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Nina Virolainen

Antimicrobial Detection Illuminated:
Developing Bioluminescent Antibiotic Biosensors Based on Bacterial Gene Regulatory Elements

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ABSTRACT

Ever since World War II, antibiotics have been medicine's number one asset in fighting microbial infection, one of the leading causes of death worldwide. Misuse of antibiotics has, however, led to rapid spread of antibiotic resistance among bacteria and ensuing development of multiple resistant pathogens. Therefore, antibiotics are rapidly losing their antimicrobial value, which can be seen a failure of society to protect one of its valuable resources.

The use of antibiotics in food production animals is strictly controlled by the European Union. Veterinary use is regulated to prevent spreading of resistance due to unwarranted use and to prevent antibiotic residues in food products. EU legislation establishes maximum residue limits (MRLs) of veterinary medicinal products in foodstuffs of animal origin, and enforces countries to establish and execute a national monitoring plan of animal products to implement food control measures. Among samples selected for monitoring, suspect noncompliant samples are screened for and then subjected to confirmatory analysis to establish the identity and concentration of the contaminant. Screening methods for antibiotic residues are typically based on microbiological growth inhibition, whereas physico-chemical methods are used for confirmatory analysis.

In this study, antibiotic whole-cell biosensor assays were examined as a novel screening method. Utilizing a tetracycline-specific bioluminescent whole-cell biosensor, a screening method for tetracycline residues in poultry meat was developed. Assay sensitization to meet the EU MRLs was achieved by improving tetracycline accumulation into the biosensor cells with a combination of membrane-permeabilizing agent polymyxin B and chelating agent EDTA. The result was a rapid, simple and cost-effective high-throughput screening method that could detect all four veterinary relevant tetracyclines and their 4-epimer metabolites in poultry meat with sensitivity below the MRLs. The study also provided proof of antimicrobial activity of tetracycline 4-epimer metabolites, a quality previously thought absent from 4-epidoxycycline.

Nisin is a lantibiotic, a peptide antibiotic produced by lactococci. The industrial use of nisin as a food preservative (E234) and maximum allowed levels set by the EU warrant developing methods for nisin quantification in foods. In this study, a bioluminescent whole-cell biosensor for nisin was constructed and utilized in determining nisin concentrations in milk. The developed assay was rapid and simple to perform, and required no sample pretreatment except dilution. Sensitivity of the assay was in the sub-picogram per ml level, exceeding the performance of all previously published methods. The assay was also used in determining nisin-production efficiency by quantifying nisin in growth medium of a nisin-producing *Lactococcus* strain. Simultaneously, nisin producers could be distinguished from non-producers. This idea was expanded in a follow-up study, which utilized the nisin biosensor in screening for nisin producers in raw milk. Screening was based on simple overlay of raw milk cultures and identification of nisin producers by a bioluminescent zone surrounding the nisinogenic colony. The seven identified nisinogenic colonies were divided in three groups by genetic fingerprinting,

and characterized as nisin variant Z producing *Lactococcus lactis* subsp. *lactis*. In addition, four nisin A producers were identified in a panel of 91 dairy lactococcal strains. Specificity studies showed that only nisin and not other bacteriocin peptides induced bioluminescence in the sensor strain. Also, all nisin-gene harboring colonies induced bioluminescence, with the exception of one lactococcal strain shown to carry a nonfunctional nisin gene.

The development of novel inducible whole-cell biosensors for different groups of antimicrobials can be limited by the lack of regulatory elements specifically responsive for these substances. In this study, we characterized DNA and ligand binding of the macrolide antibiotic-responsive repressor protein, MphR(E). The protein was modified by rational design of mutations to improve DNA affinity and dimerization. DNA and ligand binding as well as macrolide-induced dissociation from DNA were studied by fluorescence anisotropy and mass spectrometry. Mutants with improved DNA affinity and retained ligand binding and dissociation characteristics were identified. One mutant surprisingly formed a covalent dimer through disulfide bridge formation. This was shown to improve DNA affinity, but ligand binding and induction was impaired. Ligand binding spectrum of MphR(E) was shown to cover macrolides with a 14-membered lactone ring structure, but macrolides with a 16-membered ring or lincosamides showed no binding. MphR(E) and its mutants showed interesting novel characteristics that could benefit biosensor design.

In conclusion, this study shows the applicability of whole-cell biosensors in developing simple, robust and cost-effective screening methods for antimicrobials in food products. These methods show high sensitivity and specificity towards the target analyte, and can be used in semi-quantitative to quantative analysis. In addition to residue monitoring, whole-cell biosensors can be used for producer identification. The identified nisin producers can find use as protective starter cultures in fermented food production. The modified repressor MphR(E) shows promise as an improved regulator of reporter gene production in whole-cell biosensor applications, and is an example of purposeful effort to develop regulatory elements for novel biosensor designs.

TIIVISTELMÄ

Antibiootit ovat olleet tärkein bakteeri-infektioiden hoitokeino aina toisesta maailmansodasta lähtien. Bakteeri-infektiot ovat maailmanlaajuisten kuolemansyytilastojen kärkisijoilla. Antibioottien huolimaton käyttö on kuitenkin johtanut antibioottiresistenssin nopeaan leviämiseen bakteerien keskuudessa sekä useille antibiooteille resistenttien patogeenien kehittymiseen. Tämän vuoksi antibiootit menettävät nopeasti antimikrobiaalista voimakkuuttaan, mitä voidaan pitää yhteiskunnan kyvyttömyytenä suojella arvokasta pääomaansa.

Euroopan Unioni valvoo antibioottien käyttöä eläimissä, joita hyödynnetään elintarvikkeiden tuotannossa. Eläinlääketieteellistä käyttöä säädellään resistenssin leviämisen ja ruoassa esiintyvien antibioottijäämien estämiseksi. EU:n lainsäädäntö osoittaa enimmäisjäämärajat eläinlääkinnällisille aineille eläinperäisissä elintarvikkeissa ja velvoittaa jäsenvaltiot laatimaan ja toteuttamaan eläimistä saatavien elintarvikkeiden kansallisen vierasainevalvontaohjelman. Valvontaan valittujen näytteiden joukosta seulotaan näytteet, joiden epäillään sisältävän jäämiä, joiden luonne ja pitoisuus varmistetaan lisäanalyysillä. Seulonnassa käytetään tavallisesti mikrobiologiseen kasvuinhibitioon perustuvia menetelmiä, ja varmistukseen fysikaalis-kemiallisia analyysejä.

Tässä tutkimuksessa tarkasteltiin kokosolubioantureilla tehtävien antibioottimääritysten soveltuvuutta antibioottijäämien seulontamenetelmäksi. Tetrasykliinispesifistä bioluminoivaa kokosolubiosensoria käyttäen kehitettiin seulontamenetelmä tetrasykliinijäämien tunnistamiseksi kananlihasta. Määritys saatiin herkistettyä EU:n enimmäisjäämärajojen tasolle helpottamalla tetrasykliinien pääsyä bioanturisoluun solukalvon läpäisykykyä lisäävällä polymyksiini B:llä sekä kahdenarvoisia kationeja kelatoivalla EDTA:lla. Tuloksena oli nopea, yksinkertainen ja kustannustehokas seulontamenetelmä, jolla oli korkea suoritusteho. Menetelmä kykeni havaitsemaan kaikki neljä eläinlääketieteessä käytettävää tetrasykliiniä sekä niiden 4-epimeeri aineenvaihduntatuotteet kananlihassa enimmäisjäämärajat alittavissa pitoisuuksissa. Tutkimus myös tuotti todisteita tetrasykliinien 4-epimeerien antimikrobiaalisesta aktiivisuudesta, joka aiemmin arveltiin puuttuvan 4-epidoksisykliiniltä.

Nisiini on laktokokkien tuottama lantibiootti eli peptidiantibiootti. Nisiinin käyttö elintarviketeollisuudessa säilöntäaineena (E234) sekä EU:n nisiinille asettama sallittu enimmäismäärä elintarvikkeissa luovat tarpeen nisiinin määritysmenetelmille. Tässä tutkimuksessa rakennettiin bioluminoiva nisiinispesifinen kokosolubioanturi, jota käytettiin määrittämään nisiinipitoisuuksia maidossa. Kehitetty määritys oli nopea ja yksinkertainen, eikä vaatinut laimennusta monimutkaisempaa näytteen esikäsittelyä. Määrityksen herkkyys oli alle pg/ml mittaluokassa, ja se oli herkin koskaan julkaistu nisiinimääritys. Määritystä käytettiin myös nisiinintuotannon tehokkuuden arvioinnissa mittaamalla nisiinipitoisuus sitä tuottavan *Lactococcus lactis* -kannan kasvumediumista. Samanaikaisesti nisiinintuottaja voitiin erottaa nisiiniä tuottamattomista kannoista. Tätä ajatusta tarkasteltiin laajemmin jatkotutkimuksessa,

jossa nisiinibioanturia käytettiin seulomaan nisiinintuottajakantoja raakamaidosta. Yksinkertainen seulontamenetelmä perustui raakamaitobakteerien viljelmien peittämiseen ohuella bioanturikerroksella, jolloin nisiinintuottajapesäkkeiden ympärille muodostui bioluminesenssivyöhyke. Seitsemän tunnistettua nisiinintuottajapesäkettä jakautuivat geneettisen sormenjäljen perusteella kolmeen ryhmään, ja ne olivat kaikki *Lactococcus lactis* subsp. *lactis* -alalajiin kuuluvia nisiini Z -variantin tuottajia. Lisäksi 91 laktokokkikannan paneelista tunnistettin kolme nisiini A -variantin tuottajaa. Spesifisyystutkimukset osoittivat, että vain nisiini indusoi bioluminesenssin bioanturibakteerissa eivätkä muut bakteriosiinipeptidit. Lisäksi kaikki nisiinigeeniä kantavat pesäkkeet indusoivat bioluminesenssin. Poikkeuksena oli yksi laktokokkikanta, jonka todettiin kantavan toimimatonta nisiinigeeniä.

Tietyille antibioottiryhmille spesifisen vasteen antavien säätelyelementtien puute saattaa vaikeuttaa kokosolubioanturien kehittämistä. Tässä tutkimuksessa karakterisoitiin makrolidispesifisen repressoriproteiini MphR(E):n DNA- ja ligandinsitomisominaisuuksia. Proteiinia muokattiin rationaalisen mutaatiosuunnittelun keinoin tarkoituksena tuottaa DNA- ja dimerisoitumisominaisuuksiltaan parempia mutantteja. DNA- ja ligandisitoutumista sekä makrolidiligandien indusoimaa dissosiaatiota DNA:sta tutkittiin fluoresenssianisotropialla ja massaspektrometrialla. Tutkimuksessa löydettiin mutantteja, joilla oli villityypin proteiinia parempi DNA-affiniteetti sekä ennallaan säilynyt kyky sitoa ligandeja ja indusoitua sitomisen vaikutuksesta. Yksi mutanteista muodosti rikkisillan avulla kovalenttisen dimeerin vastoin odotuksia. Kovalentti dimerisaatio paransi DNA-affiniteettia, mutta haittasi ligandin sitomista sekä induktiota. MphR(E):n ligandikirjo kattoi 14-jäsenisen laktonirenkaan makrolidit, mutta ei 16-jäsenisen renkaan makrolideja eikä linkosamideja. MphR(E) ja sen mutantit osoittivat mielenkiintoisia uusia ominaisuuksia, jotka voivat hyödyttää bioantureiden suunnittelua.

Johtopäätöksenä voidaan sanoa, että tämä tutkimus osoittaa kokosolubioanturien soveltuvan yksinkertaisten, luotettavien ja kustannustehokkaiden seulontamenetelmien kehittämiseen antibioottijäämien osoittamiseen elintarvikkeista. Nämä menetelmät osoittavat suurta herkkyyttä ja spesifisyyttä analyyttimolekyyliä kohtaan, ja niitä voidaan käyttää semikvantitatiiviseen sekä kvantitatiiviseen analysiin. Jäämien havainnoinnin lisäksi bioantureita voidaan käyttää antimikrobiaalisten aineiden tuottajien tunnistamiseen. MphR(E)-mutantit ovat lupaavia paranneltua säätelykykyä osoittavia reportterigeenin tuoton repressoreita käytettäväksi bioanturisovelluksissa. Ne ovat myös esimerkki rationaalisesta säätelyelementtien parantelusta uusien bioanturisovellusten kehittämiseksi.

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LIST OF ORIGINAL PAPERS

This thesis is based on the following original papers, referred to in the thesis by the roman numerals.

I Immonen, N., Karp, M. 2007. Bioluminescence-Based Bioassays for Rapid Detection of Nisin in Food. *Biosens Bioelectron.* 22, 1982-1987.

II Virolainen, N., Guglielmetti, S., Arioli, S., Karp, M. 2012. Bioluminescence-Based Identification of Nisin Producers – a Rapid and Simple Screening Method for Nisinogenic Bacteria in Food Samples. *Int. J. Food Microbiol.* 158, 126–132

III Virolainen, N.E., Pikkemaat, M.G., Elferink, J.W., Karp, M.T. 2008. Rapid Detection of Tetracyclines and Their 4-Epimer Derivatives from Poultry Meat with Bioluminescent Biosensor Bacteria. *J. Agric. Food Chem.* 56, 11065–11070.

IV Virolainen, N., Valjakka, J., Torvinen, M., Viitamäki, J., Larjo, A., Jänis, J., Karp, M. Structural and Mutational Studies on DNA Binding and Dimerization Properties of the Macrolide Specific Repressor Protein MphR(E). *Manuscript submitted for publication.*

The experimental work was carried out under supervision of Professor Matti Karp.

Some unpublished results are also discussed in this thesis. The author has also written the following publication related to the topic of this thesis:

Immonen, N., Karp, M., 2006. A luminescent nisin biosensor. Proceedings of SPIE, 6098. Progress in Biomedical Optics and Imaging, Vol. 7, No. 21. ISSN 1605-7422.

THE AUTHOR'S CONTRIBUTION

Paper **I**:

Nina Virolainen (née Immonen) wrote the paper and is the corresponding author. She planned and conducted the experimental work and interpreted the results.

Paper **II**:

Nina Virolainen wrote the paper and is the corresponding author. She planned and performed the experimental work and interpreted the results. Genetic fingerprinting of bacterial isolates was performed by S. Guglielmetti.

Paper **III**:

Nina Virolainen wrote the paper and is the corresponding author. She planned and conducted the experimental work excluding the microbiological growth inhibition assay, and interpreted the results. M. Pikkemaat advised in manuscript writing, planning the experiments and interpreting the results.

Paper **IV**:

Nina Virolainen wrote the paper and is the corresponding author. She planned and carried out protein mutation, expression and purification, and performed the electrophoretic mobility shift assay and fluorescence anisotropy experiments. J. Valjakka instructed the rational design of the protein mutants and advised in manuscript writing. M. Torvinen performed the mass spectroscopy experiments. J. Viitamäki participated in rational design of the protein mutants and introducing these mutations. A. Larjo performed curve fitting of the polarization data. J. Jänis instructed planning the mass spectrometry experiments and participated in writing the paper regarding mass spectrometry results.

ABBREVIATIONS

16S rRNA	16S ribosomal RNA
aa-tRNA	Aminoacyl transfer RNA
ABC transporter	ATP-binding cassette transporter
ATC/DDD	Anatomical therapeutic chemical classification system with defined daily doses
BOX-PCR	Repetitive BOX element sequence-based PCR fingerprinting
CDC	Centers for Disease Control and Prevention
CMT	Chemically modified tetracycline
CTC	Chlortetracycline
DC	Doxycycline
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EU	European Union
HAI	Hospital acquired infection
IC	Induction coefficient
IP	Identification point
K_d	Dissociation constant
LAB	Lactic acid bacteria
LB	Luria-Bertani broth
M17	M17 medium by Terzaghi and Sandine
M17GCm	M17 medium supplemented with 0.5% v/v glucose and 10 μg/ml chloramphenicol
MFS	Major facilitator superfamily
MLS	Macrolides, lincosamides and streptogramins
MMP	Matrix metalloproteinase
MRSA	meticillin-resistant *Staphylococcus aureus*
NAT	Nouws antibiotic test
NICE	Nisin-controlled gene expression system
OIE	World Organization for Animal Health
OTC	Oxytetracycline
PCR	Polymerase chain reaction
PMB	Polymyxin B
RLU	Relative light unit
RNA	Ribonucleic acid
RPP	Ribosomal protection protein
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SOE-PCR	gene splicing overlap extension PCR
STAR	Screening test for antibiotic residues

TC	Tetracycline
TEV	Tobacco etch virus
TFRs	TetR family transcriptional regulators
TMS	Transmembrane sequence
TCS	Two-component system
VRE	Vancomycin-resistant enterococci
WHO	World Health Organization
WVA	World Veterinary Association

1 INTRODUCTION

Microbes are a double-edged sword to the mankind. Man has harnessed microbes for use in areas like food production, bioremediation and manufacturing valuable substances. On the other hand, microbial pathogens cause infection in humans, food animals and crops alike. One of the most important missions of human and veterinary medicine is defeating these microbial diseases. Simultaneously, medicine benefits from microorganisms as probiotics and in the form of molecules like drugs, vaccines and antibodies discovered and/or produced in microbes. Antibiotics are one such group of medicinal substances, originally produced by and discovered in microbes, and then utilized by men in an attempt to overcome microbial infection.

The widespread and often excessive use of antibiotics in health care and agriculture has led to the appearance of resistant pathogens. This is surmised to lead to an emergence of novel perilous infections and a revival of diseases that were already considered beaten. Antibiotic resistance is a common phenomenon that has evolved simultaneously with the capacity to produce antibiotics, dating back millions of years. However, due to human activity, the fraction of resistant organisms has risen above normal during the last seven decades (Martinez 2009). The problem of increasing resistance has been taken into account by international organizations and governments which have devised guidelines for antibiotic stewardship in both veterinary and human medicine. However, there still are many countries where the use of antimicrobials is controlled laxly or not at all. Resistant strains originating from these regions can stymie efforts in countries attempting to control their own antibiotic use.

EU legislation enforces countries to establish a national monitoring plan, under which a set percentage of animal products is monitored for antibiotic and other residues using screening and confirmatory methods of predetermined quality (EC 1996, 2002b). Microbial growth inhibition tests are currently the most commonly used screening method for antibiotic residues in food. These methods, however, have sensitivity and specificity problems which can lead to false negative results.

This study concentrates on a novel screening method, antimicrobial residue assays based on inducible bioluminescent whole-cell biosensors. These biosensors are living bacterial cells which have been genetically engineered to produce bioluminescence in the presence of the analyte, the antimicrobial agent. Whole-cell biosensors are an affordable screening method, which can offer sensitive and specific analyte recognition. The recognition element in the biosensor is typically a regulatory protein which recognizes the analyte and induces signal production. This study also examines the structure-function properties of a recently discovered regulatory protein and modifies it for improved biosensor performance. Biosensor assays are also applicable in recognition of novel antimicrobial agents, modes of action, and producer strains. This study uses whole-cell biosensors for rapid and specific identification of antimicrobial-producing bacteria.

2 ANTIMICROBIAL AGENTS

"The dose makes the poison", a statement made famous by the renaissance physicist and scientist Paracelsus, is still a basic principle of toxicology. In the context of antimicrobial agents, the same principle means that an antimicrobial effect is a quality possessed by any substance. Bacteria are well-known for their adaptability to any kind of conditions: living bacteria can be found in almost any corner of the world, from the deep sea to arid deserts. However, too much (or little) of anything will kill them. As an example, curing with salt, pickling with vinegar and drying with heat are used in food preservation to create conditions of high ionic strength, low pH and little moisture to inhibit bacterial growth.

Physical (heat, pressure, radiation) as well as chemical agents have antimicrobial activity. Antimicrobial agents (also known as antimicrobial substances or antimicrobials) can be defined as "natural or synthetic chemical compounds that kill or inhibit the growth of microorganisms" (Madigan and Martinko 2006). Examples of these are substances we encounter in our daily life such as ethanol, hydrogen peroxide and iodine-containing compounds used as topical antiseptics and chlorine, ozone and copper sulfate used for water disinfection.

Microbes produce a vast number of antimicrobial agents for use as signaling molecules that shape the structure of microbial communities and to outcompete organisms occupying the same ecological niche (Romero *et al.* 2011). The hormesis theory is a synthesis of these two effects: antibiotics are beneficial to bacteria at low concentrations found in most ecosystems and harmful in high concentrations used for therapy (Martinez 2009). Humans have adopted some of these substances for use as antimicrobial drugs and preservatives to inhibit bacterial disease and food spoilage. The following chapters introduce more closely two groups of antimicrobial agents that include the biosensor target analytes of this study. The first is antibiotics, an extremely important group of antimicrobials used in human and animal medicinal therapy. The second one is bacteriocins, and more exactly nisin, a lantibiotic bacteriocin widely used as a food preservative.

2.1 Antibiotics

Antibiotics are one of the most well-known and well-used groups of antimicrobial agents and the number one asset of medicine in fighting microbial infection. They represent 46% of sales of all anti-infective agents, and a total of 5% of the entire global pharmaceutical market (Hamad 2010). A textbook definition for antibiotics is "natural rather than synthetic antimicrobial compounds (that are) produced by a wide range of fungi and bacteria and inhibit or kill other microorganisms" (Madigan and Martinko 2006, p. 685).

The busiest time in antibiotic discovery commenced after World War II, when within ten years, most of the antibiotic classes still in clinical use were discovered (Table 2.1.). The "Golden Age" that began with discovery of novel natural antibiotic classes (1950-1960) and continued with development of semisynthetic antibiotics, modified derivatives of natural antibiotics, lasted until the 1980's. This era produced numerous improvements to antibiotic potency and ability to avoid resistance through chemical modification of existing antibiotic scaffolds. The number of naturally occurring antibiotics increased from ~30 known in 1945 to 150 in 1949, 450 in 1953, 1200 in 1960, 10000 by 1990 and ~16.500 by 2002 (Borders 2007). However, only around 150 of these were used in veterinary and ~100 in human therapy in 2002 (Bérdy 2005).

To overcome resistance mechanisms, development of synthetic antibiotics becomes more important as the discovery of new natural antibiotic classes is declining (Clark *et al.* 2011). Despite the effectiveness of novel methods for development of synthetic antibiotics (Clark *et al.* 2011, Sun *et al.* 2011), biosynthetic and fermentative approaches remain the most cost-effective methods for large-scale production of antibiotics (Khosla and Tang 2005). Engineered biosynthesis pathways must be therefore be developed on basis of known biosynthesis pathways of natural antibiotics, making industrial scale production of these novel molecules less straightforward (Pickens and Tang 2009, 2010, Wang *et al.* 2011).

Like any antimicrobial agent, antibiotics can have a bacteriostatic or bactericidal effect. Bacteriostatic agents inhibit bacterial reproduction without affecting viability, whereas bactericidal agents kill their target. The major classes of antibiotics with bactericidal effects are β-lactams, aminoglycosides and quinolones, whereas the remaining classes mainly have bacteriostatic effects (Kohanski *et al.* 2010b). Antibiotic-mediated cell death only begins with the primary effect of antibiotic-target interaction (see Table 2.1.). An oxidative damage cellular death pathway has been identified as a common secondary mechanism for cell death induced by bactericidal antibiotics (Kohanski *et al.* 2010b). Table 2.1 presents some of the most important antibiotic classes and their modes of action. Among these are protein synthesis inhibitors tetracyclines as well as macrolide, lincosamide, and streptogramin (MLS) class antibiotics that are more closely introduced in the following chapters. These two antibiotic classes are the target analytes of biosensor development in this study.

Table 2.1. Antibiotics classified according to the Anatomical Therapeutic Chemical Classification System with Defined Daily Doses (ATC/DDD) by the World Health Organization (WHOCC 2011).

Antibiotic class	Year of discovery	Mode of action	Representative members	Ref
Tetracyclines J01A*	1948 (Chlortetracycline)	Protein synthesis inhibitors. Inhibition of aa-tRNA binding.	Tetracycline Oxytetracycline Chlortetracycline Doxycycline	[2]
Amphenicols J01B	1947 (Chloramphenicol)	Protein synthesis inhibitors. Blocking of peptidyl transferase activity of the ribosome.	Chloramphenicol Thiamfenicol	[2]
β-lactam antibacterials: penicillins J01C	1928 (Benzylpenicillin)	Cell wall synthesis inhibitors. Inhibition of the transpeptidation reaction during peptidoglycan formation.	Benzylpenicillin Ampicillin Methicillin Cloxacillin	[2]
Other β-lactam antibacterials J01D	1948 (Cephalosporin C) 1984 (Aztreonam, monobactam) 1985 (Imipenem, carbapenem)	Cell wall synthesis inhibitors. Inhibition of the transpeptidation reaction during peptidoglycan formation.	Cephalosporins Monobactams Carbapenems	[1], [2]
Sulfonamides and trimethoprim J01E	1935 (Sulfonamido-chrysoidine)	Bacterial metabolism interference. Inhibition of synthesis of DNA precursors.	Trimethoprim Sulfonamides	[3]
Macrolides, lincosamides and streptogramins J01F	1952 (Erythromycin) 1963 (Lincomycin) 19xx (Streptogramin)	Protein synthesis inhibitors. Blocking access of peptidyl-tRNA to the ribosome → elongation reaction inhibition → dissociation of peptidyl-tRNA.	Erythromycin Tylosin Lincomycin Clindamycin Pristinamycin	[1], [2]
Aminoglycoside antibacterials J01G	1944 (Streptomycin) 1963 (Gentamicin)	Protein synthesis inhibitors. promotion of tRNA mismatching → protein mistranslation and premature termination.	Streptomycins Neomycin Kanamycin Gentamicin	[2]
Quinolone antibacterials J01M	1960 (Nalidixic acid) 1978 (Fluoroquinolones)	DNA replication inhibitors. Prevention of DNA strand rejoining by topoisomerases (DNA gyrase and topoisomerase IV).	Quinolones Nalidixic acid Flumequin Fluoroquinolones Enrofloxacin Difloxacin	[2]
Other antibacterials J01X	1939 (Tyrothricin) 1956 (Vancomycin) 1961 (Fusidic acid)	Various mechanisms	Glycopeptide antibacterials Vancomycin Lipopeptide antibacterials Daptomycin Imidazole derivatives Metronidazole Pleuromutilins Tiamulin Polymyxins Tyrothricin Colistin Polymyxin B Steroid antibacterials Fusidic acid Rifampicin Ketolide antibacterials Telithromycin Cethromycin	[1]

*ATC/DDD classification code. [1] Borders 2007, [2] Kohanski *et al.* 2010b, [3] Stokstad and Jukes 1987.

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2.1.1 Tetracycline antibiotics

The first member of the tetracycline (TC) group (Figure 2.1A), chlortetracycline, was described in 1948 under the name aureomycin (Duggar 1948). From then on, numerous novel TCs were discovered (1st generation 1948–1963) or synthesized (2nd generation 1965–1972: semisynthetic TCs), and the group rapidly increased in size until the beginning of the 1970s (Chopra and Roberts 2001). The first 3rd generation semisynthetic member to the TC family, tigecycline (Fig. 2.1C), was introduced in 1993 (Testa *et al.* 1993). It is a derivative of the TC group member minocycline, but since it is not affected by some of the key resistance mechanisms to TCs, it has also been given a first-in-class status in a new antibiotic class, glycylcyclines (Pankey 2005). Recently, two novel classes of tetracycline analogs, pentacyclines (Sun *et al.* 2011) and 8-azatetracyclines (Clark *et al.* 2011), were introduced. Both classes consist of fully synthetic molecules, and represent 4th generation tetracyclines.

