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IMPROVEMENTS IN THE ASSESSMENT OF BACTERIAL VIABILITY AND KILLING

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To my Family

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ABSTRACT

It is axiomatic that our planet is extensively inhabited by diverse micro-organisms such as bacteria, yet the absolute diversity of different bacterial species is widely held to be unknown. Different bacteria can be found from the depths of the oceans to the top of the mountains; even the air is more or less colonized by bacteria. Most bacteria are either harmless or even advantageous to human beings but there are also bacteria, which can cause severe infectious diseases or spoil the supplies intended for human consumption. Therefore, it is vitally important not only to be able to detect and enumerate bacteria but also to assess their viability and possible harmfulness. Whilst the growth of bacteria is remarkably fast under optimum conditions and easy to detect by cultural methods, most bacteria are believed to lie in stationary phase of growth in which the actual growth is ceased and thus bacteria may simply be undetectable by cultural techniques. Additionally, several injurious factors such as low and high temperature or deficiency of nutrients can turn bacteria into a viable but non-culturable state (VBNC) that cannot be detected by cultural methods. Thereby, various noncultural techniques developed for the assessment of bacterial viability and killing have widely been exploited in modern microbiology. However, only a few methods are suitable for kinetic measurements, which enable the real-time detection of bacterial growth and viability.

The present study describes alternative methods for measuring bacterial viability and killing as well as detecting the effects of various antimicrobial agents on bacteria on a real-time basis. The suitability of bacterial (lux) and beetle (luc) luciferases as well as green fluorescent protein (GFP) to act as a marker of bacterial viability and cell growth was tested. In particular, a multiparameter microplate assay based on GFP-luciferase combination as well as a flow cytometric measurement based on GFP-PI combination were developed to perform divergent viability analyses. The results obtained suggest that the antimicrobial activities of various drugs against bacteria could be successfully measured using both of these methods. Specifically, the data reliability of flow cytometric viability analysis was notably improved as GFP was utilized in the assay. A fluoro-luminometric microplate assay enabled kinetic measurements, which significantly improved and accelerated the assessment of bacterial viability compared to more conventional viability assays such as plate counting. Moreover, the multiparameter assay made simultaneous detection of GFP fluorescence and luciferase bioluminescence possible and provided extensive information about multiple cellular parameters in single assay, thereby increasing the accuracy of the assessment of the kinetics of antimicrobial activities on target bacteria.

ABBREVIATIONS

ATP	adenosine 5´-triphosphate
BL	bioluminescence
CF	correction factor
CFU	colony forming units
CL	chemiluminescence
CLSI	clinical and laboratory standards institute
CP	chromoprotein
DD-method	disk diffusion method
DNA	deoxyribonucleic acid
EGFP	enhanced green fluorescent protein
Em_{max}	emission maximum
Exc_{max}	excitation maximum
FCM	flow cytometry
$FMNH_2$	reduced flavin mononucleotide
FP	fluorescent protein
FS	forward scatter
GFP	green fluorescent protein
HTS	high throughput screening
KDa	kilodalton
lucFF	firefly luciferase
luxAB	bacterial luciferase
MAC	membrane attack complex
MBC	minimum bactericidal concentration
MCR	mixed culture recovery
MDR	multidrug resistant
MFI	mean fluorescence intensity
MIC	minimum inhibitory concentration
MPN-method	most probably number method
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NASBA	nucleic acid sequence-based amplification
PCR	polymerase chain reaction
PI	propidium iodide
PMT	photomultiplier tube
PPi	pyrophosphate
RNA	ribonucleic acid
ROS	reactive oxygen species
SDA	strand displacement amplification
SS	side scatter
TPP	three-phase-partition
VBNC	viable but non-culturable

LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following original publications, referred to in the text by Roman numerals:

I Lehtinen, J., Virta, M. & Lilius, E. M. (2003). Fluoro-luminometric real-time measurement of bacterial viability and killing. *Journal of Microbiological Methods*, 55:1, 173-186.

II Lehtinen, J., Nuutila, J. & Lilius, E. M. (2004). Green fluorescent protein-propidium iodide (GFP-PI) based assay for flow cytometric measurement of bacterial viability. *Cytometry PartA*, 60A, 165-172.

III Lehtinen, J., Järvinen, S., Virta, M. & Lilius, E. M. (2006). Real-time monitoring of antimicrobial activity with the multiparameter microplate assay. *Journal of Microbiological Methods*, 66:3, 381-389.

IV Lehtinen, J. & Lilius, E. M. (2007). Promethazine renders *Escherichia coli* susceptible to penicillin G: real-time measurement of susceptibility of bacteria by fluoro-luminometry. *International Journal of Antimicrobial Agents*, 30:1, 44-51.

In addition, some unpublished data are presented.

1. INTRODUCTION

Countless numbers of various microbiological samples are examined annually using distinct analyzing techniques, and in general each technique is most suitable for a given application. In order to relieve the incoherent situation of microbial analysis, some methods are accepted as standard reference methods and are therefore widely applied for routine use in both clinical and research laboratories. Still the heterogeneity between different methods is extensive and the need of ever-preferable methods for microbial analysis is obvious. The culture of microbial cells is mostly in a dynamic state in which both viable and dead cells occurs. Notably, different metabolic levels and vital stages of bacteria are simultaneously displayed, which reflect on such matters as the safety of food supplies for human and animal consumption as well as on the sterility and the efficacy of various pharmaceutical compounds. Accordingly, numerous antimicrobial agents, either natural or synthetic, have been found and used to eliminate bacteria or to inhibit their replication. Conventional antibiotics are most commonly used as a traditional device for restricting bacterial growth but also various disinfectants, some non-antibiotic drugs and other antimicrobials are widely employed. Typically, the number of viable micro-organisms is of particular interest. However, in some situations the number of dead cells can also be remarkable, and thus the number of both living and total microbial cells has to be defined. Simple methods which define the cell viability and the cell number with minimum delay are most desired for these purposes. Unfortunately, for most measuring techniques exploited, the time frame for obtaining results is unacceptably late. Usually it takes from several hours to days to yield reliable results, and for some situations, this delay that limits their relevance, may overcome if similar data could be obtained by the real-time analysis. Moreover, conventional methods for microbial analysis are usually laborious and in many cases do not reveal non-culturable micro-organisms.

Improvements in instrumentation and in assay reagents have offered new possibilities for following bacterial viability and killing on a real-time basis. Flow cytometry (FCM), for instance, in conjunction with reagents such as fluorescent antibodies or specific dyes with affinity to nucleic acids or cell membrane is a tool that has extensive application to microbial research. However, high cost, the complexity of instrument and the need of user-expertise have restricted the use of FCM in routine microbial analysis. On the other hand, other modern instruments such as fluorometers, luminometres and plate reading photometers are cost-effective and simple to use and are thus widely exploited for various microbiological applications. Molecular methods including the analysis of both DNA and RNA may also be applied with great sensitivity and rapidity for the assessment of bacterial viability. However, the correlation between occurrence of DNA/RNA and viability is not necessarily always well-defined which slightly diminish the power of these methods. Different multi-counter based applications, which allow several technologies such as fluorescence, luminescence and absorbance to be measured simultaneously are perhaps the most prevalent methods exploited in modern microbial research. Multi-counters are versatile instruments which enable real-time detection of parameters linked to bacterial viability

and killing making the multiparameter assays well applicable for a wide range of different microbial analyses. However, not only the incomparable progress of various measuring techniques, equipments and assay reagents but also the exploitation of different reporter proteins such as bacterial or beetle luciferaces and green fluorescent protein (GFP) have offered ever diversified alternatives for various measurements in the field of microbiology.

In this literature review, the complexity of the quantification and the enumeration of the microbial content are considered. Some of the tools and technologies currently available for the measurement of both bacterial viability and killing as well as the susceptibility of micro-organisms against various antimicrobial agents are briefly described in outline. The comparison between the advantages and disadvantages of those methods is also included in this thesis. Particularly, the benefits of real-time detection for microbial research are summarized. In addition, a short discussion on particular reporter proteins utilized in microbial research is incorporated into the review.

2. REVIEW OF LITERATURE

2.1 Bacterial viability and killing

Ever since micro-organisms were discovered humans have for variety of reasons tried to control and restrict their growth. Sometimes the presence of living cells is essential, whilst in some situations the growth of bacteria has to be completely blocked and thus the ability to measure both the viability and the killing of bacterial cells has a major role in microbiological analyses. The definition of viability, however, is a challenging task, and there is no simple answer how to define it. First it should be established how to separate viable micro-organism from nonviable ones. This question is of particular importance since one has to know a prerequisite of viability before it can be stated if bacteria are viable or not. The growth of bacteria is a fast and dynamic process, and mostly bacterial culture consists both of living and dead cells. The formation of new bacterial cells occurs typically within minutes to some hours and all environmental factors which bacteria are confronted have a distinct effect on bacterial growth and death thus making the exact definition of bacterial viability sometimes quite sophisticated (Neidhardt *et al.*, 1990; Prescott *et al.*, 2004; Roszak & Colwell, 1987). The viability is traditionally defined as the ability of bacterial cells to form colonies on solid agar plates in suitable conditions and/or to proliferate in solutions with sufficient nutrients. In contrast to viable cells, dying cells have irreversibly lost their capability of growth and multiplication. It is worthwhile to note that the formation of colonies on a plate, which is a common definition of viability, does not reveal the viability of individual cells in a sample but rather tells that there were cells in the sample that were capable to grow and divide. Moreover, the definition of cell death addresses that all vital functions of bacteria are ceased, and thus a cell, which is simply unable to grow and multiply is not necessarily dead. Yet, dead cells can produce secondary metabolites that promote the growth of other cells. However, this is not a question of active metabolism but rather a slow diffusion of dissolved cell compounds into the surroundings, otherwise those cells cannot be dead (Barer, 1997; Barer & Harwood, 1999; Nystrom, 2001; Nystrom, 2004).

