated from the mean value before and after cell sorting using the formula as stated under B.14.3.) in this experiment was 54%

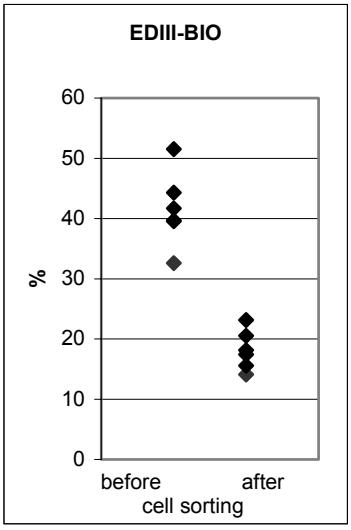


Fig. C.45. Cell sorting of a mixture of *E. coli* and *N. canis* with rRNA targeted probe E-DIII, specific for E. coli. Quantification of target cells (*E. coli*) in the sample before and after the cell sorting by analysing several microscopic fields.

Figures C.46a-c summarise the cell sorting experiments with rRNA targeted (probe DIII), plasmid targeted (probe beta lact) and chromosomal DNA targeted (probe GAPDH) probes. The exact values for each experiment are listed in the appendix (cf. F.2.).

Varying amounts of target cells were used in these experiments. A strong correlation between the percentage of target cells and the cell sorting efficiency was observed, with the highest depletion efficiencies for low initial concentrations of target cells.

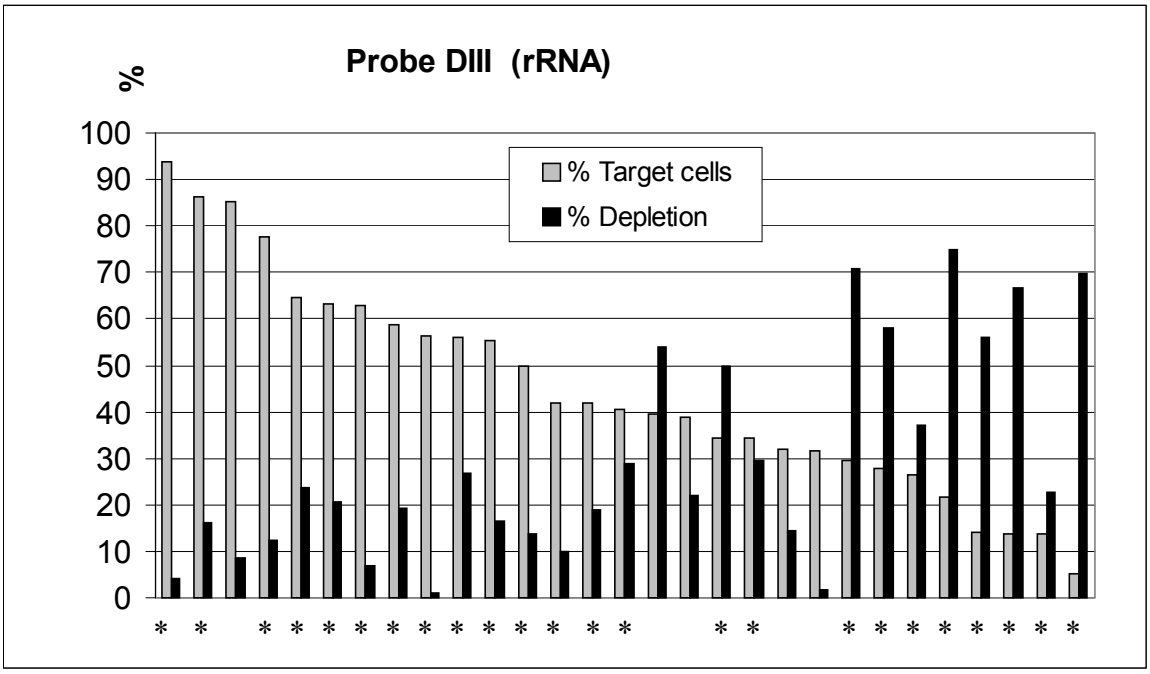


Fig. C.46.-a

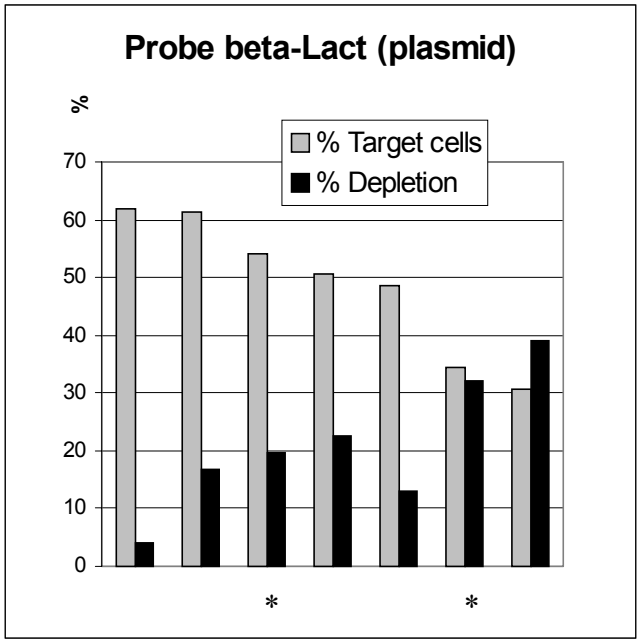


Fig. C.46.-b

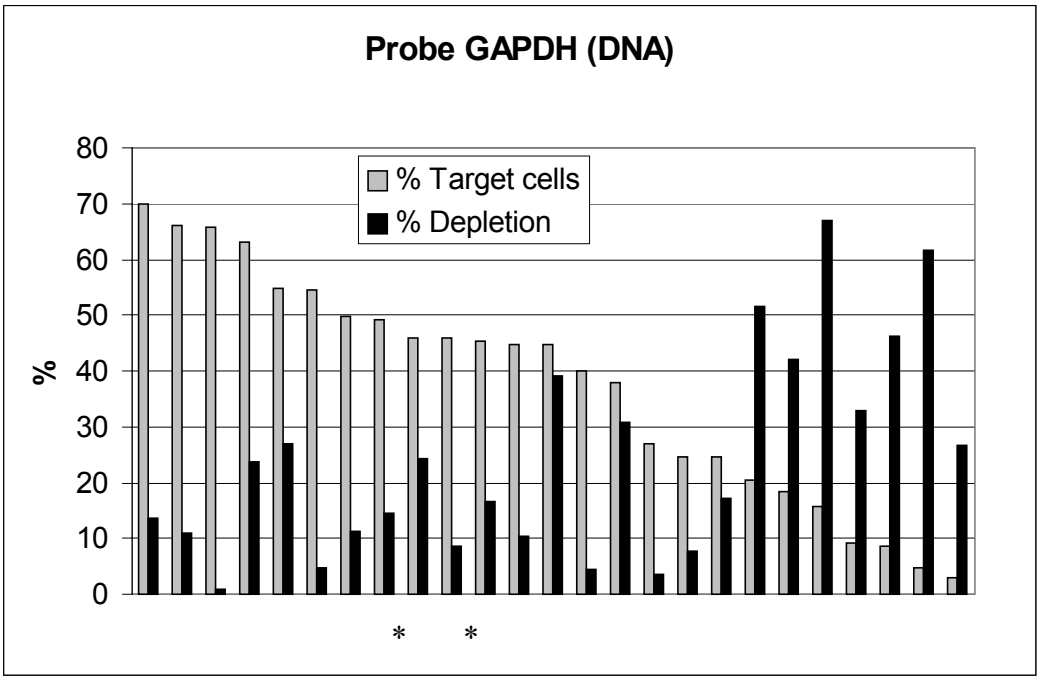


Fig. C.46.-c

Fig. C.46.a-c. Summary of cell sorting experiments – a) rRNA targeted probe DIII, b) chromosomal DNA targeted probe GAPDH, c) plasmid targeted probe betaLact. Cell sorting was done with artificial mixtures of cells further specified under F.2. Grey columns: initial concentration of target cells, black columns: percentage of cells that could be removed by cell sorting. Asterisks indicate quantification by manual cell counts.

C.6. Application of method to environmental samples

For initial evaluation and optimisation of the method artificial mixtures of cells were used. In the final stage, the method was applied to environmental samples, i.e. activated sludge from an industrial wastewater treatment plant (animal waste processing plant (Tierkörperbeseitigungsanstalt) Kraftisried, Germany).

The samples were fixed with PFA as described in Material and Methods and treated with ultrasonic sound prior to hybridisation for 30sec to disperse flocs of biomaterial and thus facilitate the access of the probes to the target cells and later the cell sorting.

The first tests with environmental samples were done with spiked samples, meaning that target cells were added to the sample before the hybridisation. Their successful capture in microplates could be monitored via PCR directly in the microplates using target cell specific primers.

Fig. C.47. shows an exemplary agarose gel of PCR in the microplates after the cell sorting, confirming that a) target cells did bind to the microplate during cell sorting and b) their DNA is still available for analysis with molecular biology methods. Activated sludge from the

Kraftisried plant was spiked with *E. coli, Pseudomonas aeruginosa* and *Klebsiella pneumoniae*, none of which occurred naturally in the plant at the time the sample was taken, and hybridised (in three independent reactions) with 23S rRNA DIII probes generated with DNA from and therefore specific for each of the three target organisms (DIII-Eco, DIII-Pae and DIII-Kpn). Sludge, that was not spiked with target cells, was hybridised with the same probes and used as a negative control,. Cell sorting in microplates coated with the respective DNA was performed and the plates were used for PCR with primers specific for *E. coli* (616Valt/Eco444), *P. aeruginosa* (616Valt/Pae153) and *K. pnemoniae (*616Valt/Kpn1129), which amplify part of the 16S rDNA. Agarose gel electrophoresis revealed successful amplification of DNA fragments of the expected size for the spiked samples, but no products were obtained from the non-spiked samples.

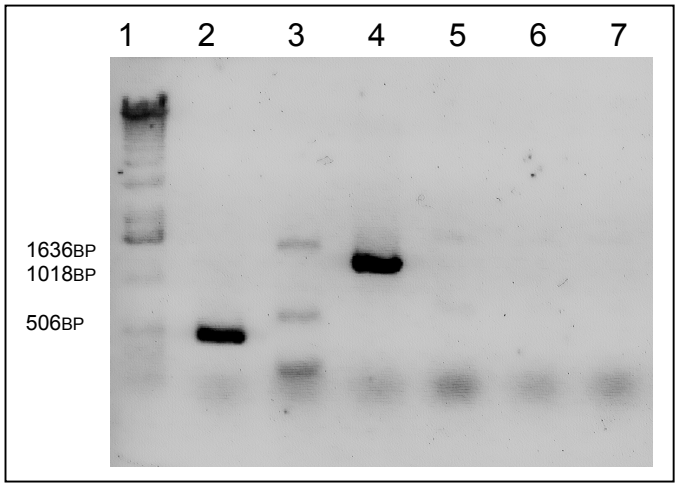


Fig. C.47. PCR from microplate after cell sorting with primers specific for *E. coli* (lanes 2 + 5*), P. aeruginosa* (lanes 3 + 6) and *K. pneumoniae* (lanes 4 + 7). Lanes 2,3,4 were from spiked samples, lanes 5,6,7 are the negative control with non-spiked samples. The size of the the PCR products corresponds to the expected length (460bp for *E. coli*, 170bp for *P. aeruginosa*, 1140b for *K. pneumoniae*). Lane 1 KBL marker.

Finally, hybridisation and cell sorting was performed with probes targeting species native in the sample without spiking.

Ultrasonically treated sludge from Kraftisried was hybridised with probe DIII-Aca, specific for *Acinetobacter calcoaceticus* and used for cell sorting. Fig. C.48. shows phase contrast images of the original sample (left image) and the cells recovered from the bottom of the microplate after cell sorting (right image). While the original sample contains a complex mixture of multiple cell types, the left image shows a clear enrichment of cells with coccoid morphology, characteristic for *A. calcoaceticus*. PCR (using genus-specific primer Aca23a and universal primer 616Valt) with the sample before cell sorting and cells recovered from the microplate confirmed the presence of *Acinetobacter* (Fig. C.49).

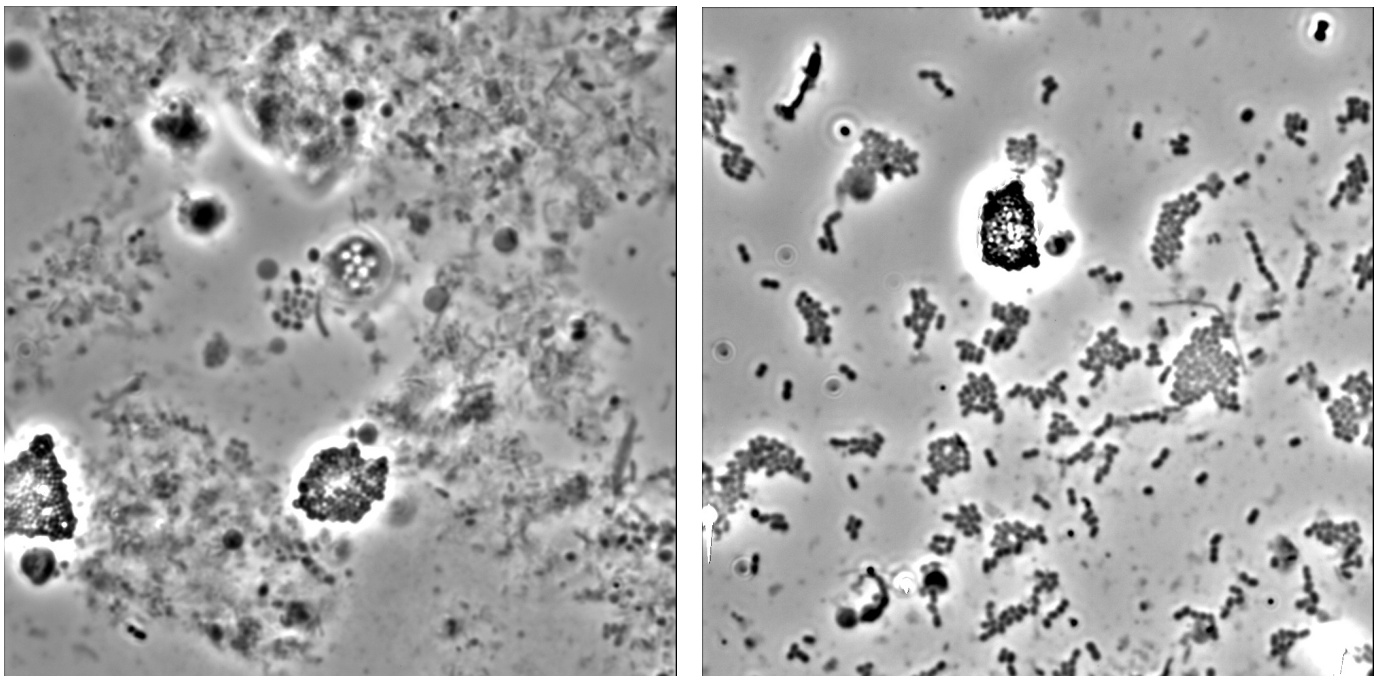


Fig. C.48. Phase contrast image of a sample before cell sorting (left) and cells that were recovered from the bottom of the microplate after cell sorting (right).

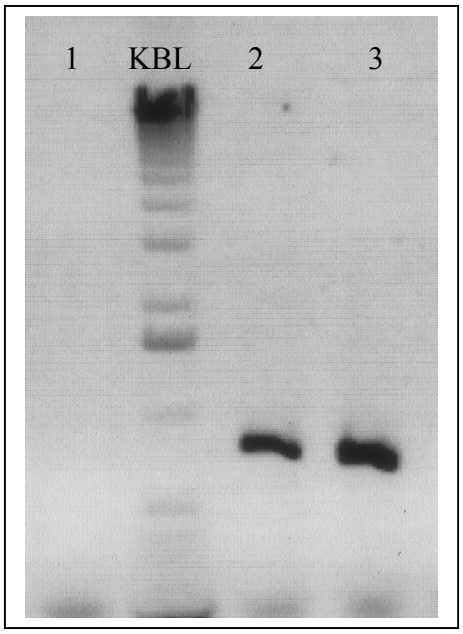


Fig. C.49. PCR using primers Aca23a/ 616Valt with sample before cell sorting (lane 2) and cells recovered from microwell (lane 3). Lane 1: negative control

D. Discussion

The aim of this work was to study the properties of polynucleotide transcript probes, extend their spectrum of applicability and finally use them for enrichment or depletion of cells.

Part I – Fluorescence *In situ* Hybridisation (FISH) with polynucleotide transcript probes

D.1. Target organisms

rRNA targeted polynucleotide transcript probes have first been used for murine leukemia cells (Baumann et al, 1988) and *S. cerevisiae* (Bertin et al., 1990). In these cases the probes comprised the major part of the complete rDNA (several kb), which was transcribed via nick translation and then degraded to smaller probes, about 100 nt in length. Hybridisation with this mixture of probe fragments resulted in high signal intensity due to multiple labelling (as compared to monolabelled oligonucleotide probes), but also had the marked disadvantage of high unspecific background due to fragments binding to conserved regions of the rRNA.