The first studies conducted on tetracyclines showed they are broad-spectrum antibiotics affecting both gram-positive and gram-negative bacteria (Paine *et al.* 1948). They exert their bacteriostatic antimicrobial activity by inhibiting protein biosynthesis. TC molecules bind the high affinity TET1 site on the 30S ribosomal subunit (Aleksandrov and Simonson 2008a). TET1 is located on the 16S rRNA, lying right above the ribosomal A site, the binding site of aminoacyl-tRNA (Brodersen *et al.* 2000). Therefore, TCs inhibit translation by allosteric blocking of aa-tRNA binding. Binding to other sites such as translation elongation factor EF-Tu and a low affinity site TET5 on the 30S subunit has been suggested, but it does not occur in significant amounts under physiological conditions (Aleksandrov and Simonson 2008a, 2008b).

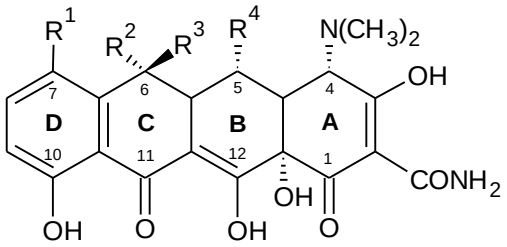
In both cytoplasm and outside the cell, TC exists in equilibrium between a neutral form and a charged Mg^{2+}-tetracycline chelate. This phenomenon plays an important role in TC antibiotic activity and its inhibition as only the chelate can bind TET1 or resistance protein expression-controlling repressor protein TetR (Lederer *et al.* 1995, Aleksandrov and Simonson 2008a). On the other hand, entry into the cell requires a dissociation step: the chelate passes the outer membrane through porins, but only the metal-free form can diffuse through the cytoplasmic membrane (Schnappinger and Hillen 1996). Therefore, the intra- and extracellular concentration of Mg^{2+} ions is of utmost importance to effectiveness of TC action.

In addition to bacteriostatic action, several non-antimicrobial effects of tetracyclines have been discovered. TCs have long been applied in treatment of dermatological conditions such as rosacea or acne, where overexpression of cellular pathways can be affected by TCs (Monk *et al.* 2011). The TC doxycycline (DC) has been found to be effective in prevention and treatment of malaria (Tan *et al.* 2011). It interferes with protein synthesis, DNA replication and transcription in the organelles apicoplasts and mitochondria of the malaria-causing parasite *Plasmodium falciparum* (Briolant *et al.* 2010).

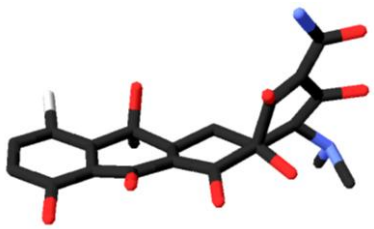
The non-antimicrobial effects of tetracyclines are typically organ protective. For instance, tetracyclines inhibit the function of matrix metalloproteinases (MMPs), scavenge reactive oxygen species and show anti-apoptotic as well as anti-inflammatory effects (Griffin *et al.* 2011). These effects together with TCs' ability to accumulate at tissue-injury sites have led to clinical trials to explore their possible beneficial effects in a wide variety of diseases (Griffin *et al.* 2010). Especially chemically modified tetracyclines (CMTs) show promise in treatment of non-microbial disease. These tetracycline analogs have been stripped of their antimicrobial activity by removing the 4-dimethylamino group but they still retain their organ protective effects (Griffin *et al.* 2011). CMTs show promise in treatment of advanced cancers, where they inhibit tumor cell proliferation and, unlike in normal cells, induce apoptosis (Lokeshwar 2011).

Tetracyclines are the most commonly used class of veterinary antibiotics: in a recent study covering 10 European countries, tetracyclines accounted for 48% of total sales of veterinary antibacterial agents in 2007 (Grave *et al.* 2010). The WHO (2009) ranking of antimicrobials important for human medicine gives tetracyclines a status as critically important antibiotics in the three-class system of critically important, highly important and important antimicrobials. Tetracyclines as well as other antimicrobials deemed critically important should be prioritized for most urgent development of risk management strategies in order to preserve their effectiveness in human medicine.

A)



B)



Generic name	Trade name	R^1	R^2	R^3	R^4
Chlortetracycline	Aureomycin	Cl	CH_3	OH	H
Oxytetracycline	Terramycin	H	CH_3	OH	OH
Tetracycline	Achromycin	H	CH_3	OH	H
Demethylchlortetracycline (Demeclocycline)	Declomycin	Cl	H	OH	H
Methacycline*	Rondomycin	H	CH_2		OH
Doxycycline*	Vibramycin	H	CH_3	H	OH
Minocycline*	Minocin	$N(CH_3)_2$	H	H	H

C)

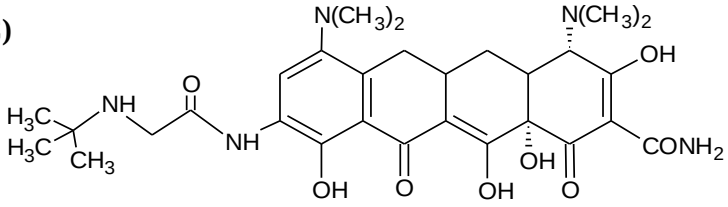


Figure 2.1. Chemical structure of tetracyclines. A) General structure for TCs and detailed structures of some representative members of the group. B) A bottom view of the chlortetracycline molecule shows the characteristic kink in the non-planar four-ring structure with carbons 1 - 3 above the plane. C) Structure of tigecycline. 3D-structure of chlortetracycline was taken from Orth *et al.* 1998, PDB file 1BJ0. *Semisynthetic molecules.

2.1.2 Macrolide, lincosamide and streptogramin (MLS) antibiotics

The first macrolide antibiotic, erythromycin A, was isolated under the name ilotycin from *Streptomyces erythreus* (McGuire *et al.* 1952), currently known as *Saccharopolyspora erythraea*. Erythromycin contains a 14-member lactone ring to which two cyclic saccharides, desosamine and cladinose, are attached (Figure 2.2A). The monocyclic lactone ring (also termed aglycone) is a structural element present in all macrolides. The highly substituted ring has between 12 to 16 members (Figure 2.2G), and one or more saccharides glycosidically attached to hydroxyl groups on either the aglycone or another saccharide (Kirst 2001). Naturally occurring macrolides have an aglycone size of 12, 14 or 16 members (Kirst 2005). Numerous semisynthetic macrolide derivatives have been constructed, some with deviating ring member numbers like the 15-membered azithromycin (Figure 2.2B).

Lincosamides and streptogramins are placed in the same antibiotic class with macrolides (WHOCC 2011). Although structurally very different, all three groups share a similar mode of action and resistance pattern (Vannuffel and Cocito 1996, Tenson *et al.* 2003, Roberts 2008). Lincosamides contain a proline residue attached by a peptide bond to a galactoside ring (Figure 2.2C) (Canu and Leclercq 2009). Streptogramins comprise of two components, streptogramin A (e.g. dalfopristin, pristinamycin II, or virginiamycin) and streptogramin B (e.g. quinupristin, pristinamycin I, or virginiamycin S) (Vannuffel and Cocito 1996) (Figures 2.2D and 2.2F). When streptogramin A or B components are applied singly, they have a bacteriostatic effect. However, together the components exert a strong synergistic bactericidal effect caused by mutual stimulation of the drug-ribosome interaction (Vannuffel and Cocito 1996, Porse and Garrett 1999).

Ketolides are the latest generation of antibiotics derived from erythromycin A by removal of the 3-L-cladinose sugar moiety and oxidation of the resulting 3-hydroxyl to a keto group (Figure 2.2E) (Douthwaite and Champney 2001). The 3-keto group is responsible for evading certain resistance mechanisms, and the C11-C12 carbamate residue as well as the groups substituting it help overcome further resistance mechanisms and enhance *in vivo* activity (Bryskier 2000).

MLS antibiotics exert their bacteriostatic activity by interacting with the 23S rRNA (especially intimately with A2058) of the 50S ribosomal subunit and to a lesser extent with L22 and L4 proteins (Bryskier 2000, Mankin 2008). This interaction blocks the peptide chain exit tunnel or directly inhibits the peptidyl transferase activity, causing dissociation of the nascent peptidyl-tRNA and inhibition of translation elongation (Tenson *et al.* 2003, Mankin 2008). In addition, macrolides and ketolides are able to inhibit the assembly of the 50S ribosomal subunit, and many carbamate ketolides inhibit assembly of the 30S subunit as well (Bryskier 2000, Douthwaite and Champney 2001). MLS antibiotics are broad-spectrum antibiotics whose activity covers both anaerobic and aerobic bacteria including gram-positive cocci and bacilli and gram-negative cocci (Canu and Leclercq 2009). Gram-negative bacilli are generally resistant with the exception of

some clinically important genera, i.e. *Bordetella*, *Campylobacter*, *Chlamydia*, *Helicobacter*, and *Legionella*.

Depending on the country, MLS is the third or fourth most used class of veterinary antimicrobials in Europe (EMA 2011). In 2009, macrolides/lincosamides accounted for 3.6% of veterinary antibiotic sales in Finland, whereas in Denmark, the corresponding figure was 13.4%. In the WHO (2009) ranking of antimicrobials important for human medicine, macrolides are not only classified as critically important, but are among the the top three critically important antimicrobial groups along with quinolones and 3rd and 4th generation cephalosporins. This is because macrolides are widely used in food animal production and are known to select for macrolide-resistant *Campylobacter* spp. in animals. At the same time, macrolides are one of few available therapies for serious *Campylobacter* spp. infections in humans. Streptogramins have been listed as critically important, and lincosamides as important to human medicine.

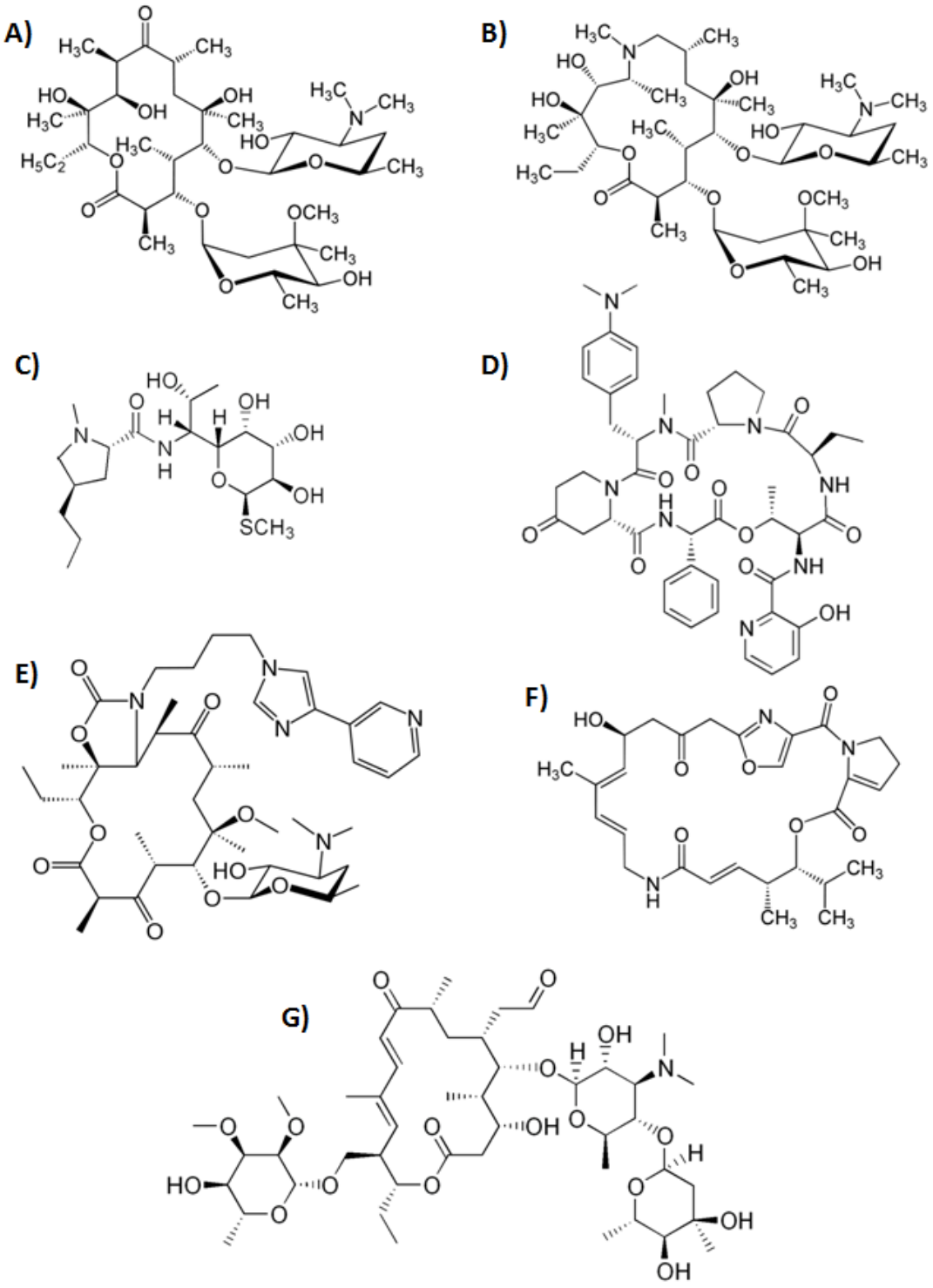


Figure 2.2. Structures of macrolide, lincosamide and streptogramin antibiotics. A) Erythromycin A, 14-membered macrolide. Of the two cyclic saccharides, desosamine is pictured above the cladinose residue.; B) Azithromycin, 15-membered macrolide; C) Lincomycin, lincosamide; D) Pristinamycin IA, streptogramin B component of pristinamycin; E) Telithromycin, 14-membered ketolide; F) Pristinamycin IIA, streptogramin A component of pristinamycin; G) Tylosin, 16-membered macrolide. The linked saccharides are mycinose (left), mycaminose (right) and mycarose linked to mycaminose.

2.2 Nisin, a lantibiotic bacteriocin

In addition to tetracycline amd MLS antibiotic analytes, this study includes development of a whole-cell biosensor assay for nisin, a lantibiotic and a bacteriocin. Like classical antibiotics, lytic agents and lysozymes, bacteriocins are antimicrobial agents produced to inhibit competing micro-organisms of the same niche, and have also been suggested to be used in acquiring transforming DNA from other species (Kreth *et al*. 2005, Riley 2011). Bacteriocins are defined as ribosomally produced protein antibiotics that have a relatively narrow killing spectrum, and to which the producer organism has an immunity mechanism (Cotter *et al*. 2005, Riley 2011). Classical peptide antibiotics are not ribosomally synthesized.

Bacteriocins are produced by a wide range of other bacteria including Gram-positive and Gram-negative species (Jack and Jung 2000). Gram-negative bacteriocins assemble into two families: high mass (30–80 kDa) colicins and low mass (1–10 kDa) microcins (Rebuffat 2011). Bacteriocins produced by lactic acid bacteria (LAB) were divided into four classes by Klaenhammer (1993), and this classification has since been modified and extended to cover all Gram-positive bacteriocins (Rea *et al.* 2011) (Table 2.2.).

Lantibiotics make up bacteriocin class Ia (Table 2.3). They are peptides ribosomally synthesized and posttranslationally modified to their active forms (Kuipers *et al.* 2011). Lantibiotics contain the unusual amino acids *meso*-lanthionine (Lan) and/or β-methyllanthionine (MeLan) (Figure 2.3A). Other unusual amino acids such as 2,3-didehydroalanine (Dha, dehydrated serine) and (*Z*)-2,3-didehydrobutyrine (Dhb, dehydrated threonine), lysinoalanine, hydroxyproline, D-cysteine etc. can be present (McAuliffe *et al.* 2001, Field *et al.* 2010). Lanthionines are embedded within cyclic lanthionine and methyllanthionine rings generated by intramolecular addition of cysteines to the dehydro amino acids (Figure 2.3B) (Chatterjee *et al*. 2005).

Table 2.2. Classification of bacteriocins produced by Gram-positive bacteria (Rea *et al.* 2011).

Class	Characteristics	Subclasses
Class I	*Post-translationally modified peptides*	
	a) Lantibiotics	Four subclasses I–IV
	b) Labyrinthopeptins	
	c) Sactibiotics	Two subclasses: single- and two-peptide bacteriocins
Class II	*Non-modified peptides*	
	a) Pediocin-like	Four subclasses I–IV
	b) Two-peptide bacteriocins	Two subclasses A and B
	c) Circular bacteriocins	Two subclasses 1 and 2
	d) Linear non-pediocin-like one-peptide bacteriocins	
Bacteriolysins	*Non-bacteriocin lytic proteins*	

Table 2.3. Classification of lantibiotics (Capstick *et al.* 2007, Goto *et al.* 2010, Rea *et al.* 2011). Modifying proteins are responsible for catalyzing the formation of lanthionine structures.

Subclass	Modifying protein	Description and characteristic members	Subclass	Modifying protein	Description and characteristic members
I	LanBC	Linear antimicrobial peptides. Secretion by ABC transporter LanT. *Nisin, subtilin*	III	RamC	Non-antimicrobial peptides involved in morphogenesis of streptomycetes. *SapB, AmfS, SapT*
II	LanM	Globular antimicrobial peptides. LanT has a dual function of secretion and modification. *Lactocin S, lacticin 3147*	IV	LanL	Non-antimicrobial peptide of unknown function. *Venezuelin*

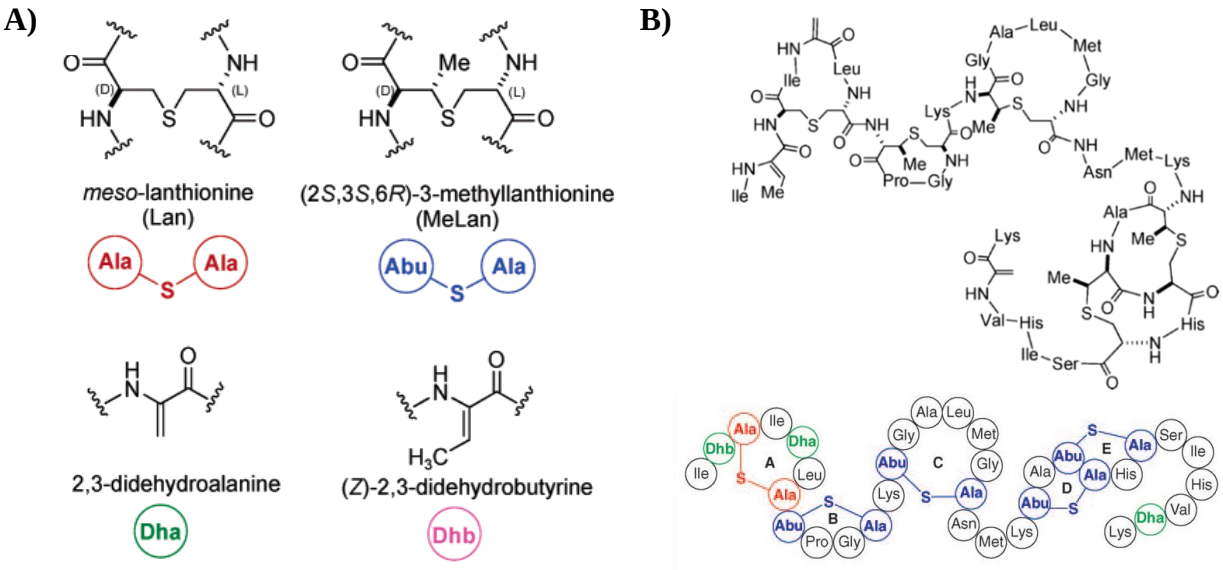


Figure 2.3. Structural motifs encountered in lantibiotics and structure of nisin. A) Abu, aminobutyrine; Ala, alanine; Dha, 2,3-didehydroalanine; Dhb, (*Z*)-2,3-didehydrobutyrine. B) The five lanthionine rings formed by addition of cysteines to Dha and Dhb residues are visible in the structure of nisin, the most studied lantibiotic. Above: molecular structure; below: ribbon structure. Adapted from Chatterjee *et al.* (2005).

Bacteriocins naturally produced by LAB are exploited by the food industry for food-grade control of bacterial spoilage (Cleveland *et al*. 2001, Chen and Hoover 2003, Cotter *et al*. 2005, Deegan *et al*. 2006, Gálvez *et al.* 2007). The use of nisin (Figure 2.3B) as a food preservative is approved in more than 80 countries (Delves-Broughton *et al*. 1996, Delves-Broughton 2005, Gálvez *et al.* 2011). In the EU, Directive 95/2/EC sets a maximum level of 3–12.5 mg/kg for nisin in various foodstuffs (EC 1995). In addition to food preservation, nisin is used or shows potential in medical applications such as contraceptive and disinfectant use, acne and mastitis treatment, tooth decay prevention and treatment of systemic, upper respiratory tract and ear infections (Reddy *et al.* 2004, Field *et al.* 2010, Dicks *et al.* 2011).

Nisin is the most studied bacteriocin, and a prototype lantibiotic. Several natural variants of nisin are known that differ in amino acid sequence but share an identical pentacyclic lanthionine ring structure: nisin A (Gross and Morell 1971), nisin Z (Mulders *et al.* 1991), nisin Q (Zendo *et al.* 2003), nisin U and nisin U2 (Wirawan *et al.* 2006), and nisin F (de Kwaadsteniet *et al.* 2008). All variants are produced by *Lactococcus lactis* except nisins U and U2 which are expressed by *Streptococcus uberis*.

Nisin exerts its antibacterial activity by forming pores in the cell membrane which leads to release of ions, amino acids, and ATP, and causes a collapse of the proton motive force and dissipation of the transmembrane pH gradient (Moll *et al.* 1997, Kuipers *et al.* 2011). Pore formation occurs through interaction with lipid II, a precursor of cell wall synthesis (Hasper *et al.* 2004). Displacement of lipid II by nisin also leads to inhibition of cell wall synthesis (Lubelski *et al.* 2008). Nisin also inhibits the outgrowth of bacterial spores, possibly through interaction of the dehydroalanine groups with spore membrane sulfhydryl groups (Morris *et al.* 1984). The antimicrobial spectrum of nisin covers a wide range of genera, including pathogens like Gram-positive *Listeria*, *Staphylococcus*, and *Clostridium*, as well as Gram-negative *Campylobacter jejuni* and *Helicobacter pylori* (Mota-Meira *et al.* 2000).

Bacteriocins have been suggested to form the next generation of antimicrobials (Gillor *et al*. 2005) since resistance to bacteriocins is infrequent and easily outcompeted by nonresistant strains (Dicks *et al.* 2011). As an example of this, nisin has been used as a food preservative for several decades without induction of widespread resistance (Christianson 2006). In addition to exploiting natural variants, bacteriocins may act as models for the design of novel antibiotics (Pag and Sahl 2002). Altered variants with enhanced antimicrobial activity have been constructed by modifying bacteriocin structural genes, and enzymes responsible for posttranslational modification have been utilized in introducing modifications in non-lantibiotic peptides to increase activity and protease resistance (Field *et al.* 2008, 2010, Kluskens *et al.* 2009, Kuipers *et al.* 2011).

3 ANTIBIOTIC USE AND ITS CONTROL

The discovery of traces of tetracycline in humal skeletal remains form Sudanese Nubia demonstrates that the ability to produce tetracycline through fermentation processes was occurring almost 2000 years ago (Bassett *et al.* 1980, Nelson *et al.* 2010). Another study of human skeletal material demonstrates possible health benefits of antibiotic use through low rate of infectious diseases in the Sudanese Nubian population (Armelagos 1969). During the post-World War II modern antibiotic era, antibiotics along with improved sanitation and application of vaccination have contributed significantly to the control of infectious diseases that were once among the leading causes of human morbidity and mortality (CDC 1999, Aminov 2010). Over the period 1937–1953, the annual decline in mortality rate in the United States increased from 2.3% to 8.2%, coinciding with commencement of clinical use of sulfonamides (1935), benzylpenicillin (1941) and streptomycin (1943) (Armstrong *et al.* 1999).

Veterinary antibiotic use has contributed to improvements in animal health and welfare and to a marked increase in productivity of livestock for human consumption (EMEA 1999). In animal husbandry, antibiotics are used for disease therapy and control as well as growth promotion. Disease control refers to prophylactic treatment of all animals in a group when one or a number of group members show signs of disease (Gustafson and Bowen 1997). Growth promotion with subtherapeutic doses of antibiotics increases viability, rate of weight gain, and reduces the amount of feed per unit of gain (Gustafson and Bowen 1997, Dibner and Richards 2005). Several mechanisms are associated with growth promotion: decreased competition of nutrients and reduction of growth-limiting microbial metabolites due to diminished numbers of gut microbiota, enhanced nutrient digestibility due to thinning of the gut wall and intestinal villi, reduction in opportunistic pathogens and subclinical infection, and finally, decreased continuous host immune stimulation by microflora (Visek 1978, Gaskins *et al.* 2002, Dibner and Richards 2005).

From the moment antibiotics were discovered, they have been used excessively and with little attention to the inevitable consequence of resistance (Dryden *et al.* 2009). Antibiotics not only act on the pathogenic bacteria causing the infection, but also on a myriad of commensal bacteria. These can then disseminate widely, creating a reservoir of resistant organisms (Wright 2007, Carlet *et al.* 2011). The first reports of resistance to antibiotics appeared shortly after the earliest clinical trials and therapeutic use of antibiotics commenced in the 1940's (Abraham and Chain 1940, Waksman *et al.* 1945, Miller and Bohnhoff 1946).

To add to the problem of increasing resistance, the discovery and development of new antibiotics has almost stopped since pharmaceutical companies withdrew from antibacterial R&D in the 1990s or earlier (Theuretzbacher 2009, Shryock and Richwine 2010). The reasons are many: focusing on products of higher profit and short-term returns, shrinking margins caused by low reimbursement rates and generic products, difficulty of creating new classes of antibiotics to meet increasing antibiotic resistance, pressure to use new compounds sparingly to avoid

resistance, and inconsistency of regulatory policies leading to uncertainty over final approval of the drug (Christoffersen 2006, Shryock and Richwine 2010). The interest of the pharmaceutical industry in antimicrobials is, however, returning in the form of premium-priced narrow-spectrum antibiotics for targeted therapy of multidrug-resistant bacteria (Theuretzbacher 2009).

3.1 Human and animal use of antibiotics in numbers

The antibiotics market amounted to global sales of US$ 25 billion in 2005 and US$ 42 billion in 2009, representing 5% of the global pharmaceutical market (Hamad 2010). Cephalosporins are the antibiotic class bringing in the highest revenue, owning more than one quarter of total global market (Figure 3.1). The total global usage of antibiotics is estimated to be between 100 000 and 200 000 tons per year (Moreno-Bondi 2009) including antibiotics used in human and veterinary medicine and as growth promoters. Veterinary antibiotics make up approximately one-third of total antibiotic sales in the EU (Moreno-Bondi 2009), yielding sales of 705 million € in 1996 (EMEA 1999).

The use of veterinary medicinal antibiotics in the EU grew from approximately 3500 tons in 1997 to almost 5400 tons in 2004, a 54% increase (Table 3.1). The majority of this increase results from the gradually enforced (from 1999 to 2006) EU ban of using antibiotics for growth promotion that led to an increase in the use of therapeutic antibiotics contrary to the intended effect (Casewell *et al.* 2003, Stolker *et al.* 2007). However, the increase in veterinary usage leveled off between years 2005–2009 (EMA 2011). Figure 3.2 presents the use of antibiotics for human and veterinary medicine in France in from 1999 to 2005. France uses the highest amount of antibiotics in veterinary medicine in the EU as the country is among the top meat producers in the Union area. Unlike in the EU, in France total veterinary usage exceeds total human medicinal use (Moulin *et al.* 2008). Tetracyclines are the most used and MLS the third most used veterinary antibiotic class in France. However, the profile of antibiotic usage varies between different EU countries. In 2009, tetracyclines and macrolides/lincosamides accounted for 13.8% and 3.6% of sales of veterinary antibiotics in Finland, respectively, whereas in the Netherlands, the corresponding sales were 52.0% and 9.2% (EMA 2011).