Variability is a special hallmark of microbial systems. The natural bacterial cultures have a remarkable capacity to display different metabolic levels and vital stages which determine the ability of bacteria to grow and reproduce. This in turn reflects on the life cycle of bacteria, which consists four different phases: lag, exponential, stationary and death (Figure 1). These phases vary considerably in length depending on the nature of micro-organisms and environmental factors such as medium, oxygen content, temperature, and so on. In lag phase of the growth bacteria display distinct metabolic activity and new cell components are synthesized, yet there is no or only modest increase in cell number and mass. The length of lag phase is greatly dependent on the conditions under which cells existed before they were introduced into medium. At the end of lag phase both the cell number and the total cell mass start to increase quickly resulting the transition to the exponential (log) phase of growth in which bacteria are

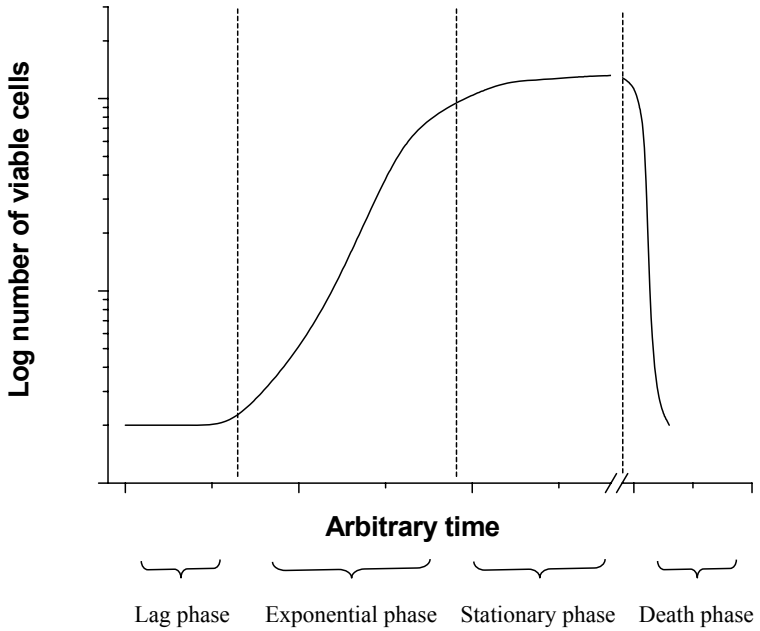

Figure 1. Typical microbial growth curve. The four phases of the growth of micro-organisms are marked on the curve. The number of viable cells can be determined by any readily measurable property of the cell culture such as its turbidity, the amount of biomass or colony forming units.

growing and dividing at the maximal rate possible. Their growth is constant throughout the phase and the cell population is most uniform in terms of chemical and physiological properties. If a culture in exponential growth is fed by fresh medium of the same composition, it will continue to grow exponentially until the maximum cell density is reached. However, under normal circumstances exponential growth is a short-term phase due to several factors that restrict bacterial growth, and eventually the growth ceases and population reach the state called stationary phase. In stationary phase the number of cells remains constant which may result from a balance between cell division and cell death, or the population may simply cease to divide although remaining metabolically active. Most micro-organisms are believed to be sited on this final state of growth (Bauman *et al.*, 2006; Neidhardt *et al.*, 1990; Prescott *et al.*, 2004; Zwietering *et al.*, 1990). Note that the metabolic activity of bacterial cells is in most cases regarded as a fundamental criterion for defining those micro-organisms, which are considered to be viable. Therefore, the cells in stationary phase exhibiting some degree of metabolic activity are denoted viable regardless of the fact that they might have irreversibly lost their ability to replicate (Nystrom, 2001). This means that the failure of bacteria to multiply does not necessarily mean that the cells are dead although cells unable to grow are traditionally called dead cells (Colwell, 2000; Roszak & Colwell, 1987). Finally, the accumulation of toxic wastes and secondary metabolites as well as depleted nutrients may induce such a cellular injury that cannot be reversed, thus causing the cell death. Death is irreversible state and easily detected by a remarkable loss both in cellular activity and integrity. A decrease in cell integrity

indicates that dead cells are usually rapidly decomposed in cell culture and therefore total cell counts decrease due to the presumable cell lysis. However, depending on several environmental factors, dying bacteria may maintain their cell integrity for undefined time which may complicate the assessment of cell death on the basis of cell lysis (Bauman *et al.*, 2006; Neidhardt *et al.*, 1990; Nystrom, 2001; Prescott *et al.*, 2004). Moreover, according to the statement that viable cells, in contrast to dead cells, are defined with capability to grow and multiply it follows that one has to wait any cell division to occur before it can be stated that a cell is alive. However, it cannot be stated that a given cell is alive only that it was alive during cell division (Kell & Young, 2000). Therefore, the whole definition of viable and dead cells is more or less retrospective and in some situations it might be troublesome to define whether an individual cell is simply alive or dead. On the other hand, continuous progress of techniques capable for measuring both the metabolic activity and cellular integrity has assisted to establish the cell viability and killing in more details.

2.1.1 Viability in contrast to culturability

Viability and culturability has often considered possessing synonymous meaning. However, as already stated in previous section, the term viability has multifarious nature and thus terms "viability" and "culturability" may or may not have an equal intent, which greatly depends on current way of definition. Growth and cell division detected by standard microbiological methods are common requirements for viable bacteria, that is, viability is equated with culturability (Kell & Young, 2000). Moreover, if viability is defined by the ability to grow, viable but non-culturable bacteria is a concept without a sense (Barer, 1997). However, non-culturable cells may possess a distinct activity and should be thus denoted viable. Culturability in turn is defined by the ability of a single cell to produce a distinct population, usually a visible colony on the plate (Bogosian & Bourneuf, 2001). Thus it follows that all culturable cells are considered to be viable, whilst all viable cells are not necessarily culturable. On the other hand, the resolution of culture based approaches may be beyond the ability for measuring microbial viability, which further complicates the separation between culturability and viability. Specifically, the inconvenience between these terms results from feature of some viable cells to enter to state where they become temporary non-culturable (for comprehensive reviews, see Barer & Harwood, 1999; Kell *et al.*, 1998; Nystrom, 2001; Nystrom, 2003; Nystrom, 2004).

The global diversity of bacteria has been estimated at $10^7 – 10^9$ species (Curtis *et al.*, 2002). Accordingly, an expanded interest in need to clarify the link between viability and culturability raises from the facts that vast majority of bacteria have never been propagated or characterized in laboratory culture (Rappe & Giovannoni, 2003), besides some bacteria may by various stimuli enter to the state in which they become temporarily non-culturable. Most assertions that viability equates culturability are currently based on culture tests. However, the introduction of new techniques, which are suitable for measuring either the activity or the integrity of different micro-organisms has facilitated the estimation of bacterial viability independently of visible cell growth, making it more and more easier to distinguish between culturable and non-culturable cells (Barer & Harwood, 1999). When culturable bacteria are subjected to

conditions not optimal for the growth, the total cell counts usually remain constant but the culturable cell counts decline. The most simple explanation of this phenomenon is that the cells are passing from viable state to the cell death. However, an alternative explanation is that the cells are indeed viable, yet they have become temporary non-culturable. Accordingly, a new term called a viable but non-culturable (VBNC) has been introduced during recent years for the apparently viable bacterial cells which have become non-culturable by certain stimulus. Bacteria possessing VBNC state fail to grow on routine bacteriological plate or solution in which they normally grow, yet these bacteria are still alive and express fluctuating metabolic activity. Bacteria may enter to the VBNC state due to various external stress factors such as coldness, deficiency in nutrients, osmotic shock, inappropriate oxygen concentration or exposure to white light (Oliver, 2005). The stress factors usually introduce injury in bacteria making them not immediately culturable or alternatively these factors are believed to trigger a specific genetic survival mechanism in bacteria that halts the growth causing bacteria to enter VBNC state (Bogosian & Bourneuf, 2001). Viable but non-culturable cells will predominantly remain in non-culturable state until exposed to factors which stimulate their resuscitation, or alternatively they gradually shift to the cell death (Kell & Young, 2000; McDougald *et al.*, 1998; Oliver, 2005). Moreover, there are several changes detected both in cellular activity (such as decrease in synthesis of macromolecules, in transport of nutrients or in cell respiration) and in cell morphology (such as reduction in cell size and in cell shape) (Oliver, 2005; Smith & Oliver, 2006). Most of these changes are analogous to comparable phenomena detected in the spore-forming bacteria, yet it is momentous to distinguish bacteria lying in VBNC state from spores (Roszak & Colwell, 1987). Notably, the bacteria lying in VBNC state are normally undetectable by standard cultivation based methods, yet some viable but non-culturable bacteria may be extremely pathogenic.

Since growth of bacteria cannot proceed in perpetuity, micro-organisms gradually lose their reproductively and bacteria enter the state called stationary phase. This state, however, is an operational definition and does not describe a specific physiological state or response of bacteria. Thus these bacteria differ physically and chemically from each other depending of time of sampling and the composition of medium (Nystrom, 2004). Bacteria lying in a stationary phase do not grow yet they possess variable degree of metabolic activity, which separates them from dead cells. Moreover, the resistance to many environmental stresses is usually increased in stationary phase, and this state actually bears many functional similarities to the starved cultures of bacteria (Vulic & Kolter, 2001). It is worth noting that the entry of bacteria into the stationary phase does not automatically denote that these bacteria will lose their culturability, yet most bacteria lying in this state are non-culturable due to the factors such as a short-term injury or cellular degeneration. On the other hand, bacteria do not remain non-culturable in perpetuity but are likely resuscitated or alternatively they enter the death phase and die within unspecified time depending of various external factors that affect their survival (Kaprelyants & Kell, 1993; Kaprelyants *et al.*, 1993; Shleeva *et al.*, 2004). Notably, some bacteria may remain in VBNC state for an outstandingly long time due to adaptive strategy to preserve some degree of viability in altered conditions. Yet any cell lying in VBNC state, unlike dying bacteria, is able to restore its

culturability if proper conditions are restored. Taken together, the viable but non-culturable definition postulates a specific program of differentiation into a long-term survival state for bacteria in altered conditions that differentiates these cells from the cells lying in a state followed by further degeneration of death. Therefore, the formation of VBNC state is not just a cytological condition but more probably it can be viewed as yet another example of a stochastic or programmed mechanism for survival in environmental conditions not suitable for cell division (Barer & Bogosian, 2004; Bogosian & Bourneuf, 2001; McDougald *et al.*, 1998; Nystrom, 2001).