Trebesius et al.(1994) adapted the method for bacteria and introduced the concept of using only a short, but highly variable part (~250nt) instead of the complete rRNA as target, thus reducing background and increasing specificity. The chosen target region was domain III of the 23S rRNA, which contains the longest stretch of high variability within the rRNA (Höpfl et al., 1989) and has previously attracted attention as a site for different stable insertions among actinomycetes (Roller et al. 1992).

Using bacteria as target organisms, a characteristic feature of polynucleotide probes, previously not observed with eukaryotes was encountered. The fluorescence signal (after detection of the digoxigenin-labelled probes with anti-digoxigenin-fluorescein) had the appearance of a “halo”, meaning it was concentrated in the periphery of the cells, with only little fluorescence inside the cells, resembling the results that can be expected using fluorescently labelled antibodies targeting surface antigens. Initially, it was assumed that the polynucleotide probes were too large to enter the cell completely and therefore bind to ribosomes located in the vicinity of the cell envelope and possibly stretch through the cell wall, leaving part of the probe outside the cell. This concept was later exploited by Stoffels et al. (1999) to enrich cells via probe mediated immobilisation (cf. C.3.).

The above mentioned experiments were restricted to gram-negative organisms. In this work the spectrum of gram-negative bacteria, which could successfully be targeted with polynucleotide probes was expanded and it was possible to extend the method to gram-positive bacteria.

Gram-positive organisms pose a certain challenge for *in situ* hybridisations due to their thick and rigid peptidoglycan layer, which may hinder the access of probes. This problem has been reported for oligonucleotide probes (Amann et al., 1995) and is much more pronounced for the complex polynucleotide probes. Supplementing the standard PFA cell fixation protocol with a lysozyme treatment prior to hybridisation efficiently weakened the cell walls to allow the binding of the probes to their targets. However, care has to be taken regarding the incubation period of the lysozyme treatment, since it can easily lead to complete cell lysis. Attempts to standardise the lysozyme treatment to get equal hybridisation results for different gram-positive bacteria failed due to the differences in lysozyme susceptibility of the organisms. While a lysozyme treatment of 5min (at 37° C) yielded satisfying hybridisation signals for *Streptomyces venezuelae* and *S. divaceus griseolus*, it led to almost complete cell lysis in the case of *B. subtilis* and *Mc. luteus* (cf. Fig. C.9-12). Other gram-positive bacteria, such as *Lactobacillus acidophilus* and *Paenibacillus polymyxa* on the other hand have been reported to give halo-like hybridisation signals without any lysozyme or other additional treatment (Fichtl, 2002).

Taken together, it has to be stated, that no universally valid protocol could be found that would result in equal permeabilisation of the cell envelope for different types of cells. This fact has to be kept in mind when analysing environmental samples, since it may lead to an underestimation of gram-positive cell numbers. This drawback of polynucleotide probes may, however, partly be compensated by their higher sensitivity in the detection of slowly growing organisms (Pernthaler et al., 2002), which, due to their low ribosome content, often elude comprehensive detection with (monolabelled) oligonucleotide probes. Here polynucleotide probes, containing 15-30 labelled nucleotides per probe molecule offer an opportunity for signal amplification, possibly further enhanced by formation of a network of probes.

Apart from prokaryotic organisms, polynucleotide-FISH could also be adapted for several eukaryotes, i.e. two mammalian cell lines and a number of yeast species.

Mammalian cells, possessing no cell wall or other rigid cell envelope, proved to be highly susceptible for polynucleotide probes, as could be expected. Positive hybridisation signals with rRNA targeted probes could already be observed after 2h hybridisation (as opposed to a minimum of 5h for bacteria or yeast cells). Due to the high permeability of the cell membrane the hybridisation signal, in contrast to bacteria, is not strictly concentrated on the outside of the cell, but also reaches into the cytoplasm.

Yeasts again are more challenging target organisms, possessing a cell wall 5-10 times as thick as that of bacteria (e.g. *S. cerevisiae*: 160nm, for comparison *S. aureus*: 20nm [Salton, 1960]) that may seriously impede the access of polynucleotide probes. Unlike bacteria, the yeast cell wall consists primarily of glucan, mannan and various proteins and is therefore insensitive to lysozyme. Here, the use of proteinase K helped to permeabilise the cell wall. Zymolyase, an enzyme preparation from *Arthrobacter luteus* which has glucanase and protease activity, has also been reported to improve cell wall permeability (Bertin et al., 1990). But even with enzyme treatment in combination with various cell fixation procedures (cf. B.10.) it was not possible to get positive hybridisation results with all yeast species studied. Of the 22 species tested, only in 7 cases was it possible to render the cells accessible for polynucleotide probes. Oligonucleotide probes, on the other hand, seem to be less problematic regarding the penetration of the cell envelope (Joao Inácio, personal communication), suggesting a careful balancing of the advantages of polnucleotide probes (high signal intensity regardless of ribosome copy number) against oligonucleotide probes (better access to the target site).

D.2. Target molecules

Apart from adapting the method for different target organisms, another aim of this work was to explore the possibility of using target molecules other than rRNA and define the limitations of the method regarding the copy number of target molecules necessary to obtain a detectable hybridisation signal.

D.2.1. rRNA

Due to its known high variability, initial studies with polynucleotide probes (Trebesius et al, 1994, Stoffels et al, 1999) focused exclusively on domain III of the 23S rRNA. In the course of analysing the nature and functional principle of polynucleotide probes, the question arose, whether other parts of the rRNA might also be suitable as target regions or whether the cell specific characteristic hybridisation signal is the result of certain features (e.g. secondary structure, target accessibility) only inherent in domain III. This question could be answered by developing probe 367 (cf. B.4.), which has approximately the same size as probe DIII (~250nt) and targets the moderately variable domain I of the 23S rRNA. The hybridisation signal of this probe has the appearance of a bright halo and is species specific within the limits of the tested organisms (*E. coli, N. canis, B.cepacia, A. calcoaceticus*) and thus equivalent to

the results obtained with probe DIII, proving the suitability of rRNA regions, other than 23S domainIII as targets for polynucleotide probes.
Further regions of the 23S rRNA were tested with the probe set 1024_1-11, comprising domains I and II, which is discussed in chapter D.4.

D.2.2. DNA: Plasmids and chromosomal DNA – RING-FISH

Ribosomal RNA has for several reasons always been a favoured target molecule for *in situ* hybridisations. It is invaluable as a phylogenetic maker, present in high copy numbers in actively growing cells and relatively stable (as compared to mRNA, which may also be present in high copy numbers, but is highly susceptible to RNase).
Stability and usefulness for phylogenetic analysis (when addressing conserved genes respectively the proteins they code for) can also be attributed to plasmid and chromosomal DNA, but due to their low copy numbers these molecules were previously regarded as unsuitable for *in situ* analysis in bacteria. In the course of this work, in exploring the limitations of polynucleotide probes, different nucleic acid targets with decreasing copy numbers were addressed, starting with rRNA (10^5-10^4 per cell), followed by plasmids (10^3-10^2) down to chromosomal DNA ($<10^1$).
Plasmids have previously been detected indirectly by using oligonucleotide probes targeting the regulatory RNA II, which is involved in the replication of ColE1 related plasmids (Juretschko et al. 1999). A strong correlation between signal intensity and plasmid copy number (and therefore also RNA II copy number) was observed, with strong signals for high copy numbers, while cells with a low number of plasmids eluded detection.
DNA targeted probes are commonly used in eukaryotes for the detection of chromosomal aberrations or chromosome mapping (e.g. Sharma et al, 2001), a technique described as "chromosomal painting". These probes usually have a length of at least 6kb up to 100kb, although there are also reports of probes targeting single-copy genes, the shortest one being 2290 bp in length (Rogan et al., 2001).
Lanoil and Giovannoni (1997) adapted the eukaryotic chromosomal painting for bacteria and adequately named it "bacterial chromosomal painting" (BCP). BCP relies on the use of the *complete* genomic DNA as a target. The probe is generated via nick translation and degraded to 100-150nt fragments prior to hybridisation. For a clearly detectable signal a hybridisation period of 48 hours is necessary. No halos were reported. Using the complete genome as target links this method to genomic DNA:DNA reassociation *in vitro*, a well-known tool for taxonomic analysis (reviewed by Grimont, 1988), which allows differentiation at species and

subspecies level (thus exceeding the resolution of rRNA analysis), but is unsuitable at higher phylogenetic levels. BCP has been shown to give specific hybridisation results in artificial mixtures of distantly related proteobacteria and to a certain extent in *S. typhimurium* strains (Lanoil and Giovannoni, 1997 and 2000).

In contrast to BCP, the polynucleotide transcript probes studied in this work target only a short fraction of plasmid or chromosomal DNA (probes ranging in size between 150 and 850nt have been tested). Specificity therefore does not rely on overall sequence similarity (in DNA:DNA reassociation the threshold between two species is drawn at 70% sequence similarity [Wayne et al., 1987]), but rather on sequence differences in a single gene or part of a gene.

The detection of single genes *in situ* has been addressed with PI-PCR (prokaryotic *in situ* PCR, Hodson et al., 1995), a prokaryotic analogue to the well established eukaryotic PRINS technique (primed *in situ* labelling, Koch et al. 1989). However, this method is impaired by the difficult balance of sufficiently permeabilising the cells to allow the uptake of primers and enzyme and at the same time preventing efflux of the PCR product.

While the permeabilisation of the cell envelope is also a concern in DNA targeted polynucleotide FISH, the problem of probe efflux has never been encountered with this technique.

To differentiate this new variation of fluorescence *in situ* hybridisation (FISH) from conventional FISH based on rRNA targeted oligonucleotide probes, a new term should be introduced. The term **RING-FISH** (*r*ecognition of *in*dividual *g*enes) is suggested. RING-FISH is defined as the use of polynucleotide transcript probes in fluorescence *in situ* hybridisation for detection of plasmid or chromosomal DNA and characterised by a ring-shaped fluorescence signal.

Crucial steps in the development of a hybridisation protocol for RING-FISH were the significant increase of hybridisation time (for BCP incubation periods of 48 hours are reported, suggesting a similar time frame for RING-FISH), probe concentration (as implied by Baumann et al. (1988), who noted an increased signal intensity with higher probe concentrations) and a decrease in stringency (with regard to the lower thermal stability of DNA:RNA hybrids as compared to RNA:RNA hybrids of rRNA targeted FISH).

While for rRNA targeted polynucleotide probes a strong signal can be observed after 4-5h hybridisation, 18-24h are necessary for plasmid and 24-30h for chromosomal DNA targeted probes. However, an extension of the incubation period beyond 30h has been found to result

in a decrease of signal intensity with a concomitant increase of unspecific signals, which might be due to a gradual degradation of the RNA probe and the subsequent unspecific binding of the shorter fragments.

In general the signal intensity with low copy numbers targets such as plasmids and chromosomal DNA is markedly weaker than with rRNA targeted probes. A positive hybridisation signal could only be observed after a significant increase of probe concentration. For rRNA targeted polynucleotide probes 250ng (5-8 times as much as for oligonucleotide-FISH, where 30-50ng are used, although with respect to the molecular weight the absolute number of probe molecules is approximately equivalent) result in a clearly visible signal. RING-FISH, on the other hand, requires 3-5µg of probe, a 100 fold increase in comparison to oligonucleotide probes. The percentage of probes involved in generating the hybridisation signal is unknown, but it has to be assumed, that the number of probe molecules contributing to the positive hybridisation signal greatly exceeds the number of available target molecules, since 10 probe molecules binding to 10 target molecules (i.e. genomic DNA) would presumably not yield a fluorescence signal strong enough to be observed with an epifluorescence microscope.

Table D.1 summarises the characteristic hybridisation conditions for poly- and oligonulceotide probes.

Probe type	**Probe target**	**Probe length (nt)**	**Copy number of target**	**Formamide in buffer (%)**	**Hybridisation period**	**Amount of probe**
oligo	rRNA	18-24	10^4-10^5	10-60	1.5-2h	0.03-0.05µg
poly	rRNA	~250	10^4-10^5	80-95	4-5h	0.25-3µg
poly	plasmid	200-850	10^2-10^3	5-20	18-24h	3-5µg
poly	genomic DNA	150-800	$<10^1$	5-15	24-30h	3-5µg

Tab. D.1. Summary of characteristic parameters for *in situ* hybridisation with poly- and oligonucleotide probes

The hybridisation signal usually has the familiar shape of a halo, although fluorescence throughout the cell has also been observed. Occasionally some cells show a very bright fluorescence signal. These cells appear brighter in phase contrast and often seem to have "burst" (Fig. D.1.).

A possible explanation may be a breakdown of cell integrity due to the long hybridisation period with a subsequent enhanced uptake of probe and an intracellular formation of a network.

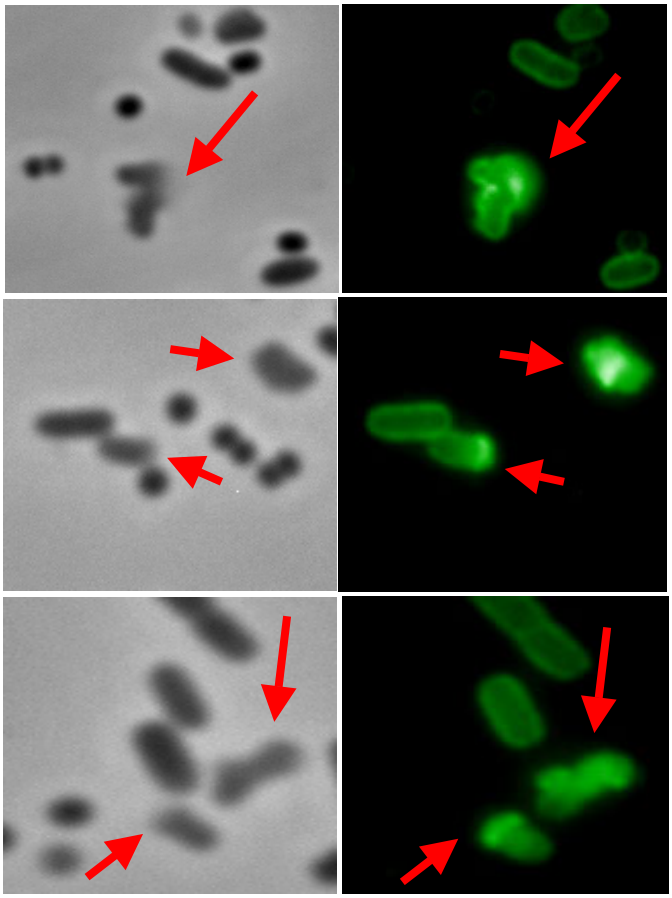


Fig. D.1. Cells that appear to have “burst” in the course of the hybridisation

As a control experiment to verify the binding of the probe to DNA instead of mRNA, hybridisations with a sense probe were performed. The positive results with this probe confirmed that the probe binds to DNA, since the sense probe, being identical to the mRNA, can only bind to the DNA.

Furthermore, hybridisations with probes targeting the human and mouse GAPDH showed a concentration of the fluorescence in the nuclear region.

Findings that are in concordance with the idea of polynucleotide probes binding to genomic DNA can also be found in literature, although they were occasionally interpreted in a different way:

Bauman et al. (1988) reported a strong cellwide fluorescence signal when using a polynucleotide probe targeting the 28S rRNA of murine leukemia cells, but a weaker signal restricted to the nuclear region with the sense probe. An RNase pretreatment of the cells also led to a signal concentrated in the nuclear region. The authors interpreted these findings as unspecific binding of the sense probe due to inverted repeats in the rRNA sequence.

In bacterial cells positive results with polynucleotide sense probes were described by Zimmermann (2002).

Specificity of the polynucleotide probes was tested using artificial mixtures of morphologically distinguishable species, i.e. *E. coli* (member of the gamma proteobacteria, rod-shaped) and *N. canis* (beta proteobacteria, coccoid) for the *E. coli* GAPDH probes and *Xanthomonas campestris* (gamma proteobacteria, rod-shaped) and *N. canis* for the *Xanthomonas* probes. Under these conditions the probes GAP-E, GAP-F, X-ca, X-ra and X-lo (B.4.) showed specific signals.

Of the ten probes designed for the *E. coli* GAPDH gene, ranging in length between 48nt (GAP-A) and 795nt (GAP-J), only the probes with an intermediate length GAP-E and GAP-F (258 resp. 338nt) were satisfyingly specific, while the shorter probes yielded unspecific signals. In hybridisations with longer probes (>400nt) the percentage of target cells detected with these probes decreased rapidly, possibly indicating insufficient cell wall permeability for these large molecules.

The *Xanthomonas campestris* probes were derived from PCR products generated with pathovar specific primers. However, while the *primers* were highly specific for their respective pathovar, the *polynucleotide probes* also bound to other pathovars. This may be explained by the binding of the primers to highly variable regions of the target genes (pathogenicity factors, that are common not only among *Xanthomonas* species), whereas the probe may also comprise more conserved regions. Elucidation of the sequence variability and fine tuning of the hybridisation paramters might eventually lead to more specific signals.

The results with DNA targeted polynucleotides presented in this work have to be regarded as introductory. Future work in this field should lead to an optimisation of the reaction parameters.

It is assumed that the positive hybridisation signal is due to the formation of a network of probes (cf. D.3.). However, with a very low copy number of target molecules the nascent network has only few anchors inside the cell and is therefore very susceptible to disturbing factors, such as slight variations in formamide/salt concentrations due to pipette inaccuracies, partially degraded probe, insufficient denaturation of probe, etc., which is reflected in a high variation of signal intensity and specificity during several experiments.