Table 3.1. Antibiotic use in the EU and Switzerland in metric tons.

Year	Human medicine	Veterinary medicine	Growth promotion	Total	Ref.
1997	7659 t (60%)	3494 t (27%)	1599 t (13%)	12 752 t (100%)	[1],[2]
1999	8528 t (65%)	3502 t (29%)	786 t (6%)	13 216 t (100%)	[2],[3]
2004	n.d.	5393 t *	n.d.	n.d.	[4]

References: [1] EMEA 1999, [2] Ungemach *et al.* 2006, [3] Moreno-Bondi 2009, [4] Kools *et al.* 2008. N.d. = not determined. *estimated use in the EU.

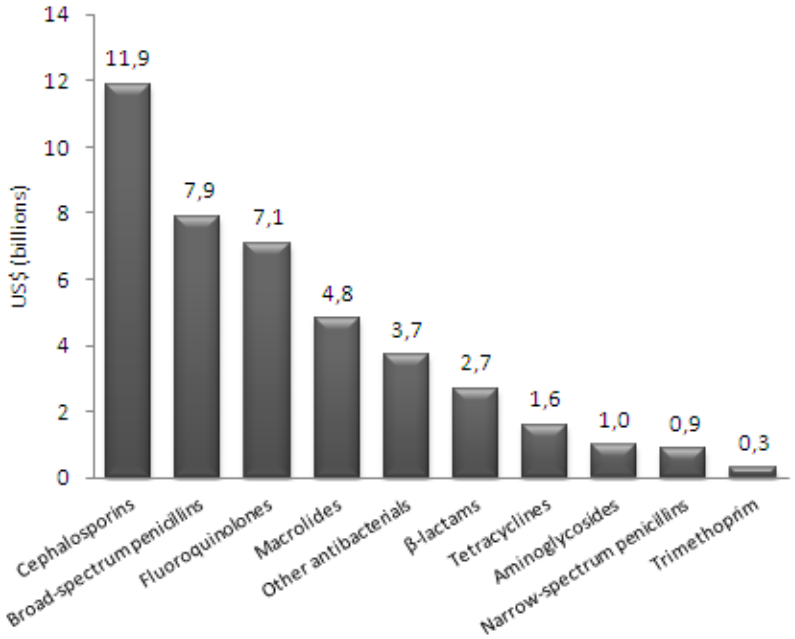


Figure 3.1. Global sales of antibiotics by class in 2009 (Hamad 2010).

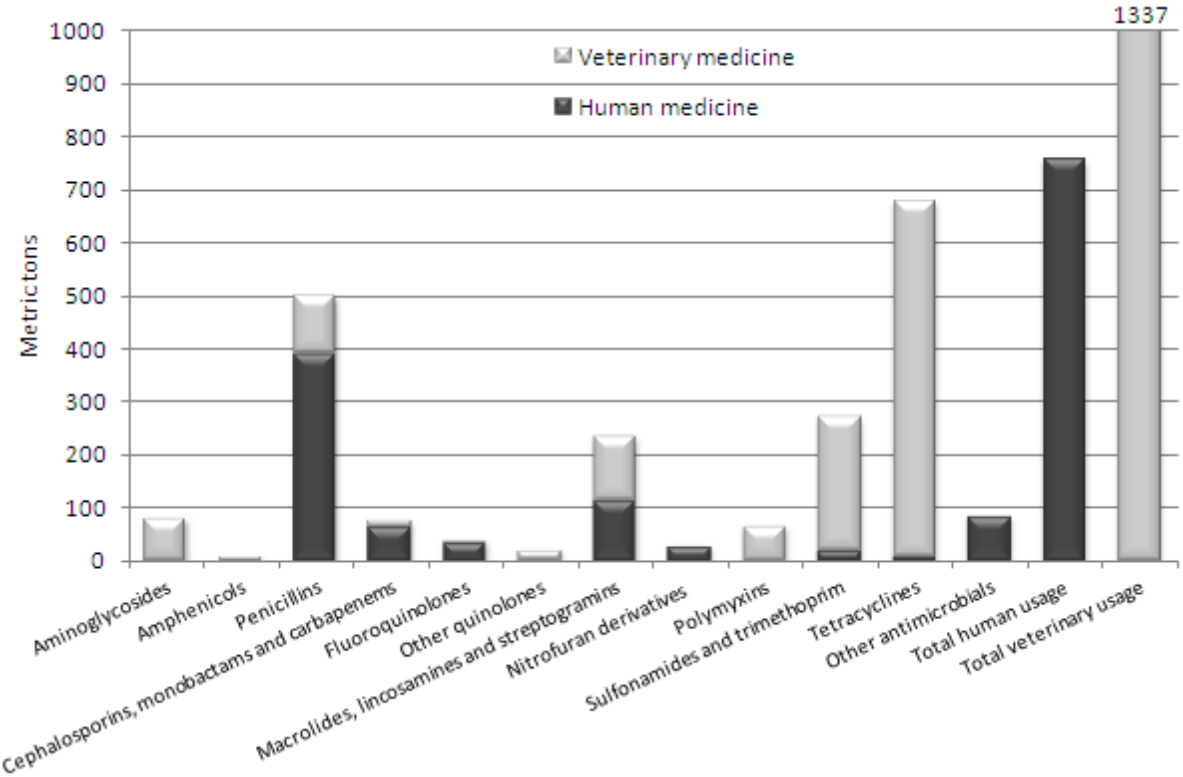


Figure 3.2. Veterinary and human medicinal usage of various antibiotic classes in France (adapted from Moulin *et al.* 2008).

3.2 Threats related to (mis)use of antibiotics

Human and animal health are integrally associated, as shown by zoonotic i.e. animal-originating pathogens such as *Listeria*, *Salmonella*, *Campylobacter* and *Escherichia coli* O157 (Aarestrup and Wegener 1999). Close relations exist between human and veterinary medicine, not just through diseases shared or transmitted from animal to human or vice versa, but also by common therapeutic agents and treatment methods (Currier and Steele 2011). Antibiotics are jointly used as a treatment for bacterial infections, and as a consequence, a pool of antibiotic resistance has emerged that has potential to spread between animals and men.

Antibiotics are misused through self-medication, prescription-free over the counter availability, and needless prescription to treat viral infections (Dryden *et al.* 2009). Also, antibiotics are overused in farmed animals through disease-control practices and increasing non-therapeutic (metaphylaxis/growth promotion) use (Carlet *et al.* 2011). It has been estimated that up to 50% of human antibiotic use and up to 80% of veterinary antibiotic use could be eliminated without serious consequence (Wise *et al.* 1998). Simultaneously, there are increasing demands for over the counter availability of antibiotics in order to reduce healthcare costs by encouraging patients to self-medicate (Dryden *et al.* 2009). Applications for prescription-free trimethoprim and nitrofurantoin in the UK were withdrawn in 2010 after much debate over associated risks (Dryden *et al.* 2009, Andalo 2010), but an application for release of trimethoprim to pharmacy availability has recently been submitted in New Zealand (Pharmacybrands Ltd. 2012).

Animals are treated to a lesser extent with antibiotics than humans: a 6.3-fold higher use of antibiotics in mg/kg of body mass per year has been estimated in humans (Ungemach *et al.* 2006). However, the conditions of antibacterial use in farm animals exert a high pressure for selection of resistance (Aarestrup and Wegener 1999, Cogliani *et al.* 2011). Over 80% of antibiotics are administered to food animals via oral flock treatment, in which whole animal herds are under long-term exposure to low levels of broad-spectrum antibiotics, and the risk for underdosing is high (Ungemach *et al.* 2006). Low concentrations of antibiotics have been discovered to cause radical-induced random mutagenesis, which in turn creates multidrug resistance to antibiotics also other than the one used for treatment (Kohanski *et al.* 2010a).

Nontherapeutic antibiotics are typically administered orally, and as antibiotics are typically poorly adsorbed in the gut, the majority is excreted unchanged in urine and feces (Sarmah *et al.* 2006). Antibiotic metabolites can also be antimicrobially potent, or can be transformed back to the parent compound (Aerts *et al.* 1995, Sarmah *et al.* 2009). Presence of antibiotic residues in urine and feces leads to spread of the drugs in the environment through wastewater or use as fertilizers. In addition, antibiotics can be disseminated in the environment through flushing of out-of-date or unused prescriptions, leakage from septic systems, land application of human, medical or agricultural waste, or direct application of antibiotics in the environment through aquaculture or plant spraying (Sarmah *et al.* 2006, Allen *et al.* 2010, Davies and Davies 2010).

These residues may assist in maintaining or developing antibiotic resistant microbial populations (Ghosh and LaPara 2007).

Not only antibiotics themselves should be considered a source of environmental pollution. Waste material from farms, homes and hospitals contains human- or animal-associated microbiota carrying antibiotic resistance determinants that can play a role in spreading of resistance in the environment (Martinez 2009, Allen *et al.* 2010). It is generally accepted that antibiotic therapy and growth promotion select for and increase the prevalence of antibiotic resistance in animal-associated microbiota (Aarestrup *et al.* 2000, Wright 2007, Davies and Davies 2010, Oliver *et al.* 2011, Carlet *et al.* 2011). It is, however, unclear whether the pool of resistance genes generated by antimicrobial use in food animals significantly influences the prevalence of therapeutic failures in humans (Cox and Ricci 2008, Wright 2010). The resistance problem in humans has mainly risen from human use, and antibiotic use in food animals may reduce the risk of zoonotic transmission of animal pathogens to humans (Gustafson and Bowen 1997, Casewell *et al.* 2003 Phillips *et al.* 2004, Cox and Ricci 2008). However, there are examples of human commensal and pathogenic isolates that are resistant to antibiotics used only in veterinary science, and evidence of transfer of human (multiresistant) pathogens to animals and vice versa (Aarestrup and Wegener 1999, van den Bogaard *et al.* 2000, Strommenger *et al.* 2006, Hunter *et al.* 2010).

Food is considered to be the most important vector for spread of resistance between humans and animals (WHO 2009). Evidence exists that ingestion of food contaminated by resistant bacteria selected in animals may lead to transfer of resistance determinants to bacteria in the human gut, or cause an infection in which therapy is compromised (Teale 2002, Cox and Ricci 2008, Allen *et al.* 2010). In addition, antibiotic residues in food products, a by-product of antibiotic use (Aerts *et al.* 1995), may allow the selection of antibiotic-resistant bacteria after the food is consumed (McDermott *et al.* 2002). There has been some concern about allergic reactions and toxicity effects caused by antibiotic residues in food, but the residue levels are generally too low to cause these adverse effects (Black 1984). Only a few reports on allergic reactions to β-lactam residues in milk exist (Dewdney *et al.* 1991).

3.3 Antibiotic resistomes

The discovery of tetracyclines in 1948 was soon followed by the first report of tetracycline resistance in 1953 (Roberts 1996). This was inevitable, since all bacteria harbor some degree of innate antibiotic resistance due to nonspecific efflux systems for expulsion of toxins, and most bacteria also have a reservoir of genes for more specific resistance towards antibiotics (Wright 2007, Allen *et al.* 2010, Davies and Davies 2010). In addition, bacteria can acquire specific and nonspecific resistance mechanisms through horizontal gene transfer: DNA can be taken up through transformation, conjugation or transduction of mobile genetic elements such as plasmids, insertion sequences, transposons and integrons (Schwarz and Chaslus-Dancla 2001, Partridge 2011). For the most part, these resistance elements originate from antibiotic producer self-protection mechanisms, and have likely been circulating in bacterial populations for millennia (Allen *et al.* 2010). However, mobile genetic elements frequently include co-selected multiple resistance genes encoding diverse modes of resistance to several antibiotic classes (Partridge 2011). These resistance plasmids and ensuing multiresistant strains are a result of a recent evolution process intensified by human activities (Davies and Davies 2010, English and Gaur 2010).

The increase in the number, diversity and range of resistant organisms has become an enormous clinical problem (Wright 2007). Selective pressure from antibiotic use has led to development of superbugs such as meticillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE), multiply resistant bacteria that are originally normal human commensal flora (Schwarz and Chaslus-Dancla 2001, Wright 2007, English and Gaur 2010). These together with natural superbugs, intrinsically multiply resistant opportunistic pathogens of environmental origin such as *Pseudomonas aeruginosa* and *Acinetobacter baumannii*, increasingly cause infections that either lead to or prolong hospitalization (Wright 2007, Davies and Davies 2010). Hospital acquired infections (HAI) caused by multiply resistant bacteria yearly affect around 7% *i.e.* over 4 million patients in the EU (ECDC 2008). Approximately 37,000 deaths are directly caused by HAIs, and they contribute to an additional 111,000 deaths. The total annual healthcare cost of nosocomial infections in the EU is estimated at € 7 billion.

The resistome is the aggregate of all antibiotic resistance mechanisms in both pathogenic and non-pathogenic bacteria (Wright 2007, Davies and Davies 2010). As an example of a resistome to an antibiotic family, the tetracycline resistome is presented in Table 3.2. The four main mechanisms of resistance are (*i*) target protection i.e. weakened interaction of drug and ribosome by ribosomal protection proteins (RPP) competing of binding site, (*ii*) efflux of TCs by integral membrane transporter pumps, (*iii*) drug inactivation by enzymatic modification and (*iv*) target modification, i.e. binding site modifying mutation of 16S rRNA (Zakeri *et al.* 2008, Thaker *et al.* 2010). Tet genes encoding TC resistance determinants are well conserved and widely expressed across various bacterial genera and species both aerobic and anaerobic as well as Gram-positive and Gram-negative (Li and Nikaido 2009, Macauley *et al.* 2007, Roberts 2003, Roberts 2005).

The list of macrolide, lincosamide, streptogramin (MLS) group resistome genes has grown from 44 known in 1999 to 66 in 2008 and 78 in 2011 (Roberts *et al.* 1999, Roberts 2008, 2011). These genes encode proteins providing four types of resistance mechanisms: (*i*) target-modifying rRNA methylases, (*ii*) efflux pump proteins, (*iii*) drug inactivating enzymes including esterases, lyases, transferases, and phosphorylases, and (*iv*) a rRNA methyltransferase. The rRNA methyltransferase has been classified separately since it confers resistance to lincosamides and streptogramin A but not macrolides. The other rRNA methylases generally give resistance to macrolides, lincosamides and streptogramin B antibiotics. In addition to the four classes mentioned above, mutant forms of 23S rRNA and ribosomal protein L4 and L22 genes have been identified that confer resistance to MLS antibiotics (Roberts 2008, Canu and Leclerq 2009) and could be included in the classification as a fifth group. Macrolide resistance is widespread in Gram-negative bacteria, most of which are intrinsically resistant to MLS antibiotics (Gibreel and Taylor 2006, Canu and Leclerq 2009). Macrolide resistance has been detected in a vide variety of Gram-positive bacteria including clinically relevant genera such as *Enterococcus*, *Streptococcus*, *Bacillus*, *Staphylococcus*, *Mycobacterium*, and *Clostridium* (Roberts *et al.* 1999, Jensen *et al.* 2002, Jalava *et al.* 2004).

Resistance to nisin is inherent in nisin producers that must harbor resistance genes to avoid auto-toxicity (Christianson 2006, Lubelski *et al.* 2008). On the nisin biosynthesis operon, *nisIFEG* genes are involved in producer self-protection. NisFEG form an ATP-binding cassette (ABC) transporter complex to extrude nisin from the membrane to the extracellular environment (Patton and van der Donk 2005, Lubelski *et al.* 2008). NisI reduces nisin concentration by sequestering nisin possibly through co-operation with NisFEG (Takala *et al.* 2004). Nisin resistance has been reported in nisin non-producing *Listeria monocytogenes* (Gravesen *et al.* 2001), *Bacillus subtilis* (Hansen *et al.* 2009, Staroń *et al.* 2011) and *Staphylococcus aureus* (Blake *et al.* 2011, Hiron *et al.* 2011). Nisin resistance is attributed to upregulation of ABC transporters, VraED in *S. aureus* and PsdAB and YvcRS in *B. subtilis*, that are involved in resistance to nisin and other peptide antibiotics in an unknown manner (Blake *et al.* 2011, Hansen *et al.* 2009, Hiron *et al.* 2011, Staroń *et al.* 2011). Bacteriocins have been suggested to form the next generation of antimicrobials (Gillor *et al*. 2005) since resistance to bacteriocins is infrequent (Dicks *et al.* 2011). However, these newly discovered peptide antibiotic resistance mechanisms may change this outlook.

Table 3.2. Tetracycline resistance mechanisms and proteins. TMS, transmembrane sequence; MFS, Major facilitator superfamily. Nomenclature and classification according to Levy *et al.* 1999, Roberts 2005 and Thaker *et al.* 2010.

Resistance type	Mode of action and details	Members	References
Target protection by ribosomal protection proteins	Weakening of the interaction between TC and ribosome causes TC dissociation. Simultaneous GTP hydrolysis leads to dissociation of RPP from the ribosome. RPPs share high homology to translation elongation factors EF-Tu and EF-G, and are proposed to be EF paralogs. Grouping based on the extent of amino acid sequence identity.	Group 1: Tet(M), Tet(O), Tet(S), Tet(W), Tet(32), Tet(36), Tet(44) Group 2: TetB(P), Otr(A), Tet Group 3: Tet(Q), Tet(T) Mosaic genes i.e. hybrids of known RPP genes also exist. Examples: Tet(O/32/O), Tet(O/W/32/O/W/O).	[1], [11], [12]
Efflux pumps	Integral membrane drug-H^+ antiporters. The exchange of antibiotic for H^+ creates a proton flow which provides energy for pumping. Members of the major facilitator superfamily (MFS) with 12–14 TSMs. Also some atypical MFS and non-MFS protein pumps	Group 1: 12 TMSs, MFS. Tet(A), Tet(B), Tet(C), Tet(D), Tet(E), Tet(G), Tet(H), Tet(Y), Tet(Z), Tet(30), Tet(31), Tet(33), Tet(39), Tet(41), Tet(42) Group 2: 14 TMSs, MFS, non-*Streptomyces* origin. Tet(K), Tet(L) Group 3: 14 TMSs, MFS, *Streptomyces* origin. OtrB, Tcr3 Group 4: 12 TMSs, atypical MFS. TetA(P), Tet(40) Group 5: ≥ 10 TMSs, atypical MFS. Tet(V) Group 6: 9 TMSs, non-MFS. Tet(35), OtrC Group 7: 14 TMSs, MFS, non-*Streptomyces* origin, not homologous to Tet(K). Tet(38)	[2], [3], [4], [5], [6], [7], [8], [11], [12]
Drug inactivation	Enzymatic inactivation by addition of a hydroxyl group to C-11a position. Mechanism also inactivates tigecycline.	Tet(X) Proposed members Tet(37) and Tet(34) (activity not confirmed)	[9], [12], [13]
Target modification	Mutant forms of the TC target, bacterial ribosome 16S rRNA.	(G → C) at position 1058 (AGA → TTC) at positions 926 - 928	[10], [12]

[1] Abril *et al.* 2010, [2] Agersø and Guardabassi 2005, [3] Brown *et al.* 2008, [4] Hansen *et al.* 1993, [5] Gordon *et al.* 2008, [6] Marshall *et al.* 1986, [7] McMurry *et al.* 1987, [8] McMurry *et al.* 1998, [9] Moore *et al.* 2005, [10] Ross *et al.* 1998, [11] Sloan *et al.* 1994, [12] Thaker *et al.* 2010, [13] Volkers *et al.* 2011.

3.4 Controlling measures on the use of antimicrobials

Growth enhancement use of antibiotics in the 1950's and 1960's led to an increase in antibiotic resistance in *Salmonella* strains associated with calf disease (EMEA 1999). Emergence of resistance led in the UK to the setting up of a Joint Committee on the Use of Antibiotics in Animal Husbandry and Veterinary Medicine, which in its 1969 report recommended antibiotics with therapeutic value should not be applied as growth promoters (House of Lords 1998). This "Swann Report" was the first action to begin the much-needed rationalization of antimicrobial use.

The World Health Organization (WHO) acknowledged the high importance of antimicrobial resistance as a threat to human and animal health by declaring antimicrobial resistance the topic of World Health Day 2011. WHO has devised a strategy for containment of antimicrobial resistance (2001a) as well as guidelines for rational drug use in humans (2002, 2007) and surveillance of antimicrobial resistance (2001b). WHO (2009) has also created a ranking of antibiotics critically important for human medicine which is intended to help develop prudent antimicrobial use in agriculture and veterinary medicine.

Responsible veterinary and agricultural use of antibiotics has been considered by international organizations such as World Organization for Animal Health (OIE), Codex Alimentarius and World Veterinary Assiciation (WVA), which have published guidelines for prudent use of antimicrobial products in food animals (Codex Alimentarius 2005, OIE 2011, WVA 2011). Guidelines for responsible use of antimicrobials in human medicine have been provided in the U.S. by CDC - Centers for Disease Control and Prevention and in the EU by the European Council (Gonzales *et al.* 2001a, 2001b, EC 2002a, Dellit *et al.* 2007). Countries like Brazil, South Korea, Canada, Australia and New Zealand have also implemented policies and programmes to prevent the emergence of resistance through antibiotic stewardship *i.e.* appropriate antibiotic use (EC 2002a, Zoutman *et al.* 2003, Oh *et al.* 2006, Pagani *et al.* 2008, Guerra *et al.* 2010).

The EU has recently devised a 5-year plan consisting of twelve key actions against antimicrobial resistance (EC 2011). The plan noted that EU recommendations for prudent use in veterinary medicine should be introduced. Also, existing resistance monitoring programs in the EU require harmonization regarding antimicrobials surveyed, definition of resistance, and epidemiological cut-off values i.e. minimum inhibitory concentrations (MIC) used for designating wild type and resistant strains (Silley *et al.* 2011).

The EU has gradually enforced a total ban on use of growth promoters in food animals, taking full effect in 2006 (EC 2003b). The ban resulted in reduced antibiotic use and antibiotic resistance in animals. A temporary increase in the use of therapeutic antibiotics and in tetracycline and sulphonamide resistant *Salmonella* in animal and human infections was seen (Stolker *et al.* 2007, Casewell *et al.* 2003), but the increase in use has since leveled out (EMA

2011), and a decrease in antimicrobial resistance ensued (van den Bogaard *et al.* 2000, Bengtsson and Wierup 2006). Another example of successful control on antibiotic use comes from Germany: the national guidelines for prudent use of antibacterials in animals implemented in 2000 led to a 73% decrease in antibiotics prescribed as antibiotic-medicated feeding stuffs, and a 57% reduction in treatment days by the year 2002 (Ungemach *et al.* 2006).

EU legislation enforces countries to establish and execute a national monitoring plan, under which a set percentage of animal products should be monitored for (antibiotic) residues and other contaminants to promote food safety by ensuring residues do not reach the consumers as well as to establish prudent use of antimicrobials (EC 1996). An EU Council Regulation for the establishment of maximum residue limits (MRL) of veterinary medicinal products in foodstuffs of animal origin became effective in 1990 and was repealed in 2009 by an update, which recognized the effect of progress in detection methods and pharmacological, toxicological and microbiological effect assessments to establishing MRLs (EC 1990, 2009). In 2010, MRLs of pharmacologically active substances were combined under a single Commission Regulation (EC 2010a). EU MRLs for macrolide/lincosamide and tetracycline antibiotics are presented in Table 3.3.

The most recent annual report on the execution of EU national monitoring plans in 2009 (EC 2010b) recounts 445.968 samples were tested under the monitoring plans, fulfilling the requirements of the minimal amount of samples to be tested (EC 1996, 1997). Of these, 155.432 samples (34.9%) were tested for presence of antibacterials, and 332 samples (0.21%) were found noncompliant i.e. containing a concentration above the MRL. Of the noncompliant samples, 109 were found in pigs, 68 in bovines, 50 in milk, 32 in poultry and the rest in sheep/goats (28), honey (23), rabbits (9), aquaculture (9), horses (2), farmed game (1), and eggs (1). Honey had the highest prevalence of noncompliance, with 23 (0.98%) samples out of 2336 testing positive.

In Finland, the EU enforced national monitoring plan of animal product contaminants is enacted by the Ministry of Agriculture and Forestry (MAF) and executed by the Finnish Food Safety Authority Evira (MAF 2007, Forsbacka *et al.* 2011). In 2005, a total of 11.209 tests were carried out. The presence of antimicrobials was tested in 6.900 samples, with 3 (0.06%) pig samples and 3 (3.61%) honey samples found noncompliant for antimicrobial substances (Saraste *et al.* 2006). Out of all samples tested, 99.7% were compliant.

Table 3.3. European Union MRLs in μg/kg for tetracyclines and macrolide/lincosamides in various edible tissues (EC 2010a). The table also indicates the species in which the use of each substance is allowed.

Group	Antibiotic	Muscle	Fat	Liver	Kidney	Milk	Eggs	Species
Tetracyclines	Tetracycline	100		300	600	100	200	All food producing species [a)]
	Oxytetracycline	100		300	600	100	200	All food producing species [a)]
	Chlortetracycline	100		300	600	100	200	All food producing species [a)]
	Doxycycline	100		300	600			Bovine [b)]
		100	300	300	600			Porcine, poultry [c)]
Macrolides	Erythromycin	200	200	200	200	40	150	All food producing species [a),d)]
	Gamithromycin		20	200	100			Bovine [b)]
	Spiramycin	200	300	300	300	200		Bovine
		200	300	400				Chicken [c), d)]
		250		2000	1000			Porcine
	Tilmicosin	75	75	1000	250			Poultry [c), d)]
		50	50	1000	1000	50		All other food producing species [a),d)]
	Tulathromycin	100		3000	3000			Bovine [b)]
		100		3000	3000			Porcine [d)]
	Tylosin	100	100	100	100	50	200	All food producing species [a),d)]
	Tylvalosin	50	50	50	50			Porcine
			50	50				Poultry [c)]
Lincosamides	Lincomycin	100	50	500	1500	150	50	All food producing species [a),d)]
	Pirlimycin	100	100	1000	400	100		Bovine

[a)] For fin fish the muscle MRL relates to 'muscle and skin in natural proportions'. MRLs for fat, liver and kidney do not apply to fin fish.
[b)] Not for use in animals from which milk is produced for human consumption.
[c)] Not for use in animals from which eggs are produced for human consumption.
[d)] For porcine and poultry species the fat MRL relates to 'skin and fat in natural proportions'.

4 DETECTION METHODS OF ANTIBIOTIC RESIDUES IN FOOD

Antibiotics are considered one of the most significant groups of food contaminants (Kantiani *et al.* 2010). Antibiotic residues in products of animal origin are a by-product of antibiotic use formed through pharmacokinetic distribution of the drug within the body based on its physicochemical properties and metabolism (Sarmah *et al.* 2006). Over time, the drug and its metabolites are cleared from the body in excretions. Other factors which determine the occurrence of residues are the route of administration, contamination of feed or water, and the physical condition of the animal (Aerts *et al.* 1995). To avoid the appearance of residues in food, withdrawal periods have been assigned to various antibiotics based on pharmacokinetic data and elimination rates from the animal body (EC 1990, 2001, 2009). The withdrawl period is the span of time until a safe level in edible tissues and other products (milk, eggs, honey) is achieved. EU MRLs are the points of reference for the establishment of withdrawal periods (EC 1990, 2001, 2009). Failure to observe withdrawal times, as well as improper treatment records, extended usage or excessive dosage of can lead to presence of antibiotic residues in concentrations above the MRLs in food products (Bovee and Pikkemaat 2009).