The loss of viability and culturability is not always simply a consequence of various external factors. Recent studies of bacterial culturability and physiology in starvation induced growth arrest have proposed a scheme which may explain the progressive decline in the culturability by the natural aging process in bacteria. In this model, the internal production of reactive oxygen species (ROS) may cause such an oxidative damage which degrades the normal metabolism of bacterial cells in stationary phase resulting slow and irreversible cellular degeneration (known as conditional senescence) and thus explain the decline in the viability and culturability of bacteria. (Fredriksson & Nystrom, 2006; Nystrom, 2001; Nystrom, 2002)

2.1.2 Diversity of total cell counts - a relation of viability, dormancy and death to living, compromised and dead cells

Discrepancies in the number of bacterial cells detected by various microbiological methods are not exceptional perception. The bacterial cells can be viewed as being in a dynamic state, that is, both viable and dead cells as well as those cells, which by traditional explication are neither simply alive or dead readily coexist. Moreover, it is a universally accepted fact that only a small portion of soil and aquatic bacteria are alive and culturable, whilst a large population of bacteria are unculturable due to dormancy or death (Bogosian *et al.*, 1998; Bogosian *et al.*, 1996; Luna *et al.*, 2002). The enumeration of living and dead bacteria is greatly affected by time of sampling and the detection technique employed (traditionally based on culture tests) which follows that calculation of the total number of bacteria is at the same time of particular interest but also a challenging assignment to fulfill.

The classification of micro-organisms into different subpopulations on the grounds of current vital stage distributes cells into three different classes – viable, dormant and dead cells. However, as already pointed the categorization of bacteria by the viability is not straightforward. Specifically, dormancy is a state with a close relation to the death and thus the definition of dormant state may be outstandingly diverse. Dormancy is mostly defined as a reversible state of metabolic shutdown, which reflects an absence of activity. Moreover, dormant bacteria are usually not immediately culturable (Bar *et al.*, 2002; Kell & Young, 2000; Kell *et al.*, 1998). Obviously, dormancy consists both of cells that have ceased growth due to injury as well as viable but non-culturable bacteria. Thus, the more specific dissection of dormancy in relation to death needs the ability to distinguish between injured cells and viable but non-culturable cells. The injured state is usually transient that results from cumulative cellular damage. It can be reversed under appropriate conditions, thereby enabling the injured cells to resume

growth. However, the shift to the death phase occurs in a point where the extent of injury is beyond the ability of single cell to resume the growth. On the other hand, the transition into viable but non-culturable state is usually a consequence of a specific process to ensure a long-term survival in altered conditions such as in starvation, yet the state may be reversed by specific resuscitation process (Barer & Harwood, 1999; Bogosian & Bourneuf, 2001; Kell *et al.*, 1998; Mukamolova *et al.*, 2003; Oliver, 2005). The discrimination between injured and VBNC cells can simply be addressed on the basis of regrowth and resuscitation as indicated in Figure 2. Specifically, a method called mixed culture recovery (MCR) has been developed to distinguish whether recovery is due to resuscitation of the non-culturable cells or simply due to regrowth of residual culturable cells (Bogosian *et al.*, 1998).

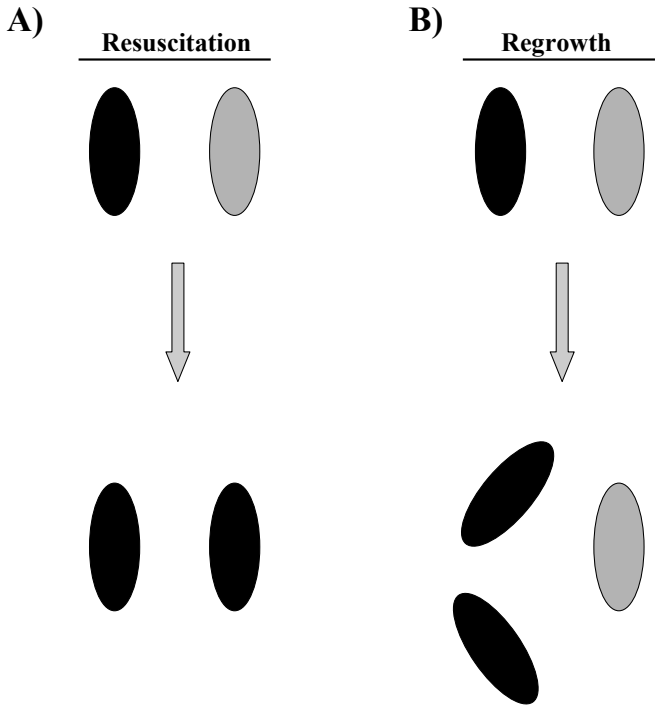

Figure. 2. A) Resuscitation versus B) regrowth. Mixtures of culturable (black) and non-culturable (grey) cells are subjected to the resuscitation. If only response is the growth of the culturable cells, then regrowth has occurred (B). If there is conversion of non-culturable cells into culturable cells without any change in cell numbers due to regrowth, then true resuscitation has occurred (A). The figure has been modified from Bogosian & Bourneuf (2001).

The discrimination between viable and dead cells is mostly based on difference in cellular integrity, in metabolic activity or in replication capability. Integrity can be investigated using different probes that have distinct penetration capability in intact and compromised cell membrane (McFeters *et al.*, 1995; Virta *et al.*, 1998). Metabolic activities of various micro-organisms, on the other hand, are often measured using specific ATP-detecting kits (Amorena *et al.*, 1999; Chu *et al.*, 2001; Rakotonirainy *et al.*, 2003; Stanley & McCarthy, 1989), whilst the replication capability can simply be

measured by traditional cultural methods (Jansson & Prosser, 1997). The bacterial cells possessing a reduction in cell membrane integrity, in metabolic activity or in replication capability are often denoted as compromised cells. However, a precise biochemical status of so-called compromised cells is still somewhat obscure (Decker, 2001) but a prospective loss of membrane integrity as well as a possible reduction in metabolic activity and in replication capability suggests that compromised cells can simply be equated with dormant cells. On the other hand, the sources of dormancy are usually far more evidenced than sources beyond the compromised cells. Therefore, it is obvious that the cells possessing a reduced vitality due to decrease in integrity or in vital cell functions should rather be denoted as dormant cells than compromised cells. Accordingly, the decision whether compromised or dormant is the more preferable term to describe the vitality of micro-organisms should be carefully considered, and all facts which may help to specify the current viability should be provided with.

2.2 Restriction of the growth of microbial cells

Our environment is surrounded by countless number of various micro-organisms, even the normal flora of humans is exceedingly complex and consist of hundreds of different bacterial species. Human organs such as skin and gut, for instance, are the natural habitats for a large and dynamic bacterial community (Batt *et al.*, 1996; Katsuyama *et al.*, 2005). Microbial cells differ from each other in their biochemical and physiological properties that all affect the susceptibility of selected micro-organisms to various antimicrobial agents, which makes the intentional restriction of their growth quite complex. Whilst most of bacteria are harmless or even extremely advantageous, there are some pathogenic and infectious bacteria present. Therefore, different methods and antimicrobial agents are needed either to eliminate the bacteria or to inhibit their activity and the growth. Several killing methods can be applied for these purposes but at the same time more effective drugs with high potency to destroy disease-causing microbes are also needed (De La Fuente *et al.*, 2006). Sometimes it is necessary to kill all bacteria but often it is sufficient to eliminate or to inhibit only harmful micro-organisms. The nature and habitat of target bacteria as well as the desired influence of the killing procedure eventually determinates the method or the antimicrobial agent to be selected for a given purpose.

2.2.1 Sterilization

Any procedure to be used to eliminate or to remove all living micro-organisms including viable spores, viruses and viroids is called a sterilization process. Sterilization is usually implemented by several physical methods such as heat, fire or ionizing radiation. Heat is probably the most commonly used method for the sterilization, yet the factors such as temperature, time of heating as well as the number and the nature of target micro-organisms affect greatly the efficiency of heat sterilization (Singleton, 1999). Accordingly, bacterial endospores may survive for long time at high temperatures, whilst vegetative cells are generally more susceptible. Moreover, younger cells are usually more readily destroyed than mature ones (Bauman *et al.*, 2006; Prescott *et al.*, 2004). Fire is used for rapid sterilization of various surfaces

and implements, whilst most disposable items are usually sterilized and destroyed by combustion. Steam sterilization is usually exploited in autoclaves in which under a pressure hot steam can reach the temperature suitable for the sterilization of most endospores resistant to normal heat sterilization. Ionizing radiation, referring mostly to *beta*- and *gamma*-rays, supplies energy for a variety of lethal (bio)chemical reactions. It is a less destructive method compared to fire and heat sterilization, thereby being well applicable for the sterilization of various plastic equipments. Sterilization by filtration can be used to remove bacteria and viruses from the solution by passing it through the membrane. Filtration is mainly used for sterilization of the heat-labile liquids. Moreover, various chemicals (sterilant) such as ethylene oxide and glutaraldehyde are highly reactive and toxic to all living tissues, and thereby may be used for different sterilization purposes. However, their use has to be strictly controlled due to general toxicity to all living things, which widely limits their all-purpose use.