At this stage, possible quantifications of cells based on DNA targeted polynucleotide probes have to be regarded with care, since 1) probably not all target cells in a sample are accessible for the probe and 2) the accessibility of the target sequence might vary depending on the growth state of the cell. In this context, it should be noted, that DAPI stains of bacterial DNA have been shown to vary greatly at different growth states (Ross et al., 1996), reflecting different copy numbers of the bacterial chromosome and the conformation of the nucleic acid (supercoiled or open circular), and thus resulting in different accessibility of the target sequence. This effect is possibly even more pronounced for DNA targeted polynucleotide probes and might still be increased by slight variations in the hybridisation conditions.

Fig. D.2 illustrates these findings, showing the results of three independent hybridisations with plasmid targeted probe betaLact (FLUOS-labelled, green). The three experiments were carried out on different days using the same batch of PFA fixed cells under identical hybridisation conditions. In all three instances the hybridisation signal was highly specific, but the percentages of detected target cells varied greatly. Probes that failed to take up the probe were highlighted by hand (red).

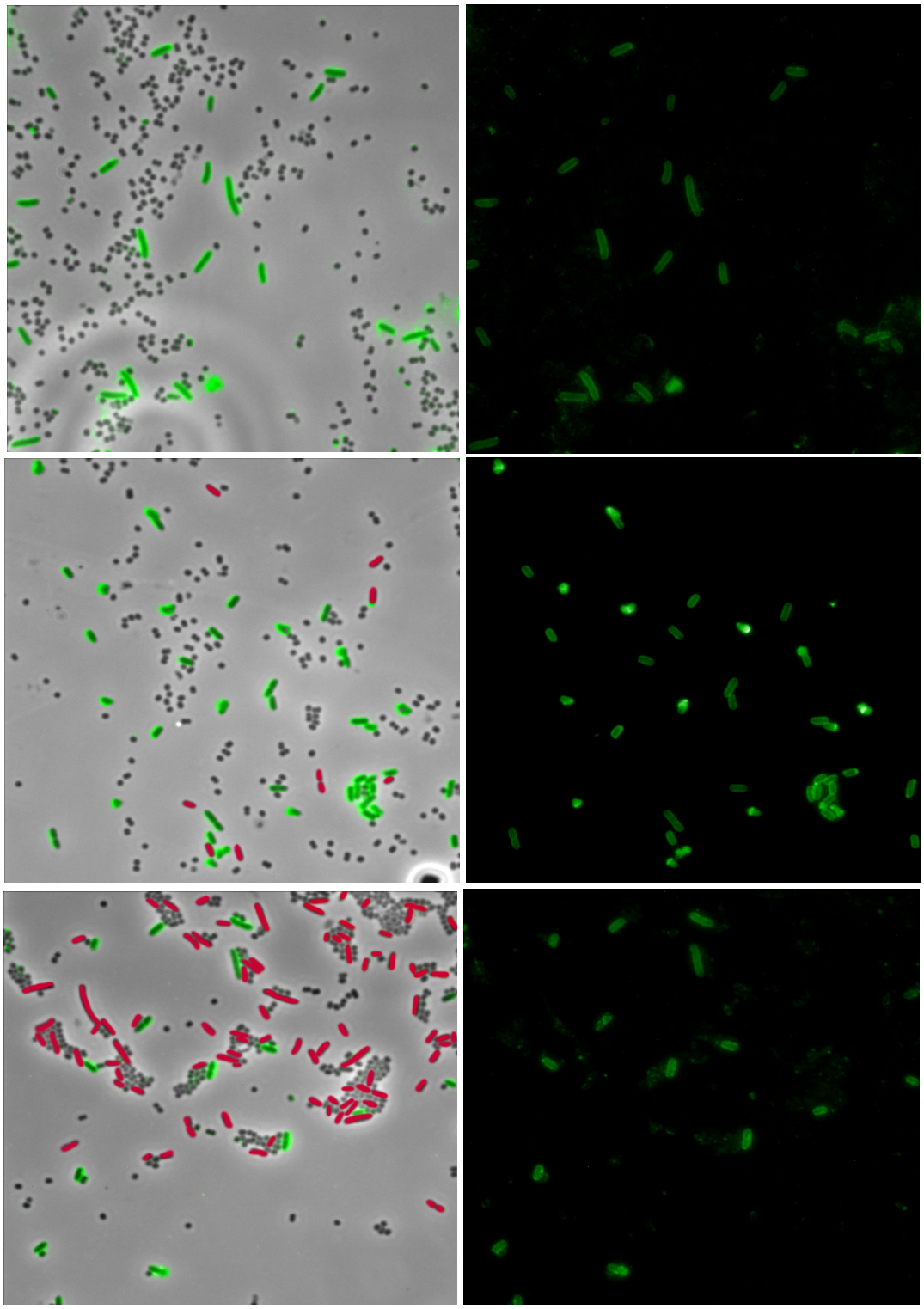


Fig. D.2. Three independent hybridisations of a mixture of *E. coli-pBBR1MCS4* and *N.canis* with plasmid targeted probe betaLact showing great differences in the pecentage of target cells detected with the probe. Comparison of epifluorecence (right) and phase contrast overlaid with epifluorescence image (left) shows the probe (labelled with FLUOS [green]) binding specifically only to *E. coli* cells, but failing to detect all target cells. *E. coli* cells showing no hybridisation signal were highlighted (manually) in red.

D.3. Network hypothesis

A characteristic feature of hybridisations with polynucleotide probes is the halo-shaped fluorescence signal, which has never been observed with oligonucleotide probes. Earlier publications (Trebesius et al., 1994, Stoffels et al., 1999) explained this phenomenon with the probes, being too long and complex to enter the cell completely, stretching through the cell envelope and binding to ribosomes that are located in the vicinity of the cell envelope (compare Fig. D.3)

This theory implies, that the probes bind only partially to their target sequence, while a large part of the probe stays outside the cell. The observation, that the halo has a slightly larger circumference than the cell itself (cf. Fig. C.17. and C.28.), seems to support this idea, as do the positive results with cell sorting (cf. C.5), which relies solely on the assumption, that part of the probe is located on the outside of the cell.

On the other hand, according to this model only very few target molecules (only those close to the cell envelope) are available for hybridisation. With this dramatic reduction of available target molecules the superiority of polynucleotide over oligonucleotide probes in the detection of cells with low ribosome content (Pernthaler et al., 2002) seems implausible.

Another point refuting the above model is the spatial extension of the polynucleotide probes in relation to the thickness of the bacterial or yeast cell wall. A 200nt probe, measuring about 0.34nm per nucleotide, would have a physical length of ca. 68nm, provided that it is completely stretched and without any secondary structures, which cannot be assumed under hybridisation conditions. A 50nt probe, such as probe 1024-1, which has been shown to give a halo signal (cf. C.2.1.2.), would have a length of just 17nm. In comparison to that, the thickness of the cell wall of *E. coli* is ca. 10nm, whereas in the yeast *S. cerevisiae* it is ca. 160nm (cf. Tab. D.2.). A halo for gram-negative bacteria hybridised with a 200nt probe might therefore seem feasible with the original theory, but the positive results with very short probes and yeasts cannot be explained.

Organism	**Thickness of cell wall**
Escherichia coli	10nm
Staphylococcus aureus	20nm
Saccharomyces cerevisiae	160nm

Table D.2. Thickness of cell walls (Salton, 1960)

To explain these findings a new model had to be devised, which was termed "network hypothesis" and is schematically depicted in Fig. D.5.
Ribosomal RNA is known to form secondary structures, consisting of stretches of intramolecular base pairing interrupted by unpaired regions, thus forming a complex structure of loops and helices (cf. Fig. D.5), which has important implications for the functionality of the ribosome. rRNA targeted polynucleotide probes comprise part of the rRNA sequence and – consisting of single stranded RNA – can therefore also be expected to have the ability to form similar secondary structures.
The first step in a polynucleotide *in situ* hybridisation is a denaturation, which should dissolve existing secondary structures of the probe. The conditions during the following hybridisation would allow renaturation of complementary regions within a probe molecule. But, considering the large number of identical probe molecules in a hybridisation, it is also conceivable, that complementary regions not within one, but between several probe molecules anneal, thereby forming a network of probes, which is connected to the target rRNA via one partially bound probe. This network is initiated inside the cell at the target site, but due to the restricted permeability of the cell envelope, only few probes can enter the cell. A chain of probes stretching through the cell envelope connects the specifically recognised intracellular target to probe networks formed outside the cell, resulting in a ring of probes around a target cell, which appears as the characteristic halo under the microscope. Due to the large excess of probe, networks will form outside the cells, but unless they are connected to an anchor of target sequence inside the cell, they will be washed away during later washing steps, ensuring specific detection of the target cells.

This hypothesis is supported by a number of findings:
1. Shortening or completely skipping the denaturation step leads to poor or nonexistent signals.

2. The diameter of the halo is slightly larger than the cell, indicating an accumulation of probe molecules outside the cell.

3. The positive results with cell sorting in DNA coated microplates (cf. C.5.) indicate the presence of probes outside the cell.

(The previous two observations could also be explained with the model proposed by Trebesius et al.)

4. The halo signal with probes of 50nt clearly contradicts Trebesius´model.

5. The possible formation of a secondary structure could be shown *in silico* for all probes used in this work (rRNA, plasmid and DNA targeted) (see Appendix for individual structures).

6. A melting profile of several polynucleotide probes in comparison to that of an oligonucleotide and genomic DNA indicated the formation of a secondary structure *in vitro* (Fig. D.6).
This experiment was done in analogy to GC determination by thermic denaturation (Marmur and Doty, 1962). In short, double stranded nucleic acids have a lower absorbance of light at 260nm than single stranded nucleic acids. Thus the melting of a double strand can be monitored with continuous absorbance measurement while increasing the temperature. The melting temperature T_m allows conclusions about the base composition, since G-C base pairs are thermically more stable than A-T base pairs. In this experiment, not the value of T_m, but only the change of absorbance at 260nm under denaturing conditions was of interest. With genomic DNA a characteristic melting curve as shown in Fig. D.6-a can be observed. Performing the same temperature gradient with several polynucleotide probes yielded similarly shaped graphs (Fig. D.6-b-d), suggesting the denaturation of double strands, whereas the oligonucleotide (Fig. D.6-.e) showed no increase in absorbance at 260nm.

7. The artificial probes Eco-5X, Eco-cX and Eco-rcX, which develop forced secondary structures with different degrees of stability, behave according to the network model, with very weak signals for probe Eco-5X up to strong, halo-shaped signals for probe Eco-rcX (cf. Fig. C.22.).

8. Oligonucleotide probes exhibit a rather weak fluorescence signal in cells with a low ribosome content (Amann et al., 1995). Cell fixation at different time points of bacterial growth (representing different metabolic states and therefore different ribosome contents) and subsequent hybridisation with oligo- and polynucleotide probes showed a sharp decrease of signal intensity at higher cell densities for oligonucleotide probes, but no significant change for polynucleotide probes (Fig. D.4.). Assuming a network as the origin of the halo signal, a

10-fold reduction in target molecules would not lead to a decrease of signal intensity, since the number of probe molecules contributing to the signal is not equivalent to the number of target molecules.
Analogous to cells with different ribosome contents, experiments with the plasmid targeted probe betaLact, applied to plasmids ranging in copy number between $1x10^1$ and $5x10^2$ (C.2.2.) showed no significant change in signal intensity.

9. With low copy number targets such as plasmid and especially genomic DNA, the number of probe molecules involved in signal formation has to exceed the number of target molecules for a signal to be detectable.

10. Unlike oligonucleotide probes, where an increase of probe concentration does not necessarily increase signal intensity, there is a strong correlation of probe concentration and signal intensity in hybridisations with polynucleotide probes, especially for low copy targets.
Allowing the formation of a network requires a large excess of probe, with concentrations 100 times higher than in oligo-FISH. Reducing the amount of polynucleotide probe results in a prompt decrease of signal intensity (cf. C.4.2.3.).

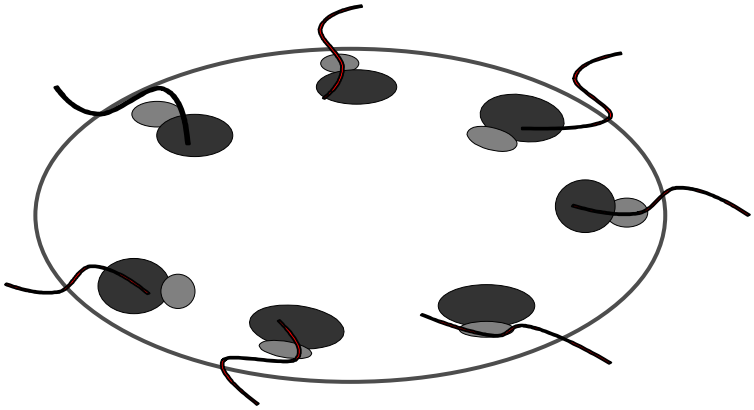


Fig. D.3. Model for the origin of the halo signal of polynucleotide probes according to Trebesius et al, 1994 and Stoffels et al., 1999. Due to their size, the probes cannot enter the cells completely and therefore stretch through the cell envelope with part of the probe remaining outside the cell.

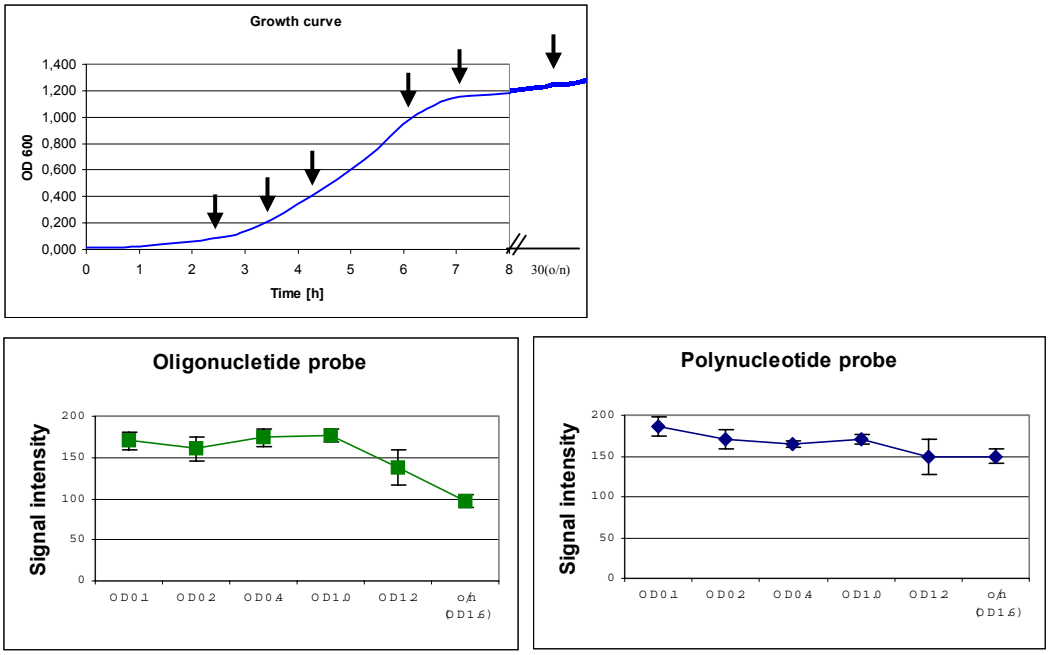
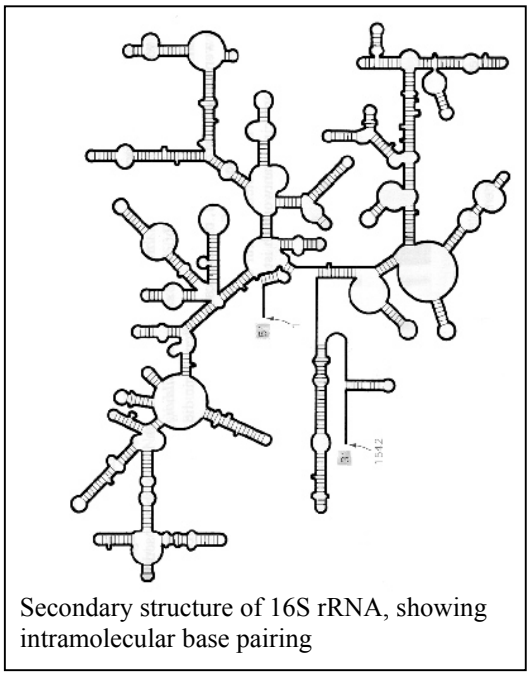
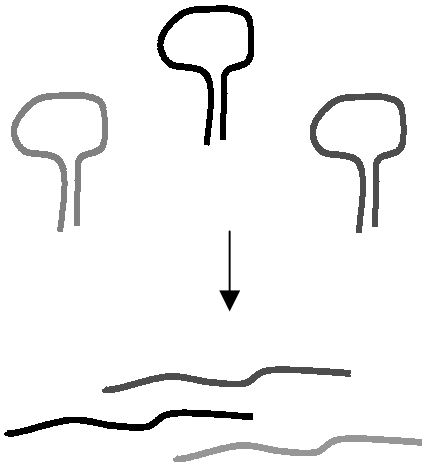


Fig.D.4. Cell fixation at different time points of bacterial growth, reflecting different ribosome content (upper graph) and hybridisation with oligo- and polynucleotide probes shows a sharp decrease of signal intensity for oligonucleotide probes, but almost constant signal intensities for polynucleotide probes at increasing cell densities.