European Commission Decision 2002/657/EC lays down performance and validation criteria for the screening and confirmatory methods used in national residue monitoring programs (EC 2002b). Methods of analysis of antimicrobials can be grouped in three categories: microbiological, immunochemical, or physicochemical (EC 2010b). Microbiological methods are fast screening methods which allow a high sample throughput but limited information is obtained about substance identity and its concentration in the sample. Immunochemical methods are rapid, selective, and sensitive and are widely applied in some areas of residue analysis, typically in screening for substances that cannot be discerned by microbiological growth inhibition. Physicochemical methods allow an accurate identification and quantification of the substance, and are therefore applied in confirmatory analysis of suspect samples identified by screening methods.

4.1 Confirmatory analysis of antibiotic residues

Physicochemical methods are typically used in confirmatory analysis of the presence and concentration of antibiotic residues in products of animal origin after they have been indicated by a screening test (EC 2010b). A confirmatory test involves a more sophisticated testing method providing full or complementary information enabling the substance to be identified precisely and confirming that the MRL has been exceeded (EC 2010b). Confirmatory methods are typically not suitable for screening since they are time-consuming, expensive, and require complex laboratory equipment as well as trained personnel (Cháfer-Pericás *et al.* 2010). Also, they typically require extensive sample-preparation based on liquid and solid-phase extraction and multi-step clean-up (Kinsella *et al.* 2009).

European Commission Decision 2002/657/EC lists suitable methods for quantitative confirmatory analysis of antibiotic residues (EC 2002b). These consist of chromatographic separation in combination with detection (Table 4.1.). The Decision also introduces identification points (IP), the basic idea of which is that a laboratory is allowed to use any spectrometric technique or combination of techniques to earn a minimum number of IPs necessary for proper identification of a component (Stolker *et al.* 2000). The minimum amount of IPs for identification of antimicrobials is three. As a consequence, methods based on chromatographic analysis followed by mass spectrometric detection are becoming the norm in confirming antibiotic residue identity and determining concentration (Stolker *et al.* 2007, McGlinchey *et al.* 2008, Boscher *et al.* 2010).

Alternative physicochemical methods for confirmatory analysis include capillary electrophoresis, which has been used to detect antibiotics in food matrices (García-Ruiz and Marina 2006). However, although the technique is less expensive and has higher separation efficiency than HPLC methods, the lower sensitivity of capillary electrophoresis may prevent detection at MRL (Hernández *et al.* 2003, McGlinchey *et al.* 2008). Immunoanalytical methods such as radioimmunoassays, fluoroimmunoassays, and the most commonly used enzyme-linked immunosorbent assays (ELISA) are quantitative and have a high sensitivity, capacity for high-

Table 4.1. Suitable confirmatory methods for veterinary drugs or contaminants in products of animal origin according to European Commission Decision 2002/657/EC. (EC 2002b).

LC or GC with mass-spectrometric detection	2-D TLC-full-scan UV/VIS
LC or GC with IR spectrometric detection	GC-electron capture detection
LC-full-scan DAD	LC-immunogram
LC -fluorescence	LC-UV/VIS (single wavelength)

LC, liquid chromatography; GC, gas chromatography; IR, infrared spectrometry; DAD, diode array detection; TLC, thin layer chromatography; UV/VIS ultraviolet/visible spectrophotometry; HPTLC, high-performance thin layer chromatography.

throughput, and often do not require complex sample clean-up (Pastor-Navarro *et al.* 2009). However, immunoassays are generally group-specific by nature, and therefore cannot offer direct identification of the analyte due to cross-reactivity towards structurally similar antibiotics (Korpimäki *et al.* 2004). Therefore, they are often better suited for use as screening methods.

As mentioned above, LC-MS methods form the majority of routine confirmatory methods, but other methods are also validated along the guidelines. Table 4.2 presents a selection of validated confirmatory methods for tetracycline and macrolide/streptogramin/lincosamide residues and briefly describes sample preparation and clean-up steps necessary for each method. In addition to the extraction and filtration steps described in Table 4.2, solvent changes by evaporation were often a part of the cleanup process, and the final step before LC analysis. Most methods described in Table 4.2 represent multiresidue methods which can detect antibiotics from other classes than TC and MLS groups (Granelli and Branzell 2007, Carretero *et al.* 2008, Chico *et al.* 2008, Kaufmann *et al.* 2008, Stolker *et al.* 2008, Granelli *et al.* 2009, Peters *et al.* 2009, Boscher *et al.* 2010, Dasenaki and Thomaidis 2010, Lopes *et al.* 2012, Tang *et al.* 2010), but results regarding only these two classes are concentrated upon. The methods mainly originate from different EU countries and have been validated for various food matrices in order to be applied in carrying out the national residue monitoring plan.

European Commission Decision 2002/657/EC states that as a part of assay validation, a decision limit (CCα) must be established for confirmatory methods used for identification and quantification of substances with an established MRL. CCα is the limit at and above which a sample is considered to be noncompliant with an error probability α of 5%. Therefore, it can be concluded with 95% certainty that a sample is non-compliant. Also a detection capability (CCβ) must be established for confirmatory methods, although it is considered more important in validation of screening methods. Methods used for residue detection of substances with an established MRL, such as antibiotics, must have a detection capability with a false compliant rate < 5% (EC 2002b). In other words, CCβ is the smallest amount of analyte that can be detected in a sample with 95% confidence. CCβ must be less than or equal to the MRL for less than 5% of noncompliant samples to give a false compliant result.

To ease the comparison of various methods and their qualification for MRL standards, CCβ has been given in Table 4.2 as a fraction of MRL. CCα corresponds to limit of quantification and CCβ to limit of detection which were used in assay validation prior to Decision 2002/657/EC and determined using various methods. The guidelines for establishing CCα and CCβ in Decision 2002/657/EC remove this variation.

Table 4.2. An overview of confirmatory methods validated according to Commission Decision 2002/657/EC for macrolide/lincosamide/streptogramin (MLS) and/or tetracycline (TC) group antibiotics.

Analytical method	Sample matrix	Sample preparation	Sample clean-up	Recovery (%)	CCβ (xMRL)	Analytes	Reference
UHPLC-MS/MS	Chicken muscle	LE with 1% acetic acid in ACN/H_2O (80:20 v/v); LLP by $MgSO_4$ and sodium citrate	Dispersive SPE with PSA sorbent; filtration	75–117	1.1–2	4 M	Lopes *et al.* 2012
UHPLC-MS/MS	Milk	LE with ACN	Filtration	58–128 (MLS)	n.r.	11 M, 2 L,1 S	Tang *et al.* 2012
HPLC-ESI-MS/MS	Animal feed	Ultrasonic LE CH_3OH/ CH_3CN/McIlvaine buffer pH 4.6 (37.5:37.5:25 v/v/v)	Dispersive SPE with PSA sorbent	51–63 (TC) 88–109 (MLS)	n.r.	3 TC 3 M, 1 L,1 S	Boscher *et al.* 2010
LC-ESI-MS/MS	Fish tissue	Ultrasonic LE with ACN/ MeOH (1:1 v/v) + 0.05% v/v formic acid	Filtration	40–100	0.1–0.3	5 TC	Dasenaki & Thomaidis 2010
LC-MS/MS	Milk and bovine muscle	Pressurized LE with ASE 200; LE of lipids with ether	Filtration	70–93	1.1–1.6	5M, 2L	Juan *et al.* 2010
HPLC-DAD	Bovine muscle	LE with MeOH/succinic acid buffer (1:1 v/v)	Column MCAC; McIlvaine buffer pH 3 elution; cartridge SPE; MeOH elution	91–104	1.2–1.6	4 TC, 3 epi-TC	Cristofani *et al.* 2009
LC-MS/MS	Bovine and porcine muscle	LE with MeOH/H_2O (7:3 v/v)	-	61–70 (TC) 75–84 (M)	1.4–1.9 (TC) 1.3–1.4 (M)	4 TC 4 M	Granelli *et al.* 2009
HRLC-TOF-MS	Muscle (various species), fish tissue (various species), eggs	LE with ACN/H_2O (6:4 v/v)	Column SPE; MeOH/ethyl acetate (1:1 v/v) elution for egg or MeOH/ACN (1:1 v/v) elution for muscle and tissue	63–120 (TC) 69–261 (ML)	≤2 (TC and ML)	4 TC 6M, 2L	Peters *et al.* 2009
LC-MS/MS	Bovine and porcine muscle	Pressurized LE with ASE 200	-	73–86 (TC) 82–91 (ML)	1.1–1.2 (TC) 1.1–1.2 (ML)	3 TC 4 M, 1 L	Carretero *et al.* 2008
UHPLC-ESI-MS/MS	Chicken muscle	LE with MeOH/H_2O (7:3 v/v)	Filtration	87–97	1.1–1.2 (TC) 1.1–1.2 (M)	4 TC 4 M	Chico *et al.* 2008

Analytical method	Sample matrix	Sample preparation	Sample clean-up	Recovery (%)	CCβ (xMRL)	Analytes	Reference
UPLC-TOF-MS	Muscle, kidney, liver	LE with ACN/succinate buffer (1:1, v/v); LLP with $(NH_4)_2SO_4$	Cartridge SPE; elution with ACN and ACN/succinate buffer (2:1 v/v)	60–148 (TC) 16–121 (ML)	1.1–1.4 (TC) 0.4–1.3 (ML)	6 TC 11 M, 5 L	Kaufmann *et al.* 2008
UHPLC-TOF-MS	Milk	LE with ACN	Column SPE; MeOH elution	n.r.	1.3–1.4 (TC) 0.3–1.3 (ML)	4 TC 6 M, 2 L	Stolker *et al.* 2008
LC-DAD	Liver and kidney (various species)	LE with McIlvaine buffer pH 3.5	Cartridge SPE; MeOH elution	40–88	1.0–1.3	7 M	Berrada *et al.* 2007
HPLC-DAD	Milk	LE with TFA/ oxalate buffer (1:10 v/v)	Cartridge SPE; elution with CH_3OH/CH_3CN/0.01 M oxalic acid (30:30:40 v/v/v)	98–111	1.0–1.1	7 TC	Samanidou *et al.* 2007
LC-MS/MS	Muscle and kidney (various species)	LE with MeOH/H_2O (7:3 v/v)	-	26–62 (TC) 44–104 (M)	Below MRL	4 TC 4 M	Granelli & Branzell 2007
HPLC-DAD	Bovine muscle	LE with citrate buffer pH 4	Cartridge SPE; elution with CH_3OH/CH_3CN/0.05 M oxalic acid (30:30:40 v/v/v)	91–104	1.1–1.2	5 TC	Samanidou *et al.* 2005

ACN, acetonitrile; ASE 200, accelerated solvent extraction system; epi-TC, 4-epimer metabolite of a tetracycline; HRLC, high-resolution liquid chromatography; LE, liquid extraction; LLP, liquid-liquid partitioning; LOD, limit of detection; LOQ, limit of quantification; MCAC, metal chelate affinity chromatography; n.r., not reported; PSA sorbent, primary-secondary amine sorbent; PVDF, polyvinylidene difluoride; SPE, solid-phase extraction; TFA, trifluoroacetic acid; TOF, time-of-flight; UHPLC, ultra high performance liquid chromatography.

4.2 Screening methods for antibiotic residues

Screening is used for large sample numbers to pinpoint suspect noncompliant samples to be subjected to confirmatory analysis. Methods used for screening can detect an analyte or a family at MRL level, and provide semi-quantitative or qualitative results (Cháfer-Pericas *et al.* 2010). Main requirements for a screening method include rapid analysis, ease of use, low set-up and running costs, high-throughput capacity, repeatability as well as high sensitivity (low amount of false negatives) and specificity (low amount of false positives) (Toldrá and Reig 2006).

Methods used for screening for antibiotic residues include immunoanalytical methods and biosensors, as well as methods typically used for confirmatory analysis (LC-MS, LC-UV/VIS, LC-fluorescence, LC-DAD) (Situ and Elliott 2005, Toldrá and Reig 2006, Peters *et al.* 2009, Cháfer-Pericas *et al.* 2010, Stolker *et al.* 2010, Verdon 2009). The majority of screening tests is, however, based on microbial growth inhibition. In 2001–2003, 15 EU reference laboratories reported 53% of muscle sample screening was performed using microbiological methods, and the second most common method, ELISA, was used in 21% of cases (Verdon 2009). A disadvantage of microbiological assays is that they cannot establish the identity of a compound, although they can be fairly group-specific (Pikkemaat *et al.* 2008, 2009a). However, they are cost-effective in situations where the bulk of samples is expected to be compliant (Pikkemaat 2009). Microbial growth inhibition assays are also suited for high-throughput, require no high-tech equipment or specialized technicians, and due to their general nature may detect unknown or new compounds lacking from the confirmatory method toolkit (Bovee and Pikkemaat 2009).

Growth-inhibition assays mainly come in two formats: the tube test and the (multi-)plate assay. In the tube test, growth of indicator bacteria in the test medium causes a pH-indicator color change which is absent during growth-inhibition (Le Breton *et al.* 2007, Gaudin *et al.* 2008). Simple use and commercial availability of tube tests has caused them to be widely applied both in the laboratory and in the field, where residue detection is necessary e.g. because inhibition of starter cultures of fermented milk products can cause major economic losses (Mitchell *et al.* 1998, Bovee and Pikkemaat 2009). A plate test comprises of an agar plate inoculated with the test organism (Ferrini *et al.* 2006, Gaudin *et al.* 2010). Diffusion of analyte into the agar causes a growth inhibition zone whose diameter is depends on analyte concentration. Detection of all veterinary relevant antibiotics requires multi-plate assays with conditions suitable on each plate for detection of one or select groups of antibiotics (Pikkemaat *et al.* 2009b).

According to European Commission Decision 2002/657/EC, a screening method must have a CCβ with a false compliant rate of < 5% (EC 2002b). A guideline document by EU Reference Laboratories describes in detail screening method validation through determination of stability, applicability and ruggedness, as well as selectivity and specificity (Anon 2010). Stability of the analyte and standard samples must be determined under various storage conditions. Applicability refers to usability in various sample matrixes, and ruggedness to the method's ability to

withstand minor variations occurring during laboratory analysis, such as age of reagents, temperature fluctuations, personnel changes etc. Selectivity and specificity refer to the power of discrimination between the analyte and coexisting (related) substances. Table 4.3 presents the few microbial screening methods validated along these guidelines.

Establishment of MRLs and performance criteria of analytical methods (EC 1990, 2002b, 2010a) was followed by a critical evaluation of the commonly used screening methods (Pikkemaat *et al.* 2008, 2009a, 2011, Gaudin *et al.* 2010). As Decision 2002/657/EC allows screening method development following the validation criteria, national monitoring plans are based on a variety of screening methods. The EU Four-Plate Test (EU4pt) (Bogaerts and Wolf 1980) was considered a gold standard for a long time, but has now been deemed insufficiently sensitive although it is still widely in use (Berendsen *et al.* 2011). Two commonly used commercial tube tests, Premi® Test and Delvotest® SP-NT, lack sufficient detection capability of several antibiotic groups, including tetracyclines (Le Breton *et al.* 2007, Gaudin *et al.* 2008). In addition, Premi® Test suffers from a comparatively high false-positive rate (Pikkemaat *et al.* 2011).

Validation of the Screening Test for Antibiotic Residues (STAR) used by the French national residue monitoring plan showed the CCβ values of most antibiotics tested, including tetracyclines and macrolides/lincosamides, were above the EU MRL values (Gaudin *et al.* 2004, 2010). In addition, group specificity was not achieved. Improved group-specificity was attained in the six-plate Combined Plate Microbial Assay (CPMA), but false compliant rate of < 5% was achieved only partially (Ferrini *et al.* 2006). The Nouws Antibiotic Test (NAT) used by the Dutch national residue monitoring plan yields group-specific detection and shows below- or near-MRL sensitivity towards most veterinary antibiotics (Pikkemaat *et al.* 2008, 2009a). However, since initial screening is performed to renal pelvis fluid extracted from kidney, high residue levels occurring solely in muscle may never make it to the post-screening step (Pikkemaat *et al.* 2011). NAT has a higher workload due to the extra post-screening step, which the CPMA test has been designed to avoid (Ferrini *et al.* 2006). On the other hand, post-screening reduces the number of samples subjected to costly confirmatory analysis (Pikkemaat *et al.* 2009a, 2009b).

The Finnish national monitoring plan uses microbial growth inhibition for the majority of screening: out of 6.900 samples tested for the presence of antimicrobials in 2005, 83.6% were processed with microbial growth inhibition and 16.4% with physicochemical methods (Saraste *et al.* 2006). For porcine and bovine kidney or muscle samples, a two-plate test was used with *Bacillus subtilis* BGA as the indicator organism, and Delvotest®SP-NT for milk samples. In the view of studies discussed above, these methods are not likely to detect all noncompliant samples. The widespread use of insufficiently sensitive methods is reflected by a proficiency test involving 23 laboratories performing residue screening in the EU (Berendsen *et al.* 2011). The false negative rate for microbial methods was 73% compared to 22% for chemical methods, and only 39% of the laboratories identified the test samples correctly.

Table 4.3. Microbial growth-inhibition screening methods validated according to CD 2002/657/EC. Detection capability CCβ is reported as a fraction of the MRL for only tetracycline (TC) and macrolide/lincosamide (ML) antibiotics.

Test	Test format	Indicator organism	Analytes	Selective conditions and supplements	Sample matrixes	CCβ (xMRL)	References
Two-plate microbiological method	Multi-plate	*Bacillus subtilis* BGA *Bacillus subtilis* BGA	TC,Q S	pH 6, NaOH pH 7.5, TMP, PABA	Shrimp	0.28–0.65 (4TC)	Dang *et al.* 2010
Screening Test for Antibiotic Residues (STAR)	Multi-plate	*Bacillus subtilis* BGA *Kocuria rhizopila* ATCC 9341 *Bacillus cereus* ATCC 11778 *Escherichia coli* ATCC 11303 *Bacillus stearothermophilus* ATCC 10149	TC, AF M, C TC Q, C B, S, C, Q, ML, AG, AF	pH 8 pH 8 pH 6 pH 8 -	Milk[2], muscle	1–2.5 (2TC) 2 – 5 (2M, 1L)	Gaudin *et al.* 2010
Explorer®	Microtiter plate	*Geobacillus stearothermophilus*	S, TC, M, AG	-	Eggs	7.5–20 (4TC) 2–2.5 (2M)	Gaudin *et al.* 2009
Premi®Test	Tube	*Bacillus stearothermophilus*	S, TC, M, AG	-	Eggs	1–10 (4TC) 0.25–0.5 (2M)	Gaudin *et al.* 2009
Nouws Antibiotic Test (NAT) Post-screening[1]	Multi-plate	*Bacillus cereus* ATCC 1178 *Kocuria rhizopila* ATCC 9341 *Yersinia ruckeri* NCIM 13282 *Bacillus pumilus* CN 607	TC B, ML Q AG	pH 6, 30 °C pH 6, TY, CX, 30 °C pH 6.5, 30 °C pH 8, 37 °C	Muscle, kidney	0.25–0.5 (4TC) 0.07–1 (5M, 2L)	Pikkemaat *et al.* 2009a, 2011
Nouws Antibiotic Test (NAT) Initial screening[1]	Multi-plate	*Bacillus cereus* ATCC 1178 *Kocuria rhizopila* ATCC 9341 *Yersinia ruckeri* NCIM 13282 *Bacillus pumilus* CN 607 *Bacillus subtilis* BGA	TC B, ML Q S,D AG	pH 6, CAP, 30 °C pH 8, 37 °C pH 6.5, 30 °C pH 7, TMP, 37 °C pH 8, 37 °C	Renal pelvis fluid	0.013–0.08 (4TC) 0.5–4 (5M, 2L)	Pikkemaat *et al.* 2008
Delvotest® SP-NT	Tube	*Bacillus stearothermophilus*	B, S, C, AG, TC	-	Milk	2 (1TC)	Le Breton *et al.* 2007

AG, aminoglycosides; AF, amphenicols; B, β-lactams; C, cephalosporines; CAP, chloramphenicol; CX, cloxacillin; D, diaminopyridines; M, macrolides; ML, macrolides and lincosamides; n.r., not reported; PABA, 4-aminobenzoic acid; Q, quinolones ; S, sulfonamides; TC, tetracyclines; TMP, trimethoprim; TY, tylosin.

[1]NAT consists of an initial screening performed to renal pelvis fluid samples, and a corroborating post-screening step for suspect samples using one of the four plates described and muscle or kidney fluid as sample.

[2]STAR has been validated for milk (Gaudin *et al.* 2004) following an intralaboratory procedure and not Decision 2002/657/EC criteria. Validation for muscle was done following 2002/657/EC.

5 BIOSENSORS FOR DETECTION OF ANTIBIOTICS IN FOOD

Biosensors are an emerging class of methods suitable for screening purposes. By definition, biosensors combine a biological recognition element with a transducer to produce a measurable signal proportional to the concentration of the analyte (Velasco-Garcia and Mottram 2003, McGrath *et al.* 2012). Figure 5.1 presents a general working principle of biosensors and the types of recognition elements and transducers typically used in biosensors.

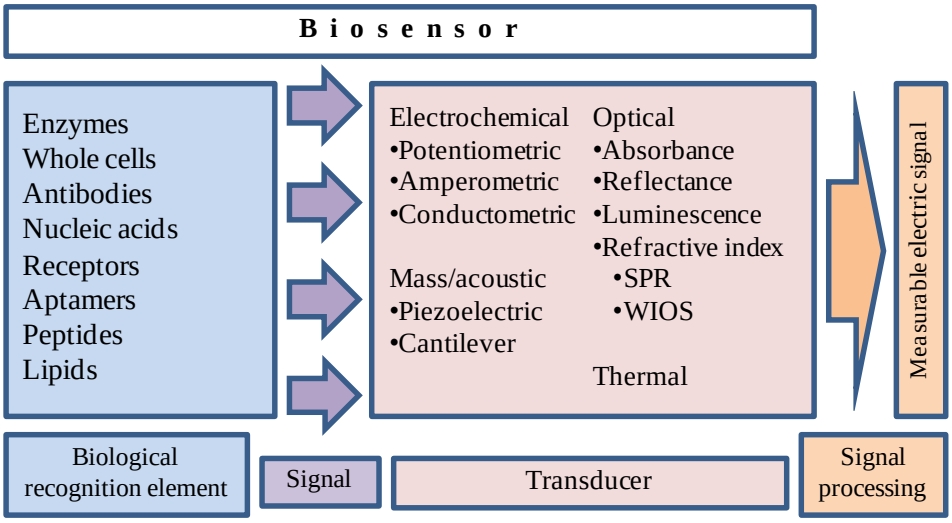


Figure 5.1. The principle of biosensors. Biosensors combine a biomolecule-based recognition element with a transducer, which converts the signal triggered by the recognition event to a quantifiable electric signal (Velasco-Garcia and Mottram 2003, McGrath *et al.* 2012). SPR, surface plasmon resonance; WIOS, wavelength-interrogated optical system.

Although biosensors are mostly used for antibiotic detection in the environment, they are also increasingly used in screening for antibiotic residues in food, currently in 8% of screening cases (Cháfer-Pericas *et al.* 2010, Reder-Christ and Bendas 2011). Table 5.1 gives an overview of biosensors developed for antibiotic detection in food. Biosensor assays have a high capacity for automatization and high-throughput, produce results rapidly, and typically require no or very simple sample pretreatment (Toldrá and Reig 2006, Huet *et al.* 2010). Limitations of biosensor methods include instability of the biorecognition element due to conditions like pH, ionic strength and temperature it is exposed to during immobilization and the assay (Cháfer-Pericas *et al.* 2010). Even so, biosensor methods often are robust enough to allow regeneration, so successive cycles of analysis can be performed with the same recognition molecules (Caldow *et al.* 2005, McGrath *et al.* 2005, Marchesini *et al.* 2007, Fernández *et al.* 2009, Adrian *et al.* 2009). Surface plasmon resonance (SPR) appears to be the transducer of choice for antibiotic detection, as it is utilized in 49% of published detection methods (Reder-Christ and Bendas 2011). SPR allows easy-to-use, real-time, label-free studying of biomolecular interactions (Huet *et al.* 2010).

In accordance with the role of biosensors as an emerging screening tool, a few biosensor screening methods have been validated according to 2002/657/EC (Ashwin *et al.* 2005, Caldow *et al.* 2005, Stead *et al.* 2011). Biosensor methods are typically suitable only for screening due to cross-reactivity within antibiotic groups. However, a biosensor using a ssDNA aptamer as the recognition element has been reported to specifically detect tetracycline among tetracycline antibiotic family members (Kim *et al.* 2010). An interlaboratory study compared a SPR biosensor screening assay for fluoroquinolones in various food matrices with established microbiological growth inhibition and LC-MS/MS methods (Weigel *et al.* 2009). The study demonstrated that unlike the microbiological assay, the biosensor method correctly identified all samples and demonstrated advantages in sensitivity and analysis time. However, assay costs were higher using the biosensor assay (30–50 €/sample) than the microbiological method (5–15 €/sample), which may curb the interest in SPR-based biosensor screening methods.

To establish biosensors as a screening method for antibiotic residues they have been studied in combination with confirmatory methods for simultaneous development of a comprehensive detection procedure. Ashwin *et al.* (2005) developed an SPR biosensor screening and LC-MS/MS confirmatory method for chloramphenicol residues in four different food matrices and performed validation of the method according to 2002/657/EC. Marchesini *et al.* (2007) developed a dual SPR biosensor assay, where suspect samples from the first round of SPR are subjected to HPLC fractionation, a second round of SPR, and finally LC-ESI-TOF-MS confirmatory analysis to identify and quantify residues in positive fractions harboring fluoroquinolone receptor binding activity.

Future directions in antibiotic biosensor development include assay multiplexing and portable devices for field use (Huet *et al.* 2010). As an example of multiplexing, recently developed SPR biosensor microarrays simultaneously detect on a single sensor chip two aminoglycoside antibiotics or compounds from four major antibiotic families: aminoglycosides, sulfonamides, amphenicols, and fluoroquinolones (Rebe Raz *et al.* 2008, 2009). A biosensor based on a wavelength-interrogated optical system (WIOS) transducer can simultaneously detect sulfonamide, fluoroquinolone, β-lactam and tetracycline antibiotics on a multianalyte sensor chip (Adrian *et al.* 2009). Portable multiplex SPR biosensors have been developed for *on-site* analysis of milk samples for fluoroquinolone family compounds or sulfonamide, chloramphenicol, and fluoroquinolone residues (Fernández *et al.* 2010, 2011). Commercialization of biosensors requires wireless technology, automatization and miniaturization, which also must be future directions of antibiotic biosensor development (Luong *et al.* 2008).

Proteins, i.e. enzymes and bioreceptors have traditionally been used as biological recognition elements in antibiotic biosensors. A new type of recognition element, a DNA-based aptamer was recently introduced in tetracycline detection (Kim *et al.* 2010). Proteins can also be modified for improved biosensor performance: a fluorescein-labeled β-galactosidase mutant with reduced catalytic activity was used as a recognition element for β-lactams in a fluorescence-based biosensor (Chan *et al.* 2004).

Table 5.1. An overview of biosensors for antibiotic detection. The references were chosen on basis of demonstrated applicability in a food matrix except Kim *et al.* (2010) who introduced a new interesting recognition element, a ssDNA aptamer, in antibiotic biosensors.