2.2.2 Disinfection

In contrast to the sterilization, a disinfection procedure is purposed to destroy, inactivate or remove micro-organisms that may cause disease without necessarily affecting the other organisms present. The procedure is well applicable for the treatment of non-living objects or surfaces. Various chemicals (disinfectants) are most commonly utilized for disinfection processes, yet some physical methods can be exploited in certain purposes. Disinfectants intended for general use should be able to kill a wide range of different pathogens. However, any given disinfectant is usually more active against certain micro-organism(s) than it is against others. Furthermore, the activity of disinfectants may vary remarkably depending on factors such as dilution, temperature, pH, the duration of treatment and the presence of other compounds, for instance detergents (Prescott *et al.*, 2004; Singleton, 1999). Note that the sanitiziation refers to the procedure in which the microbial population is reduced to the levels that are proved to be safe by public health standards, thereby reflecting a close relation to the disinfection. Chemical disinfectants include compounds such as phenol, chlorine, quaternary ammonium compounds, hypochlorous acid, and so on. On the other hand, physical methods such as fire and boiling water have been used for disinfection purposes from very ancient times, and heating is still probably one of the most popular ways to destroy various viruses, bacteria and fungi. Ultraviolet radiation is also a physical method, which can be exploited for disinfection purposes. Whilst UV-radiation has a rather poor power of penetration, it passes bacterial cell membrane, thereby inducing DNA damages on bacteria. UV-radiation is most suitable for the disinfection of different surfaces in enclosed areas such as worktop of laminar hoods.

2.2.3 Antisepsis

Antisepsis is the disinfection of living tissues, and thus the properties of disinfectants are applicable to the chemicals (antiseptics) used for antisepsis. Antiseptics can be used either prophylactically to prevent infection or therapeutically to treat infection (Singleton, 1999). Antiseptics are generally not as toxic as disinfectants so that host tissues are not injured all too much (Prescott *et al.*, 2004). Most commonly used antiseptics are phenolic antiseptics such as dettol and hexachlorophene, common alcohols such as ethanol and

isopropanol and some halogens such as iodine. Also different heavy metals such as mercury, arsenic and copper have traditionally been used for antisepsis, yet the use of these metals have mostly been replaced with other less toxic compounds.

2.2.4 Antibiotics

Micro-organisms produce an extraordinary array of microbial defence systems, which include classical antibiotics, metabolic by-products, lytic agents and numerous exotoxins as well as bacteriocins. Among these, antibiotics are the most important group of chemicals that have been used to treat and prevent the infections critical to human and animal health. Originally antibiotic meant any microbe-derived product that uniquely has a capability to kill or to inhibit certain micro-organisms. However, the term antibiotic is now also expanded on semi-synthetic and wholly synthetic antimicrobial substances. The first antibiotics were introduced in early 1930s. Since that the number and the use of different antibiotics has notably increased, although virtually all current antibiotics with clinical importance were already identified during the "golden" period of antimicrobial discovery between the 1940s and 1960s (Carrasco *et al.*, 2002; Chopra *et al.*, 2002). The influence of antibiotics can either be bacteriostatic or bactericidal. Static effect means that the antimicrobial compound inhibits the growth of microbe, and if the antimicrobial agent is removed the micro-organism will mainly recover and resume the growth. A cidal agent, on the other hand, kills the target bacteria but its activity is strongly dependent on concentration used and thus the action may be only static if too low levels of antibiotic is administered. Some cidal antibiotics act against the cell membrane or cell wall leading to the rapid cell lysis, and are therefore known as bacteriolytic agents (Prescott *et al.*, 2004). None of any antibiotic is effective against all bacteria but vary considerably in its range of effectiveness. Some antibiotics are effective only against narrow range of microbes, whilst others have influence on broad range of micro-organisms. Thus antibiotic chemotherapy should be settled not only on the grounds of the suitable drug dosage but also the micro-organisms in focus should be closely considered to ensure the most efficient treatment. The methods available for the assessment of microbial susceptibility to given antimicrobial compounds are in particular significance in the field of clinical microbiology, and the efficacy of antibiotics or any other antimicrobial agents against a given pathogen can be described by quantities such as minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). The MIC is the lowest concentration of selected drug that prevents visible growth of a particular pathogen, whilst the MBC is the lowest drug concentration that kills all (usually >99.9%) of the target pathogens (Mims, 2004).

Different antibiotics have different mechanism of action which are responsible for the transmission of antimicrobial effects, yet the specificity of any antibiotic is much determined by the site of action of given drug. The efficacy of a single drug is dependent on several factors, and they all have to be taken into account in order to achieve a potent antimicrobial effect. Specifically, each drug should reach the site of infection with maximal efficacy indicating that both the proper route of administration (oral, injection by intramuscularly, intravenous drip or direct distribution on skin) and the speed of uptake are crucial. Moreover, the concentration of an antibiotic should always exceed the MIC value of susceptible pathogens and the rate at which antibiotic is removed or eliminated from the body should not reduce the amount of drug below

that concentration. Furthermore, each drug should retain its active form so that the environmental factors such as pH and temperature, having a direct effect on the activity of drugs, should be noticed when selecting a proper antibiotic for the therapy. Sometimes two or more antibiotics can simultaneously be administrated, and depending on an effect produced, synergism or antagonism between antibiotics may occur. Synergistically acting antibiotics produce an effect which is greater than the sum of the effects of individual antibiotics, whilst the antagonism is an inverse of synergism (Mims, 2004; Prescott *et al.*, 2004). In Table 1 the different mechanisms of antimicrobial drug action with some examples of antibiotics or groups of antibiotics suitable for treating diseases are summarized. It is worth noting that one of the most troublesome feature of antibiotic chemotherapy over the past half-century has been the development of antibiotic resistance. Although resistance has been a continuing problem ever since antibiotics were introduced, resistance is rapidly expanded during past decades due to continued growth of micro-organisms in environments containing various antibiotics (Wright, 2007). On the other hand, also such bacteria have been found that survive in the presence of a given antibiotic, yet being not simply resistant to that antibiotic. Obviously these cells (specifically called as persisters) are in dormant state. Dormancy poses a metabolic shutdown and inhibits cell-wall synthesis, translation and topoisomerase activity, which renders cells tolerant to the antibiotic action. Persisters are outstandingly reviewed by Lewis (2007).

Table 1. The mechanisms of action of specific chemotherapeutic agents.

Antibiotic / group of antibiotics	Mechanism of action	Reference
	Cell membrane disruption:	
polymyxin B	Polymyxin B binds and penetrates the cell membrane leading its rapid disruption	(Storm *et al.*, 1977)
	Cell wall synthesis inhibition:	
penicillins such as ampicillin penicillin G	These antibiotics bind and inhibit enzymes involved in the cross-linking of the polysaccharide chains of the bacterial cell wall peptidoglycan leading lysis of dividing bacteria	(Nathwani & Wood, 1993)
cephalosporins such as cephalexin	Same as above	(Klein & Cunha, 1995)
	Nucleic acid synthesis inhibition:	
fluoroquinolones such as ciprofloxacin	These antibiotics inhibit bacterial DNA gyrase and thus interfere with DNA replication, transcription and other DNA involving activities	(Maple *et al.*, 1990)
nalidixic acid	Nalidixic acid inhibits DNA replication by binding to the DNA gyrase	(Singh & Sachdev, 1988)
rifampin	Rifampin blocks RNA synthesis by binding to the RNA polymerase	(Alsayyed, 2004)
	Protein synthesis inhibition:	
aminoglycosides such as kanamycin streptomycin	These antibiotics bind 30S subunit of the bacterial ribosome causing the inhibition of protein synthesis as well as misreading of mRNA	(Gonzalez & Spencer, 1998)
tetracyclines such as tetracycline	These antibiotics bind also 30S subunit of the bacterial ribosome and thus interfere with aminoacyl-tRNA binding	(Chopra & Roberts, 2001)
macrolides such as erythromycin	These antibiotics bind to the 23S rRNA molecule in the large (50S) subunit of the bacterial ribosome inhibiting peptide chain elongation	(Katz & Ashley, 2005)
	Metabolic antagonism:	
Sulfonamides such as sulfanilamide	These antibiotics inhibit folic acid synthesis and thereby block nucleotide and protein synthesis in bacteria	(Smith & Powell, 2000)
Folic acid analogs such as trimethoprim	Same as above	

2.2.5 Non-antibiotic drugs

The emergence of antibiotic resistance throughout the world is a potentially serious threat to public health, and it is mainly caused by an excessive and inappropriate use of antibiotics that has led to the increased race between the development of new antibiotics and the emergence of drug-resistant pathogens. Antibiotic resistance impedes proper treatment of infections and causes substantial economical losses in health care (Finch, 2002; Livermore, 2004), thereby producing a significant need for the discovery of new types of antimicrobial agents. Accordingly, much of research has been lately invested in various resources to provide more possibilities to control the growth of microbes and reverse the resistance. However, only limited number of new class of antibiotics has been introduced since the golden era of antibiotic discovery (Barrett & Barrett, 2003; Wright, 2007). On the other hand, there are several different non-antibiotic preparations available to control the microbes. Specifically, non-antibiotic preparations are defined as medicinal compounds, which are employed for the treatment of a variety of non-infectious diseases. These preparations exhibit in vivo a direct or indirect antimicrobial properties (Kristiansen, 1992). Whilst some non-antibiotic drugs may exhibit a direct antimicrobial activity, thereby being well applicable for the treatment and prevention of infections, most of these drugs are used in combination with conventional antibiotics. The combination has often an increased activity compared to the individual drugs. Moreover, some non-antibiotic drugs can actually restore the susceptibility of certain micro-organisms or even render bacteria susceptible to specific antibiotics to which resistance was previously demonstrated. Non-antibiotic drugs cover various divergent pharmacological classes including compounds such as antihistamines (Rajyaguru & Muszynski, 1998), barbiturates (Cederlund & Mardh, 1993), psychotropics (Amaral *et al.*, 2004), antihypertensives (Chakrabarty *et al.*, 1993), anesthetics (Rajyaguru & Muszynski, 1998), phytomedicines (Kamiji & de Oliveira, 2005), probiotics (Banerjee & Lamont, 2000), phages (Carson & Riley, 2003) and antioxidants (Kamiji & de Oliveira, 2005). In near future the use of non-antibiotic drugs will probably further increase in clinical microbiology, which hopefully gives more alternatives to classical antibiotic chemotherapy and helps to overcome resistance which results from the incredible ability of bacteria to adapt for different antimicrobial agents.