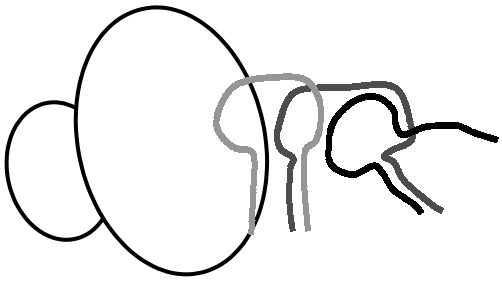


Secondary structure of 16S rRNA, showing intramolecular base pairing

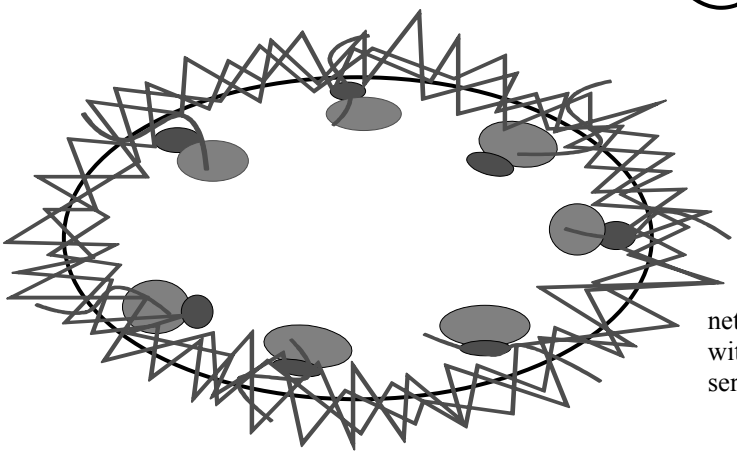


polynucleotide probes with simplified secondary structure

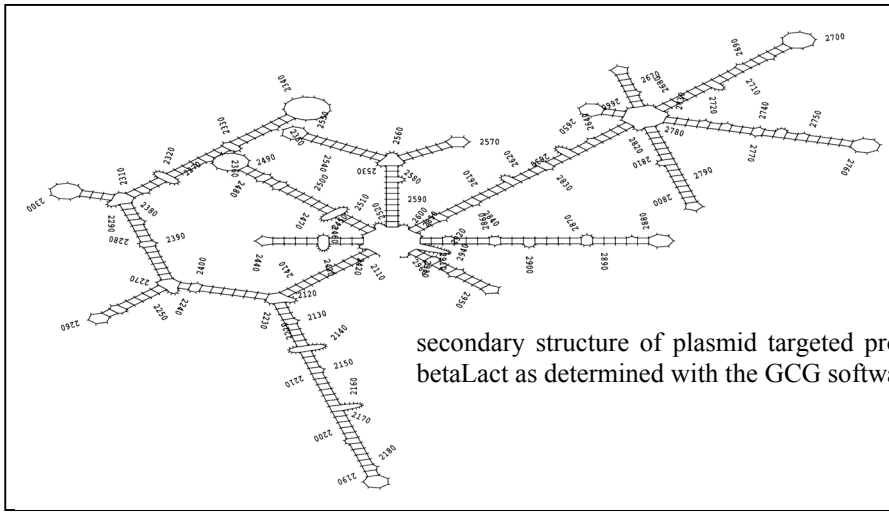
denaturation step in hybridisation protocol dissolves secondary structures



renaturation of probes during the hybridisation; part of a probe molecule binds specifically to target region in ribosome, while the unbound part interacts with other probe molecules to form a network



network of probes around a cell with probes bound to the ribosomes serving as anchors



secondary structure of plasmid targeted probe betaLact as determined with the GCG software

Fig. D.5. Schematic illustration of the network hypothesis

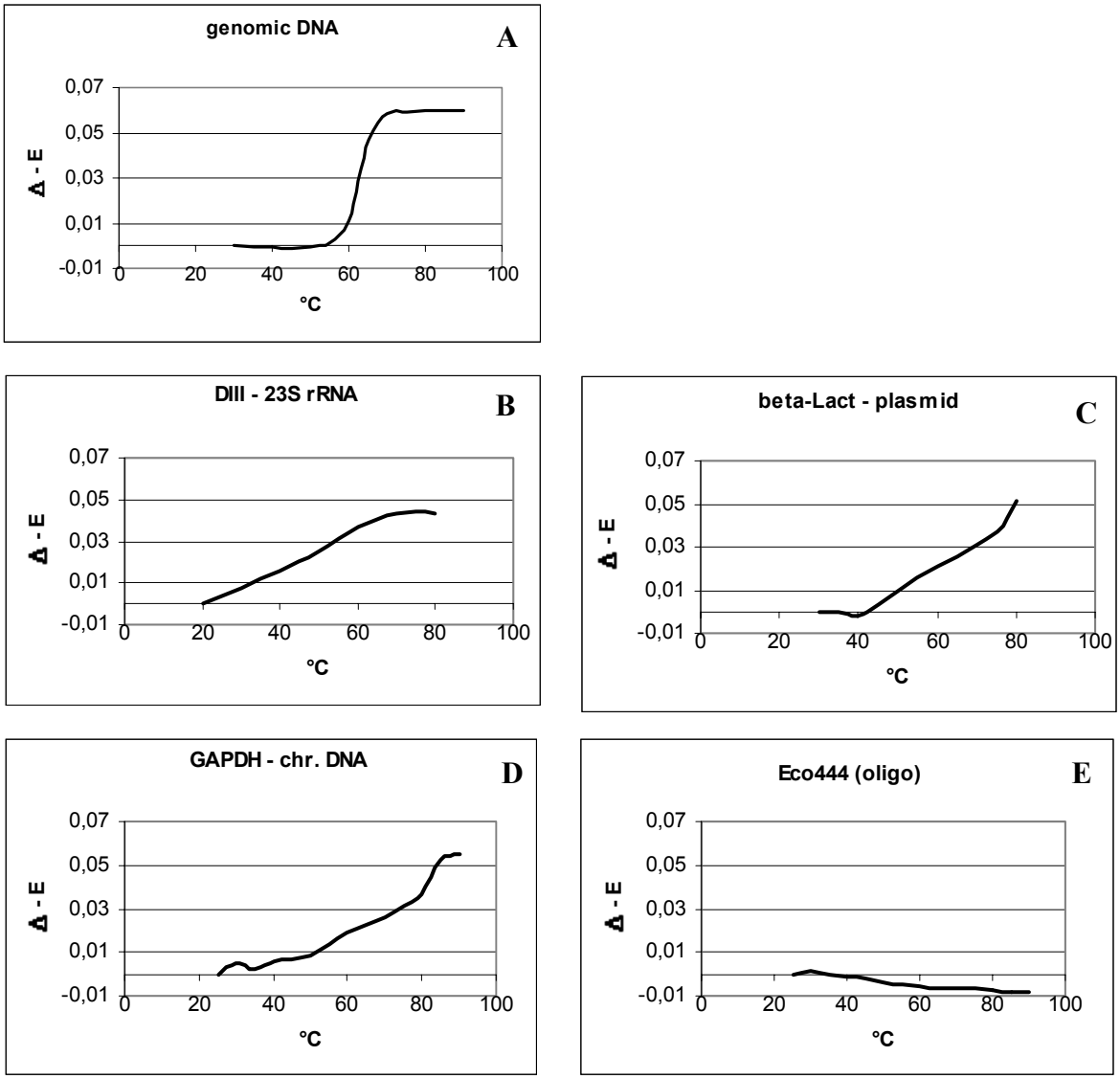


Fig. D.6-A-E. Melting profile of several polynucleotide transcript probes compared to genomic DNA and an oligonucleotide probe.

D.4. Probe design

The concept of a network offers a wide range of possibilities for probe design.

A drawback of the early polynucleotide probes with a length of 200-250nt is their restricted specificity due to the presence of highly conserved regions scattered throughout the rRNA.

As shown with probe 1024 (cf. C.2.1.2.), a probe length of less than 100nt can be sufficient to result in a halo signal (which is desired in the context of cell sorting, since it indicates the presence of probes or parts of probes on the outside the cell), provided that the probe sequence permits the formation of secondary structures. Artificial probes, consisting of a

chain of several oligonucleotides (oligo-oligomer, e.g. probe EcoX [C.2.1.3.]) or of an oligonucleotide connected to an arbitrary tail sequence (oligo-tail probes, K. Fichtl, unpublished), designed to be able to form secondary structures, are conceivable. In the field of rRNA targeted probes, this approach would combine the advantages of oligonucleotides (i.e. high specificity and the possibility to create probes for different phylogenetic levels) and polynucleotides (high signal intensity independent of ribosome content and the possibility for cell sorting).

In the context of cell sorting in DNA-coated microplates, oligo-tail probes might be useful with a universal tail, connected to different oligonucleotides, allowing the simultaneous capture of different species (identified via the specific oligonucleotide) in one microplate, coated with the universal tail.

D.5. Possible applications

With the feasibility to detect individual genes *in situ*, this new technique offers a wide variety of applications.

- Using DNA targeted polynucleotide probes in combination with rRNA targeted olignucleotide probes, detection of specific genes in environmental or clinical samples, e.g. pathogenicity factors, antibiotic resistance, etc., with simultaneous phylogenetic identification of the organisms carrying these genes is possible.
- The metabolic and genetic potential of uncultured members of bacterial communities can be analysed using conserved structural genes and operons as probe targets (e.g. nitrogen fixation, bacterial photosynthesis, carbon fixation, etc.).

In contrast to FISH-MAR (a combination of fluorescence *in situ* hybridisation and microautoradiography, which allows monitoring the uptake of radioactively labelled substrate plus identification of the respective organisms via FISH [Ouverney et al., 1999, Lee et al., 1999]), RING-FISH can detect the presence of certain genes, irrespective of whether they are metabolically active or inactive under the given environmental conditions.

- Cases of horizontal gene transfer could be disclosed by detecting the respective gene and simultaneously identifying the organism harbouring it.

Part II – Cell sorting with polynucleotide probes

D.6. Comparison of cell sorting methods

Based on the insights gained from analysing the nature of polynucleotide probes as described in the previous chapters, is was possible to modify and improve a method for cell sorting developed by Stoffels et al. (1999).

While the method described by Stoffels (cf. chapter C.3, method A) proved very efficient in artificial samples it is nevertheless rather tedious, requiring a lot of hands-on time and - being based on the use of columns in a magnetic field - is unsuitable for the simultaneous processing of a larger number of samples. Another drawback is the high amount of cells lost in the column (up to 90%), which might be problematic when an enrichment of cells, that are rather underrepresented in a sample, is to be achieved.

By transferring the cell sorting process to streptavidin-coated microplates (cf. C.3, method B), the problem of cell loss and the hands-on time could be reduced, while at the same time offering the possibility for automation of the procedure.

A constraint common to both methods A and B, is the necessity to label the polynucleotide probes with biotin, since in both cases the eventual cell separation relies on the binding of streptavidin to biotin. This involves the risks of unsatisfying cell sorting results due to insufficient labelling or the unspecific binding of streptavidin to biotin occuring naturally in the sample. Using DNA coated microplates for the cell sorting (cf. C.3., method C) abrogates this problem, since in this case the separation of cells is completely independent of probe labelling. For detection of the cells after the cell sorting (either for microscope analysis or in the microplate) probes can be labelled with biotin or other substances if desired. Quantification is done with oligonucleotide probes.

The concept of DNA coated microplates also allows the simultaneous hybridisation with different probes and the subsequent cell separation in several microplates coated with DNA corresponding to the different probe sequences.

Rendering the labelling of probes unnecessary greatly reduces the costs of the method, since the labelled UTP, which is incorporated during the *in vitro* transcription of the probe, is generally the most cost intensive part of the procedure.

Cell sorting in microplates presents a low-cost alternative to flow cytometry based methods (e.g. Wallner et al., 1995), requiring only standard equipment (PCR cycler, oven).

D.7. Cell sorting in DNA-coated microplates

Cell sorting in DNA coated microplates constitutes a combination of several established methods, i.e.

- the concept of polynucleotide probe based cell sorting as described by Stoffels et al. (1999), with part of the probe remaining outside the cell
- the coating of microplates with DNA and subsequent hybridisation in the microplate (Ezaki et al., 1989)
- "panning" (Wysocki et al, 1978), a cell sorting technique developed for lymphocytes, which relies on antibodies binding to cell surface antigens and capture of the thus labelled cells in microplates coated with antibodies directed against the Fc region of the primary antibodies.

Combining these assays required evaluation and optimisation of different reaction parameters.

D.7.1. Cell fixation

Permeabilising the cell envelope through cell fixation is a prerequisite for the cell sorting, since only cells accessible for the probe, can be addressed in the cell sorting process.

Different approaches for cell fixation and postfixational treatment of cells were described and discussed in chapters C.1.2./C.1.3. and D.1. An over-fixation of the cells has to be avoided since it might lead to the disruption of the cell structure. The appearance of a halo signal is regarded as the optimal balance of cell wall permeability and cell integrity. However, in an inhomogenous sample containing both gram-positive and gram-negative organisms, this balance is difficult to achieve.

D.7.2. Hybridisation parameters

The parameters most vital for the cell sorting process were found to be the stringency of the two hybridisation steps and the amount of probe in relation to the amount of target cells.

The cell sorting in DNA coated microplates relies on two hybridisation steps, which differ greatly in their stringency. While the first hybridisation requires rather stringent conditions to ensure specific binding of the probes, the second hybridisation has to be carried out under more relaxed conditions to allow all labelled cells to bind to the microplate.

Polynucleotide probes have to be employed in large excess to allow the formation of a network (cf. D.4). This has to be taken into account, if the target cells constitute a high percentage of the sample.

D.7.3.. Cell sorting efficiency

The efficiency of the cell sorting process greatly depends on the amount of target cells in the sample (cf. Fig. C.46). This could be attributed to the saturation of the surface of the microplate or a too weak binding to the plate due to an incomplete network of probes around the target cells if too many cells compete for a limited amount of probe.

But even under optimal conditions, a 100% cell sorting cannot be achieved. This is mostly due to the fact, that not all cells are able to take up the probe, a problem more pronounced when using DNA targeted probes (where the target accessibility might be reduced when the DNA is in supercoiled state during the stationary phase of the cell), than with rRNA targeted ones. Microscopic analysis of the supernatant recovered from the microplate (which should be depleted in target cells) after cell sorting showed no cells with halos, indicating either the successful immobilisation of all target cells that had taken up the probe in the microplate or the degradation of the polynucleotide probe.

D.8. Environmental samples

Experiments with activated sludge from a waste water treament plant proved the general applicability of the method for complex environmental samples. A significant, but not 100% enrichment, respectively depletion of target cells could be observed. However, exact quantification of the cell sorting efficiency was not feasible, since comprehensive staining of the cells with oligonucleotide probes (necessary for the LSM –based quantification) after the cell sorting was not possible. The cause may lie in a partial degradation of the rRNA in the course of the two hybridisation steps (1. binding of the probe to the target cells and 2. cell sorting). Weak fluorescence signals of the oligonucleotide probes (as compared to conventional oligonucleotide-FISH with samples that had not undergone cell sorting) have also been observed after cell sorting with artificial cell mixtures, but since in those cases cells harvested and fixed during the log-phase (and thus with a high ribosome content) were used, quantification was still possible.

Nevertheless the DNA of the cells is still intact and available for analysis as was confirmed by the positive PCR results.

D.9. Possible applications

Cell sorting in microplates could prove useful whenever enrichment or depletion of cells is desired but no FACS (fluorescence activated cell sorter) or similar expensive equipment is available.

It is not even necessary for the cell sorting to know the sequence of the polynucleotide probe. Using a library of rDNA clones, derived from an environmental sample, it is possible to generate probes by using universal primers binding to conserved regions of the rDNA but flanking a highly variable region. The resulting probe will therefore be specific only for the organism whose DNA was used. In the context of the cyclic rRNA approach to study the phylogenetic composition of environmental samples, as postulated by Amann et al. (1995), this cell sorting technique might prove useful to enrich rare species in a sample, which might otherwise elude the attention and make them available for further analysis.

Using DNA probes targeting conserved regions of a gene and performing the hybridisation under relaxed conditions it could be possible to identify new members of a gene family and at the same time isolate the organisms carrying them.

In screening projects with environmental samples it can serve to either enrich a group of species to facilitate further analysis, or to remove a (possibly dominating) population, that is of no interest in the given context, thereby – if a screening project can be compared to the search for the famous needle in the haystack – reducing the size of the haystack.

E. Summary

The work presented here deals with *in situ* hybridisation techniques using polynucleotide probes and applications thereof.

Fluorescence *in situ* hybridisation (FISH) using rRNA targeted oligonucleotide probes is a standard method for identification of microorganisms in environmental samples. A few years ago polynucleotide probes (generated via *in vitro* transcription) were introduced, which are superior to oligonucleotide probes regarding their signal intensity, thus allowing the detection of organisms with a low number of ribosomes. One characteristic feature of hybridisations with polynucleotide probes is the so called "halo", i.e. a concentration of the fluorescence signal in the cell periphery.