Biological recognition element	Recognition event	Tranducer	Sample matrix	Sample pretreatment	Analyte	Sensitivity (LOD)	Reference
DD-carboxypeptidase	Inhibition	SPR	Milk	Dilution	7 B	0.5 – 2 x MRL	Gustavsson *et al.* 2004
β-lactamase	Hydrolysis	Amperometric pH detector	Milk	Protein removal by salting-out	1 B	2000 x MRL	Chen *et al.* 2010
Polyclonal antibody	Binding	SPR	Honey	LLP with potassium buffer/hexane (5:3 v/v), SPE, MeOH elution	TY	2.5 μg/kg[1]	Caldow *et al.* 2005
Polyclonal antibody	Binding	SPR	Chicken muscle	Homogenization, ultrafiltration, clean-up with SPE, MeOH/ACN (8:2) elution	6 FQ	< MRL	Marchesini *et al.* 2007
Antibody	Binding	SPR	Milk, honey, prawn, porcine kidney	Varies depending on the matrix, includes LE, LLP, SPE	CAP	0.3 – 0.7 x MRPL[2]	Ashwin *et al.* 2005
Monoclonal antibody	Binding	SPR	Milk	Hydrolysis of the β-lactam ring	9 B	≤ MRL	Gaudin *et al.* 2001
Monoclonal antibody	Binding	SPR	Chicken serum	Dilution	8 S	0.07 – 0.2 x MRL	Haasnoot *et al.* 2003
Monoclonal antibody	Binding	Bioluminescence	Milk	-	2 B, 1 C, 2 S, 3 AG, 2 M	0.002 – 0.8 x MRL	Knecht *et al.* 2004
Monoclonal antibody	Binding	SPR	Milk	Dilution and filtration	1 FQ, 1 S, CAP	0.02 x MRL 3.6 x MRPL[2]	Fernández *et al.* 2010
Antibody and receptor	Binding	WIOS	Milk	Dilution	1 S,1 FQ, 1 B, 1 TC	0.005 – 0.8 x MRL	Adrian *et al.* 2009
Repressor protein	Binding	SPR	Milk, honey	Dilution (honey); heating and centrifugation (milk)	7 TC, 3 epi-TC	0.15–0.25 x MRL	Moeller *et al.* 2007
SBP (sulfonamide binding protein)	Binding	SPR	Porcine muscle	Homogenization, liquid extraction	20 S	0.2 x MRL	McGrath *et al.* 2005
ssDNA aptamer	Binding	Voltammetric	Buffer	-	1 TC	4 μg/kg	Kim *et al.* 2010
Microbial cell	Growth inhibition	Potentiometric CO_2 detector	Milk	-	7 Q, 3 TC	0.25 – 0.8 x MRL	Pellegrini *et al.* 2004

ACN, acetonitrile; AG, aminoglycosides; B, β-lactams; C, cephalosporines; CAP, chloramphenicol; epi-TC, 4-epimer metabolite of a TC, FQ, fluoroquinolones; LE, liquid extraction; LLP, liquid-liquid partitioning; LOD, limit of detection, M, macrolides; MeOH, methanol; Q, quinolones, S, sulfonamides; SPE, solid-phase extraction SPR, surface plasmon resonance; TC, tetracyclines; TY, tylosin; WIOS, wavelength-interrogated optical system.

[1]No EU MRL for tylosin in honey has been set (EC 2010a) so no detectable residues are allowed.

[2]The use of chloramphenicol in treatment of food animals in the EU is prohibited. Therefore, an MRL is not set. The EU minimum required performance limit (MRPL) for chloramphenicol residues in food products of animal origin is 0.3 μg/kg (EC 2002b, 2003a).

5.1 Whole-cell biosensors

Whole-cell biosensor assays are an emerging bioactivity-based screening method for antibiotic residues (Bovee and Pikkemaat 2009). The principle is more widely applied in environmental monitoring (Daunert *et al.* 2000, Köhler *et al.* 2000, Nivens *et al.* 2004, Harms *et al.* 2005, Woutersen *et al.* 2011) but food control applications are increasing (Kurittu *et al.* 2000a, 2000b, 2000c, D'Souza 2001, Hakovirta *et al.* 2006, papers **I**, **III**). In whole-cell biosensors, the living cell functions as the biological recognition element, which in the event of biosensing produces a specific signal to be transduced into a quantifiable electrical signal (Daunert *et al.* 2000, D'Souza 2001).

Whole-cell biosensor bacteria can be divided in systems with constitutive or inducible expression (Daunert *et al.* 2000, Woutersen *et al.* 2011). The former has a high continuous expression of signal, which decreases under toxic conditions ("turn off"). This type of detection is highly nonspecific, as signal decrease is a result of any type of cytotoxic effect (Andrew and Roberts 1993, Vesterlund *et al.* 2004). Inducible expression, however, is more specific, as transcription of the reporter gene occurs only when the stimulus is present (Figure 5.2). Specificity is achieved by employing a promoter-regulatory protein pair which recognizes and reacts to the stimulus ("turn on") (Daunert *et al.* 2000, Su *et al.* 2011).

Inducible whole-cell biosensors can be further divided into effect- and compound-specific sensors (Daunert *et al.* 2000, Yagi 2007, Woutersen *et al.* 2011). The former are stimulated by a a change in a physicochemical condition (pH, temperature, osmotic pressure, electron potential) or specific type of toxicity (DNA, protein or membrane damage or oxidative stress) by coupling the reporter gene to a promoter involved in the stress response (van der Meer *et al.* 2007, Shapiro

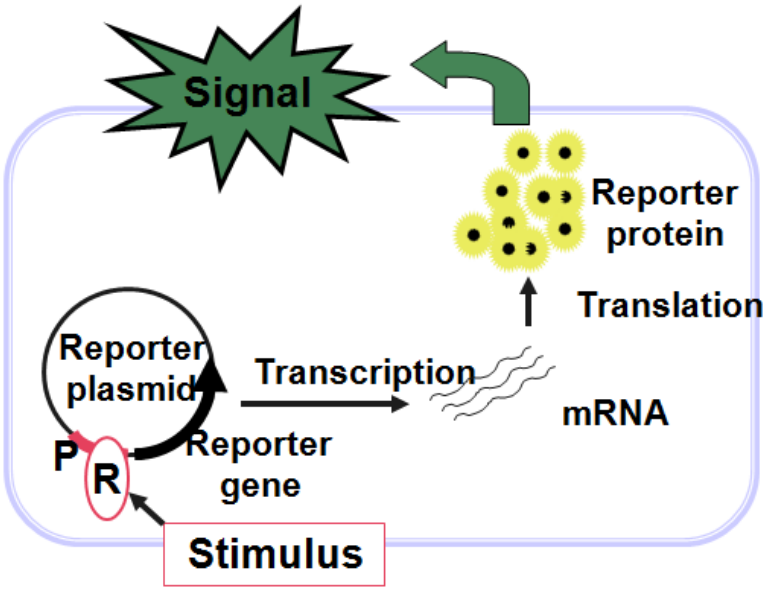


Figure 5.2. Operating principle of an inducible bacterial whole-cell biosensor. The stimulus induces reporter gene expression, which leads to quantifiable signal. Regulatory protein R limits induction to occur from promoter P only when the stimulus is received. Induction involves relieving repression or activating transcription.

and Baneyx 2007). Compound-specific sensors react to a single compound or group of compounds with similar chemical characteristics or mode of action (Korpela *et al.* 1998, Wahlström and Saris 1999). Response of the sensor strains correlates with the concentration and potency of inducing compounds (Hakovirta *et al.* 2006, Möhrle *et al.* 2007).

Whole-cell biosensor assays offer possibility for more cost-effective and accurate group-specific detection than microbial growth-inhibition methods, and are better suited for high-throughput due to assay miniaturization from agar plates to microtiter plates (Chafer-Pericas *et al.* 2010, Pikkemaat *et al.* 2010). Also, growth inhibition on agar plates is typically visualized after overnight incubation, whereas whole-cell biosensor assays can be performed within hours (Bovee and Pikkemaat 2009, Woutersen *et al.* 2011). Whole-cell biosensors can equal growth inhibition assays in below-MRL sensitivity and simplicity in sample preparation (Pikkemaat *et al.* 2010).

Biosensor assay ruggedness is advanced by cell preservation methods such as lyophilization, vacuum drying, and immobilization in biocompatible polymers (Bjerketorp *et al.* 2006). These methods facilitate reagent-like use of the biosensor cells (Kurittu *et al.* 2000a, 2000b, 2000c). The fact that whole-cell biosensors inherently produce the necessary assay components, and just need the presence of the analyte (and sometimes substrate) to induce signal production, further enhances assay ruggedness (Su *et al.* 2011). The renewable storage of assay components within the biosensor cell helps overcome instability problems encountered with using purified biomolecules such as enzymes as recognition elements in biosensors (Yagi 2007)

There are, however, some intrinsic disadvantages to using whole-cell biosensors. When purified biomolecules are used for recognition, conditions can be optimized for the biosensing event (Pellinen *et al.* 2004, 2006, Weber *et al.* 2005, Link *et al.* 2007, Adrian *et al.* 2009). In contrast, biosensor cells continuously sense their local environment, and bioassay variation is caused by responses to diverse intra- and extracellular factors such as cell concentration, growth stage and metabolic activity, nutrient availability, temperature, pH, oxygen content, inducer type and bioavailability as well as duration of induction (Wahlström and Saris 1999, Reunanen and Saris 2003, Hakovirta *et al.* 2006, Marqués *et al.* 2006, Shapiro and Baneyx 2007). However, with standardization of assay conditions and applying homogeneous biosensor cell material through lyophilization, reproducible results can be achieved (Kurittu *et al.* 2000a, 2000b, 2000c, Smolander *et al.* 2009).

Whole-cell biosensors have a narrower detection range than biomolecule-based antibiotic assays since toxicity of the analyte to the cell at high concentrations causes a characteristic hook effect seen as a bell-shaped dose-response curve (Galluzzi and Karp 2006). Assay conditions must therefore be optimized so that the dynamic range of the assay meets the MRL (Kurittu *et al.* 2000b, 2000c).

Because in biosensor cells the biorecognition elements typically reside within the cell, the analyte must first pass the diffusional barrier cell wall – a rate-limiting step in the biosensing reaction leading to lowered sensitivity (van der Meer *et al.* 2004). Utilizing permeabilizing agents or host strains with a defective outer membrane permeability barrier can facilitate more efficient analyte entry into cell (Möhrle *et al.* 2007, Kumar *et al.* 2008). Bacterial cells also have group-specific and multidrug mechanisms of antimicrobial resistance, which may hinder intracellular accumulation of the antibiotic analyte (Roberts 2003, 2005, 2008, Thaker *et al.* 2010). Choosing or creating host strains deficient in antimicrobial resistance mechanisms alleviates this problem (Shapiro and Baneyx 2002).

The choice of reporter gene is yet another factor affecting whole-cell biosensor performance. The most commonly used reporters luciferase (bacterial or eukaryotic), green fluorescent protein (GFP) and the enzyme β-galactosidase all have their advantages and disadvantages when compared to each other (Table 5.2). Light can be measured from bacterial cells non-invasively and sensitively, and because of their high sensitivity and fast response times, luciferase reporters have found use in numerous biosensors, especially online monitoring systems (Nivens *et al.* 2004, van der Meer *et al.* 2004, Woutersen *et al.* 2011). GFP and β-galactosidase both suffer from a high cellular background. However, they benefit from higher stability compared to luciferases and require no ATP for signal production (Köhler *et al.* 2000, Yagi *et al.* 2007).

Table 5.2. A comparison of reporter genes and proteins commonly used in whole-cell biosensors. Adapted from Daunert *et al.* 2000, van der Meer *et al.* 2004, Köhler *et al.* 2000, Yagi 2007.

Gene	Protein	Origin	Advantages	Disadvantages
lux	Bacterial luciferase	Luminescent bacteria	Rapid response High sensitivity No exogeneous substrate requirement	Heat lability Oxygen and ATP requirement
luc	Insect luciferase	Firefly, click beetle	Rapid response Very high sensitivity Heat resistance	Exogeneous substrate requirement Oxygen and ATP requirement
gfp	Green fluorescent protein	*Aequorea victoria* (jellyfish)	No substrate or ATP requirement Limited oxygen requirement High stability	Low sensitivity Lag time before expression Slow maturation Autofluorescence background
lacZ	β-galactosidase	*Escherichia coli*	Detection by naked eye Good stability No ATP requirement	Exogenous substrate requirement Modest sensitivity Endogenous background

5.2 Regulatory elements utilized in whole-cell biosensors

Proteins are intimately involved in control of gene expression in bacteria. There are few examples of transcription initiation control mechanisms relying on other molecular classes, such as RNA secondary structure-based cis-acting riboswitches (Waters and Storz 2009), but transcription initiation is a realm of proteins. For example, approximately 8% (380) of *Escherichia coli* genes are involved in transcription and regulation (Ishihama 2010). The majority of these (300) encode DNA-binding and RNA-polymerase binding transcription factors which have been divided into 54 families.

Negative and positive regulators of transcription, i.e. repressor and activator proteins, are applied in inducible whole-cell biosensors (Korpela *et al.* 1998, Wahlström and Saris 1999, Su *et al.* 2011, papers **I**, **III**). The specific recognition function of these control elements innately combines with an activation or derepression function, the effect of which can be seen as induction of gene expression. A regulatory protein must specifically bind to a promoter to exert its control on transcription (Orth *et al.* 2000, Schumacher *et al.* 2002). These regulator-promoter pairs act as *in vivo* regulatory circuits of reporter genes in whole-cell biosensors (Daunert *et al.* 2000). The gene regulatory elements used in this study are members of the TetR family of transcriptional regulators (TFRs) (papers **III**, **IV**) or two-component signal transduction systems controlling lantibiotic biosynthesis (papers **I**, **II**). Therefore, these two regulatory protein groups are introduced more closely.

5.2.1 The TetR family transcriptional regulators

Expression of resistance determinants related to TC efflux (see table 3.2) is typically controlled by TetR repressors (Agersø and Guardabassi 2005, Brown *et al.* 2008, Hansen *et al.* 1993, Thaker *et al.* 2010). As the most well characterized member, TetR gives its name to an entire protein family: the TetR family transcriptional regulators (TFRs). TFRs bind to the upstream operator region of genes and negatively regulate protein expression by repression (Noguchi *et al.* 2000, Orth *et al.* 2000, Schumacher *et al.* 2002, Ramos *et al.* 2005). They are all alpha-helical homodimeric proteins with a signal-receiving domain and a DNA-binding domain for transduction of the signal (Yu *et al.* 2010). In addition to antibiotic resistance determinants, TFRs control genes whose products are involved in biosynthesis of antibiotics, osmotic stress, pathogenicity of gram-negative and gram-positive bacteria, morphogenesis, biofilm formation, nitrogen uptake and catabolic pathways such as the citric acid cycle (Ramos *et al.* 2005, Yu *et al.* 2010).

The DNA-binding structure of TetR is presented in Figure 5.3A. TetR binds tetracycline as a TC-Mg^{2+} complex (Kisker *et al.* 2000, Lederer *et al.* 1995). The binding of tetracycline to the signal-receiving domain of TetR leads to a conformational change that weakens the repressors interaction with DNA and results in dissociation from the operator region, which allows efflux protein synthesis (Orth *et al.* 1998, 1999). The 15 bp palindromic *tetO* operator binds one TetR dimer (Orth *et al.* 2000). Two copies of this operator exist in the intergenic region separating the *tetR* and the divergently oriented *tetA* gene that codes a TC efflux pump (Ramos *et al.* 2005). TetR binds to these operators and prevents transcription from both promoters. TetR controls tetracycline-inducible expression of bacterial luciferase operon (TetR/P_{tetA}::*luxCDABE* construct) in the tetracycline biosensor (Korpela *et al.* 1998) used in paper **III**.

Resistance to macrolide antibiotics is conferred by various mechanisms such as target modification, efflux and inactivation (Roberts 2003, 2005, 2008). Macrolide resistance module *mphR*(E)-*mph*(E)-*mrx*(E) encodes 2'-phosphotransferase I Mph(E), an inactivating phosphorylase, and Mrx(E), a putative hydrophobic transmembrane transport protein (Szczepanowski *et al.* 2007). Expression of these genes is controlled by repressor MphR(E), a TFR. In paper **IV**, structural and functional characterization of MphR(E) was based on a homology model built using the structure of a related protein MphR(A) (Figure 5.3B) as a reference. The entire macrolide resistance operon *mphR*(E)-*mph*(E)-*mrx*(E) is related to resistance module *mph*(A)-*mrx*(A)-*mphR*(A) with genes sharing sequence identity (34–40%) and identical functions (O'Hara *et al.* 1989, Noguchi *et al.* 1995, 2000). MphR(A) binds a 35 bp promoter upstream from *mph*(A) (Noguchi *et al.* 2000), whereas MphR(E) controls expression from a 52 bp promoter upstream from *mph*(E) (Szczepanowski *et al.* 2007). Degenerated palindromic motifs of 26 bp reside within promoters of both MphR(E) and MphR(A). They are similar in length to *qac* operator, which binds two QacR dimers for repression (Schumacher *et al.* 2002). Therefore, it is possible that two MphR repressors bind per operator.

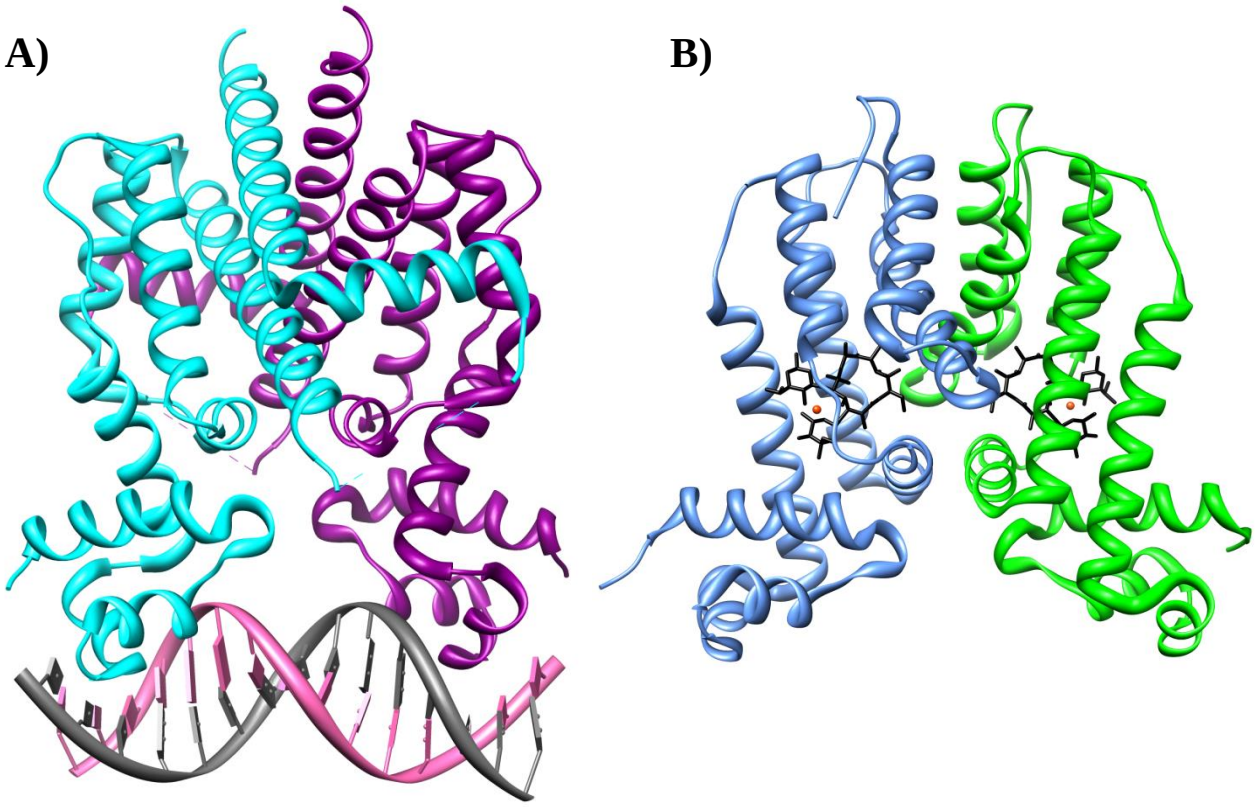


Figure 5.3. Structures of TFRs TetR and MphR(A). A) TetR in complex with operator DNA (PDB accession code 1QPI, Orth *et al.* 2000). The two TetR monomers are shown in cyan and magenta, and DNA strands in grey and pink. DNA binding domains sit in the major groove of operator DNA when the repressor is in the uninduced state i.e. not bound to ligand. B) MphR(A) in complex with two erythromycin ligands (PDB accession code 3FRQ, Zheng *et al.* 2009). The two MphR(A) monomers are shown in blue and green, and erythromycins in black. Two chloride ions modeled in proximity of the ligand binding sites are shown in orange. In MphR(A), ligand binding causes a structural change which increases the distance of the two DNA binding domains by 3.4 Å, and leads to induced state and dissociation from DNA. Images were created with UCSF Chimera (Pettersen *et al.* 2004; http://www.cgl.ucsf.edu/chimera/).

5.2.2 Nisin biosynthesis gene cluster regulators

The lantibiotic biosynthesis operons generally contain genes coding for the prepeptide (*lanA*), enzymes responsible for the modification reactions (*lanBCM*), processing proteases for removal of the leader peptide (*lanP*), regulatory proteins (*lanRK*), an ABC superfamily transport protein involved in translocation (*lanT*), and immunity proteins (*lanIFEG*) (Chen and Hoover 2003). The biosynthesis, regulation and immunity machinery responsible for nisin production is presented in Figure 5.4. Lantibiotic gene clusters may be chromosomal or present on plasmids. The nisin gene cluster is located on chromosomal conjugative transposons such as *Tn5276*, *Tn5301*, *Tn5306*, and *Tn5307* (Horn *et al.* 1991, Thompson *et al.* 1991, Lubelski *et al.* 2008).

Two component systems (TCSs) are the chief mechanism used by bacteria for sensing their environment (Rodrigue *et al.* 2000). Most species contain more than a dozen TCSs that regulate processes such as metabolism, motility, osmoregulation, transport, virulence and development (West and Stock 2001, Gao and Stock 2009). The signaling pathways defined by TCSs consist of four steps. A homodimeric sensor histidine kinase is autophosphorylated by ATP at a histidine residue (Casino *et al.* 2009). The phosphoryl group is then relayed to an aspartate on a cognate response regulator which then interacts with DNA, RNA or protein targets, triggering cellular responses (Gao and Stock 2009). Finally, signaling is terminated by dephosphorylation by an intrinsic or histidine kinase -induced autophosphatase activity (Casino *et al.* 2009).

The expression of genes nisABTCIPRK and nisFEG under their respective promoters *nisA* and *nisF* is controlled by nisin itself via the two-component system NisRK (Lubelski *et al.* 2008). The membrane kinase NisK autophosphorylates upon interaction with extracellular nisin, and phosphorylates the activator NisR, which then induces transcription from P_{nisA} and P_{nisF} (Figure 5.4). The NIsin-Controlled gene Expression system (NICE) exploits nisin mediated auto-induction to facilitate efficient over-expression of genes (Kuipers *et al.* 1998, Mierau and Kleerebezem 2005). The system consists of *Lactococcus lactis* host strains expressing *nisRK* genes, and plasmids containing the *nisA* or *nisF* promoter fragments, followed by convenient cloning sites to introduce the gene(s) of interest. NICE has been used for expression of a multitude of homologous and heterologous proteins of both Gram-positive and Gram negative origin even in an industrial scale (Mierau and Kleerebezem 2005, Zhou *et al.* 2006). Since the NICE system is extremely tightly controlled and provides a linear dose–response relationship, it was an ideal host system to be used in construction of the bioluminescent nisin biosensor strain in paper **I**.

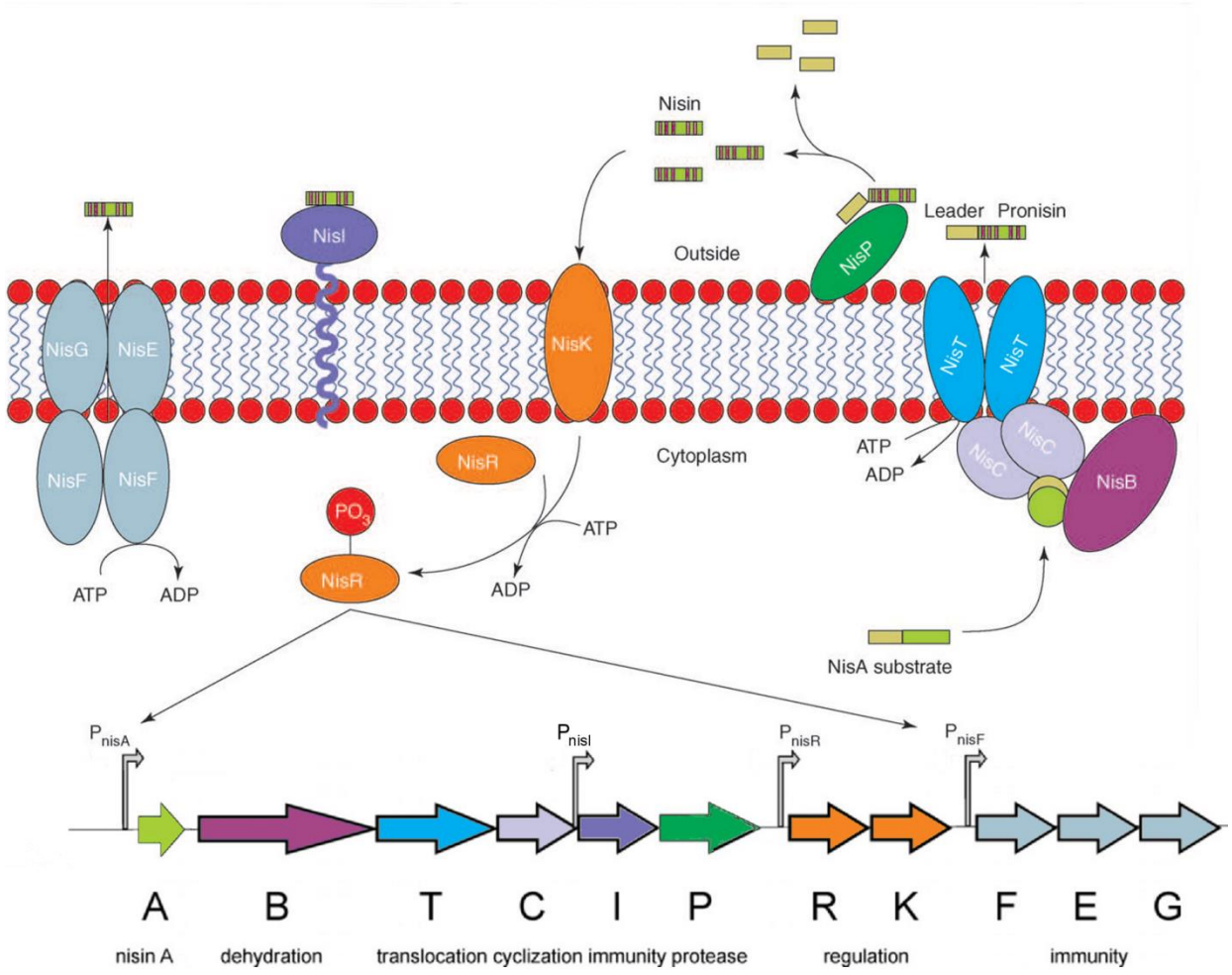


Figure 5.4. Biosynthesis, regulation and immunity machinery responsible for nisin production. In the nisin gene cluster, *nisA* is the structural gene, *nisBTCP* are involved in modification, translocation and processing, *nisIFEG* in immunity and *nisRK* in regulation. Extracellular changes in nisin concentration result in autophosphorylation of NisK and transfer of a phosphoryl group from NisK to an aspartate residue on NisR. The activated NisR then induces transcription of *nisABTCIPRK* and *nisFEG*. In the absence of extracellular nisin, *nisI* and *nisRK* are expressed from independent *nisI* and *nisR* promoters. NisA is processed by a dehydratase (NisB) and cyclase (NisC) multienzyme complex to generate the lanthionine rings in the mature polycyclic peptide. The peptide is then transported out of the cell by NisT in an ATP-dependent manner, and the leader sequence is removed by the membrane-anchored protease NisP to generate the active antimicrobial agent. NisI and NisFEG are involved in producer self-protection. NisI sequesters nisin to reduce its concentration while NisFEG forms an ABC transporter complex to extrude nisin from the membrane to the extracellular environment. Adapted from Patton and van der Donk 2005, Lubelski *et al.* 2008.