2.3 Traditional culture based methods to measure bacterial viability

The most obvious way to measure the microbial content of various samples is probably through direct counting. However, the direct counting yields the result of all cells, whether alive or dead, whilst the true cell viability remains unclear. Therefore, more specific methods have been developed for revealing the micro-organisms that display some signs of viability. Since a variety of different methods are currently available for this purpose, it is possible to study micro-organisms at unparalleled levels of detail, which brings new challenges in understanding the information in all details. Viability, as a rule, is linked with the ability of bacteria to grow and reproduce, and thus the quantification of bacterial growth is a major issue for revealing whether micro-organisms are alive or not. Moreover, most bacterial cells may produce variety of

metabolites during their growth, which has a significant effect on the microbiological quality of different products, thereby affecting the safety of different food supplies and pharmaceuticals. Accordingly, numerous assays have broadly been exploited for the detection of viability by cultural means. However, as previously discussed in section "viability in contrast to culturability" there are some situations in which viability is not detectable by cultural methods. Therefore, conventional cultural assays are not necessarily the most feasible choices for each application. Nevertheless, plate counting and optical density measurements are still probably the most commonly used methods for the detection of cell number and cell growth, which in most cases reveal viability with sufficient accuracy.

2.3.1 Viable counting methods

Traditionally, the number of viable micro-organisms is evaluated by plate counting. Plate counting refers to the technique in which a diluted sample is spread over a solid agar followed by case-specific incubation at an appropriate temperature. Under given cultural conditions each micro-organism develops a distinct colony on the plate, and the initial number of viable organisms in the sample can be calculated from the number of colonies formed multiplied by dilution factor (Li *et al.*, 1996; Madigan *et al.*, 1997). Whilst plating techniques are simple and relative applicable methods for detecting viable micro-organisms from various samples of soil, water and food, they still contain several insufficiencies that easily lead to inaccurate results. Firstly, viability in this context is connected to the ability of micro-organisms to grow on plate under given cultural conditions. Therefore, only culturable cells are detected, which means that most dormant bacteria as well as viable but non-culturable bacteria and micro-organisms with lag periods greater than the incubation period are not detected (Barer & Harwood, 1999; Jansson & Prosser, 1997). Secondly, laboratory media and cultural conditions are mostly selective and none can support the growth of all micro-organisms. Moreover, if possible cell clumps are not broken up and the micro-organisms in a sample well dispersed on plate, too low cell counts will presumably result (Jansson & Prosser, 1997). Since it is not likely that each colony formed on a plate arose from an individual cell, the results of plate counting are usually represented in the terms of colony forming units (CFU) rather than the direct number of viable micro-organisms. In order to improve the reliability of plate counting, the number of colonies formed on a plate (CFU) should be settled approximately between 50 and 250 CFUs. However, in most cases multiple dilution and numerous plates are needed to confirm the desired CFU, which denotes that plate counting is a labour-intensive and cumbersome method. Furthermore, results are mostly read after a long incubation, typically over night but sometimes several days or even weeks are needed.

Membrane filter method resembles conventional plate counting technique. The method is based upon the use of highly porous membrane incubated on agar plate. The porous membrane simply retains bacteria from a sample to the plate as high volumes of liquid is passing through it. The major advantage of this method over plate counting is the ability to easily process large sample volumes with relative low number of micro-organisms (Jansson & Prosser, 1997; Madrid & Felice, 2005). Another related approach for the assessment of bacterial viability is the MPN (most probably number)

method. It provides an estimate of the number of viable micro-organisms that are capable to grow in selective liquid growth medium. The method is based on a 10-fold dilution series, each dilution being inoculated into a separate tube of growth medium. The inoculated samples are incubated and the number of positive tubes are recorded for determining the MPN of micro-organisms. The method is particularly useful for the detection of low cell numbers or for micro-organisms that grow rather poorly on agar plates (Madrid & Felice, 2005).

2.3.2 Optical density measurements

An increase, both in total cell mass and cell number, can readily be estimated by measuring the turbidity of a cell suspension using instrument such as spectrophotometer (Dalgaard & Koutsoumanis, 2001). The fact that cells scatter light enables the optical measurement of turbidity of the cell culture, thereby offering an alternative to plate counting technique for the detection of cell growth. Since microbial cells are relative constant in size, the amount of light scattered is almost directly proportional to the concentration of cells present in a sample. The basis of spectrophotometric measurement derives from the Beer's law, and the actual measurement can be performed either by measuring the primary beam of light that passes into the sample without deviation to reach the detector (transmittance) or by measuring the amount of scattering light (absorbance) (Harris & Kell, 1985; Hobson *et al.*, 1996; Madrid & Felice, 2005). Spectrophotometric measurement of cell density is a rapid and sensitive method for detecting cell growth as well as to calculate the total number of cells that are present in a sample. Whilst the extent of light scattering is almost linearly related to the cell number at low absorbance levels, the high cell concentration causes distorted absorbance readings which can, however, be corrected by sample dilution. On the other hand, very low cell contents cannot be detected, and in fact the cell medium appears slightly turbid not until the cell concentrations reaches approximately ten million (10^7) cells per ml. Further increase in cell density results greater turbidity and more light is scattered so that the absorbance reading given by spectrophotometer increases, that is, the transmittance decreases. Note that cells grown in medium containing high carbohydrate or fat source have frequently a high turbidity resulting higher absorbance readings than normally would be expected (Hobson *et al.*, 1996; Prescott *et al.*, 2004). Taken together, the cell growth and the cell number can readily be measured by spectrophotometry providing that the absorbance reading of cell culture is in detectable, linear level. Moreover, the automated measurement systems, which allow on-line monitoring of large sample amounts have increased the accuracy and speed of spectrophotometric estimation of bacterial growth during recent years (Metris *et al.*, 2003).

2.4 Other methods for assessing viability and killing of microbial cells

Despite the enormous importance of cultural methods for the assessment of bacterial viability and killing, there are several other methods currently available for that purpose, and in most cases these methods reveal viability with greater accuracy and specificity than conventional cultural methods. The ability to grow and reproduce is a

most common explication of viability, yet viable cells possess other signs of viability such as cell membrane integrity and metabolic activity, which can also be followed. However, a wide diversity of methods available for the quantification of bacterial viability and killing from divergent samples poses significant variances in the interpretation of results, thus final results are exceedingly dependent on the method chosen. Obviously, the methods that enable simultaneous measurement of multiple viability parameters give more reliable results than single parameter assays, which makes multiparameter assays more prevalent choices for various applications. However, these assays are not an unquestionable choice for each application, particularly when same results are obtained with more simple methods or when highly specific technology is required. Examples of some non-cultural methods currently available for the assessment of bacterial viability and killing are briefly outlined in following sections.

2.4.1 Microscopy

Microscopic detection has traditionally been applied for direct enumeration of microbial cells in a sample, and a normal light microscope represents probably the most conventional instrument to be used for that purpose. On the other hand, more sophisticated instruments such as fluorescence microscopes share the same optical principles of common microscopy but differs in sample handling and in the operation of instrument. Notably, suitable filter sets for fluorochromes to be visualized are needed in order to enable the discrimination between different vital stages. Fluorochrome is a fluorescent dye, which holds affinity to the certain cell compounds such as nucleic acids, lipids and proteins, and is therefore suitable for staining micro-organisms. Fluorescent staining has notably improved both the direct counting of bacteria (Maruyama *et al.*, 2004) and the quantification of bacterial viability (Auty *et al.*, 2001; Burnett & Beuchat, 2002; Gatti *et al.*, 2006; Lopez-Amoros *et al.*, 1997). Fluorescent staining typically separates bacteria according to the differences in membrane integrity, which allows the dye(s) to be bound to the different cell component as a function of membrane integrity. Conventional epifluorescence microscopy (Ercolini *et al.*, 2006) and confocal scanning laser microscopy (Auty *et al.*, 2001; Biggerstaff *et al.*, 2006) are probably the most commonly employed direct microscopic methods used for differentiating between different vital stages of stained microbial cells. The common basis of fluorescence microscopy is very simple, whilst a technical structure of the instrument is more sophisticated. Briefly, the fluorescence microscopy produces an image of the microbes labelled with a compound exhibiting fluorescence after exposing to suitable exciting light. The image is detected through an eyepiece, often connected to computer that enables electronic reconstruction and the storage of a picture (Herman *et al.*, 1998). Fluorescence microscopy allows the direct enumeration of viable and non-viable micro-organisms in less than 30 minutes as compared to cultural methods, which may easily require over night incubation or even more. Moreover, fluorescence microscopy is capable for enumerating bacteria that exist in the viable but non-culturable state (Madrid & Felice, 2005). Whilst microscopic methods are successfully applied in microbiological research to produce detailed information about the integrity of target micro-organisms, their wide-ranging use is unlikely due to various disadvantages. The microscopic

detection of cell viability is exceedingly labour-intensive because individual cells have to be stained with appropriate fluorescent reagent and often fixed to suitable sheet for the examination. The design and interpretation of experiments needs an user-expertise, and besides, it may be very time consuming. Also the selection of optical filter sets is a critical step for adapting a fluorescence microscope to particular fluorochrome, and the filters from different manufactures are rarely compatible. Moreover, only one or few cells can mostly be detected simultaneously, which makes the detection of large sample amounts inconvenient.

2.4.2 Flow cytometry

Flow cytometry (FCM) is an analytical instrument that allows a rapid analysis of thousands of cells per second. FCM highly resembles microscopy with advantages of automation, objectivity and speed (Veal *et al.*, 2000). The flow cytometric instrumentation is based on the optical analysis of individual cells. Cells suspended in a buffer are introduced in to the flow cell with flowing sheet fluid. The suitable light source (mostly an assortment of lasers) is used to illuminate cells as they pass individually through a beam of light focused on flow cell. Light scattered by cells, consisting of forward scatter (FS) and side scatter (SS), is collected by suitable filter units, amplified by photomultiplier tubes and send to data processing unit. Forward scattered light, collected in the same direction as the illuminating light, is related mainly to cell size, and side scattered light, collected at an angle of 90^{o}, gives an information of surface properties and internal structure of cells. Additional information is obtained through fluorescence emission by staining cells with different dyes exhibiting bright fluorescence when illuminated with suitable excitation light. A schematic illustration of light detection in FCM is given in Figure 3. Conventional FCM simply analyses and discards each cell but more sophisticated instruments have sorting ability for separating individual cells of interest for further analysis. Fundamental explanation about flow cytometry in microbiology can be found from Alvarez-Barrientos *et al.* (2000); Davey (2002); Shapiro (1995); Veal *et al.* (2000).