In the first stage of this work several parameters of the hybridisation with polynucleotide probes, such as cell fixation, stringency, length and secondary structure of the probes were explored and optimised. Furthermore the method was applied to several organisms differing in the structure of their cell envelope (i.e. gram-positive and gram-negative bacteria, yeasts and mammalian cell lines). With the insights gained from these analyses a hypothesis was postulated that explains the origin and nature of the characteristic halo signal and has important implications for future probe design. This "network hypothesis" was supported by various control experiments.

On the basis of this hypothesis it was possible to develop polynucleotide probes targeting plasmids and even chromosomal DNA – target molecules that have previously been thought to be absolutely unsuitable for detection with *in situ* hybridisation due to their low copy number. This modified FISH technique now for the first time permits the detection of individual genes in single cells *in situ* and has therefore been termed RING-FISH (recognition of individual genes). In combination with classic rRNA targeted oligonucleotides this method offers a wide range of possible applications, including the analysis of metabolic activity of certain species within a bacterial community or the detection of horizontal gene transfer.

In the second stage of this work an existing method for cell sorting, which is based on polynucleotide probe mediated immobilisation of cells, was advanced and optimised. The method was tested with rRNA as well as plasmid and DNA targeted probes and might prove useful in the isolation of new members of a gene family if conserved domains of a gene are used as probe target.

Zusammenfassung

Die vorliegende Arbeit befaßt sich mit *in situ* Hybridisierungstechniken für fluoreszenz-markierte Polynukleotidsonden und deren mögliche Anwendungen.

Fluoreszenz-in-situ-Hybridisierung (FISH) mit rRNA gerichteten Oligonukleotidsonden ist eine bewährte Methode zur Identifizierung von Mikroorganismen in Umweltproben. Vor einigen Jahren wurden erstmals auch (durch *in vitro* Transkription generierte) Polynukleotid-sonden verwendet, die den Oligonukleotisonden in punkto Signalintensität überlegen sind und somit auch eine Detektion von Organismen mit niedriger Ribosomenzahl gestatten. Ein charakteristisches Merkmal von Hybridisierungen mit Polynukleotidsonden ist die Ausbil-dung eines sogenannten "Halos", d.h. eine Konzentration des Fluoreszenzsignals in der Zellperipherie.

Im Rahmen dieser Arbeit wurden zunächst verschiedene Parameter der Hybridisierung mit Polynukleotidesonden, wie Zellfixierung, Stringenz der Hybridisierung, sowie Länge und Sekundärstruktur der Sonden untersucht und optimiert und die Methode mit einigen Organis-men, die sich im Aufbau ihrer Zellhülle stark unterscheiden (grampositive und -negative Bakterien, Hefen, Säugetierzellen), getestet. Anhand dieser Erkenntnisse wurde eine Hypo-these formuliert, die die Ausbildung des charakeristischen Halos erklärt und weitreichende Implikationen für die weitere Entwicklung von Sonden hat. Diese "Netzwerk"-Hypothese konnte durch verschiedene Kontrollexperimente unterstützt werden.
Basierend auf dieser Hypothese war es möglich, Polynukleotidsonden zu entwickeln, die gegen Plasmide und sogar chromosomale DNA gerichtet sind, Zielmoleküle, die bisher auf-grund ihrer niedrigen Kopienzahl als gänzlich ungeeignet für eine Detektion mittels *in situ* Hybridisierung galten. Diese modifizierte FISH-Methode gestattet nun erstmals den *in situ* Nachweis eines spezifischen Gens in einer einzelnen Zelle und wird deshalb als RING-FISH (recognition of individual genes) bezeichnet. In Kombination mit klassischen rRNA gerichteten Oligonukleotidsonden bietet die Methode ein breites Anwendungsspektrum, wie z.B. die Aufklärung der Stoffwechselfunktion einzelner Spezies in einer komplexen Bakterienpopulation oder den Nachweis von horizontalem Gentransfer.

Im zweiten Teil dieser Arbeit wurde eine bereits bestehende Methode zur Zellsortierung, die sich auf eine durch Polynukleotidsonden vermittelte Immobilisierung von Zellen stützt, weiterentwickelt und optimiert. Die Methode wurde, zusätzlich zu rRNA- , auch mit Plasmid- und DNA-gerichteten Sonden getestet und könnte, bei Verwendung von konservierten Domänen als Sondenziel, Anwendung finden bei der Isolierung neuer Mitglieder einer Genfamilie

F. Appendix

F.1. Sequence of plasmid PCR2.1TOPO

AGCGCCCAATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCATTAATGCAGCTGGCACGACAGGTTTC
CCGACTGGAAAGCGGGCAGTGAGCGCAACGCAATTAATGTGAGTTAGCTCACTCATTAGGCACCCCAGGCTT
TACACTTTATGCTTCCGGCTCGTATGTTGTGTGGAATTGTGAGCGGATAACAATTTCACA**CAGGAAACAGCT**
ATGACCATGATTACGCCAAGCTTGGTACCGAGCTCGGATCCACTAGTAACGGCCGCCAGTGTGCTGGAATTC
GCCCTTAAGGGCGAATTCTGCAGATATCCATCACACTGGCGGCCGCTCGAGCATGCATCTAGAGGGCCCAAT
TCGCCCTATAGTGAGTCGTATTACAATTCA**CTGGCCGTCGTTTTAC**AACGTCGTGACTGGGAAAACCCTGGC
GTTACCCAACTTAATCGCCTTGCAGCACATCCCCCTTTCGCCAGCTGGCGTAATAGCGAAGAGGCCCGCACC
GATCGCCCTTCCCAACAGTTGCGCAGCCTGAATGGCGAATGGGACGCGCCCTGTAGCGGCGCATTAAGCGCG
GCGGGTGTGGTGGTTACGCGCAGCGTGACCGCTACACTTGCCAGCGCCCTAGCGCCCGCTCCTTTCGCTTTC
TTCCCTTCCTTTCTCGCCACGTTCGCCGGCTTTCCCCGTCAAGCTCTAAATCGGGGGCTCCCTTTAGGGTTC
CGATTTAGAGCTTTACGGCACCTCGACCGCAAAAAACTTGATTTGGGTGATGGTTCACGTAGTGGGCCATCG
CCCTGATAGACGGTTTTTCGCCCTTTGACGTTGGAGTCCACGTTCTTTAATAGTGGACTCTTGTTCCAAACT
GGAACAACACTCAACCCTATCGCGGTCTATTCTTTTGATTTATAAGGGATTTTGCCGATTTCGGCCTATTGG
TTAAAAAATGAGCTGATTTAACAAATTCAGGGCGCAAGGGCTGCTAAAGGAACCGGAACACGTAGAAAGCCA
GTCCGCAGAAACGGTGCTGACCCCGGATGAATGTCAGCTACTGGGCTATCTGGACAAGGGAAAACGCAAGCG
CAAAGAGAAAGCAGGTAGCTTGCAGTGGGCTTACATGGCGATAGCTAGACTGGGCGGTTTTATGGACAGCAA
GCGAACCGGAATTGCCAGCTGGGGCGCCCTCTGGTAAGGTTGGGAAGCCCTGCAAAGTAAACTGGATGGCTT
TCTTGCCGCCAAGGATCTGATGGCGCAGGGGATCAAGATCTGATCAAGAGACAGGATGAGGATCGTTTCGCA
TGATTGAACAAGATGGATTGCACGCAGGTTCTCCGGCCGCTTGGGTGGAGAGGCTATTCGGCTATGACTGGG
CACAACAGACAATCGGCTGCTCTGATGCCGCCGTGTTCCGGCTGTCAGCGCAGGGGCGCCCGGTTCTTTTTG
TCAAGACCGACCTGTCCGGTGCCCTGAATGAACTGCAGGACGAGGCAGCGCGGCTATCGTGGCTGGCCACGA
CGGGCGTTCCTTGCGCAGCTGTGCTCGACGTTGTCACTGAAGCGGGAAGGGACTGGCTGCTATTGGGCGAAG
TGCCGGGGCAGGATCTCCTGTCATCTCGCCTTGCTCCTGCCGAGAAAGTATCCATCATGGCTGATGCAATGC
GGCGGCTGCATACGCTTGATCCGGCTACCTGCCCATTCGACCACCAAGCGAAACATCGCATCGAGCGAGCAC
GTACTCGGATGGAAGCCGGTCTTGTCGATCAGGATGATCTGGACGAAGAGCATCAGGGGCTCGCGCCAGCCG
AACTGTTCGCCAGGCTCAAGGCGCGCATGCCCGACGGCGAGGATCTCGTCGTGATCCATGGCGATGCCTGCT
TGCCGAATATCATGGTGGAAAATGGCCGCTTTTCTGGATTCAACGACTGTGGCCGGCTGGGTGTGGCGGACC
GCTATCAGGACATAGCGTTGGATACCCGTGATATTGCTGAAGAGCTTGGCGGCGAATGGGCTGACCGCTTCC
TCGTGCTTTACGGTATCGCCGCTCCCGATTCGCAGCGCATCGCCTTCTATCGCCTTCTTGACGAGTTCTTCT
GAATTGAAAAAGGAAGAGTAT**GAGTATTCAACATTTCCG**TGTCGCCCTTATTCCCTTTTTTGCGGCATTTTG
CCTTCCTGTTTTTGCTCACCCAGAAACGCTGGTGAAAGTAAAAGATGCTGAAGATCAGTTGGGTGCACGAGT
GGGTTACATCGAACTGGATCTCAACAGCGGTAAGATCCTTGAGAGTTTTCGCCCCGAAGAACGTTTTCCAAT
GATGAGCACTTTTAAAGTTCTGCTATGTCATACACTATTATCCCGTATTGACGCCGGGCAAGAGCAACTCGG
TCGCCGGGCGCGGTATTCTCAGAATGACTTGGTTGAGTACTCACCAGTCACAGAAAAGCATCTTACGGATGG
CATGACAGTAAGAGAATTATGCAGTGCTGCCATAACCATGAGTGATAACACTGCGGCCAACTTACTTCTGAC
AACGATCGGAGGACCGAAGGAGCTAACCGCTTTTTTGCACAACATGGGGGATCATGTAACTCGCCTTGATCG
TTGGGAACCGGAGCTGAATGAAGCCATACCAAACGACGAGAGTGACACCACGATGCCTGTAGCAATGCCAAC
AACGTTGCGCAAACTATTAACTGGCGAACTACTTACTCTAGCTTCCCGGCAACAATTAATAGACTGGATGGA
GGCGGATAAAGTTGCAGGACCACTTCTGCGCTCGGCCCTTCCGGCTGGCTGGTTTATTGCTGATAAATCTGG
AGCCGGTGAGCGTGGGTCTCGCGGTATCATTGCAGCACTGGGGCCAGATGGTAAGCCCTCCCGTATCGTAGT
TATCTACACGACGGGGAGTCAGGCAACTATGGATGAACGAAATAGACAGATCGCTGAGATAGGTGCCT**CACT**
GATTAAGCATTGGTAACTGTCAGACCAAGTTTACTCATATATACTTTAGATTGATTTAAAACTTCATTTTTA
ATTTAAAAGGATCTAGGTGAAGATCCTTTTTGATAATCTCATGACCAAAATCCCTTAACGTGAGTTTTCGTT

Probe Eco2.1 targeting the multiple cloning site; primers M13-V/M13-R

Probe betaLact targeting the beta lactamase gene; primers betaLact-V/ betaLact-R

CCACTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAGATCCTTTTTTTCTGCGCGTAATCTG
CTGCTTGCAAACAAAAAAACCACCGCTACCAGCGGTGGTTTGTTTGCCGGATCAAGAGCTACCAACTCTTTT
TCCGAAGGTAACTGGCTTCAGCAGAGCGCAGATACCAAATACTGTCCTTCTAGTGTAGCCGTAGTTAGGCCA
CCACTTCAAGAACTCTGTAGCACCGCCTACATACCTCGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCCAG
TGGCGATAAGTCGTGTCTTACCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCGCAGCGGTCGGGCTG
AACGGGGGGTTCGTGCACACAGCCCAGCTTGGAGCGAACGACCTACACCGAACTGAGATACCTACAGCGTGA
GCATTGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGCAGGGTCGGAAC
AGGAGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGTATCTTTATAGTCCTGTCGGGTTTCGCCACCT
CTGACTTGAGCGTCGATTTTTGTGATGCTCGTCAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAACGCGGC
CTTTTTACGGTTCCTGGCCTTTTGCTGGCCTTTTGCTCACATGTTCTTTCCTGCGTTATCCCCTGATTCTGT
GGATAACCGTATTACCGCCTTTGAGTGAGCTGATACCGCTCGCCGCAGCCGAACGACCGAGCGCAGCGAGTC
AGTGAGCGAGGAAGCGGAAG

F.2. Cell sorting experiments – summary

Date	%Target cells before cell sorting	% Target cells after cell sorting	%Depletion	Cells *	Probe	Method of quantification
09.03.2000	13,70	10,60	22,6	E/N	E-DIII	manual cell count
10.03.2000	86,30	72,23	16,3	N/E	N-DIII	manual cell count
14.06.2000	40,40	28,68	29	A/E	A-DIII	manual cell count
15.06.2000	5,30	1,60	69,8	E/N	E-DIII	manual cell count
16.06.2000	93,90	90,14	4	N/E	N-DIII	manual cell count
21.08.2000	56,00	40,99	26,8	E/N	E-DIII	manual cell count
22.08.2000	63,30	50,32	20,5	N/E	N-DIII	manual cell count
24.08.2000	62,80	58,53	6,8	A/E	A-DIII	manual cell count
25.08.2000	50,00	43,10	13,8	A/E	A-DIII	manual cell count
26.08.2000	64,50	49,21	23,7	A/E	A-DIII	manual cell count
06.09.2000	77,50	67,97	12,3	A/E	A-DIII	manual cell count
07.09.2000	58,90	47,59	19,2	A/E	A-DIII	manual cell count
15.09.2000	14,10	6,20	56	E/N	E-DIII	manual cell count
16.09.2000	21,50	5,40	74,9	E/N	E-DIII	manual cell count
17.09.2000	13,80	4,60	66,7	E/N	E-DIII	manual cell count
18.09.2000	27,70	11,61	58,1	E/N	E-DIII	manual cell count
14.12.2001	34,40	17,20	50	E/N	E-DIII	manual cell count
15.12.2001	34,40	24,20	29,7	E/N	E-DIII	manual cell count
20.12.2001	41,90	37,70	10	E/N	E-DIII	manual cell count
21.12.2001	41,90	34,00	18,9	E/N	E-DIII	manual cell count
06.02.2002	26,40	13,60	37,1	E/N	E-DIII	manual cell count
07.02.2002	29,40	8,60	70,7	E/N	E-DIII	manual cell count
13.02.2002	56,20	55,70	0,9	E/N	E-DIII	manual cell count
14.02.2002	55,30	46,20	16,5	E/N	E-DIII	manual cell count
21.02.2002	34,40	23,40	32,1	pUN/N	beta-Lact	manual cell count
22.02.2002	54,10	43,40	19,7	pBBR/N	beta-Lact	manual cell count
21.02.2002	45,90	34,80	24,2	E/N	GAPDH-V	manual cell count
22.02.2002	45,30	37,80	16,6	E/N	GAPDH-V2	manual cell count
04.03.2002 (OT3)	32,04	27,43	14,39	E+KR	Eco-DIII	LSM
04.03.2002 (OT2)	30,75	18,71	39,15	pCR/N	beta-Lact	LSM
06.03.2002 (OT4)	85,15	77,96	8,44	P+KR	Pae-DIII	LSM
06.03.2002 (OT5)	40,04	38,31	4,32	E/N	GAP2	LSM
07.03.2002 (OT6)	62,01	59,54	3,98	pCR/N	beta-Lact	LSM
08.03.2002 (OT7)	49,28	42,12	14,53	E/N	GAP1	LSM
08.03.2002 (OT7)	44,64	27,19	39,09	E/N	GAP2	LSM
08.03.2002 (OT7)	49,88	44,31	11,17	E/N	GAP3	LSM

Date	%Target cells before cell sorting	% Target cells after cell sorting	%Depletion	Cells	Probe	Method of quantification
08.03.2002 (OT7)	45,81	41,92	8,49	E/N	GAP4	LSM
08.03.2002 (OT7)	44,79	40,11	10,45	E/N	GAP5	LSM
11.09.2002 (OT78)	61,25	50,94	16,8	pCR/N	beta-Lact3	LSM
14.03.2002 (OT9)	39,57	18,16	54,11	E/N	E-DIII	LSM
14.03.2002 (OT9)	38,81	30,22	22,12	E/N	E-DIII	LSM
14.03.2002 (OT9)	48,55	42,29	12,89	pCR/N	beta-Lact	LSM
14.03.2002 (OT9)	50,55	39,14	22,57	pBBR/N	beta-Lact	LSM
16.04.2002 (OT11)	31,70	31,18	1,64	Eco+KR	Eco-DIII	LSM
25.04.2002 (OT16)	4,67	1,79	61,67	E/A	GAP1	LSM
25.04.2002 (OT16)	8,62	4,65	46,10	E/A	GAP2	LSM
25.04.2002 (OT16)	15,79	5,21	66,97	E/A	GAP3	LSM
25.04.2002 (OT16)	3,05	2,24	26,64	E/A	GAP4	LSM
25.04.2002 (OT16)	9,33	6,27	32,78	E/A	GAP5	LSM
29.05.2002 (OT24)	20,54	9,98	51,41	E/N	GAP-A	LSM
29.05.2002 (OT24)	18,50	10,69	42,22	E/N	GAP-B	LSM
29.05.2002 (OT24)	24,52	20,31	17,18	E/N	GAP-C	LSM
29.05.2002 (OT24)	26,93	26,01	3,41	E/N	GAP-G	LSM
26.06.2002 (OT32)	38,00	26,30	30,79	E/Pae	GAP-A	LSM
26.06.2002 (OT32)	69,80	60,20	13,75	E/Pae	GAP-B	LSM
26.06.2002 (OT32)	63,00	48,10	23,65	E/Pae	GAP-C	LSM
26.06.2002 (OT32)	54,60	52,00	4,76	E/Pae	GAP-E	LSM
26.06.2002 (OT32)	54,90	40,10	26,96	E/Pae	GAP-F	LSM
26.06.2002 (OT32)	65,70	65,10	0,91	E/Pae	GAP-H	LSM
26.06.2002 (OT32)	66,20	58,90	11,03	E/Pae	GAP-J	LSM
26.08.2002 (OT73)	24,70	22,80	7,69	E/Kpn	GAP-F	LSM