5.3 Inducible whole-cell biosensors for antibiotic detection

Inducible whole-cell biosensors for antibiotics include effect-specific and compound-specific sensors (Table 5.3). Effect-specific biosensors are induced by a stress reaction caused by the mechanism of action of different antibiotic classes. A panel of *Escherichia coli* based biosensors includes strains induced by cold shock response to translation inhibition (*cspA* promoter; amphenicol and tetracycline antibiotics), heat shock response to translation inhibition (*ibp*; aminoglycosides), SOS response to DNA replication inhibition (*sulA*; quinolones) and heat shock response to membrane damage and peptidoglycan synthesis interference (*P3rpoH*; β-lactams, polymyxins) (Bianchi and Baneyx 1999, Shapiro and Baneyx 2002, 2007). A similar system based on *Bacillus subtilis* whole-cell biosensors responds to antibiotic interference of the five major biosynthetic pathways of bacteria: biosynthesis of DNA, RNA proteins, cell wall, and fatty acids (Hutter *et al.* 2004a, Urban *et al.* 2007). The antibiotic-inducible promoters were found by analyzing upregulated genes in an expression profile database of *B. subtilis* 168 (Hutter *et al.* 2004a, 2004b) and therefore the regulatory proteins and pathways are not known.

Effect-specific biosensors can be used in seeking entirely new antibiotic mechanisms of action: bacterial cell division inhibiting compounds were discovered with a *B. subtilis* biosensor featuring two reporter genes to facilitate differentiation of specific and nonspecific inhibitors (Stokes *et al.* 2005). A downside of effect-specific biosensors regarding antibiotic residue detection is that they can detect analytes other than antibiotics that induce the same effect. For example, DNA damaging agent-detecting SOS response biosensors are also induced by substances like formaldehyde and hydrogen peroxide (Norman *et al.* 2005, Biran *et al.* 2011).

Compound-specific biosensors for antibiotics offer more specific identification (Table 5.3). These whole-cell biosensors typically detect analytes in a group-specific manner, i.e. are responsive to a group of structurally similar antibiotics instead of a single compound. This kind of behavior is an advantage in screening as all or several compounds of an antibiotic family can be detected simultaneously (van der Meer *et al.* 2004). Several TetR-based tetracycline-specific whole-cell biosensors have been constructed (Table 5.3). Some of these have also been applied for TC detection in a food matrix. Hansen and Sørensen (2000) demonstrated applicability of a β-galactosidase-expressing biosensor in TC detection in incurred milk samples, whereas the bioluminescent biosensor by Korpela *et al.* (1998) has been applied in milk (Kurittu *et al.* 2000b, 2000c), porcine serum (Kurittu *et al.* 2000a), fish tissue (Pellinen *et al.* 2002), and poultry tissue samples (Pikkemaat *et al.* 2010, paper **III**).

Table 5.3. An overview of effect- and compound-specific inducible whole-cell biosensors for antibiotics.

Type	Analyte	Effect/Regulatory protein	Promoter	Reporter gene(s)	Host	Limit of detection[1]	Induction time	Remarks	Reference
Effect-specific	Fluoro-quinolones	DNA replication interference	*pL*	*lux*CDABE	*E. coli*	n.d.	60–90 min	Heat induces *pL* → reporter plasmid replication, whose inhibition decreases signal.	Anko *et al.* 2002
	DNA damaging agents	SOS response	*cda* *recA* *sulA* *umuDC*	*gfp*	*E. coli*	n.d.	90–120 min		Norman *et al.* 2005
	Different antibiotic classes	Translation inhibition DNA replication interference	cspA sulA	*lucFF* *lucR1*	*E. coli*	1000 CAP 50 OFL	3 h	Dual reporter strain *cspA*::*lacZ* *sulA*::*lucR1* detected both effects.	Shapiro and Baneyx 2007
	Vast majority of antibiotic classes	DNA biosynthesis RNA biosynthesis Protein biosynthesis Cell wall biosynth. Fatty acid biosynth.	*yorB* *yvgS* *yheI* *ypuA* *fabHB*	*lucFF*	*B. subtilis*	n.d.	3 h 90 min 4 h 1 h 3 h	The panel targets the majority of antibiotic classes. However, e.g. aminoglycosides did not induce *yheI*.	Urban *et al.* 2007
	Different antibiotic classes	DNA damage Heat-shock Quorum sensing	*rec*A *grp*E *las*I	*lux*CDABE	*E. coli*	50–5x10^5	n.r.	Response signature of growth and/or luminescence inhibition and induction indicates mode of action.	Eltzov *et al.* 2008
	β-lactams, glycopeptides D-cycloserine, bacitracin	Cell wall stress	*pbp2* *tcaA* *vraSR* *sgtB* *lytR*	*lacZ*	*S. aureus*	n.d.	2 h		Steidl *et al.* 2008
	DNA damaging agents	SOS response	*sulA*	*phoA*	*E. coli*	50 MMC 1700 NA	2 h	Sensitivity-enhancing host knockout mutations: enhanced cell membrane perme-ability (*rfaE*), inhibited DNA damage repair (*umuD*, *uvrA*).	Biran *et al.* 2011
Compound-specific	Tetracyclines	TetR	*tetA*	*lux*CDABE	*E. coli*	6	90 min		Korpela *et al.* 1998
	Tetracyclines	TetR	*tetA*	*lac*ZYA *lux*CDABE *gfp*	*E. coli*	10 < 10 < 20	3 h 50 min 16 h		Hansen and Sørensen 2000
	Tetracyclines	TetR	*tetA*	*gfp*	*E. coli*	50	16 h	FACS-optimized GFP mutant.	Hansen *et al.* 2001

Type	Analyte	Effect/Regulatory protein	Promoter	Reporter gene(s)	Host	Limit of detection[1]	Induction time	Remarks	Reference
Compound-specific	Tetracyclines	TetR	*tetA*	*gfp*	*E. coli*	5-16	18 h	Extended range biosensor created by insertion of *tet*(M) resistance gene.	Bahl *et al.* 2005
	Tetracyclines	TetR	*tetA*	*lacZ* *nuoA* *selA*	*E. coli*	110 2.6 1450	2 h	Amperometric detection of cell respiration which is affected by expression of reporter genes.	Song *et al.* 2012
	Macrolides	MphR(A)	*mphA*	*lux*CDABE	*E. coli*	0.008	2 h		Möhrle *et al.* 2007
	Vancomycin	VanRS	*vanH*	*lacZ*	*B. subtilis*	100–1000	4 h	Also responsive to other glycopeptides, β-lactams, D-cycloserine, bacitracin, fosfomycin.	Ulijasz *et al.* 1996
	β-lactams	AmpR	*ampC*	*lux*CDABE	*E. coli*	2.5–2500	3 h		Valtonen *et al.* 2002

CAP, chloramphenicol; *gfp*, green fluorescent protein gene; FACS, fluorescence-activated cell sorting; *lac*ZYA, *lac* operon encoding β-galactosidase (*lacZ*), β-galactoside permease (*lac*Y) and β-galactoside transacetylase (*lac*A); *luc*FF, *Photinus pyralis* firefly luciferase gene; *luc*R1, a red-shifted variant of firefly luciferase; *lux*CDABE, *Photorhabdus luminescens* or *Vibrio fischeri* bacterial luciferase operon encoding luciferase (*lux*AB) and fatty acid reductase complex (*lux*CDE); MMC, mitomycin C; n.d., not determined, NA, nalidixic acid; *nuoA*, NADH dehydrogenase I subunit A gene; OFL, ofloxacin; *phoA*, alkaline phosphatase gene; *selA*, selenocysteine synthase gene.

[1]In ng/ml. For effect-specific biosensors, limit of detection is not relevant since multiple classes of antibiotics cause induction with various potencies.

6 NISIN DETECTION METHODS

Nisin is approved as a food preservative in over 80 countries, and it is used particularly in processed cheese, dairy products and canned foods (Delves-Broughton et al. 1996, Delves-Broughton 2005, Gálvez *et al.* 2011). Nisin is not, however, considered natural when it is applied in concentrations that exceed what is found in food naturally fermented with a nisin-producing starter culture. Various countries have set maximum levels of nisin in foods (Cleveland *et al.* 2001). Therefore, several methods for nisin detection and quantification have been developed. In the EU, Directive 95/2/EC sets a maximum level of 3–12.5 mg/kg for nisin in various foodstuffs (EC 1995). An ISO standard for the determination of the nisin A content in cheese by LCMS and LCMS/MS has also been published (ISO 2009).

An overview of nisin detection methods is presented in Table 6.1. A growth-inhibition-based agar diffusion assay originally introduced in 1964 for nisin detection is still widely in use (Tramer and Fowler 1964, Fowler *et al.* 1975). Although improved versions of the assay have been developed (Wolf and Gibbons 1996, Pongtharangkul and Demirci 2004), several parameters such as sample diffusion properties, choice of indicator organism and assay media as well as subjectivity in determining inhibition zone size affect the sensitivity and accuracy of this method. Consequentially, alternative methods for nisin detection and quantification have been devised. These include physico-chemical methods based on capillary zonal electrophoresis (Rossano *et al.* 1998), micellar electrokinetic chromatography (Soliman and Donkor 2010), LC-MS/MS (Schneider *et al.* 2011), immunochemical methods (Suárez *et al.* 1996, Bouksaim *et al.* 1998, Daoudi *et al.* 2001, Aly *et al.* 2011) and microbiological methods such as turbidometric bioassays (Turcotte *et al.* 2004) and flow cytometry (Budde and Rasch 2001). Not all methods utilize food samples, but nisin standards diluted in buffer.

Most sensitive nisin detection and quantification is, however, achieved by nisin-inducible whole-cell biosensor assays. The whole-cell biosensor assays based on nisin-inducible bioluminescence (Wahlström and Saris 1999, paper **I**) and fluorescence (Reunanen and Saris 2003, Hakovirta *et al.* 2006) involve reporter genes encoding luciferase (*luxAB* or *luxABCDE*) or green fluorescent protein (*gfp*) placed under control of a nisin-inducible promoter. Maturation of fluorescent reporter proteins takes longer than luciferase enzyme maturation, and sensitivity is typically lower in fluorescent whole-cell biosensors due to interfering background autofluorescence (Hakkila *et al.* 2002). These effects were seen in fluorescent nisin biosensor assays, which required overnight incubation and removal of light-absorbing supernatant from assay wells prior to fluorescence measurement. Bioluminescent nisin sensors offer the most sensitive nisin detection ever obtained (paper **I**).

Table 6.1. An overview of nisin quantification methods for food samples.

Category	Method	Analyte[1]	Sample matrix	Sample pretreatment	LOD	Recovery (%)	Reference
Physico-chemical	Capillary electrophoresis	Nisin	Milk	Nisin spiking, dilution, ACN extraction, centrifugation, suspension and filtration	10.000 µg/l	n.d.	Rossano *et al.* 1998
	Micellar electrokinetic chromatography	Nisin	Milk, cream, yogurt, yogurt drink, processed cheese, beer, wine, salad dressing, canned tomatoes	Homogenization, (ultra)filtration, centrifugation, nisin spiking	300–800 µg/l	90–104	Soliman and Donkor 2010
	LC-MS/MS	Nisin A, Z	Processed cheese	Dilution, homogenization, acid extraction by heating, centrifugation and filtration	n.d. (at least 50 µg/l)	49–69	Schneider *et al.* 2011
Immuno-chemical	Immunoblot assay	Nisin Z	Milk and whey	Nisin spiking, dilution, EDTA and Triton X-100 addition, heating, dilution	155 µg/l	n.d.	Bouksaim *et al.* 1998
	Competitive ELISA	Nisin Z	Milk and whey	Nisin spiking, acidification, heating, centrifugation, filtration	91–106 µg/l	89–98	Daoudi *et al.* 2001
	Competitive ELISA	Nisin	Cheese	Dilution, homogenization, centrifugation	626 µg/kg	98–120	Aly *et al.* 2011
Micro-biological	Growth inhibition (agar diffusion)	Nisin	Nisin-containing food material (not specified)	Dilution, homogenization, acid extraction by heating, centrifugation and filtration	< 30.000 µg/l	n.d.	Fowler *et al.* 1975
Biosensor	Bioluminescent whole-cell	Nisin	Milk	Nisin spiking	0.075 µg/l	n.d.	Wahlström and Saris 1999
	Fluorescent whole-cell	Nisin	Milk, processed cheese, salad dressing	Nisin spiking, dilution	45–1000 µg/kg	n.d.	Reunanen and Saris 2003
	Fluorescent whole-cell	Nisin	Milk, processed cheese, salad dressing, canned tomatoes, liquid egg	Nisin spiking, dilution	0.2 –9 µg/kg	n.d.	Hakovirta *et al.* 2006
	Bioluminescent whole-cell	Nisin	Milk	Nisin spiking, dilution	0.003 µg/l	25–36	Paper **I**

ACN, acetonitrile; ELISA, enzyme-linked immunosorbent assay; n.d., not determined; LOD, limit of detection.

[1]When only nisin is indicated as the analyte, the study did not take into account detection efficiency of nisin variants.

7 AIMS OF THE STUDY

This study aims to develop rapid, sensitive, inexpensive and simple whole-cell biosensor based methods for screening for antimicrobial residues and producers of antibiotic molecules. These methods can be applicable in a variety of sample matrices such as different foodstuffs. Recognition of the presence of the analyte in the sample induces bioluminescence production within the biosensor cells. Assay simplicity is underlined by no need to add exogenous substrates, as the enzyme and substrate needed for the bioluminescence reaction are produced endogenously within the biosensor cells. Furthermore, the reagent-like use of biosensor cells in a lyophilized form removes the need to culture them separately for each assay.

Tetracyclines are the most commonly used class of veterinary antibiotics (Grave *et al.* 2010). This study involved assessing the functionality of a previously developed TetR repressor protein regulated tetracycline biosensor (Korpela *et al.* 1998) in screening for tetracycline residues in poultry muscle. Simultaneously, the effects of various tetracyclines and tetracycline metabolites in the biosensor assay were determined.

Nisin is an antimicrobial peptide naturally produced by some *Lactococcus* and *Streptococcus* strains. The use of nisin as a food preservative is limited by the EU to 3–12.5 mg/kg in various food products (EC 1995), placing a demand for effective and sensitive nisin assays. A nisin-specific biosensor strain was constructed and used in this study for detection, quantification and screening purposes.

Macrolides are the fourth most used group of veterinary antimicrobial agents in the EU (Grave *et al.* 2010). A novel macrolide-specific repressor protein MphR(E) (Szczepanowski *et al.* 2007) is a candidate for development of macrolide-responsive biosensors. In the first ever study on MphR(E) structure and function, we analyzed and sought to modify DNA binding characteristics of the repressor protein to improve its performance as a biosensor regulatory element.

Specific aims for the study:

- Construction of a nisin biosensor, and its use in detection of nisin in food samples (**I**)
- Utilization of the nisin biosensor in screening for nisin producers in food samples (**II**)
- Development of a screening method for tetracycline and their 4-epimer metabolite residues in poultry meat and comparison with a microbiological growth inhibition assay (**III**)
- Studying the structure-function relationships of the macrolide specific repressor protein MphR(E) and developing improved versions of the repressor for biosensor use by rational mutagenesis (**IV**)

8 MATERIALS AND METHODS

8.1 Bioluminescent biosensor organisms (I, II, III)

The bioluminescence reaction of bacteria [1] involves the oxidation of reduced riboflavin phosphate ($FMNH_2$) and a long-chain aldehyde conjoined with emission of blue-green light (Meighen 1991).

$FMNH_2 + RCHO + O_2 \rightarrow FMN + H_2O + RCOOH + h\nu\ (490\ nm)$ [1]

On bacterial luciferase operons, the luciferase enzyme performing the oxidation reaction is coded by *luxAB*, and the fatty acid reductase complex responsible for synthesizing the fatty aldehyde substrate by *luxCDE* (Meighen 1993). Paper **III** used a tetracycline biosensor strain (Korpela *et al.* 1998) harboring a sensor plasmid that contains bacterial luciferase operon *luxCDABE* from *Photorhabdus luminescens* (Meighen and Szittner, 1992). In papers **I** and **II**, we used a modified version *luxABCDE* of the *P. luminescens* luciferase operon that has been altered to be functional in Gram-positive bacteria (Francis *et al.* 2000). The structures of the sensor plasmids used in **I**, **II** and **III** and the regulated bioluminescence response pathways are described in Figures 8.1 and 8.2.

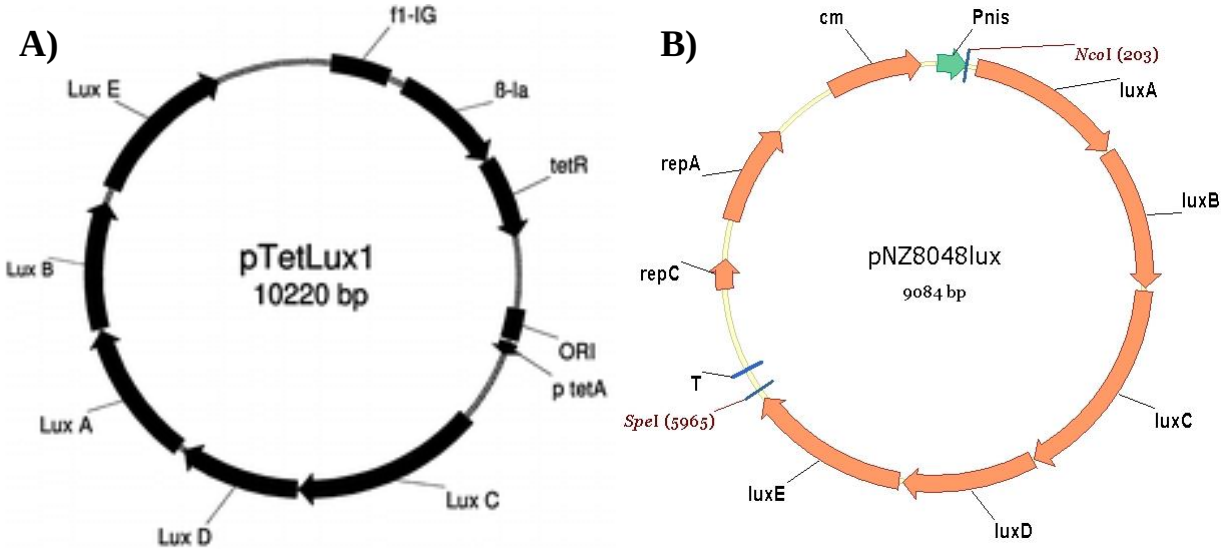


Figure 8.1. Sensor plasmids used in this study. A) The tetracycline biosensor used in paper **III** had been constructed by transforming the host strain *E. coli* K-12 M72 [Sm^R*lacZ*(Am)Δ*bio-uvrB*Δ*trpE42*(λN7(Am)*N53*(Am)*cI857*-ΔH1)] with pTetLux1 (Korpela *et al.* 1998). The plasmid contains a gene encoding the repressor protein TetR, which controls transcription from *tetA* promoter. B) Plasmid pNZ8048 was constructed in paper **I** for use as a sensor plasmid in host strains *L. lactis* NZ9800 and NZ9000 (Kuipers *et al.* 1993, 1998). The two resulting biosensor strains were denoted NZ9800lux and NZ9000lux.

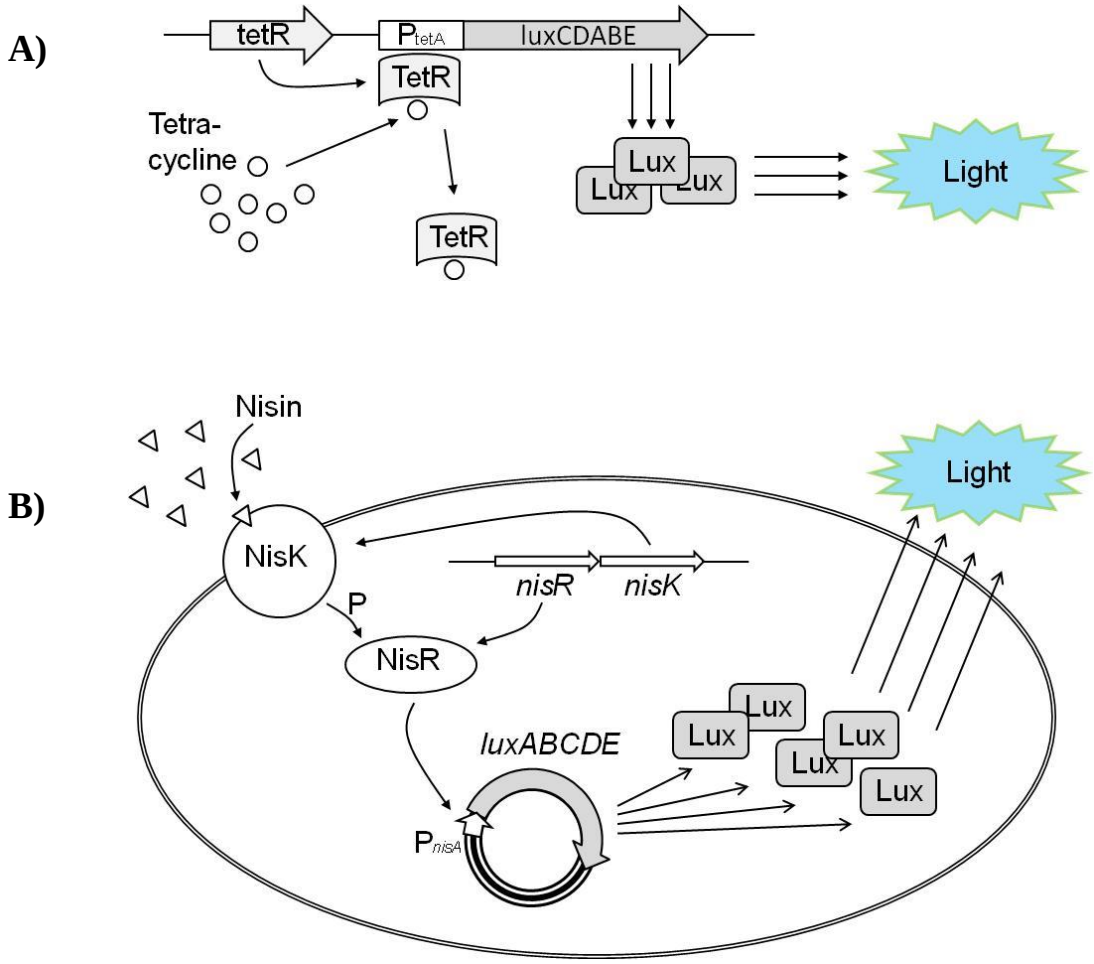


Figure 8.2. Regulation of bioluminescence response pathways in the whole-cell biosensors used in this study. A) All biosensing elements of the tetracycline biosensor are present on the same plasmid. Repression of *luxCDABE* transcription by TetR is lifted in the presence of TC molecules. This results in expression of the luciferase enzyme and concomitant bioluminescence. B) Nisin-induced expression of luciferase requires regulatory protein genes *nisRK* of the host chromosome (Mierau and Kleerebezem 2005). NisK is a histidine kinase present at the cytoplasmic membrane. Upon nisin binding, it activates the response regulator NisR through phosphorylation, and *luxABCDE* expression is induced from *nisA* promoter.

8.2 Cultivation and lyophilization of biosensor cells (I, II, III)

For use in bioassays, *E. coli* K-12(pTetLux1) was cultivated in Luria-Bertani broth (LB) supplemented with 100 μg/ml ampicillin (**III**), and NZ9800lux and NZ9000lux in M17 broth (Terzaghi and Sandine 1975) supplemented with 0.5% w/v glucose and 10 μg/ml

chloramphenicol (M17GCm) (**I**, **II**). The biosensors were used in the bioassays either as freshly cultivated or reconstituted lyophilized cells. Lyophilization was performed with cells grown to late logarithmic phase, which were then harvested and suspended in LB or M17G supplemented with 10% lactose. The cell suspension was distributed in glass vials and lyophilized under vacuum following a 24 h (**III**) or 96 h (**I**) procedure (Sidyakina and Golimbet 1991). Reconstitution of biosensor cells was performed by adding fresh medium in the vial and incubating at room temperature.

8.3 Sample preparation for biosensor assays (I, II, III)

A nisin preparation containing 2.5% nisin in milk solids and NaCl was used as standard material in the nisin bioassays (**I**). The powder was dissolved in 0.1% Tween 80. Nisin standards were prepared as serial dilutions in 0.1% Tween 80 or low-fat milk. To identify nisin producers, samples collected from culture media of lactococci were serially diluted in 0.1% Tween 80 and compared to nisin standards in a nisin biosensor assay.

To screen for nisin producers (**II**), raw milk was diluted in peptone water and plated on M17G agar supplemented with 1% w/v lactose (M17GL). Mixed and isolated cultures of raw milk lactic streptococci were used as sample material. A panel of 91 *Lactococcus* strains was cultured on M17GL prior to screening. Bacteriocin producers tested in the screening assay were cultured on media suitable for each species. To characterize the bacteriocin produced by strain SL149, a sample of its culture medium and a nisin-containing sample were run on a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel and subjected to a nisin bioassay.

Tetracycline-spiked poultry meat fluid was used as sample material in the tetracycline assays (**III**). Organically produced chicken meat was spiked with TC parent compounds, 4-epimer metabolites, or H_2O to obtain blank material. After mincing and incubating to obtain uniform antibiotic distribution, the meat was heated at 64 °C and centrifuged to collect the resulting fluid.

8.4 Characterization of nisin producers (II)

Nisin producers identified using a nisin biosensor overlay were characterized by PCR amplification and sequencing of the 16s rRNA and nisin genes (**II**). In addition, *his* operon amplification product length and genetic fingerprinting by repetitive BOX element sequence-based PCR (BOX-PCR) and random amplified polymorphic DNA (RAPD) were used for characterization.

8.5 MphR(E) repressor protein expression and mutation (IV)

In paper **IV**, MphR(E) gene was PCR-amplified from plasmid pUC18-*mphR*(E)-*mph*(E)-*mrx*(E) (Szczepanowski *et al.* 2007) and inserted into plasmid pAK400c (Santala and Lamminmäki 2004). The primers used added an N-terminal 6 x histidine tag and a tobacco etch virus (TEV) protease site to facilitate tag removal. Rational design of mutations was based on a 3D model of MphR(E) built with Swiss-pdbViewer v4.0.3 (Guex and Peitsch 1996, 1997) using the crystal structure of a related protein, MphR(A) (Zheng *et al.* 2009), as a template. Mutations were introduced into *mphR*(E) by gene splicing overlap extension PCR (SOE-PCR) using mutagenic primers designed for each individual mutation.

MphR(E) expression was performed in *E. coli* XL1-Blue by an overnight IPTG-induction of cells cultured to late logarithmic phase. The protein was purified with Ni-NTA affinity chromatography and digested with TEV protease obtained from expression plasmid pMHT238Δ (Blommel and Fox 2007) harboring strain *E. coli* DH5α. Protein purity and success of digestion were verified by SDS-PAGE and mass spectrometry.