The heterogeneity of microbes themselves is enormous, which complicates the direct assessment of bacterial viability by FCM. However, using specific flow cytometric applications, the determination of basic cell functions such as reproductive ability, metabolic activity and membrane integrity can successfully be revealed as shown by Alvarez-Barrientos *et al.* (2000); Caron *et al.* (1998); Hewitt & Nebe-Von-Caron (2001); Shapiro & Nebe-von-Caron (2004); Vives-Rego *et al.* (2000). Moreover, it is relatively easy to yield multiparameter data for each cell in the sample of interest using fully exploited FCM. This provides a clear insight into population heterogeneity and helps to discriminate between bacteria with different vital stages. Accordingly, flow cytometric multiparameter analysis at the single cell level is without a question among the most practicable methods applied in modern microbiology. This field has been comprehensively reviewed for example by Davey *et al.* (1999); Nebe-von-Caron *et al.* (2000); Shapiro (2000); Shapiro & Nebe-von-Caron (2004); Winson & Davey (2000). The flow cytometric assessment of bacterial viability is usually based on fluorescent staining that allows the distinguishment between viable, dead and dormant cells (Alvarez-Barrientos *et al.*, 2000; Davey & Kell, 1996; Veal *et al.*, 2000). The dyes

applied in flow cytometric analyses possess an affinity to specific cell compounds, and most dyes are actually the same as used in microscopic detection described in previous section. Using divergent staining applications it has been measured both the metabolic activity (Nebe-von-Caron *et al.*, 2000) and the membrane integrity (Gregori *et al.*, 2001; Nebe-von-Caron *et al.*, 2000). The metabolic activity is most easily measured by detection of biosynthesis, whilst the cell membrane integrity is detected simply by measuring dye-retention or dye-exclusion from viable and non-viable bacteria. On the other hand, the flow cytometric assessment of the reproductive growth does not require specific staining but is easily demonstrated by direct counting of cells against reference particles (Nebe-von-Caron *et al.*, 2000; Virta *et al.*, 1998). Overall, flow cytometric instrumentation is a powerful technique to be used in microbiological analytics, yet it contains features which can be considered as serious disadvantages. The first is the substantial cost of the instrument which can easily be over 100 000 euros for typical laser-based FCM. The second disadvantage is that because FCM is extremely sophisticated instrument, the need of skilled and well-trained users to obtain the optimal performance throughout the analyses is highly evident (Davey & Kell, 1996; Vives-Rego *et al.*, 2000)

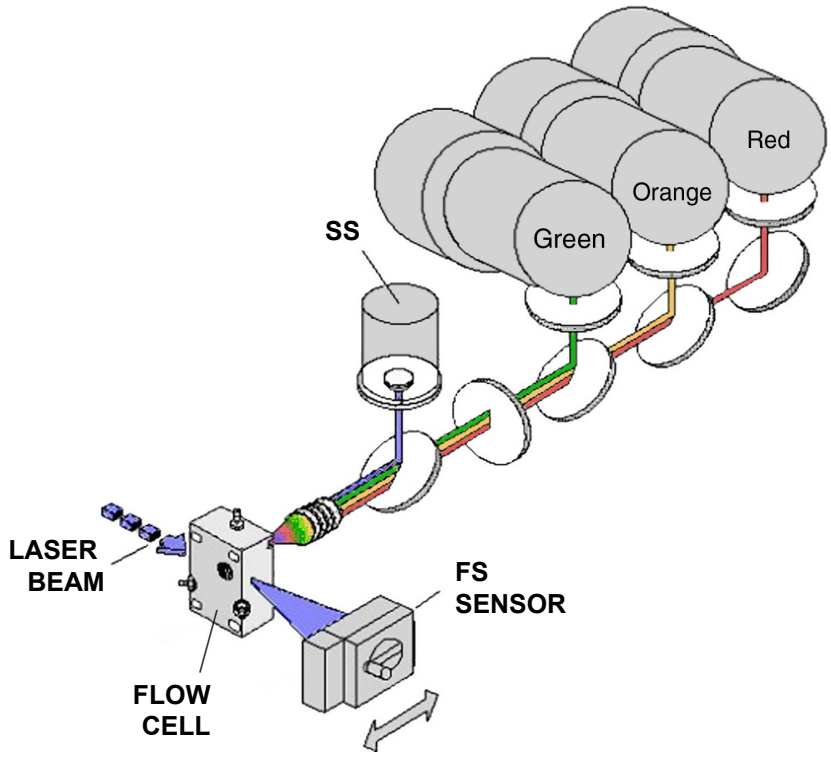

Figure 3. The principle of light detection in FCM, an example of SS- and FS sensors as well as of filter settings of Coulter EPICS XL flow cytometry for fluorescence analysis of bacterial cells. Fluorescence from bacterial cells stained with suitable fluorochrome(s) is amplified by photomultiplier tube(s). Specifically, PMT1, PMT2 and PMT3 amplify green, orange and red fluorescence light collected through suitable bandpass-filters, respectively.

2.4.3 Fluorometry

Fluorometric measurement of cell viability and killing is rapid, reliable and simple technique that allows the detection of microbial cells directly from their environment. The technique is based on the detection of various fluorescent compounds, which exhibit fluorescence when suitable energy is supplied by electromagnetic radiation (Jameson *et al.*, 2003). Fluorometric assessment of bacterial viability is not related to the capability of bacteria to form colonies but it correlates directly with a cell's physiological states such as metabolic activity, cell respiration, intracellular pH and membrane potential or integrity (Breeuwer & Abee, 2000; Prakash Singh, 2006; Roth *et al.*, 1997). The fundamental principles of fluorescence and fluorometric measurements are far established and can be summarized as described by Brehm-Stecher & Johnson (2004). Briefly, fluorescence is a special form of generic phenomenon called luminescence in which light is emitted by given substance. Specifically, light is generated when electrons in a fluorophore are raised in a higher-energy state (excited), due to hit by photons emitted from external light source, followed by the return to the lower-energy state accompanied by the emission of light as a fluorescence. For the fluorometric detection of bacteria, the cells are labelled with suitable fluorochrome(s) and excitation light is focused on cell suspension. An appropriate wavelength for excitation is isolated with the aid of suitable optical excitation filter or monochromator, and the emission following the excitation is then filtered by suitable emission filter, respectively. The detection and quantification of fluorescence is finally fulfilled by photomultiplier tube (PMT) to produce an electric current, which can be recorded. Note that the intrinsic properties of fluorochrome such as its excitation and emission spectra, quantum yield and efficiency, molar absorbance coefficient and photostability along with environmental factors such as local pH and charge concentrations greatly affect to final fluorescence emitted from cell suspension (Mason, 1999). Although micro-organisms contain intrinsic molecules such as aromatic amino acids and flavins that exhibit natural fluorescence, most fluorometric methods utilize various external dyes to discriminate between viable, dormant and dead cells. Dyes are targeted to specific cell element, and if a compound is not fluorescent as such, the binding to the target usually evokes its conversion into fluorescent form. It is worthwhile to note that most dyes employed in fluorometric assays are similar or completely same as used in microscopic and flow cytometric applications. Some of the most prevalent fluorescent dyes exploited in various bacterial viability assays are listed in Table 2. On the other hand, green fluorescent protein (GFP) and related fluorescent reporter proteins are very popular alternatives to conventional dyes for the fluorometric assessment of cell viability as shown by Changsen *et al.* (2003); Lowder *et al.* (2000); Webb *et al.* (2001). Since the use of reporter proteins in various viability assays is discussed in more details later in this review, their significant role in fluorometric assays is only mentioned here.

Numbers of fluorescence based assays for the assessment of bacterial viability have been introduced over recent years. Bacterial metabolic activity, for instance, can easily be measured by fluorometry using various intra- or extracellular fluorescent probes (Clarke *et al.*, 2001; Wos & Pollard, 2006). However, the connection between

metabolic activity and viability is sometimes incoherent as the degree of metabolic activity varies in cells which are not simply alive or dead as already discussed in previous sections. Consequently, other markers of viability have much been exploited. The detection of membrane potential by various fluorescent molecules is one acceptable alternative. It separates viable bacteria from nonviable ones according to the plasma membrane potential using positively and negatively charged fluorescent molecules, which have divergent distribution between viable and nonviable cells. Typically, bacteria possessing membrane potential accumulate the cationic molecules, whilst negative charged molecules are accumulated only in cells with dissipated membrane potential. However, it is still questionable whether cells without membrane potential can simply be denoted as nonviable (Breeuwer & Abee, 2000). A similar approach for fluorometric membrane potential assay is the determination of cell membrane integrity, a character being the most important criterion for distinguishing between intact and damaged cells (Nocker *et al.*, 2006). The principle of assessment of membrane integrity is the finding that viable cells with intact membrane exclude fluorescent dyes that easily penetrate dead or membrane-damaged cells. On the other hand, most membrane integrity assays currently utilize dual staining technique in which one dye stains all cells regardless of membrane integrity, whilst the intensity of other dye increases along with decreasing membrane permeability. Various dyes with distinct membrane affinities have successfully been exploited during recent years for the fluorometric assessment of viability of various micro-organisms including bacteria (Alakomi *et al.*, 2005; Helander & Mattila-Sandholm, 2000) and yeast cells (Bowman *et al.*, 2002). Note that whilst fluorometric analyses may be disturbed by factors such as autohydrolysis, background fluorescence or quenching of fluorescence signal, the advantages of fluorometric detection are generally more obvious. Firstly, fluorescence offers good sensitivity and specificity. Secondly analyses are not as laborious and do not require as sophisticated equipments as fluorescent microscopy and flow cytometric instrumentation. Thirdly, a large number of fluorescent dyes are developed for different applications. Moreover, a diversified range of experience in the field of fluorescence is well evident and readily available.