*A = *Acinetobacter calcoaceticus*

E = *Escherichia coli*

N = *Neisseria canis*

Pae = *Pseudomonas aeruginosa*

Kpn = *Klebsiella pneumoniae*

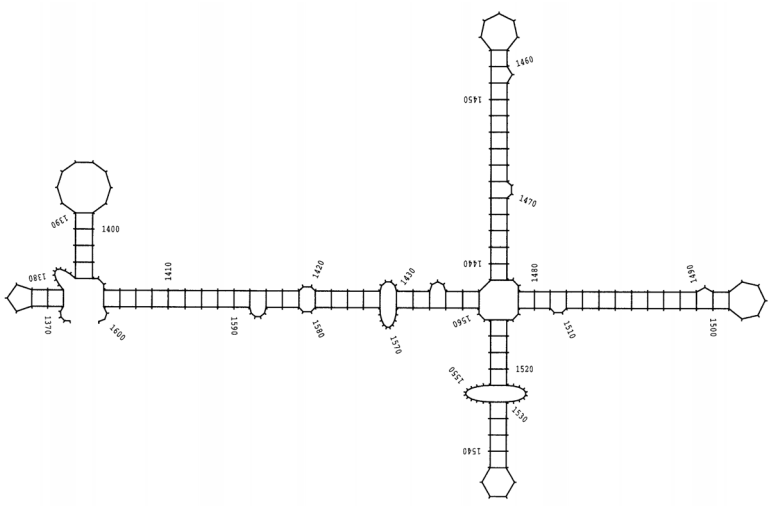
pCR = *E. coli* carrying plasmid pCR 2.1 TOPO

pBBR = *E. coli* carrying plasmid pBBR MCS4

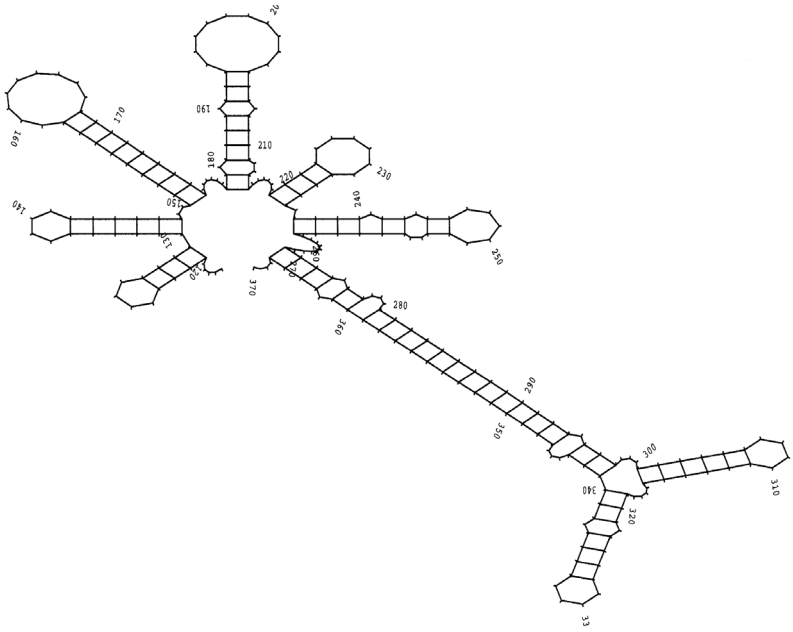
pUN = *E. coli* carrying plasmid pUN 121

F.3. Secondary structure

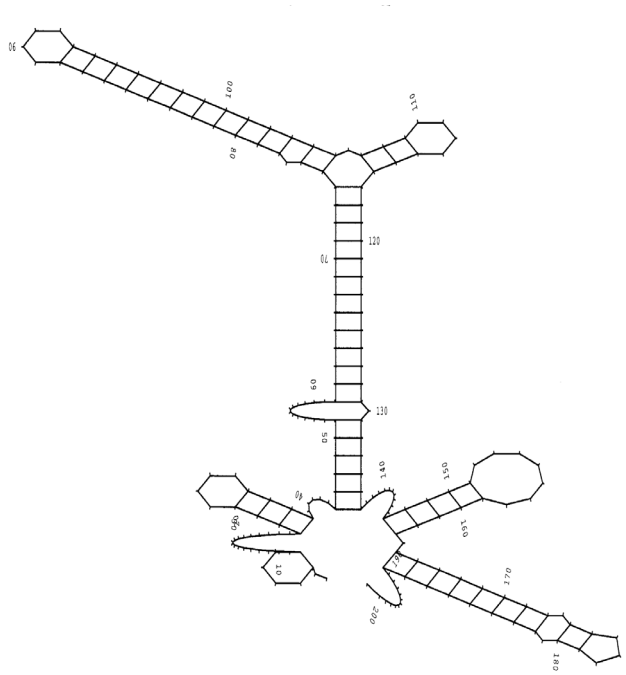
Secondary structure of the polynucleotide probes used in this work as determined by the GCG Software (Wisconsin, USA). In cases where the software calculated several possible secondary structures only the one with the highest energy value is shown.



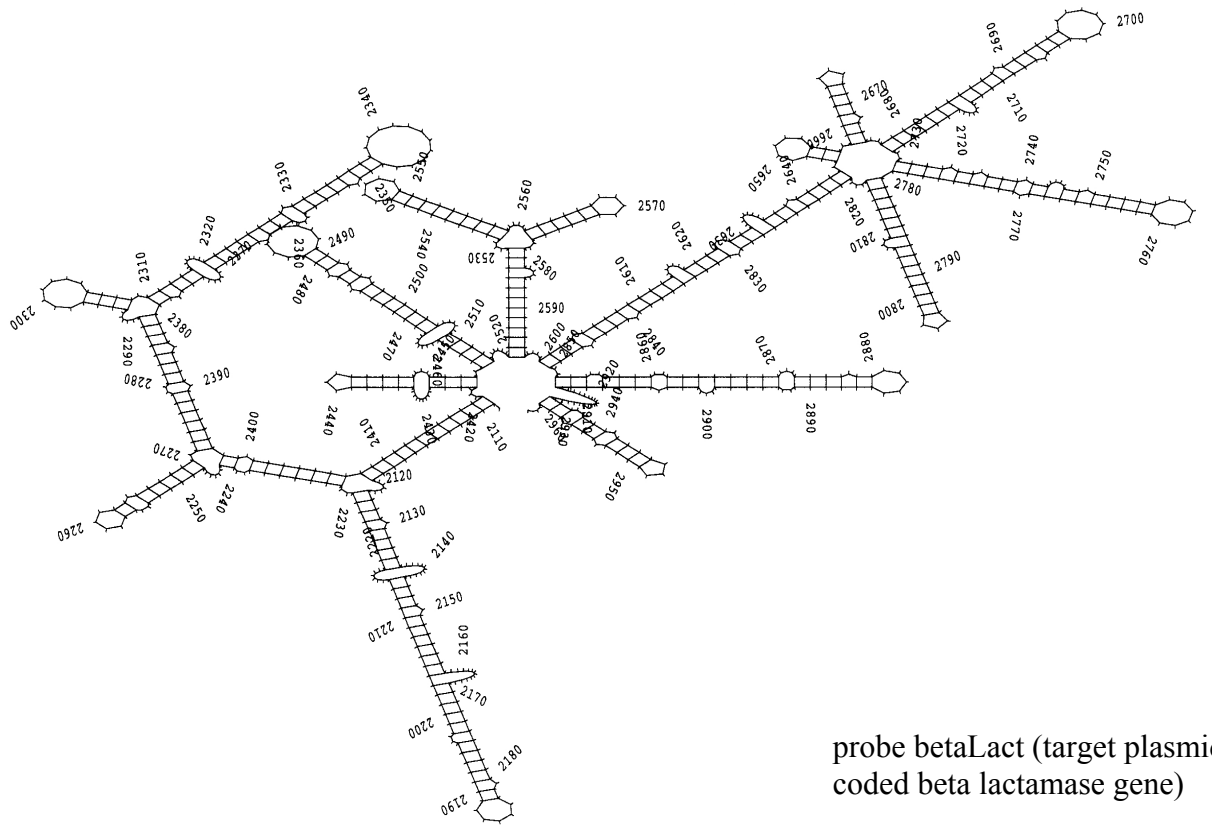
probe DIII (target 23S rRNA, domainIII)



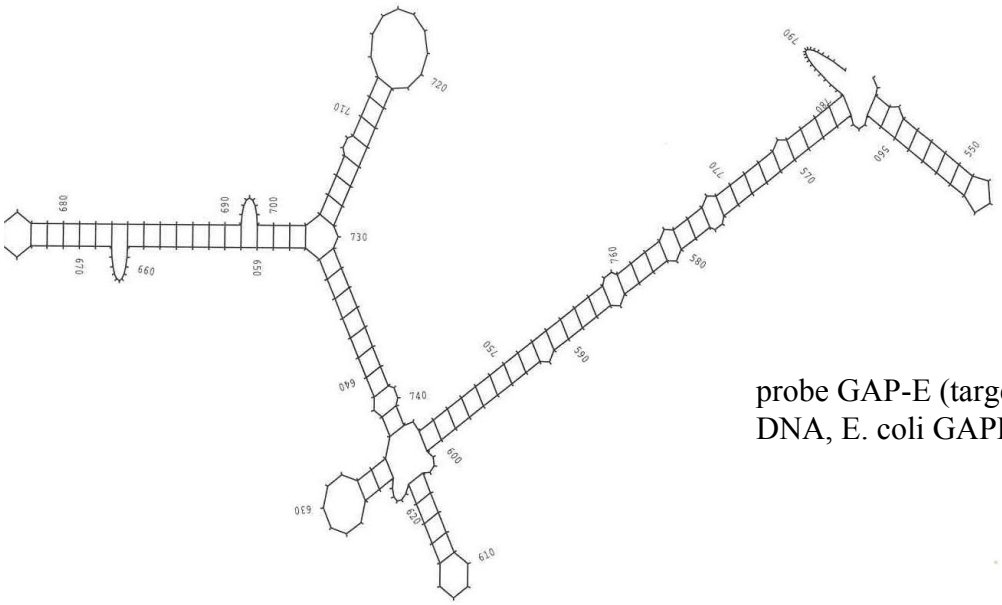
probe 367 (target 23S rRNA, domain I)



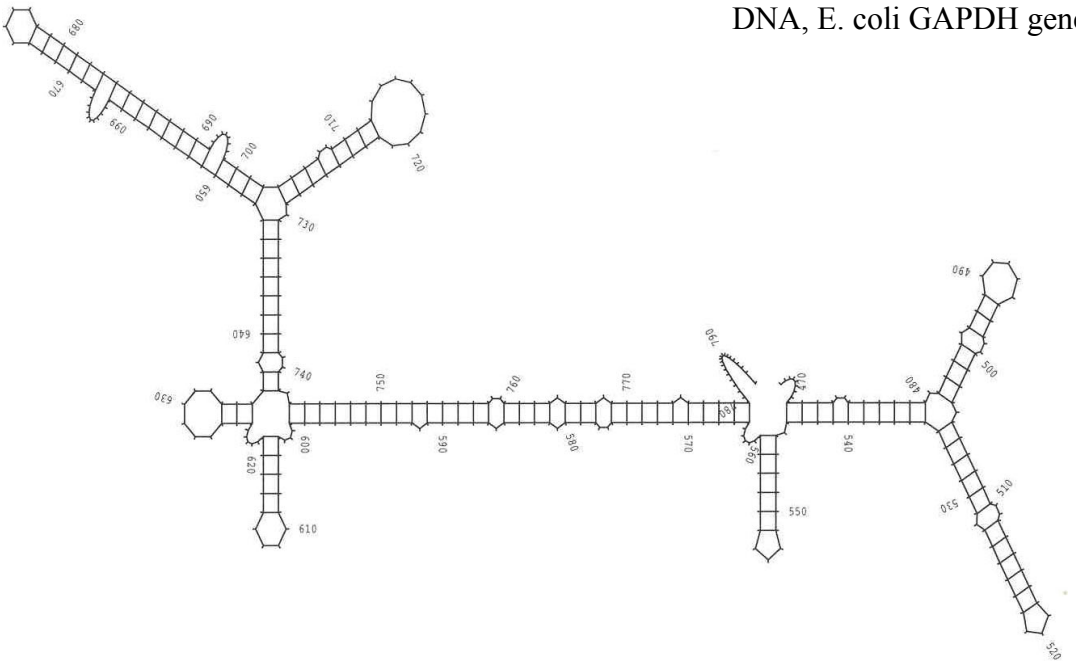
probe Eco2.1 (target plasmid pCR 2.1 TOPO, multiple cloning site)



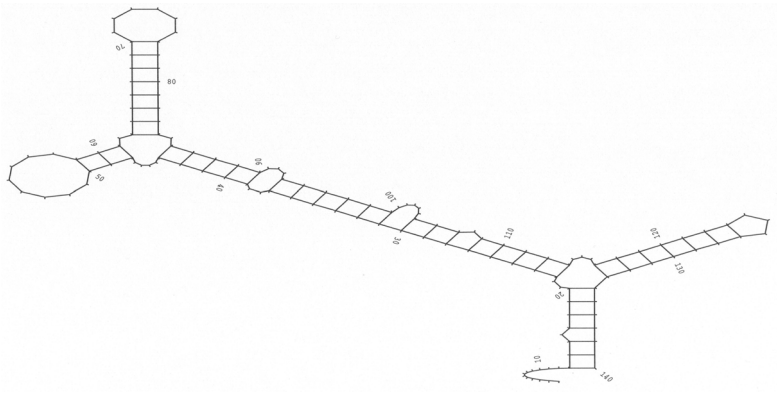
probe betaLact (target plasmid coded beta lactamase gene)



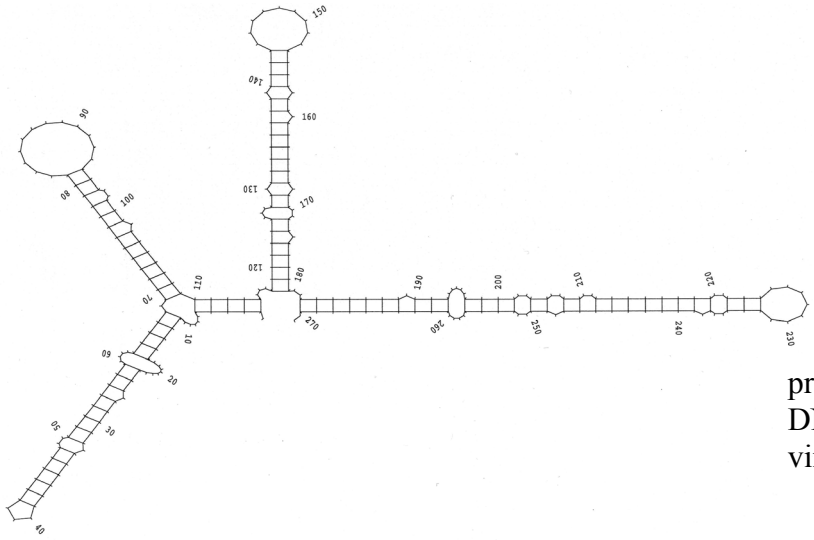
probe GAP-E (target: chromosomal DNA, E. coli GAPDH gene)



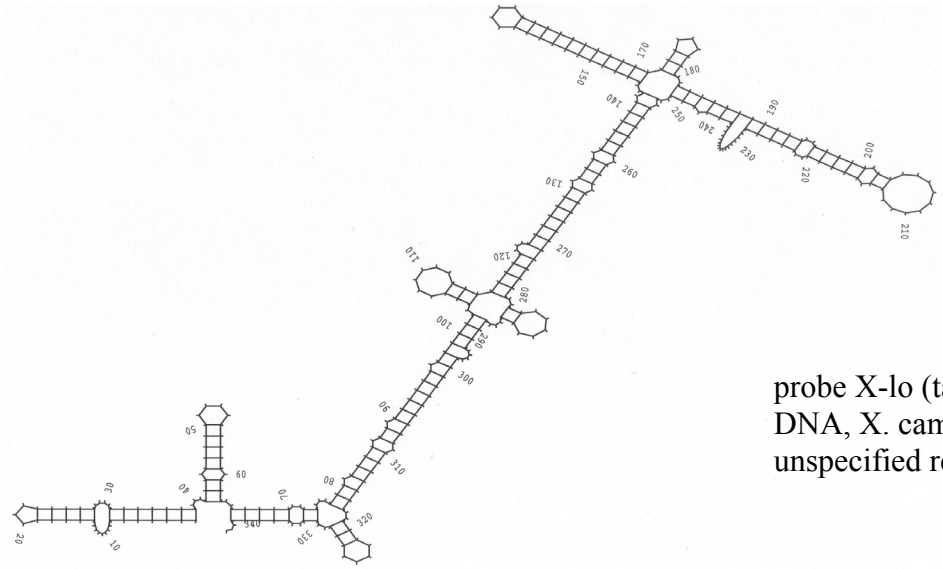
probe GAP-F (target: chromosomal DNA, E. coli GAPDH gene)



probe X-ca (target: chromosomal DNA, X. campestris pv. campestris, xpso gene)