8.6 Bioluminescence and fluorescence anisotropy data collection and analysis (I, II, III, IV)

Bioluminescence signal from whole-cell biosensor assays was read with multidetection microplate readers: Plate CHAMELEON^{TM} (Hidex) in paper **I**, and Synergy HT (BioTek Instruments Inc.) in paper **III**. The screening application in paper **II** utilized *in vivo* bioluminescence imaging by Xenogen IVIS Lumina II (Caliper Life Sciences) to detect nisin production-indicating bioluminescence.

Induction coefficients (IC) (in paper **I** referred to as induction factor, IF) were calculated (**I**, **III**) from bioluminescence data using equation 2:

$$IC = \frac{B_S}{B_B} \qquad [2]$$

where B_S is the measured bioluminescence signal in relative light units (RLU) from a sample and B_B the measured bioluminescence signal in relative light units (RLU) from a blank sample containing all assay components except the analyte.

Limit of detection was determined in paper **I** as the concentration of nisin at which the IC exceeded the value two. In paper **III**, the limit of detection was the tetracycline concentration at which the IC exceeded the value three.

In paper **IV**, fluorescence anisotropy was used to determine dissociation constant (K_d) of the interaction of MphR(E) wild type or mutant protein with fluorescently labeled $P_{mph\text{-}mrx}$ promoter DNA. Data was obtained with Plate CHAMELEON™ equipped with polarizing excitation and emission filters and fit into equation 3:

$$A = A_f + (A_b - A_f) \times \frac{(L_T + K_d + R_T) - \sqrt{(L_T - K_d - R_T)^2 - 4L_T R_T}}{2\,L_T} \quad [3]$$

Where A = the experimental anisotropy; A_f = the anisotropy for the free ligand; A_b = the anisotropy for the fully bound ligand; L_T = the total added concentration of ligand; R_T = The total added concentration of receptor. The ligand is the fluorescein-labeled promoter DNA, and the receptor the MphR(E) repressor.

8.7 Mass spectrometry experiments (IV)

In paper **IV**, mass spectrometric experiments were performed with a 12-T hybrid quadrupole Fourier transform ion cyclotron resonance (FT-ICR) instrument (Apex-Qe™; Bruker Daltonics, Billerica, MA, USA) equipped with an Apollo-II (Bruker) electrospray ionization (ESI) source. Prior to mass measurements, all protein samples were desalted by buffer exchanging to 10 mM ammonium acetate pH 6.9 and concentrated if necessary. Mass spectra were measured using either denaturing solvent conditions (acetonitrile/water/acetic acid, 49.5:49.5:1.0, v/v) or native conditions (500 mM ammonium acetate pH 7.0). To study ligand binding, a small aliquot of the protein sample was mixed with a ligand to obtain 1:1 or 1:2 molar ratios with respect to monomeric protein and directly measured.

9 RESULTS AND DISCUSSION

9.1 Biosensor assay protocols (I, II, III)

A biosensor assay in 96-well format was developed for nisin (**I**) and tetracyclines (**III**). Figure 9.1 presents the final, optimized assay protocols. Both assays utilized lyophilized biosensor cells as a reagent-like component, making assay execution rapid and simple. Samples and standards were dispensed on the plate along with the biosensor cells for bioluminescence induction. Total assay durations were three (**I**) and four (**III**) hours, including sample preparation during cell reconstitution. As the biosensors utilized *P. luminescens* bacterial luciferase operon, the long-chain fatty aldehyde substrate is synthesized innately within the biosensor cells, and no addition of exogenous substrate was therefore required in the assay. The nisin biosensor constructed in paper **I** was utilized in paper **II** to develop a rapid and simple plate overlay assay for screening for nisin producers in mixed cultures of food sample bacteria, or isolated cultures of putative nisin producers (Figure 9.2). The assay produced results within one hour of application of the overlay.

The biosensor assay for nisin (**I**) is simple to perform compared to other nisin quantification methods presented in Table 6.1. There is minimal sample pretreatment involved, only dilution with an acidic, detergent containing solution. The protocols of the other nisin whole-cell biosensor assays require addition of an exogenous substrate (Wahlström and Saris 1999) or overnight induction and a complex medium removal and freeze-thaw treatment before signal measurement (Reunanen and Saris 2003, Hakovirta *et al.* 2006).

Exogenous substrate additions are a source of assay variation due to flash kinetics of the bacterial bioluminescence reaction (Meighen and Hastings 1971). Bioluminescence measurement from each sample should be performed with an identical delay after substrate addition, but in practice this is difficult to carry out. The assay by Wahlström and Saris (1999) did not utilize lyophilized cells, which may cause growth-stage related variance in luciferase expression (Galluzzi and Karp 2007). The biosensor assays based on green fluorescent protein reporter (Reunanen and Saris 2003, Hakovirta *et al.* 2006) use end-point analysis of fluorescence signal from stationary phase cells, and therefore are not affected by growth stage. However, GFP reporter matures slowly and therefore requires an overnight incubation (Hakkila *et al.* 2002). GFP-based assays are also less sensitive than luciferase assays due to background autofluorescence and relatively low fluorescence intensity (Daunert *et al.* 2000). These properties are a drawback to using these biosensors in nisin producer identification, as substrate additions and overnight incubations would complicate the simple overlay assay protocol developed in paper **II**. Hu *et al.* (2009) also used a biosensor based on the NICE system (see chapter 5.2.2) in identification of nisin producers. However, their assay included using culture supernatants of putative nisin producers as samples, and an SDS-PAGE analysis to detect

expression of reporter protein. The overlay assay (**II**) does not require pure cultures, but instead, nisin producers can be identified within mixed cultures. The bioluminescence signal can be recorded directly from the plate, and no separate detection assay is needed.

In addition to poultry muscle (**III**), the tetracycline biosensor has been applied for milk (Kurittu *et al.* 2000b, 2000c), porcine serum (Kurittu *et al.* 2000a), and fish tissue samples (Pellinen *et al.* 2002). All these assays include simple sample pretreatment, and the assay is performed using lyophilized sensor bacteria in buffered media and in the presence of a chelating agent. In paper **III**, the assay was further sensitized with membrane permeabilizing agent polymyxin B, since the EDTA addition alone did not sufficiently promote analyte entry into cells. This was likely because of the relatively high Mg^{2+} content of poultry meat (270 µg/g) compared to e.g. milk (150 µg/g).

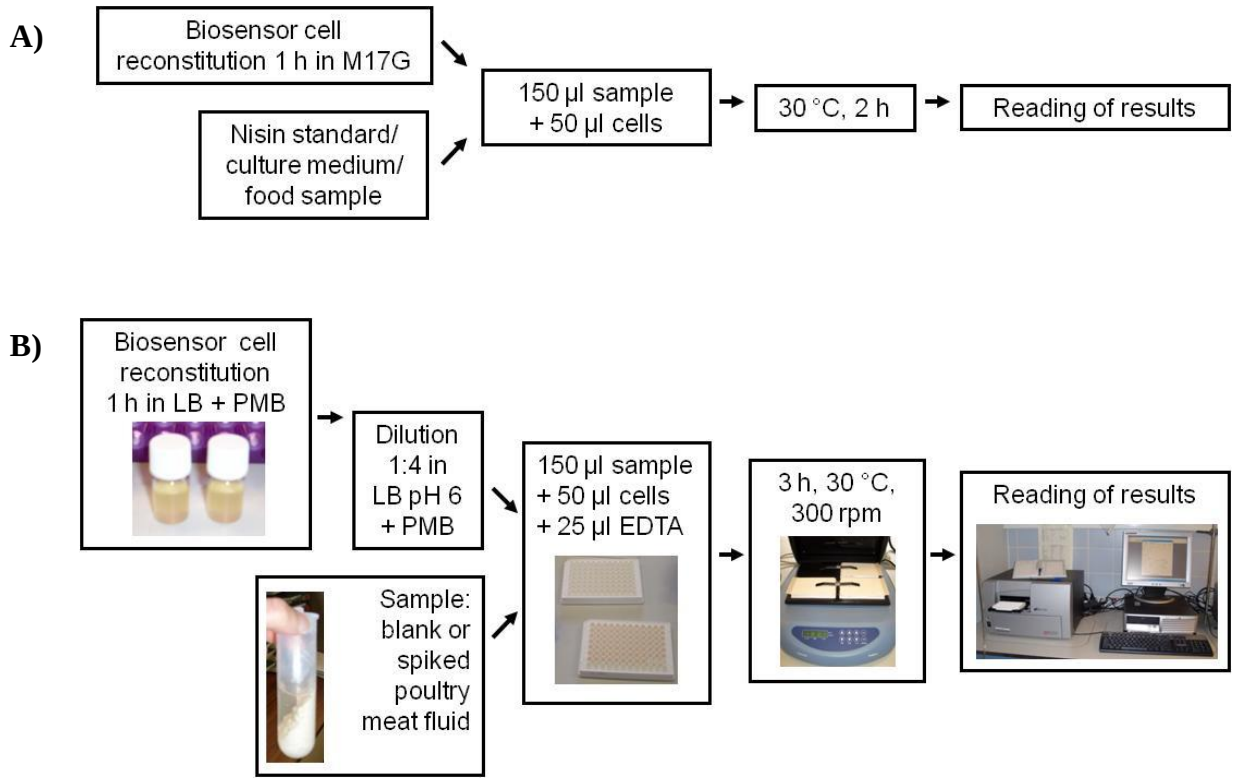


Figure 9.1. 96-well format biosensor assays developed in this study. A) Nisin bioassay. B) Tetracycline bioassay for tetracycline residues in poultry meat. M17G, M17 broth supplemented with 0.5% w/v glucose; LB, Luria-Bertani broth; PMB, polymyxin B; EDTA, ethylenediaminetetraacetic acid.

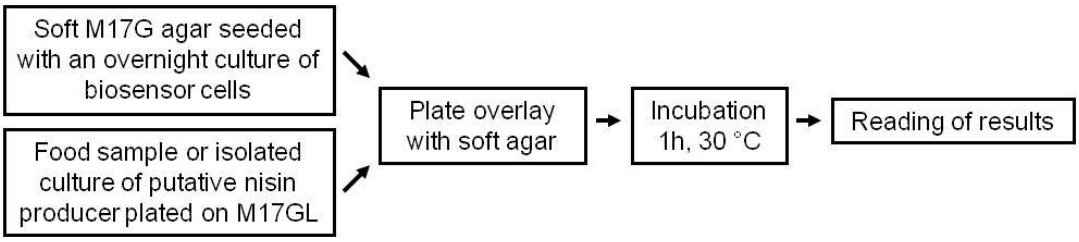


Figure 9.2. Nisin biosensor overlay assay for screening for nisin producers. The bioassay was performed on mixed or isolated cultures of putative nisin producers plated on agar and overlaid with a thin layer of soft agar seeded with NZ9800lux nisin biosensor.

9.2 Biosensor assay performance (I, II, III)

In paper **I**, two nisin biosensor strains, NZ9800lux and NZ9000lux, were constructed of two different *L. lactis* host strains, NZ9800 and NZ9000. The performance of NZ9800lux exceeded that of NZ9000lux in the speed of response, span of constant induction level, width of linear range, low level of background signal, and compatibility with lyophilization (Figures 1, 2, and 3 in **I**). NZ9000lux did exhibit slightly better sensitivity than NZ9800lux (0.03 pg/ml vs. 0.1 pg/ml, respectively), but the superiority of NZ9800lux in other aspects made it the prime choice for use in bioassays. NZ9800lux had a wider linear range and higher induction coefficients than NZ9000lux likely because the activity of nisin immunity proteins NisIFEG in NZ9800lux (Kuipers *et al.* 1993) protects the biosensor cell from the antimicrobial effect of nisin. NisI is expressed without nisin autoinduction from an internal promoter preceding the *nisI* gene (Li and O'Sullivan 2008), so the protective effect is constantly present. NZ9000lux does not harbor any nisin resistance genes (Kuipers *et al.* 1998) which would render some of the nisin unavailable for induction, and therefore the sensitivity is higher in this strain.

The nisin biosensor assay was optimized for food samples using low-fat milk as a test matrix (**I**). The sensor behaved similarly in milk and in 0.1% Tween 80 pH 2.5 used as nisin standard diluent, but showed lower sensitivity and slower signal development due to opaqueness of milk (Figures 4 and 5 in **I**). However, diluting milk samples 1:4 in 0.1% Tween 80 pH 2.5 prior to the assay somewhat restored assay performance. The nisin bioassay was also applied in detecting nisin concentrations in culture medium samples, and simultaneously to identify a nisin-producing strain among three *L. lactis* strains (Table 1 in **I**). Nisin expression level was determined from serial dilutions of growth medium. The nisin concentration produced by strain *L. lactis* 20729 after 7 h cultivation was 4.53 µg/ml in M17G broth and 0.14 µg/ml in milk, remaining constant until 48 h of cultivation. This experiment established the use of the bioassay in determining nisin

concentration in a food matrix. The difference in nisin concentration between the two culture media was determined to be caused by differences in stationary phase bacterial concentration: 1.2×10^{10} cfu/ml in M17G medium vs. 1.2×10^{8} cfu/ml in milk. Also, low recoveries (25–36%) from spiked and diluted milk samples (= internal standardization) suggested lowered nisin bioavailability due to association with milk components. Measuring only the bioavailable fraction of the analyte is an inherent quality of whole-cell biosensors since only this soluble fraction can exert an inducing effect on the cell (Hansen and Sørensen 2001, van der Meer and Belkin 2010). It is both an advantage and disadvantage, since the bioavailable fraction is the part of the analyte molecule population that has the potential to have a (toxic) effect on living cells and organisms, but it is an underestimation of the total analyte concentration whose determination is often required by food safety regulations.

The nisin biosensor assay (**I**) is the most sensitive nisin assay ever reported (see Table 6.1). Extreme sensitivity facilitates extensive dilution of the sample prior to nisin quantification, minimizing the effect of possible interfering factors present in the sample matrix. Simple pretreatment of samples under mild conditions should also protect nisin from degradation (Schneider *et al.* 2011). However, the low recovery from internal standards (**I**) suggests dilution of food samples with 0.1% Tween 80 pH 2.5 does not extract nisin sufficiently, although the detergent is present in the diluent to reduce nisin adsorption. It has been known for a long time that nisin adsorption to food components such as proteins lowers nisin bioavailability in growth inhibition assays, and therefore a sample pretreatment protocol (acid extraction) was devised for food samples (Tramer and Fowler 1964, Fowler *et al.* 1975). Modified versions of this protocol have been used in several studies including growth inhibition assays (Wolf and Gibbons 1996), immunoassays (Daoudi *et al.* 2001) and LC-MS/MS (Schneider *et al.* 2011). Other pretreatment protocols have also been developed (Rossano *et al.* 1998, Bouksaim *et al*. 1998, Soliman and Donkor 2010, Aly *et al.* 2011). In a micellar electrokinetic chromatography method for nisin quantification, addition of internal standard was performed after sample pretreatment, resulting in high recoveries (Soliman and Donkor 2010), whereas nisin addition before sample pretreatment led to lower recoveries (Schneider *et al.* 2011). Wahlström and Saris (1999) claimed no nisin was lost when dose-response of nisin-spiked milk samples was compared to nisin standards in a bioluminescent whole-cell biosensor assay. However, the limit of detection was higher and the linear range of the assay shifted towards higher concentrations in milk, suggesting lowered bioavailability. Similar results were obtained in paper **I**. Therefore, low recoveries can be present in other nisin whole-cell biosensor assays which use a similar dilution approach (Wahlström and Saris 1999, Reunanen and Saris 2003, Hakovirta *et al.* 2006). Dose-response curves in paper **I** were determined using milk samples spiked to each individual concentration to achieve a uniform sample matrix effect, and to ensure no nisin is lost during dilution, so the curve should reflect real bioavailable nisin concentrations.

Whole-cell biosensors are typically used in quantitative to semiquantitative analysis of analyte concentrations. The nisin biosensor was used for quantitative analysis of nisin concentrations in

growth medium and milk (**I**). The effect of bioavailability and ensuing low recoveries in milk samples cast a shadow of doubt on whether the results of milk sample analysis truly are quantitative. Recoveries were not determined for growth medium samples in paper **I**, so it is not known whether the results reflect actual nisin concentrations in the samples. Using a growth inhibition assay, Li and O'Sullivan (2002) have determined a maximum nisin concentration of 2.9 μg/ml for a 10 h old culture of *Lactococcus lactis* subsp. *lactis* DSM-20729 (ATCC 11545) in identical culture conditions to paper **I**. The concentration then decreased to 1.9 μg/ml in a 48 h old culture. These values are similar to those determined in paper **I**, a maximum value of 8.58 μg/ml at 6 h and 4.61 μg/ml at 48 h. Therefore, the recovery from growth medium samples is likely not as low as from milk samples, and the quantification results are more reliable. A more efficient extraction protocol would likely increase recovery from milk samples and facilitate quantification.

There are examples of whole-cell biosensors used for quantitative detection of nisin and tetracycline antibiotics in food samples. The nisin bioassay developed by Reunanen and Saris (2003) was used for nisin quantification in sausage (Reunanen and Saris 2004). In another study, the tetracycline biosensor also used in paper **III** was utilized in determining tetracyclines in fish tissue (Pellinen *et al.* 2002). The results from the biosensor assay correlated well with HPLC analysis, showing whole-cell biosensors can be applied in quantitative determination of antibiotic residues. However, in a follow-up study of paper **III**, Pikkemaat *et al.* (2010) used the tetracycline biosensor assay in routine screening analysis of poultry muscle samples, and came to the conclusion that the assay only gave qualitative results. This was due to absorption of the bioluminescence signal by haemoglobin, the concentration of which varied from sample to sample. This could be overcome by utilizing mutant versions of bioluminescent reporter proteins with emission maxima not overlapping with the hemoglobin absorption spectrum. Such mutants of *Photinus pyralis* (firefly) luciferase have been created by Shapiro *et al.* (2005) and of GFP by Heim and Tsien (1996). Also, mathematical methods could be used to account for the bioluminescence signal lost due to haemoglobin. Another source of variation in tetracycline screening analysis was batch variation in ampoules of lyophilized biosensor cells. This can be overcome by more careful assay standardization, *i.e.* balancing the optical density of cells in the assay.

In papers **I** and **III**, the performance of freshly cultured and lyophilized K-12(pTetLux1) and NZ9800lux cells was remarkably similar, facilitating reagent-like use of lyophilized biosensor cells in the nisin and tetracycline bioassays (Figures 4 in **I** and 1 in **III**). Similar behavior of cultured and lyophilized cells in a biosensor assay has previously been reported (Kurittu *et al.* 2000a) and lyophilization is widely used as a preservation method for whole-cell biosensors (Bjerketorp *et al.* 2006).

In paper **III**, the tetracycline bioassay at first failed to detect all four veterinary relevant tetracyclines (DC, doxycycline; CTC, chlortetracycline; TC, tetracycline; OTC, oxytetracycline) at levels below the EU maximum residue limits (MRL) (EC 1990, 1999, 2010a). Thus, a novel

method for whole-cell bioassay sensitization was developed using polymyxin B (PMB) and EDTA, two substances which cooperate to increase cell wall permeability and facilitate analyte entry into the biosensor cell (Figures 1 and 2 in **III**). The divalent cation dependent uptake mechanism of tetracycline into Gram negative cells is presented in Figure 9.3. In addition to facilitating tetracycline uptake through the cytoplasmic membrane by chelating excess Mg^{2+} ions (Schnappinger and Hillen 1996), EDTA chelation removes stabilizing divalent cations from the outer membrane (Daugelavičius *et al.* 2000), further aiding tetracycline entry into biosensor cells. PMB also removes outer membrane stabilizing cations, but its main antibiotic effect is forming pores in the outer membrane, and at high concentrations also in the cytoplasmic membrane (Daugelavičius *et al.* 2000).

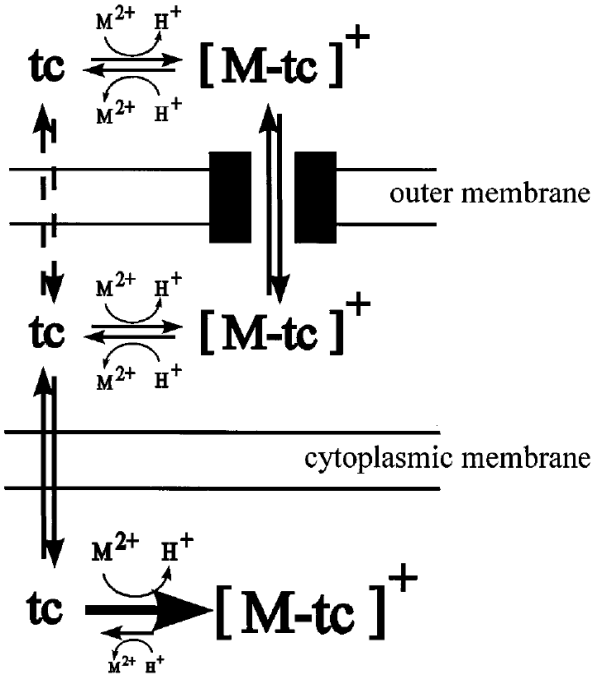


Figure 9.3. The divalent cation (M^{2+}) dependent uptake mechanism of tetracycline into Gram negative cells (Schnappinger and Hillen 1996). Tetracycline is thought to pass the outer membrane of gram-negative bacteria through the porins OmpF and OmpC chelating a M^{2+} ion as $[M\text{-}tc]^+$. The cationic $[M\text{-}tc]^+$ accumulates in the periplasm. After dissociation of the $[M\text{-}tc]^+$ complex the uncharged tetracycline is able to diffuse through the cytoplasmic membrane. In the cytoplasm the chelate must reform as only the chelate can bind the ribosome or TetR (Lederer *et al.* 1995, Aleksandrov and Simonson 2008a).

In the optimized assay, 0.5 µg/ml PMB was used during biosensor cell reconstitution and dilution prior to the assay, and a final concentration of 25 mM EDTA was dispensed to the microtiter plate wells with the tissue fluid samples (Tables 1 and 2 in **III**). In addition, the reconstitution medium was phosphate buffered to pH 6 to prevent the alkaline EDTA solution from elevating the pH above the intracellular pH, because TCs accumulate in the compartment with higher pH (Schnappinger and Hillen 1996). The dose-response curves of the four tetracyclines showed detection limits well below the MRL of 100 µg/kg set for muscle tissue (EC 1990, 1999, 2010a) (Figure 2 in **III**). Detection limits achieved in the assay were 5 µg/kg for DC, 7.5 µg/kg for CTC, and 25 µg/kg for TC and OTC. In other tetracycline bioassays, the limit of detection was 20 µg/kg for TC and slightly higher for OTC in fish muscle tissue, and 6 µg/l for TC, 25 µg/l for OTC, and 3 µg/l for DC and CTC in milk using the same biosensor strain (Kurittu *et al.* 2000c, Pellinen *et al.* 2002).

The tetracycline biosensor assay interestingly provided proof of induction capacity and antibiotic effect of tetracycline 4-epimer metabolites, including 4-epidoxycycline previously thought ineffective (Figure 3 in **III**). The results were confirmed by a microbiological growth inhibition assay routinely used in monitoring tetracycline residues in tissue fluid samples (Pikkemaat *et al.* 2008) (Figure 4 in **III**). The bioluminescence signal and growth inhibition was larger than would be inflicted by the parent compound present in the 4-epimer sample as an impurity. Also, the assay conditions favor epimerization instead of reversion back to parent compound (Anderson *et al.* 2005). The study on antibiotic nature of TC metabolites is of interest since 4-epidoxycycline was included in the provisional EU maximum residue limit list but left out from the final version (EC 1990, 1999, 2010a) due to assumed biological inactivity (Croubels *et al.* 1998a). It was also assumed that unlike other 4-epimers, 4-epiDC is not formed during sample preparation (EMEA 1997). 4-epimer metabolites of the other three veterinary tetracyclines are therefore listed as MRL marker residues along with the parent compound (EC 2010a). However, increasing evidence suggests 4-epimer metabolites including 4-epiDC are also formed *in vivo* (Croubels *et al.* 1998b).

In paper **II**, the overlay assay for screening for nisin producers showed bioluminescence induction in the area surrounding a nisin producing colony within 1 h of application of the sensor layer, but no induction around a nisin non-producer (Figure 1 in **II**). The assay was used for detection and isolation of nisin producers in mixed cultures of lactic streptococci originating from raw bovine milk (Figure 2A in **II**). However, after overnight incubation of the overlaid plates, growth inhibition zones were visible around colonies other than the nisin producers. Therefore, nisin producer screening assay specificity was verified by identifying bioluminescence-inducing nisin producers and otherwise antagonistic *i.e.* inhibition zone-producing colonies among 144 raw milk lactic streptococcal isolates and a panel of 91 lactococcal strains (Figure 2B in **II**). PCR-amplification of the nisin structural gene in a total of 53 antagonistic isolates showed that only the bioluminescence-inducing colonies harbored the nisin gene. An exception to the rule was strain *Lactococcus lactis* subsp. *lactis* SL149, which

carried a modified nisin Z gene (Figure 3 in **II**). The other four lactococcal panel strains produced nisin A, and all seven raw milk isolates produced nisin Z (four isolates from mixed cultures and three colonies from lactic streptococcal isolates) (Table 1 in **II** and Figure 9.4). BOX-PCR and RAPD experiments (Supplementary Figures 1A and 1B in **II**) showed the genetic fingerprints of the seven raw milk isolates divided them in three groups, so it was not a question of one multi-isolate. Nisin producing raw milk isolates were identified as *Lactococcus lactis* subsp. *lactis* by 16S-rRNA gene sequencing and his operon amplification (Table 1 and Supplementary Figure 1C in **II**).

Screening assay specificity was also tested with 11 strains producing reuterin or bacteriocins other than nisin. None of these strains induced bioluminescence, indicting nisin specificity of the assay (Supplementary Figure 2 in **II**). The antagonistic substance produced by strain SL149 was shown to be of lower molecular weight than nisin by SDS-PAGE and overlay nisin bioassay (Figure 4 in **II**). Based on these results and verified assay specificity, the substance in question was not a non-inducing nisin variant resulting from modifications in the nisin Z gene, but some other bacteriocin. Possible candidate bacteriocins lacticin 481, lacticin 3147 and bacteriocin J46 were identified by a search in two bacteriocin databases, BACTIBASE (Hammami *et al.* 2010) and BAGEL2 (de Jong *et al.* 2010).

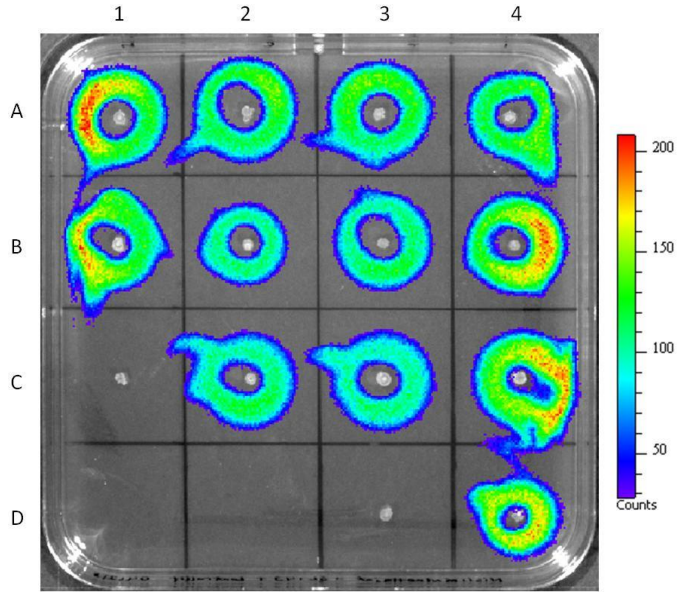


Figure 9.4. Bioluminescence induction by nisin-producers. Nisin production in all eleven nisin-producing strains identified in paper **II** is indicated by bioluminescence production in the nisin biosensor layer after 1 h induction. As expected, strain SL149 (position C1) and nisin non-producer NZ9000 (D3) did not induce bioluminescence. **A1**, RM-III2; **A2**, RM-III1; **A3**, RM-II; **A4**, RM-I; **B1**, SD12; **B2**, IV-17; **B3**, III-32; **B4**, I-12; **C1**, SL149; **C2**, SL29; **C3**, SL28; **C4**, SD14; **D3**, NZ9000 (nisin non-producing control); **D4**, 20729 (nisin producing control). Unpublished data.