Table 2. Examples of fluorescent molecules used to study different microbial criteria / parameters associated with bacteria and their viability.

Criteria / parameter	Dye	Ex_{max}(nm)[a]	Em_{max}(nm)[a]	Reference
Direct counting, identification and cell morphology analysis	4,6-diamino-2-phenylindole (DAPI)	350	470	(Davey & Kell, 1996; Lowder & Oliver, 2001)
	Acridine orange (AO)	440-470	650	(Davey & Kell, 1996; McFeters *et al.*, 1995)
	Carboxyfluorescein (CFDA)	492	517	(Fuller *et al.*, 2000; Joux & Lebaron, 2000)
	Fluorescein isothiocyanate (FITC)	495	525	(Alvarez-Barrientos *et al.*, 2000)
Membrane Integrity	1-*N*-phenylnaphthylamine (NPN)	330-350	415	(Helander & Mattila-Sandholm, 2000)
	Ethidium bromide (EB)	510-518	595-605	(Alvarez-Barrientos *et al.*, 2000; Caron *et al.*, 1998 ; Joux & Lebaron, 2000)
	Propidium iodide (PI)	535-537	617-625	(Alvarez-Barrientos *et al.*, 2000; Joux & Lebaron, 2000; Virta *et al.*, 1998)
	Propidium monoazide (PMA)	541-551	590	(Nocker *et al.*, 2006)
	SYTO 9	480	537	(Biggerstaff *et al.*, 2006; Virta *et al.*, 1998)
	SYTO 13	488-489	505-509	(Biggerstaff *et al.*, 2006)
	SYTO BC	488	530	(Lebaron *et al.*, 1998)
	SYTOX Green	502-504	523-525	(Alvarez-Barrientos *et al.*, 2000; Joux & Lebaron, 2000; Roth *et al.*, 1997)
	SYTOX Orange	548	571	(Biggerstaff *et al.*, 2006)
	TOTO-3	642	660	(Alvarez-Barrientos *et al.*, 2000)
	(Fluorescein diacetate (FDA[b]))	473	514	(Joux & Lebaron, 2000; McFeters *et al.*, 1995)
Respiration	5-cyano-2,3-ditolyl tetzolium chloride (CTC[c])	450-550	580-660	(Davey & Kell, 1996; Joux & Lebaron, 2000; Stellmach, 1984)
Enzyme activity	5-cyano-2,3-ditolyl tetzolium chloride (CTC[c])	450-550	580-660	(Davey & Kell, 1996; Joux & Lebaron, 2000; Kaprelyants & Kell, 1993)
	Fluorescein diacetate (FDA[b])	473	514	(Joux & Lebaron, 2000; McFeters *et al.*, 1995
Membrane potential	DiBAC4(3)	488-493	516-525	(Davey & Kell, 1996; Joux & Lebaron, 2000; Lopez-Amoros *et al.*, 1997)
	Rhodamine123 (Rh 123)	507-560	529-580	(Davey & Kell, 1996; Joux & Lebaron, 2000; Kaprelyants & Kell, 1992)
pH gradient	BCECF	460-510	520-610	(Alvarez-Barrientos *et al.*, 2000; Breeuwer & Abee, 2000)
	SNARF-1	510	587-635	(Alvarez-Barrientos *et al.*, 2000; Breeuwer & Abee, 2000)

[a] Maximal excitation and emission wavelengths display some variability depending on conditions used. [b] FDA is mainly a marker of active metabolism but its accumulation into the cells depends upon an intact membrane thus being also a marker of membrane integrity. [c] Specifically CTC can be utilized as well in respiration and enzyme activity analyses

2.4.4 Luminometry

Luminometric measurement of cell viability and killing shares some similarity with fluorometric measurements. It is a rapid and simple method, which allows the detection of microbial cells directly from their environment by measuring the emission of light that is proportional to the viability status of target cells. Most luminometric assays are based on two phenomena called chemiluminescence (CL) and bioluminescence (BL) that are also special forms of luminescence. Specifically, chemiluminescence is the emission of light that is produced as a result of energy released in a chemical reaction without emission of heat. Bioluminescent reaction resembles much chemiluminescence but the production and emission of light occurs at a living organism as a result of biologically catalyzed chemical reaction, which converts chemical energy into light. In both reactions, the energy released is used to generate an intermediate or product in an electronically excited state, which then emits a photon. It is worth noting that the emission of light occurs at temperatures, which separates this type of emission from incandescence. The light emission in chemi- and bioluminescent reactions does not come from or depend on light absorbed in a reaction, as in fluorescence (photoluminescence), yet most of the photons generated in reactions get absorbed, reflected or lost before leaving the reaction vessel. The background in luminescent measurements is usually negligible and the detectability of signal is high, which can be observed as down as to the few emitted photons per reaction. Therefore, the sensitivity of luminometric measurements is usually better than those of other spectroscopic techniques, which makes luminometric measurements good alternatives for various bacterial viability assays. More detailed description about chemiluminescence and bioluminescence in general can be found from Hastings & Johnson (2003); McCapra (2000); Roda *et al.* (2004); Stanley (2000); Wilson & Hastings (1998).

The quantification of light involves the conversion of emitted photons into an electrical signal that determines the basic design of all luminometers. For low light detection, as it is a case in luminometric measurements where most of photons do not reach the detector, the most commonly used device for photon-counting is the photomultiplier tube. Other main parts of typical luminometry are measuring chamber and reagent injector(s). Measuring chamber has to be absolutely light tight place to avoid external light to reach PMT. Notably, the light emission occurs mostly uniformly in all directions, and thus only a fraction of the light reach the detector directly. Therefore, special optics such as reflecting surfaces (mirrors) should be used in chamber for increasing the amount of photons that reach the detector. Injectors are used to add reagent(s) starting the reaction. They are essential for the measurement when a flash type luminescence is measured, that is the light emission reach its peak typically after 1 sec or less, and most of the light is emitted within 2 to 3 sec. However, in the case of glow kinetics, the injectors are irrelevant since emission is long-lived. (Berthold *et al.*, 2000)

Various different luminometric applications for the assessment of bacterial viability have been introduced during past decades (Chu *et al.*, 2001; Duncan *et al.*, 1994; Wheat *et al.*, 1989). Chemiluminescent methods can be used to detect viability of micro-organisms by measuring active oxygen species as a luminol enhanced

chemiluminescence (Yamashoji *et al.*, 2004). However, the assessment of viability by luminometric means is more often based on the detection of a wide range of substances of biological interest, thereby referring to bioluminescence. Vast majority of these substances are important components of different metabolic reactions. Thus it can be stated that most bioluminescence based viability assays actually reveal metabolically active cells (Marques *et al.*, 2005; Roda *et al.*, 2004). However, the connection between metabolic activity and viability could present a problem since luminometric assays may be unable to detect cells whose activity is reversibly diminished but which are still alive. On the other hand, all bacteria that display some metabolic activity are detected by luminometric means. Notably, these cells may have lost their capability to grow, thereby being unculturable which denotes that these cells are undetectable by traditional cultural techniques. There are several different substances such as NAD(P)H, $FMNH_2$ and PP_i which are crucial for light-producing reactions, yet the most commonly detected molecule is ATP found in all living cells (Chu *et al.*, 2001; Mason, 1999). The significant role of ATP in cellular metabolism makes it necessary for the cell to maintain a rather constant intracellular concentration of ATP. This denotes that most viability assays are coupled either directly or indirectly to the quantification of cellular ATP. Accordingly, there are several different preparations available which are capable to monitor ATP, or alternatively many different naturally bioluminescent organisms can be utilized for the quantification of ATP (Lundin, 2000). Most ATP-detecting kits contain reagent(s) that initializes a chemical reaction which produces light in the presence of ATP. In most cases light is originated from the same reactions, which take place in naturally bioluminescent organisms. ATP-detecting kits are broadly applied for the assessment of viability of different bacterial species (Romanova *et al.*, 2003; Valat *et al.*, 2003) as well as various fungal (Yoshida *et al.*, 1997) and yeast (Thomsson *et al.*, 2005) cells. Majority of bioluminescence based viability assays are, however, connected to natural bioluminescence systems found from many different organisms, from bacteria and fungi to insects, fishes and many more (Greer & Szalay, 2002). In most assay systems particular genes responsible for bioluminescence are directly incorporated into target micro-organisms or alternatively target organism is naturally bioluminescent. Light is produced as a result of reaction catalyzed by various luciferases such as bacterial (*luxAB*) or beetle (*luc*) luciferases, and the viability of target micro-organisms is calculated according to the light emission. Luciferases are discussed more detailed later in this literary review. Overall, luminometric methods are well-established techniques for the assessment of bacterial viability. They offer significant advantages over other detection methods including elements such as sensitivity, rapidity and simplicity. Moreover, luminometric assays have wide dynamic range and high detectability. Additionally some systems may produce a very long-lived light (glow reactions) that can last for hours or even days. Finally, no known hazards are reported with the reagents used in luminometric assays.