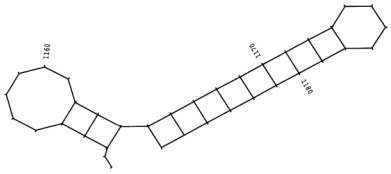


probe X-ra (target: chromosomal DNA, X. campestris pv. raphani, virB11 gene)

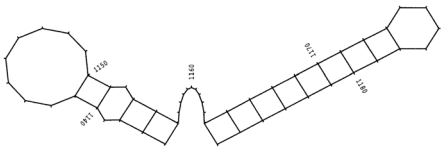


probe X-lo (target: chromosomal DNA, X. campestris pv. lobelia, unspecified region)

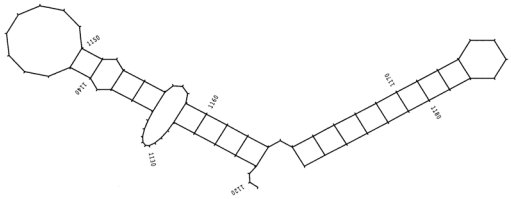
probe 1024_1-11 (target: 23S rRNA, domain I-III)



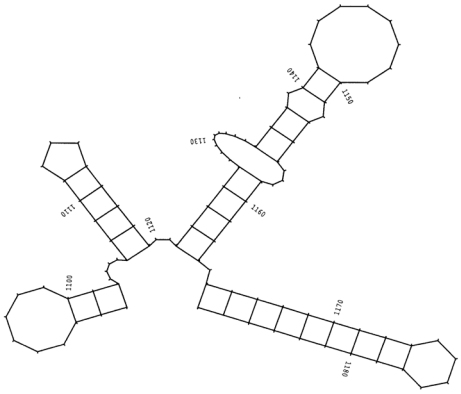
1024-1 (length 50 nt)



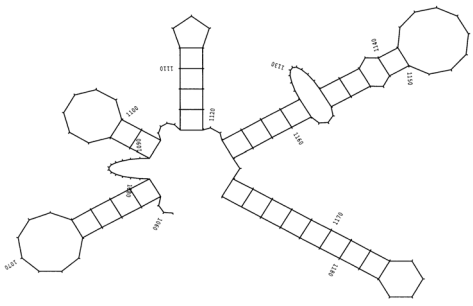
1024-2 (length 65 nt)



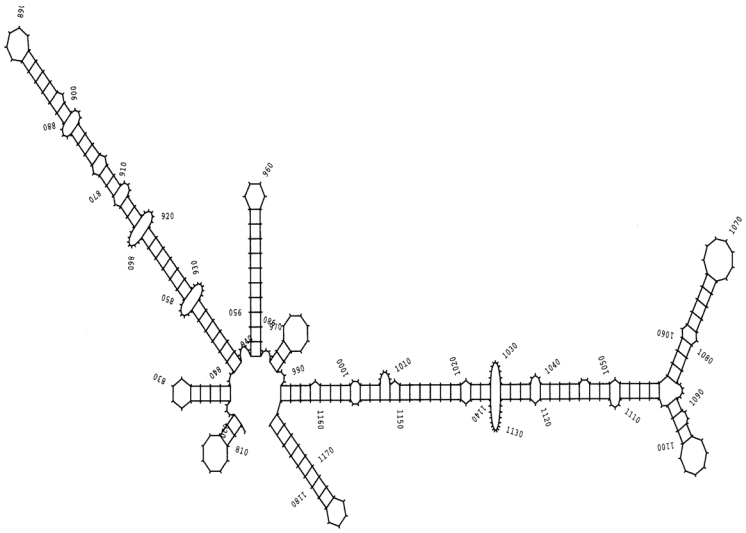
1024-3 (length 82 nt)



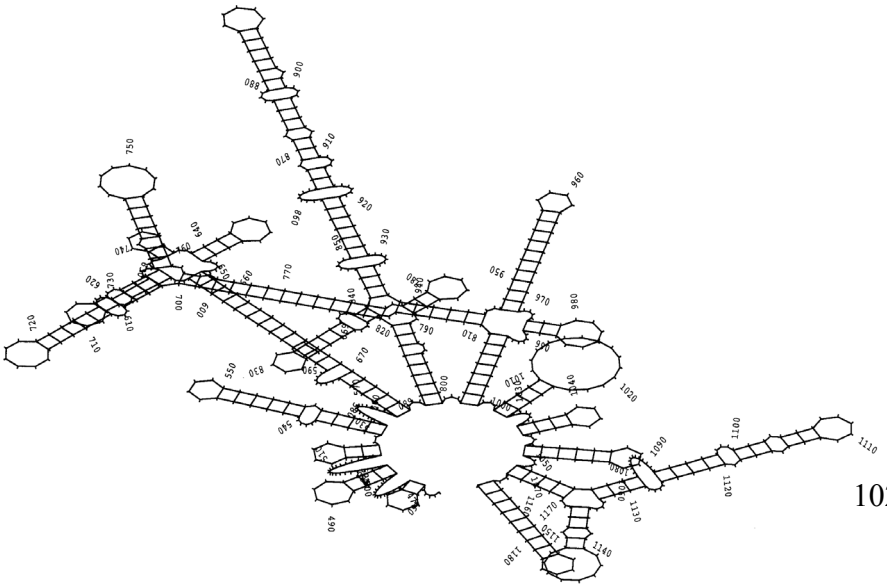
1024-4 (length 110 nt)



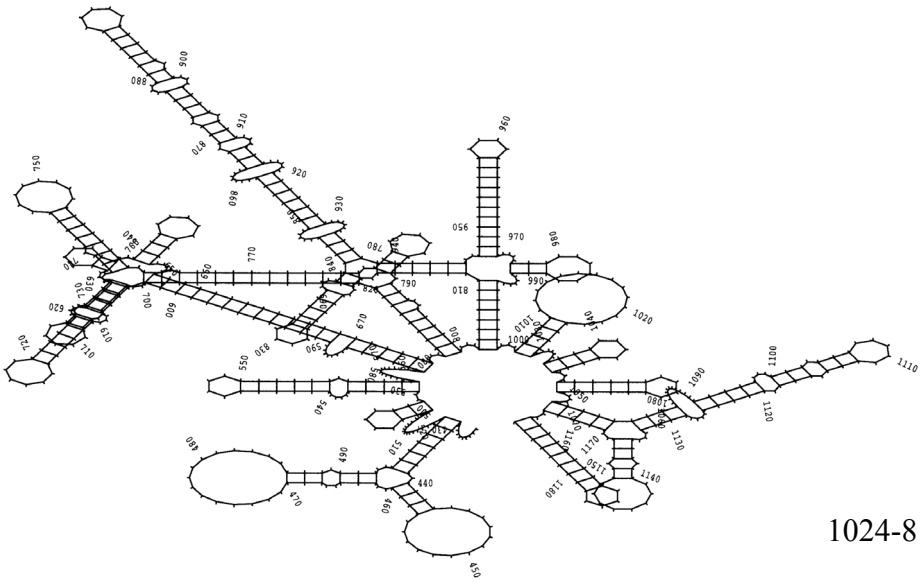
1024-5 (length 142 nt)



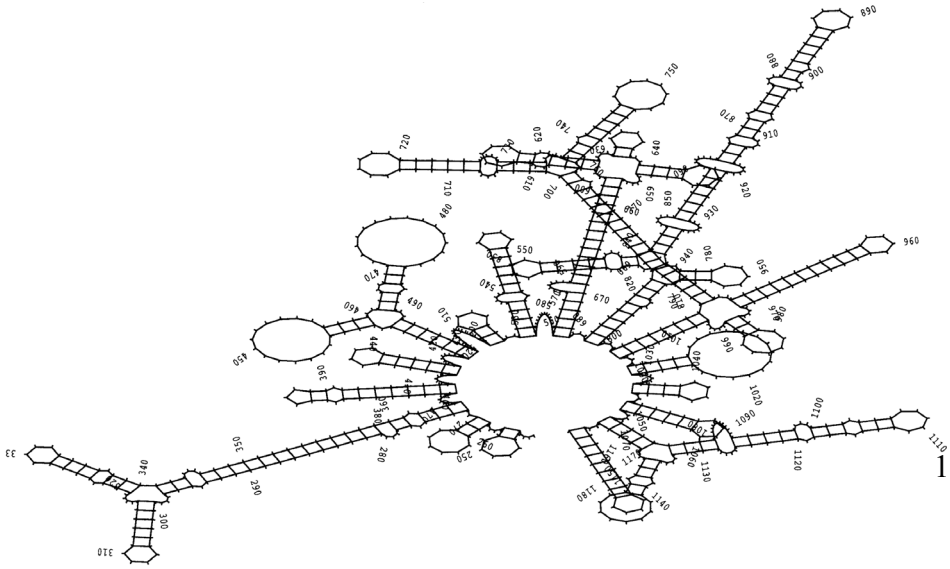
1024-6 (length 394 nt)



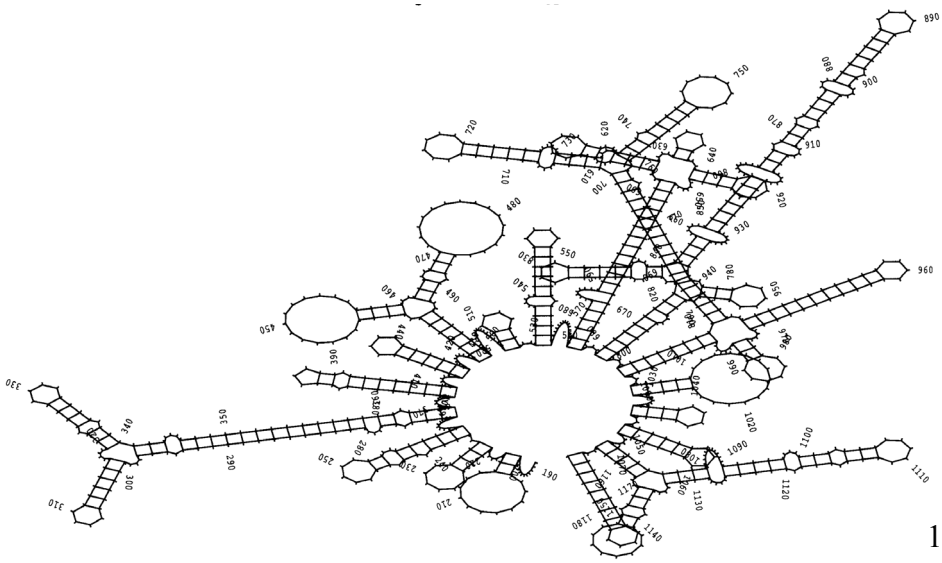
1024-7 (length 744 nt)



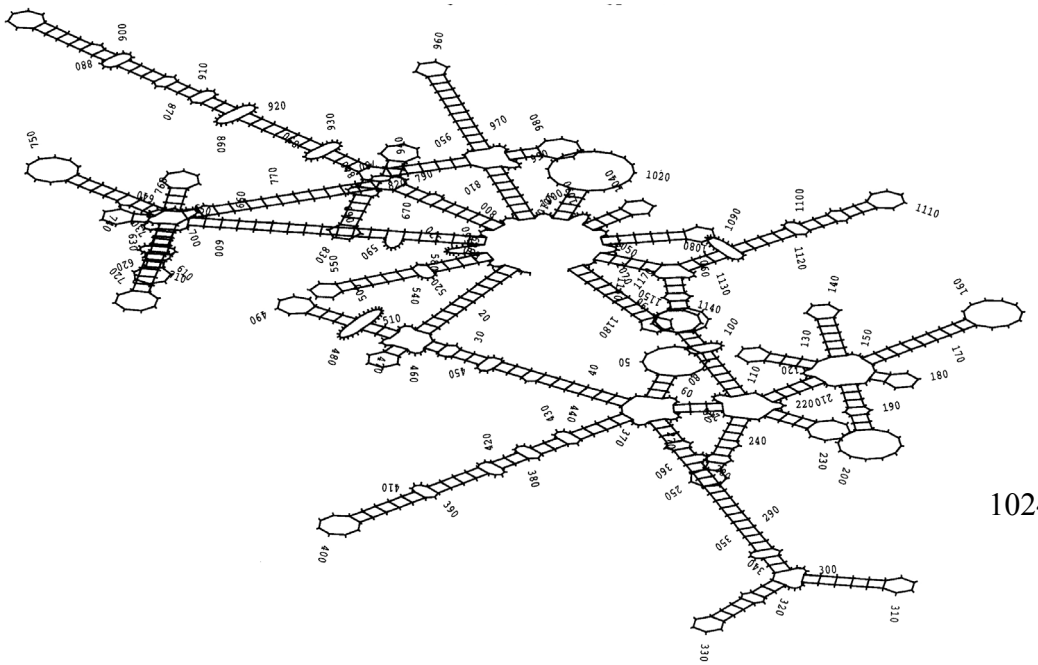
1024-8 (length 778 nt)



1024-9 (length 960 nt)



1024-10 (length 1012 nt)



1024-11 (length 1188 nt)

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G. References

Amann, R.I., Binder, B.J., Olson, R.J., Chrisholm, S.W., Devereux, R., Stahl, D.A. (1990): Combination of 16S rRNA-Targeted Oligonucleotide Probes with Flow Cytometry for Analyzing Mixed Microbial Populations. Appl Environ Microbiol **56**, 1919-1925

Amann,R., Springer,N., Ludwig,W., Görtz,H.-D., Schleifer, K.H. (1991): Identification and phylogeny of uncultured bacterial endosymbionts. Nature **351**, 161-164

Amann,R., Ludwig,W., and Schleifer, K.H. (1995): Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. Microbiol Rev **59**, 143-169

Antoine, R., Locht, C. (1992): Isolation and molecular characterization of a novel broad-host-range plasmid from *Bordetella bronchiseptica* with sequence similarities to plasmids from gram-positive organisms. Mol Microbiol **6** (13), 1785-1799

Ashkin,A., Dziedzic,J.M. (1987): Optical trapping and manipulation of viruses and bacteria. Science **235**, 1517-1520

Bauman, J.G.J., Bentvelzen, P. (1988): Flow cytometric detection of ribosomal RNA in suspended cells by fluorescence *in situ* hybridisation. Cytometry, **9**, 517-524

Bakermans, C., Madsen, E.L. (2002): Detection in coal tar waste-contaminated groundwater of mRNA transcripts related to naphthalene dioxygenase by fluorescence *in situ* hybridisation with tyramide signal amplification. J Mircrobiol Meth, **50**, 75-84

Beimfohr,C., Krause, A., Amann,R., Ludwig,W., Schleifer, K.H. (1993): *In situ* identification of *Lactococci*, *Enterococci* and *Streptococci*. System Appl Microbiol **16**, 450-456

Ben Ali, A., Wuyts,J., De Wachter, R., Meyer, ., Van de Peer, Y. (1999): Construction of a variability map for eukaryotic large subunit ribosomal RNA. Nuc. Acids. Res. **27** (14) 2825-2831

Berger, B.R., Christie, P.J. (1994): Genetic complementation analysis of the *Agrobacterium tumefaciens* virB operon: virB2 through virB11 are essential virulence genes. J Bacteriol. **176** (12), 3646-3660

Bertin, B., Broux, O., Van Hoegaerden, M. (1990): Flow cytometric analysis of yeast by in situ hybridization with a fluorescent ribosomal RNA probe. J Microbiol Meth **12**, 1-12

Birnboim,H.C., Doly,J. (1979): A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nuc Acids Res, nov 24, **7** (6) 1513-1523

Bremer, H., Dennis, P.P. (1987): Modulation of chemical composition and other parameters of the cell by growth rate. pp.1537-1542, in F.C. Neidhardt, J.L. Ingraham, K.B. Low, B. Magasanik, M. Schaechter, H.E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.

Brown, E.W., Davis, R.M., Gouk, C., van der Zwet,T. (2000): Phylogenetic relationships of necrogenic *Erwinia* and *Brenneria* species as revealed by glyceraldehyde-3-phosphate dehydrogenase gene sequences. Int.J.Syst.Evol.Microbiol. **50**, 2057-2068

Brosius,J., Dull,T.J., Sleeter,D.D., Noller,H.F. (1981): Gene organisation and primary structure of a ribosomal RNA operon from *Escherichia coli*. J Mol Biol **148**, 107-127

Cao, T.B., Saier, M.H.Jr. (2001): Conjugal type IV macromolecular transfer systems of gram-negative bacteria: - organismal distribution, structural constraints and evolutionary conclusions. Microbiol **147**, 3201-3214

Christensen,B., Torsvik,T., Lien,T. (1992): Immunomagnetically capured thermophilic sulfate-reducing bacteria from the north sea oil field waters. Appl Environ Microbiol **58**, 1244-1248

Clark,J.M., Switzer,R.L. (1977): Experimental Biochemistry. 2nd edition, W.H. Freeman and Company, San Francisco

Connon, S.A., Giovannoni, S.J. (2002): High-throughput methods for culturing microorganisms in very-low-nutrient media yield diverse new marine isolates. Appl Environ Microbiol. Aug, **68**(8), 3878-85.