9.3 Regulatory element properties and performance (I, II, III, IV)

The extremely high sensitivity of the nisin biosensor assay (**I**) when compared to other nisin detection and quantification assays (see Table 6.1) is indicative of the sophisticated ability of the NisRK two-component system to identify nisin and rapidly relay the response to induce transcription of genes under control of the *nisA* promoter. The bioluminescence signal from biosensor strains NZ9000lux and NZ9800lux was detectable in less than ten minutes from induction (Figure 2 in **I**). This response time time is extremely fast, since *e.g.* a DNA damage responsive *E. coli* biosensor with a *recA'*::*luxCDABE* reporter construct expressed detectable mitomycin-induced bioluminescence after approximately one hour (Vollmer *et al.* 1997), and a multidrug-responsive *Staphylococcus aureus* QacR/*qca*::*luxCDABE* biosensor after 30 min from benzalkonium chloride induction (Galluzzi and Karp 2007). Both host strains used in constructing the nisin biosensor strains harbor chromosomal *nisRK* genes. The chromosomal location of these genes was proven beneficial by Hakovirta *et al.* (2006) whose fluorescent nisin biosensor based on NZ9000 host strain was more sensitive than another biosensor strain where *nisRK* were present on the biosensor plasmid along with the promoter::reporter gene construct (Reunanen and Saris 2003). The same study deemed the *nisA* promoter stronger than P_{nisF}, which ensures more efficient reporter protein expression. P_{nisA} is also used in the NZ9000lux and NZ9800lux biosensor strains (**I**).

Using the nisin producer screening assay, producers of nisin variants A and Z were identified, therefore showing the NisRK two-component regulatory protein system reacts to both variants (Table 1 in **II** and Figure 9.4). The NisRK system should respond to the other known nisin variants nisin F, nisin Q, nisin U and nisin U2 as well, since these variants have been shown to induce expression from P_{nisA} via NisRK (Wirawan *et al*. 2006, Piper *et al.* 2011). Specificity tests with 11 strains producing reuterin or bacteriocins other than nisin showed no reaction to these substances, demonstrating the NisRK regulatory system recognizes nisin in a very specific manner (Supplementary Figure 2 in **II**). Similar results have been obtained by Wahlström and Saris (1999) and Kuipers *et al.* (1995) who reported lantibiotics such as subtilin, the structurally closest analog of nisin, as well as carnocin, sakacin A, lacticin 481, and Pep5, and the antimicrobial peptide lactococcin A do not induce transcription through the NisRK system. The control exerted by NisRK and P_{nisA} was very tight, as the background expression of *luxABCDE* was only 0.02–0.05% at maximum response (**I**), whereas whole-cell biosensors generally display background expression levels of about 1–5% of the maximum response (van der Meer *et al.* 2004).

Variations in binding affinity of different tetracyclines to TetR are relayed as changes in the biosensor bioluminescence signal strength. The induction potency of different tetracyclines increased in the order OTC < TC < CTC < DC, whereas the 4-epimer metabolites had a slightly different order 4-epiOTC < 4-epiDC < 4-epiTC < 4-epiCTC (Figures 2 and 3 in **III**). All epimers were weaker inducers than the parent compounds. These differences reflect the variations in

binding affinity of the TC analogs to TetR repressor that controls bioluminescence induction. TetR has been determined to bind to TC and OTC with similar affinity, whereas CTC and DC bind TetR with higher and 4-epiTC with lower affinity (Lederer *et al.* 1996). Also TetR induction efficiency of the TC analogs varied according to binding affinity (Lederer *et al.* 1996), an effect seen in paper **III** as well. The background expression level in the tetracycline biosensor was 2–4% of maximum, which is a typical value for whole-cell biosensors (van der Meer *et al.* 2004).

In paper **IV**, DNA affinity affecting mutations were rationally designed in the erythromycin specific repressor MphR(E) based on a homology model (Figure 2 in **IV**) built using the crystal structure of a related repressor MphR(A) (Zheng *et al.* 2009) as a reference. Structure-function studies of MphR(E) and its mutants showed variations in DNA binding affinity (Table 1 and Figure 4 in **IV**). Among the seven rationally designed mutants studied, there were three with increased (K, KC and CG), one with similar (Y) and three with decreased (H, N, L) affinity to operator DNA. However, mutation T35K alone can likely be accredited with producing the improved affinity of double mutant KC, as the affinity of these two mutants was similar. Mutants K, KC and CG showed approximately an 1.3-fold improvement in DNA binding affinity when compared to wild type MphR(E) which bound operator DNA with a dissociation constant K_d of 289 ± 20 nM. MphR(E) bound its operator DNA with a higher affinity than the related repressor MphR(A) (K_d of 574 ± 29 nM) (Zheng *et al.* 2009) but with a much lower affinity than TetR (K_d of 0.2 nM) (Kamionka *et al.* 2006). Despite of the relatively low affinity towards operator DNA, MphR(A) has been successfully used as the recognition element in an *E. coli* whole-cell biosensor responsive to macrolides (Möhrle *et al.* 2007). However, in addition to inserting the entire *mphA* operon (Figure 1 in **IV**) in the host strain chromosome, an extrachromosomal vector for overexpression of MphR(A) had to be included in the biosensor strain for sufficient repression of *luxCDABE* expression.

In addition to improved DNA affinity, mutant CG was observed to form a covalent dimer through disulfide formation (Table 1 and Supplementary Figure S2 in **IV**). This result was interesting, since in the homology model of MphR(E), the cysteine residues forming the disulfide bond are approximately 20 Å apart, and should therefore not be able to interact. This indicates that the *in vivo* structure of MphR(E) deviates from the model. However, the mutations designed to affect DNA binding did cause affinity changes and there is significant sequence conservation between the DNA binding helix-turn-helix motifs of MphR(E) and MphR(A) (Figure 3 in **IV**) (Szczepanowski *et al.* 2007, Zheng *et al.* 2009), so the model is likely to reflect *in vivo* structure of MphR(E) in these regions. However, the KC double mutant designed to exhibit the affinity improving effect of mutation T35K as well as to form an intermonomeric disulfide bond did not dimerize covalently. Instead, native mass spectrometry showed mutants K and KC form noncovalent dimers (Figure 7 in **IV**). The homology model placed the L182C mutation site in the dimerization interface between the two monomers, but because a disulphide was not observed in

the experiments, it is possible the homology model is not able to predict the protein fold correctly in this region.

Ligand binding properties of MphR(E) were also studied in paper **IV**. The repressor protein did not bind lincosamide ligands lincomycin and clindamycin or 16-membered macrolides tylosin and spiramycin. Instead. MphR(E) bound 14-membered macrolides erythromycin, oleandomycin, and clarithromycin (Figures 5, 6 and 8 in **IV**). Szczepanowski *et al.* (2007) show the *mph(E)* operon (Figure 1 in **IV**) gives resistance to 14-membered erythromycin and roxithromycin as well as to 15-membered azithromycin, so MphR(E) is likely to be induced by this ring structure as well. Conversely, the *mph(E)* operon gave very weak resistance to tylosin which is likely due to leaky expression of resistance genes without MphR(E) induction. Table 9.1 presents the ligand binding spectrum of MphR(A). It is wider than the spectrum of MphR(E), with effective binding of 12- to 16-membered macrolides. Binding of MphR(E) to 12-membered macrolides has not been studied. The EU MRL values have been set for four 16-membered, two 15-membered, and one 14-membered macrolide (EC 1990, 1999, 2010a). Therefore, if used as a biosensor recognition element, MphR(E) cannot respond to all relevant macrolides. Instead, it can be used to distinguish between ligands with varying ring structures.

Mutants K, KC and CG all bound erythromycin but with slightly (K and KC) or significantly (CG) lower affinity than wild type MphR(E) (Table 1 and figures 6-7 in **IV**). It was not surprising that covalent dimerization of CG would affect ligand binding properties as well, but the adverse effect of the DNA binding domain mutation T35K was less expected. Mutation L182C, however, did not affect ligand binding even though it was located in the ligand binding domain. A study of disulfide bonds designed in the TetR structure showed that intermonomeric disulfides can be rationally designed in the dimer without adversely affecting DNA binding or

Table 9.1. Ligand binding spectrum of MphR(A).

Macrolide	**Lactone ring members**	**MphR(A) binding**	**Ref.**	**Macrolide**	**Lactone ring members**	**MphR(A) binding**	**Ref.**
Azithromycin	15	+	[1]	Kitasamycin	16	+	[4]
		++	[2]	Methymycin	12	+++	[3]
		++	[3]	Narbomycin	14	+	[3]
Clarithromycin	14	+++	[1]	Oleandomycin	14	+++	[1]
		+++	[2]			++	[2]
		++	[3]			++++	[3]
Erythromycin	14	++++	[1]			+++	[4]
		+++	[2]	Picromycin	14	+	[3]
		+++	[3]	Roxithromycin	14	++	[1]
		+++	[4]			++	[2]
Josamycin	16	+	[4]	Tylosin	16	++	[2]

[1] Weber et al. 2004; [2] Link et al. 2007;[3] Möhrle et al. 2007; [4] Noguchi et al. 2000.

inducibility (Tiebel *et al.* 1998). However, ligand binding affinity of TetR was improved by some intermonomeric disulfides and weakened by some. Fluorescence anisotropy experiments (Figure 5 in **IV**) showed that CG was the only mutant with weakened inducibility: not even 25-fold molar excess of erythromycin was enough for complete dissociation from operator DNA. The other mutants had an inducibility similar to wild type MphR(E). Disulfide bond formation over such a long distance (20 Å as indicated by the homology model) may distort repressor structure more than intermonomeric bonding occurring over a shorter distance (6–8 Å) in TetR (Tiebel *et al.* 1998).

In the crystal structure of MphR(A), a chloride ion was located in close proximity to the ligand binding site (Zheng *et al.* 2009). Therefore, the effect of chloride ion on ligand binding by MphR(E) was determined (Figure 6d in **IV**). The chloride ion did not bind MphR(E) and no changes in the ligand binding interaction were seen, so it seems to have no part in MphR(E) function. In TetR, the Mg^{2+} ion of the TC-Mg complex is primarily responsible for the structural change required for induction (Orth *et al.* 1999). A new mode of negative allosteric modulation of ligand binding was suggested by the mass spectrometry results (Figures 6-7 in **IV**), as the abundance of the two-ligand complex was lower than expected if the K_d values were the same for the two binding sites. Fluorescence anisotropy results indicated both ligand binding sites must be occupied for full induction of MphR(E) (Figure 5 in **IV**). This is consistent with results from TetR (Orth *et al.* 1999, Kamionka *et al.* 2006). The majority of TFRs bind two ligands per dimer, but some only one. A single molecule of a bulky ligand can cause the structural change required for induction, but two molecules of smaller ligands are required (Itou *et al.* 2010).

Pathogenic origin of a protein may cause doubts about whether it is suitable for use in e.g. biosensor applications. MphR(E) gene harboring plasmid pRSB111 was isolated in wastewater, and therefore the host organism is not known (Szczepanowski *et al.* 2007). Plasmid pRSB111 is a member of the incompatibility group IncP-1, which consists of broad host-range conjugative plasmids that are deemed potent vehicles for the spread of antibiotic resistance within and between bacterial communities (Schlüter *et al.* 2007). Plasmid pRSB111 is closely related to prototype IncP-1β plasmid pB3, whose plasmid-host history suggests its ancestry consists of two putative hosts: *Ralstonia solanacearum*, a phytopathogen, and *Eggerthella lenta*, a gut commensal which rarely causes infection (Norberg *et al.* 2011). The result was based on genomic signature analysis of conserved backbone regions descending from parental plasmids, which have evolved in different hosts. Therefore, MphR(E) is not of human pathogen origin. Like all Inc-P1 plasmids, pRSB111 is self-transmissible, and efficient transfer from *Escherichia coli* to *Escherichia coli*, *Pseudomonas* sp. and *Ralstonia eutropha* has been shown (Szczepanowski *et al.* 2007).

MphR(E) mutants with improved DNA affinity can benefit whole-cell biosensor design, as higher DNA affinity can be expected to lead to more efficient repression of the reporter gene and lowered background signal values. Mutant K also showed ligand binding and induction properties almost similar to wild type MphR(E) (**IV**). In contrast, mutant CG showed improved

DNA affinity but impaired ligand binding and induction. In a whole-cell biosensor setting, this would likely result in tightened repression but also induction at higher macrolide concentrations, i.e. lower sensitivity. However, the use of mutant CG as the recognition element might lead to a wider assay dynamic range as dissociation from DNA occurs on a wider range of macrolide concentrations than normal. TetR mutants with enhanced DNA affinity were characterized by repression of β-galactosidase activity in a whole-cell biosensor (Helbl and Hillen 1998, Helbl *et al.* 1998). Therefore, enhanced transcriptional control by the TetR mutants was verified in an *in vivo* gene regulatory circuit. This remains to elucidated for MphR(E) mutants. It is not self-evident that regulatory proteins with enhanced function improve biosensor functioning: mutants of the DmpR regulatory protein with enhanced ligand binding did not functionalize into elevated transcription initiation (Skärfstad *et al.* 2000).

9.4 Applicability of biosensor assays in screening (I, II, III)

The functionality of the nisin bioassay as a screening test was first shown by identifying a nisin-producing strain among three *L. lactis* strains (**I**). Since it is laborious to perform screening through a full bioassay with culture medium dilutions as samples, a simple nisin producer screening assay in plate overlay format was developed (**II**). The bioluminescence induction surrounding a nisin-producing colony is detected directly from the plate without a separate assay, and results are obtained very rapidly, within one hour. High-throughput capacity of the screening overlay assay was increased by employing a plate divided in a grid of thirty-six 2 cm x 2 cm squares, on which inoculations of isolated cultures were made. In paper **II**, the specificity of the screening assay for nisin was also confirmed, further corroborating its reliability. The PCR methods used for nisin producer identification falsely recognized one strain as a nisin producer, but the nisin screening assay and confirmatory studies verified the strain as a nisin non-producer that harbors a non-functional nisin structural gene giving a positive PCR result. Presence of nonfunctional nisin genes has been shown previously (Moschetti *et al.* 1996, Vuyst 1994). The phenomenon can be due to dysfunction or absence of the nisin biosynthesis operon genes or proteins since production of nisin is a concerted action of a number of gene products (Lubelski *et al.* 2008). Unlike growth inhibition assays, the overlay assay directly identifies the antimicrobial in question is indeed nisin, and avoids the PCR-based method pitfall related to nonfunctional nisin genes. The overlay assay is expected to be responsive to all known natural nisin variants, but it cannot identify the nisin variant in question. Therefore, a confirmatory method such as the LC-MS method by Zendo *et al.* (2007) could be employed for variant identification.

Since inducible whole-cell biosensor response to analytes is typically group-specific and depends on potency of the inducer, it is generally not possible to identify the analyte. However,

Smolander *et al.* (2009) have developed an algorithm which allows direct identification of β-lactams inducing bioluminescence in a compound-specific biosensor. By following response trajectories over 300 minutes, it was possible to differentiate between 15 β-lactams. The classification system is scalable to larger sets of antibiotics of the same class, or antibiotics of other classes than β-lactams. This kind of an approach has potential in screening for new antibiotics and identification of antibiotic residues. It could be employed in direct identification of nisin variants (**II**) or tetracyclines (**III**) causing induction in the screening assays.

Nisin and tetracycline biosensors were readily lyophilized for reagent-like use in the assay, and performed similarly to freshly cultured cells (**I**, **III**). In paper **II**, freshly cultured NZ9800lux cells were used in the assay, but there is no impediment to using lyophilized cells in the overlay. Both nisin and tetracycline biosensors express inherent bioluminescence without the addition of exogenous substrates. This further simplifies their use in screening. The nisin biosensor was detected to maintain a constant induction level for several hours (Figure 1 in **I**), which adds to its applicability in screening. Unlike growth inhibition methods, whole-cell biosensor assays are suitable for assay miniaturization (van der Meer 2004). A plate assay format is as an essential prerequisite for a rapid, inexpensive high-throughput screening system.

In a follow-up study of paper **III**, Pikkemaat *et al.* (2010) used the tetracycline biosensor assay in routine screening analysis of poultry muscle samples. The method was determined to be specific and robust. It correctly identified noncompliant samples, but indicated more suspect samples than a microbial inhibition test. This can be avoided by adjusting the cut-off value selected for differentiating between suspect and compliant samples. The whole-cell biosensor assay was faster, more sensitive and more cost-effective than the microbiological assay. Market price per sample was 15 € with the microbiological and 7.5 € with the biosensor assay.

10 CONCLUSIONS

Whole-cell biosensors are a valid alternative in development of antibiotic detection and screening methods. The tetracycline and nisin assays developed in this study (**I**, **II**, **III**) are simple and rapid to perform, have good high-throughput capacity, and need minimum sample pretreatment and no exogenous substrate additions. These methods show potential in replacing the less specific and sensitive, very laborious and voluminous microbial growth inhibition assays used in screening for antibiotic residues and nisin producers as well as nisin quantification. The biosensor bacteria are compatible with lyophilization, facilitating ready availability and reagent-like use.

The nisin assay (**I**) shows extremely high sensitivity towards it analyte due to the strict control and efficient transcription activation by the NisRK two-component regulatory system and *nisA* promoter. It is the most sensitive nisin detection method ever published. Of the two nisin biosensor strains, the performance of NZ9800lux exceeded that of NZ9000lux in most aspects. Most importantly, NZ9800lux showed a wider linear range and higher induction coefficients due to a lower background signal. NZ9000lux did have higher sensitivity towards nisin due to the absence of nisin immunity proteins, but the difference to NZ98000lux was negligible.

The nisin bioassay (**I**) can be used for quantitative determination of nisin concentrations in simple sample matrices such as growth medium. However, low recoveries from milk suggest a more efficient nisin extraction protocol is needed for complex sample matrices.

The nisin biosensor strain was successfully used in screening for nisin producers in raw milk and among a panel of lactococcal strains (**II**). The assay could identify nisinogenic bacteria in mixed cultures after a simple plating and overlay protocol. Four nisin A producers were identified among the lactococcal panel strains, and seven nisin Z producers in raw milk. The seven nisin Z producers were not a multi-isolate, as they divided in three groups by genetic fingerprinting.

The screening assay proved very specific for nisin, as other bacteriocins and substances produced by antagonistic bacteria did not induce bioluminescence (**II**). The results were verified by PCR amplification which showed only strains harboring the nisin structural gene induced bioluminescence. An exception was a strain that was antagonistic and harbored a modified nisin gene but did not induce bioluminescence. With additional experiments, this strain was confirmed to produce a bacteriocin of lower molecular weight than nisin, and not a non-inducing variant of nisin.

A whole-cell biosensor assay for tetracycline residues in poultry muscle tissue was developed in paper **III**. An assay sensitization method was developed to lower the detection limit of all veterinary relevant tetracyclines to below-MRL concentrations. Sensitization was performed using membrane permeabilizing and chelating agents polymyxin B and EDTA. Together these

facilitate analyte entry into the biosensor cell and promote tetracycline accumulation into the intracellular compartment.

Veterinary tetracycline 4-epimer metabolites were shown (**III**) to harbor induction capacity of the tetracycline biosensor as well as antimicrobial activity which has previously been thought absent for 4-epidoxycycline. Therefore, the inclusion of this metabolite as a marker residue for doxycycline MRLs should be reconsidered.

The tetracycline biosensor assay showed acharacteristic induction potency for each tetracycline antibiotic and 4-epimer tested. These differences reflect variations in the binding affinity of each substance to the recognition element TetR.

This study included the first ever structure-function study of the macrolide-specific repressor protein MphR(E) (**IV**). DNA affinity affecting mutations were rationally designed on basis of a homology model of MphR(E). Of the seven designed mutants, three (K, KC and CG) showed improved affinity, one similar, and three decreased affinity towards operator DNA as compared to wild type MphR(E). One source of affinity improvement was the T35K mutation present in two mutants. The third mutant CG unexpectedly showed covalent dimerization which was accredited for improved affinity.

The ligand binding spectrum of MphR(E) covered macrolides with a 14-membered lactone ring structure, but not lincosamides or 16-membered ring macrolides (**IV**). MphR(E) mutants K and KC showed slightly impaired ligand binding properties, but induction *i.e*. dissociation from operator DNA occurred with similar efficiency as wild type MphR(E). However, mutant CG had significantly impaired ligand binding properties and induction capability. Covalent dimerization is likely to cause structural changes that affect the ligand binding site and allosteric regulation of DNA binding.

The ligand binding experiments suggested a novel type of negative allosteric modulation of ligand binding among TetR family of transcriptional regulators (**IV**). Chloride ions had no effect on ligand binding by MphR(E). However, both ligand binding sites in the homodimer must be occupied for induction.

11 RECOMMENDATIONS FOR FURTHER STUDY

MphR(E) mutants designed in this study (paper **IV**) can benefit macrolide specific (whole-cell) biosensor design as higher operator DNA affinity can result in more efficient regulation. However, their functionality in an *in vivo* gene regulatory circuit will not necessarily reflect the effects seen *in vitro* and needs to be determined. The biosensor design can be a whole-cell, or the repressor-protein interaction can be used as the biosensing system without the surrounding cell. Such biosensor systems have previously been constructed for tetracyclines (Pellinen *et al.* 2006) and tetracyclines, macrolides and streptogramins (Weber *et al.* 2005). They are based on immobilizing the operator DNA in a well, and allowing the DNA binding reaction of the repressor protein to occur in the presence of varying amounts of the antibiotic analyte. After removing antibiotic-bound repressor from the well by washing, the DNA-bound repressor remaining in the well is quantified. Antibiotic concentration can then be derived from the result.

MphR(E) is one example of an antibiotic specific regulator that has not been utilized in biosensor designs. Regulatory systems specific for one antibiotic group are relatively rare, but necessary in inducible compound-specific biosensor development. When bacteria are treated with sublethal concentrations of antibiotics, they alter global transcription patterns by repressing or activating expression. For example, in *Salmonella typhimurium*, as many as 5% of promoters may be affected (Goh *et al.* 2002). Through these kinds of experiments, new regulator-operator pairs could be identified for biosensing applications. Also, known regulatory proteins can be modified for altered ligand specificity to include or exclude certain molecules in the ligand spectrum (Scholz *et al.* 2003, Hakkila *et al.* 2011)

A multiplate approach is used in microbial growth inhibition assays for simultaneous identification of several compound groups and preliminary classification of the inhibiting antibiotic residue (Pikkemaat *et al.* 2008, 2009a, 2011, Gaudin *et al.* 2010). A similar approach could be used with inducible whole-cell biosensors. Since a compound-specific biosensor does not exist for each antibiotic group, a panel of biosensor bacteria responsive to various antibiotic groups through stress reactions and compound-specific reactions would help in classifying the residue conclusively. Such an approach has been introduced for selected classes of toxic compounds (Belkin *et al.* 1997), and also for antibiotics (Bianchi and Baneyx 1999, Shapiro and Baneyx 2002, 2007, Hutter *et al.* 2004a, Urban *et al.* 2007), but these biosensor panels are not able to conclusively classify the residue. Therefore, compound specific biosensors could be incorporated for more accurate classification. A recent study by Melamed *et al.* (2012) combined a panel of antibiotic-inducible effect-specific whole-cell biosensors and an algorithm-based approach to compute patterns of response by various antibiotics to derive the identity of the inducing antibiotic. This kind of an approach can reduce the need for compound-specificity.

Im paper **II**, the *Lactococcus lactis* subsp. *lactis* strain SL149 was shown to harbor a modified nisin Z gene, and to produce a bacteriocin-like antagonistic substance with a lower molecular

mass than nisin. It would be of interest to find out the reason behind hampered expression of the modified nisin Z gene by PCR amplification and sequencing of the nisin biosynthesis operon to see if the operon is intact. Also, the expression of the modified nisin Z should be verified by mutating a functional nisin structural gene, and the resulting gene product tested for NisRK induction and antimicrobial activity. The nisin producer screening assay could also be applied in *e.g.* monitoring population dynamics of nisin producers used as protective starter cultures in production of fermented foods. It was also suggested in paper **II** that biosensors responsive to other bacteriocins could be constructed by utilizing auto-inducible regulation of their biosynthetic gene clusters. Such a biosensor already exists for subtilin, the structurally closest homolog of nisin (Burkard *et al.* 2007).

The assay sensitization method developed in paper **III** could be utilized as a universal method to facilitate analyte entry into whole-cell biosensors. The sensitization method can in principle be adjusted for any analyte and host cell by the right choice of permeabilizing and chelating agent concentrations and activities. Polymyxin B is effective against Gram negative bacteria (Daugelavičius *et al.* 2000), but permeabilizing antibiotics effective towards Gram positives are known, such as gramicidin S (Kondelewski *et al.* 1996). It would be of interest to test various sensitization methods on various host organisms and analytes.

The nisin bioassay (**I**) showed low analyte recoveries from complex food matrices. Development of a more efficient extraction protocol is therefore necessary. Generally good results have been obtained with nisin extraction protocols based on acid extraction, since unlike most proteins, nisin is highly soluble at pH 2 (Cleveland *et al.* 2001). At low pH, nisin can even withstand heating to 121 °C without losing its activity (Noonpakdee *et al.* 2003). A combination of acidic pH and heating should remove most of the assay interfering molecules with which nisin interacts. Nisin shows interaction with both food proteins and fats (Aasen *et al.* 2003), so separating nisin from lipids should be taken into consideration when designing the extraction protocol. The extraction protocol should be validated for various food matrices in which nisin is typically used as a preservative and/or nisin producers are present.

A follow-up study of paper **III** comparing the tetracycline whole-cell biosensor assay with microbiological inhibition assays and LC-MS/MS detection of tetracyclines has confirmed the value and applicability of the biosensor approach in routine analyses of poultry muscle samples (Pikkemaat *et al.* 2010). In the future, validation for use in routine analysis of samples from other food-producing species and tissues listed in the EU MRLs (EC 2010b) should be performed. Validation should be performed according to European Commission Decision 2002/657/EC (EC 2002b) and following the guideline document by EU Reference Laboratories which describes in detail screening method validation through determination of stability, applicability and ruggedness, as well as selectivity and specificity (Anon 2010). In this way, whole-cell biosensors could gain a foothold among screening methods available for antibiotic residue analysis.

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I

Bioluminescence-based bioassays for rapid detection of nisin in food

Immonen, N., Karp, M.

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II

Bioluminescence-based identification of nisin producers – a rapid and simple screening method for nisinogenic bacteria in food samples

Virolainen, N., Guglielmetti, S., Arioli, S., Karp, M.

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III

Rapid detection of tetracyclines and their 4-epimer derivatives from poultry meat with bioluminescent biosensor bacteria

Virolainen, N.E., Pikkemaat, M.G., Elferink, J.W., Karp, M.T.

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IV

Structural and mutational studies on DNA binding and dimerization properties of the macrolide specific repressor protein MphR(E)

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