Cryer,D.R., Eccleshall,R., Marmur,J. (1975): Isolation of yeast DNA. 39-44. in Prescott D.M. (ed.). Methods in Cell Biology. Vol. XII

Daims, H., Brühl, A., Amann, R., Schleifer, K.-H., Wagner, M. (1999): The Domain-specific Probe EUB338 is Insufficient for the Detection of all Bacteria: Development and Evaluation af a more Comprehensive Probe Set. System Appl Microbiol **22**, 434-444

Daims, H., Ramsing, N.B., Schleifer, K.H., Wagner, M. (2001): Cultivation-independent, semiautomatic determination of absolute bacterial cell numbers in environmental samples by fluorescence *in situ* hybridization. Appl Environ Microbiol **67** (12), 5810-5818

DeLong,E.F., Wickham,G.S., Stahl,D.A. (1989): Phylogenetic strains: ribosomal RNA-based probes for the identification of single microbial cells. Science **243**, 1360-1363

DeLong, E.F., Taylor, L.T., Marsh, T.L., Preston, C.M. (1999): Visualization and enumeration of marine planktonic archaea and bacteria by using polyribonucleotide probes and fluorescence *in situ* hybridization. Appl Environ Microbiol **65,** 5554-5563

DeLong, E.F.,. Pace, N.R (2001): Environmental Diversity of Bacteria and Archaea. Syst. Biol. **50**(4), 470-478

Ezaki, T., Hashimoto, Y., Yabuuchi, E. (1989): Fluorometric DNA-DNA hybridization in microdilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. Int J Syst Bacteriol **39**, 224-229

Felsenstein,J. (1981) : Evolutionary trees from DNA sequences : a maximum-likelyhood approach. J Mol Evol **17**, 368-376

Fichtl, K. (2002): Taxonspezifische Abreicherung von Bakterien mittels rRNS-gerichteter Polynukleotidsonden, Diploma thesis, Technical University Munich.

Fuchs, B.M., Wallner, G., Beisker, W., Schwippl, I., Ludwig, W., Amann, R. (1998) : Flow cytometric analysis of the *in situ* accessibility of Escherichia coli 16S rRNA for fluorescently labeled oligonucleotide probes. Appl Environ Microbiol. **64** (12), 4973-4982

Fuchs, B.M., Syutsubo, K., Ludwig.W., Amann, R. (2001): *In situ* accessibility of *Escherichia coli* 23S rRNA to fluorescently labelled oligonucleotide probes. Appl Environ Microbiol **67** (2) 961-968.

Giovannoni,S.J., DeLong, E.F., Olsen,G.J., Pace,N.R. (1988): Phylogenetic groupspecific oligodeoxynucleotide probes for identification of single microbial cells. J Bacteriol **170**, 720-726

Grimont, P.A.D. (1988): Use of DNA reassociation in bacterial classification. Can J Microbiol **34**, 541-546

Hodson, R.E., Dustman, W.A., Garg, R.P., Moran, M.A. (1995): *In situ* PCR for visualization of microscale distribution of specific genes and gene products in prokaryotic communities. Appl Environ Microbiol, **61** (11), 4074-4082

Höpfl, P., Ludwig,W., Schleifer, K.H., Larsen, N. (1989) The 23S ribosomal RNA higher-order structure of Pseudomonas cepacia and other prokaryotes. Eur. J. Biochem. **185**,355-364

Hu, N.T., Lee, P.F., Chen, Ch. (1995): The type IV pre-pilin leader peptidase *of Xanthomonas campestris pv. campestris* is functional without conserved cysteine residues. Mol Microbiol **18** (4), 769-777

Jukes, T.H., Cantor C.R. (1969): Evolution of protein molecules. In H.N. Munro (ed.): Mammalian protein metabolism III:21-132. Academic Press, New York

Juretschko, S., Schönhuber, W, Kulakauskas, S, Ehrlich, D.S., Schleifer, K.-H. (1999): *In situ* detection of E. coli cells containing ColE1-related plasmids by hybridization to regulatory RNA II. Syst Appl Microbiol, **22**, 1-8

Juretschko, S., Loy, A., Lehner, A., Wagner, M. (2002): The Microbial Community Composition of a Nitrifying/Denitrifying Activated Sludge from an Industrial Sewage Treatment Plant Analyzed by the Full-Cycle rRNA Approach. System. Appl. Microbiol. **25**, 84-99

Keller, G.H., Manak, M.M. (1989): DNA probes. Stockton Press, New York

Koch, J.E., Kolvraa, S., Petersen, K., Gregersen, N., Bolund, L. (1989): Oligonucleotide priming methods for chromosome-specific labelling of alpha satellite DNA *in situ*. Chromosoma, **98**, 259-265

Kovach, M.E., Elzer, P.H., Hill, D.S., Robertson, G.T., Farris, M.A., Roob, M.R., Peterson,K.M. (1995): Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. Gene **166**, 175-176

Krall, L., Wiedemann, U., Unsin, G., Weiss, S., Domke, N., Baron, C. (2002): Detergent extraction identifies different VirB protein subassemblies of the type IV secretion machinery in the membranes of *Agrobacterium tumefaciens*. Proc Nat Acad Sci **99** (17), 11405-11410

Lanoil, B.D., Giovannoni, S.J. (1997): Identification of bacterial cells by chromosomal painting. Appl Environ Microbiol**, 63** (3), 1118-1123

Lanoil, B.D., Carlson, C.A., Giovannoni, S.J. (2000): Bacterial chromosomal painting for *in situ* monitoring of cultured marine bacteria. Environ. Microbiol**. 2** (6), 654-665

Lathe, R. (1985): Synthetic oligonucleotide probes from amino acid sequence data. Theoretical and practical considerations. J Mol Biol **183**, 1-12

Lee, N., Nielsen, P.H., Andreasen, K.H., Juretschko, S., Nielsen, J.L., Schleifre, K.H., Wagner, M. (1999): Combination of fluorescence *in situ* hybridisation and microautoradiography – a new tool for structure-function analyses in microbial ecology. Appl Environ Microbiol, **65** (3), 1289-1297

Liaud, M.F., Valentin, C., Matrin,W., Bouget,F.Y., Kloareg, B., Cerff,R. (1994) : The evolutionary origin of red algae as deduced from the nuclear genes encoding cytosolic and chloroplast glyceraldehyde-3-phosphate dehydrogenases from *Chondrus crispus*, J Mol Evol **38**, 319-327

Lawrence,J.G., Ochman,H., Hartl, D.L. (1991): Molecular and evolutionary relationships among enteric bacteria. J Gen Microbiol, **137**, 1911-1921

Ludwig,W., Dorn,S.,Springer,N.,Kirchhof,G., Schleifer, K.H. (1994): PCR-based preparation of 23S rRNA-targeted group-specific polynucleotide probes. Appl Environ Microbiol **60**, 3236-3244

Ludwig,W., Strunk, O. (1996): ARB: A software environment for sequence data. http://www.arb-home.de

Ludwig,W., Strunk,O., Klugbauer,S., Klugbauer,N., Weizenegger,M., Neumaier,J., Bachleitner,M., Schleifer,K.H. (1998): Bacterial phylogeny based on comparative sequence analysis. Electrophoresis **19**, 554-568

Luk, J.M.C., Lindberg, A.A. (1991): Rapid and sensisitive detection of *Salmonella* (O:6.7) by immunomagnetic monoconal antibody-based assays. J Immunol Meth**, 137**, 1-8

Manz, W., Amann, R., Ludwig, W., Wagner, M., Schleifer, K.-H. (1992): Phylogenetic Oligodeoxynucleotide Probes for the Major Subclasses of Proteobacteria: Problems and Solutions. System Appl Microbiol **15**, 593-600

Marmur, J. (1961): A Procedure for the Isolation of Deoxyribonucleic Acid from Micro-organisms. J Mol Biol **3**, 208-218

Marmur, J., Doty, P. (1962): Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. J Mol Biol **5**, 109-118

McGraw, N.J., Bailey,J.N., Cleaves,G.R., Dembinsky, D.R., Gocke,C.R., Jollife,L.K., McWrite,R.S., McAllister,W.T. (1985): Sequence and analysis of the gene for bacteriophage T3 RNA polymerase. Nucl Acids Res **13**, 6753-6766

Mehlen, A. (2003): Stammspezifische Identifizierung pathogener und biotechnologisch relevanter Bakterien durch in vitro Amplifikation und Detektion charakteristischer Genfragmente. PhD thesis, Technical University Munich

Melchior, W.B., von Hippel, P.H. (1973): Alteration of the Relative Stability of dA•dT and dG•dC Base Pairs in DNA. Proc Nat Acad Sci **70**, 298-302

Nilsson,B., Uhlen,M., Josephson,S., Gatenbeck,S., Philipson,L. (1983): An improved positive selection plasmid vector constructed by oligonucleotide mediated mutagenesis. Nuc.Acids Res. **11** (22), 8019-8030

Olsen, G.J., Lane, D.J. Giovannoni, S.J., Pace, N.R. (1986): Microbial Ecology and Evolution: A Ribosomal RNA Approach. Ann Rev Microbiol, **40**, 337-365

Ouverney, C.C., Fuhrman, J.A. (1999): Combined microautoradiography – 16S rRNA probe technique for determination of radioisotope uptake by specific microbial cell types *in situ*. Appl Environ Microbiol, **65** (4), 1746-1752

Pernthaler, A., Preston, C.M., Pernthaler, J., DeLong, E.F., Amann, R. (2002): Comparison of Fluorescently Labeled Oligonucleotide and Polynucleotide Probes for the Detection of Pelagic Marine Bacteria and Archaea. Appl. Environ. Microbiol. **68**, 661-667

Petroni, G., Spring, S., Schleifer, K.-H., Verni, F., Rosati,G. (2000): Defensive extrusive ectosymbionts of Euplotidium (Ciliophora) that contain microtubule-like structures are bacteria related to Verrucomicrobia. PNAS **97**, 1813-1817

Porter, K.G., Feig, Y.S. (1980): The use of DAPI for identifying and counting aquatic microflora. Limnol Oceanogr **25**, 943-948

Rogan, P.K., Cazcarro, P.M., Knoll, J.h.M. (2001): Sequence-based design of single-copy genomic DNA probes for fluorescence *in situ* hybridisation. Gen Res **11**, 1086-1094

Roller, C., Ludwig,W., Schleifer, K.H. (1992): Gram-positive bacteria with a high G+C content are characterized by a common insertion within their 23S rRNA genes. J Gen Microbiol, **138** (Pt 6), 1167-75.

Ross, J., Boon, P.I., Sharma, R., Beckett, R. (1996): Variations in the fluorescence intensity of intact DAPI-stained bacteria and their implications for rapid bacterial quantification. Lett Appl Microbiol **22**, 283-287

Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf,S.J., Higuchi,R., Horn,G.T., Mullis,K.B. (1988): Primer-directed enzymatic amplification of DNA with a thermostabile DNA polymerase. Science **239**, 487-491

Saitou, N., Nei, M. (1987): The neighbour joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol **4**, 406-425

Salton, M.R.J. (1960): Microbial Cell Walls. John Wiley and Sons, Inc., New York, London

Sanger,F., Nicklen,S., Coulson,A. (1977): DNA sequencing with chain terminating inhibitors. Proc Natl Acad Sci USA **74**, 5463-5467

Schlaeper, B.S., Zuber,H. (1992): Cloning and sequencing of the genes encoding glyceraldehyde-3-phosphate dehydrogenase, phosphoglyserate kinase, and triose phophate isomerase (gap operon) from mesophilic *Bacillus megaterium*: comparison with corresponding sequences from thermophilic *Bacillus* species, Gene **122**, 53-63

Schönhuber, W., Fuchs, B., Juretschko, S., Amann, R. (1997): Improved sensitivity of whole-cell hybridization by the combination of horseradish peroxidase-labeled oligonucleotides and tyramide signal amplification. Appl Environ Microbiol, ***63*** (8), 3268-3273

Sharma, A.K., Sharma, A. (2001): Chromosome painting – principles, strategies and scope. Meth Cell Science, **23**, 1-5

Skjerve, E., Rörvik, L.M., Olsvik, Ö. (1990): Detection of *Listeria monocytogenes* in food by immunomagnetic separation. Appl Environ Mircobiol, **54**, 287-297

Spring, S., Lins, U., Amann, R., Schleifer, K.-H., Ferreira, L.C.S., Esquivel, D.M.S., Farina, M. (1998): Phylogenetic affiliation and ultrastructure of uncultured magnetic bacteria with unusually large magnetosomes. Arch. microbiol. **169**, 136-147

Stoffels,M., Ludwig,W., Schleifer,K.H. (1999): rRNA probe-based cell fishing of bacteria. Environ Microbiol **1** (3) 259-271

Suggs, S.V., Hirose, T., Miyake, T., Kawashima, E.H., Johnson, M.J., Itakura, K., Wallace, R.B. (1981): Use of synthetic oligodeoxyribonucleotides for the isolation of specific cloned DNA sequences. In D.D. Brown, and C.F. Fox (eds.): Developmental biology using purified genes 683-693. Academic Press, New York

Tolkien, J.R.R. (1954): The Lord og the Rings. George Allen and Unwin (Publishers) Ltd., Great Britain

Trebesius, K.H., Amann,R., Ludwig,W., Muhlegger,K., Schleifer,K.H. (1994): Identification of whole fixed bacterial cells with nonradioactive 23S rRNA-targeted polynucleotide probes. Appl Environ Microbiol **60**, 3228-3235

Wagner, M., Erhardt, R., Manz, W., Amann, R.I., Lemmer, H., Wedi, D., Schleifer, K.H. (1994): Development of an rRNA targeted oligonucleotide probe specific for the genus *Acinetobacter* and its application for *in situ* monitoring in activated sludge. Appl Environ Microbiol **60**, 792-800

Wagner, M., Schmid, M., Juretschko, S., Trebesius, K.H., Bubert, A., Goebel, W., Schleifer, K.H. (1998): *In situ* detection of a virulence factor mRNA and 16S rRNA in *Listeria monocytogenes*. FEMS Microbiol Lett, **160**, 159-168

Wahl, G.M., Berger, S.L., Kimmel, A.R. (1987): Molecular hybridization of immobilized nucleic acids: Theoretical concepts and practical considerations. Methods in Enzymology **152**, 399-407

Wallner,G., Erhardt,R., Amann,R. (1995): Flow cytometric analysis of activated sludge with rRNA-targeted probes. Appl Environ Microbiol **60**, 792-800

Watson, J.D., Crick, F. (1953): Molecular structure of nucleic acids. Nature **171**, 737-738

Wayne, L.G., Brenner, M., Colwell, R.R., Grimont, P.A.D., Kandler, O., Krichevsky, M.I., Moore, L.H., Moore, W.E.C., Murray, R.G.E., Stackebrandt, E., Starr, M.P., Trüper, H.G. (1987): Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. Int J Syst Bacteriol, **37**, 463-464

Wisotzkey, J.D., Jurtshuk, Jr.P., Fox, G.E. (1990): PCR amplification of 16S rDNA from lyophilized cell cultures facilitates studies in molecular systematics. Curr Microbiol **21**, 325-327

Woese, C.R. (1987): Bacterial evolution. Microbial Reviews **51**, 221-271

Wysocki, L.J., Sato, V.L. (1978): "Panning" for lymphocytes: A method for cell selection. Proc Natl Acad Sci USA**, 75** (6), 2844-2848

Zimmermann, F.J. (2002): Oligo- und Polynukleotidsonden-gestützte Charakterisierung und Anreicherung bisher nicht kultivierbarer Acidobacteria. PhD thesis, Technical University Munich

Zlotnik H, Fernandez MP, Bowers B, Cabib E. (1984): Saccharomyces cerevisiae mannoproteins form an external cell wall layer that determines wall porosity. J Bacteriol **159** (3), 1018-26.

Zuckerkandl, E., Pauling, L. (1965): Molecules as documents of evolutionary history. J Theoret Biol, **8**, 357-366

Zwirglmaier,K., Wassill,L., Ludwig,W., Schleifer, K.H. (2001): Subtraction hybridisation in microplates: An improved method to generate strain specific PCR primers. System Appl Microbiol **24**, 108-115

Zwirglmaier, K., Ludwig, W., Schleifer, K.H. (2003): Improved fluorescence in situ hybridization of individual microbial cells using polynucleotide probes: The network hypothesis (in press, System Appl Microbiol **26**)

Zwirglmaier, K., Ludwig, W., Schleifer, K.H.: Recognition of individual genes in a single bacterial cell by fluorescence in situ hybridization – RING-FISH (submitted)

Zwirglmaier, K., Ludwig, W., Schleifer, K.H.: An improved method for polynucleotide probe based cell sorting using DNA coated microplates (submitted)

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