2.4.5 Molecular methods

Molecular methods for the assessment of bacterial viability are currently of particular interest. These methods are used to detect the presence of nucleic acids, either DNA or RNA. Traditionally polymerase chain reaction (PCR) has been used to detect DNA and

to illustrate cell viability. The presence of intact DNA sequences was initially believed to reveal cell viability with the assumption that DNA would be degraded in dead cells more rapidly than other cellular components (Jamil *et al.*, 1993). However, DNA is relatively stable molecule and it may persist in a detectable form long after all viable cells have been eradicated (Deere *et al.*, 1996). During recent years PCR technique has also been applied in combination with some live-dead discriminating dyes that inhibit amplification of DNA from dead cells, thereby increasing the reliability of molecular methods (Lee & Levin, 2006; Rudi *et al.*, 2005). Nevertheless, traditional PCR is relative poor technique to distinguish dead cells from living ones, and thus alternative techniques such as RT-PCR, NASBA and SDA, based on the detection of other molecules than DNA, are rather exploited. Both ribosomal (r)RNA and messenger (m)RNA can be used to distinguish between viable and dead cells. Initially rRNA was utilized to assess viability but it can also persist for lengthy periods in dead cells similarly with DNA. On the other hand, mRNA is degraded rapidly upon cell death (half-life from seconds to some minutes), thus the presence of mRNA is believed to be a good indicator of viable cells (Simpkins *et al.*, 2000). However, it has been shown that under suitable circumstances also mRNA may persist in a detectable form for hours after cell death (Birch *et al.*, 2001). Nevertheless, a technique called reverse transcription PCR (RT-PCR) can be used to amplify mRNA, thereby assessing cell viability. Reverse transcription produces a DNA copy of the mRNA target which is further amplified by PCR. Notably, PCR can amplify any single target molecule, and if all traces of genomic DNA are not removed, for instance using DNase treatment, contaminating DNA will melt during denaturation step (typically at temperatures which are above 80 ^{o}C), thereby producing a false positive signal (Birch *et al.*, 2001; Keer & Birch, 2003; Simpkins *et al.*, 2000). Alternative transcription-based amplification techniques to RT-PCR are nucleic acid sequence-based amplification (NASBA) originally described by (Guatelli *et al.*, 1990) and strand displacement amplification (RT-SDA) (Hellyer & Nadeau, 2004; Hellyer *et al.*, 1999). NASBA utilizes three enzymes to mimic retroviral replication in order to amplify RNA target in an isothermal reaction at 41 ^{o}C. It overcomes the main weakness of RT-PCR because possible DNA contaminants are not amplified in reaction to produce a positive signal since RNA target is amplified far below the melting point of DNA (Birch *et al.*, 2001; Keer & Birch, 2003; Simpkins *et al.*, 2000). Both NASBA and SDA are successfully applied in clinical diagnostics (de Oliveira *et al.*, 2006; Hellyer *et al.*, 1999; Maher *et al.*, 2001) as well as in food analysis (Bentsink *et al.*, 2002; Guy *et al.*, 2006) to quantify and detect different viable pathogens. The main advantages of molecular methods over other methods mainly arise from improved speed and sensitivity of molecular detection. However, the increased sensitivity may widen the possible discrepancy between actual viability and viability assessed by the molecular methods as the nucleic acid content of cells varies greatly in different conditions. Accordingly, the molecular methods cannot easily differentiate between culturable and viable but non-culturable cells. Thus, the detection of a positive signal does not necessarily indicate the presence of viable cells due to unpredictable stability of DNA or RNA in certain situations. On the other hand, the absence of viable cells is usually evidenced by a negative amplification but it should be noticed that injured or stressed cells do not necessarily contain or may contain extremely low levels of mRNA so that the overall result is interpreted incorrectly as a negative (Birch *et al.*, 2001; Keer & Birch, 2003).

2.4.6 Scintillation counting

The world is full of electromagnetic radiation. This radiation is composed of photons, and each photon has a quantum of energy whose value is dependent on the wavelength of the radiation. Ionizing radiation occurs at very short wavelengths, which means that the energy of radiation is high. Ionizing radiation is mostly composed of *alfa*-, *beta*- and *gamma*-rays that are emitted during radioisotope decay. At the end of 19th century the first compound emitting invisible radiation was discovered and very soon the term radioactive was attached to these compounds named as radioisotopes (Kolar & Den Hollander, 2004). Since then various radiochemical assays for multiple applications are developed. Also microbial analysis exploits different radiolabelling techniques. Specifically, the technique has successfully been applied for the assessment of bacterial viability during past decades (Ahrenholtz *et al.*, 1994; Friedlander, 1978; Tesh *et al.*, 1986). Most assays detect the release of radioactively labelled DNA, which is believed to be a direct evidence of cell death (Ahrenholtz *et al.*, 1994; Friedlander, 1978). On the other hand, the release of other radioactively labelled macromolecules from microbial cells can also be followed (Tesh *et al.*, 1986; van Langevelde *et al.*, 1998). However, it is somewhat unsure to what extent the degradation and release of these molecules from microbes can be associated with cell death as discussed in previous sections. Therefore, the direct permeability of cell membrane, i.e. cell membrane integrity, has also been measured with the radiolabelling techniques (Abramov *et al.*, 1996). Radioactivity is mostly measured by various scintillation counting systems that use photomultiplier tubes (PMTs) to detect radiation (Hwang *et al.*, 2004). PMTs can be located close together over sample area in scintillation counter, which provides low background and high counting efficiency (Hyypia *et al.*, 1990). Various radioisotopes can be utilized in radiochemical assays for the assessment of cell death, isotopes such as ^{3}H, ^{14}C, ^{32}P, ^{35}S and ^{125}I are probably most frequently used. Whilst radiochemical assays may detect radioactively labelled macromolecules with high detection efficiency, the widespread use of this technique for the assessment of bacterial viability remains quite low due to several disadvantages. Radiolabelling techniques always produce harmful radiation, although radioactive compounds mostly utilized in various assays radiate with relatively low intensities. Also, the labelling of microbes with radioactive compounds and the quantification of labelling degree may be extremely troublesome so it is somewhat unknown to what extent emitted radiation indeed correlates with bacterial viability. Therefore, other techniques already discussed in previous sections are mostly more preferable alternatives for divergent assessment of bacterial viability.

2.4.7 Multiparameter data-analyses by multi-label counter devices

The methods described in previous sections are routinely used in various viability assays, yet their measurement capability is restricted only to single technology. The inability to utilize multiple technologies simultaneously in one assay may diminish the importance of information gathered on viability. Therefore, several manufacturers have developed high performance plate reader instruments, which are capable to measure more than just one technology in single assay. These equipments called routinely multi-counters give extensive information on multiple cellular parameters in one assay, thereby predicting

cell viability more accurately than single technology assays (Rudney & Staikov, 2002). Fluorescence, luminescence, absorbance and scintillation counting are technologies most probably found from various multi-counters. Also other technologies such as fluorescence polarization and time-resolved fluorescence are often available. Direct use of multiple technologies in one assay makes it possible to measure simultaneously all important parameters linked to viability of micro-organisms (Qazi *et al.*, 2004). These include parameters such as cell membrane potential or integrity, metabolic activity and multiplication capability. Simultaneous detection of multiple viability parameters substantially improves the detection of actual cell growth and activity as well as the assessment of total cell counts and possible cell lysis compared to single measurement. Notably, multi-counters enable kinetic measurements on a real-time basis, which makes assays suitable for high throughput screening applications (Huang *et al.*, 2006). Multi-counters are mainly applied for running diverse analyses of pharmacodynamics of various antimicrobial agents (Beard *et al.*, 2002; Huang *et al.*, 2006), and the use of technique will likely increase in the field of applied and clinical microbiology in near future. However, in most situations the viability and killing of various micro-organisms can still be determined with sufficient accuracy by single technology assay. Thus, the use of multiparameter data-analysis does not necessarily give any additional value for the measurement. Therefore, the selection between multi-counter device and more simple one-technology equipment is much case-dependent, and the final choice is based on factors such as the instruments available and especially how detailed information is required from a given analysis.

2.5 Standard methods for assessing the susceptibility of microbial cells

Susceptibility testing is indicated for any organism that causes an infection process requiring antimicrobial chemotherapy, and the results of the test may provide an answer how to control the growth and multiplication of a given organism. Accordingly, traditional culture based methods are broadly exploited techniques not only for the assessment of bacterial viability but also to determine bacterial susceptibility for numerous antimicrobials. Traditionally plate counting techniques as well as other methods capable to enumerate cell growth, such as spectrophotometry, have particular importance in the field of susceptibility testing. To perform the susceptibility test, a series of tubes or plates is prepared with a broth or agar to which variable concentrations of the antimicrobial agents to be tested are added. The tubes or plates are then inoculated with a suspension of target organism and after appropriate incubation time, the tests are examined and the susceptibility of tested organism is determined. However, the final result of susceptibility testing is significantly influenced by methodology used, and thus CLSI (the Clinical and Laboratory Standards Institute, formerly NCCLS) has specified among others the standard micro- and macrodilution broth methods as well as agar dilution and disk diffusion methods that should be utilized as standard reference methods for the determination of the in vitro activity of an antimicrobial agent against a given micro-organism that grows aerobically (NCCLS, M2-A8. 2004; NCCLS, M7-A6. 2003). Moreover, if the simultaneous action of two drugs against a single micro-organism is going to be

evaluated, a checkerboard method can be utilized (Eliopoulos & Moellering, 1996; Isenberg, 2003). The quantification of susceptibility of various micro-organisms is probably one of the most important tests performed in clinical microbiology, particularly when the nature of infection is due to multidrug resistant (MDR) bacteria or the causative organism is thought to be capable of acquiring resistance to commonly used antibiotics. Susceptibility tests are also important in studies of epidemiology and in drug discovery. However, susceptibility testing rarely provides kinetic information on the antimicrobial activities of various compounds against given micro-organisms. Thus, alternative methods are required if the kinetics of antimicrobial action on target bacteria are going to be revealed.

2.5.1 Broth dilution methods

Broth dilution procedures, consisting of both micro- and macrodilution methods, are probably the most commonly used techniques throughout the world to reveal the susceptibility of target bacteria against various antimicrobial agents. These methods are standardized by CLSI so that susceptibility readings can reliably be compared between different laboratories (NCCLS, M7-A6. 2003). In both methods serial twofold dilutions of antimicrobials to be tested are prepared in the broth medium following the incorporation of inoculate of target bacteria into broth. Thereafter, the inoculated broth should be incubated at appropriate temperature for a certain period of time. Since testing conditions have a significant effect on results obtained, the preparation of test including cultivation of target micro-organism, the size of inoculum, incubation time and current temperature should be carefully considered and kept constant for each bacterial strain throughout the testing. Microdilution broth method (Amsterdam, 1996; NCCLS, M7-A6. 2003) is called “micro” because it involves the use of small volumes of growth medium dispensed in plastic microdilution trays (usually 96-well plate). Specifically, each well should contain total volume of 0.1 ml of broth containing twofold dilutions of antimicrobials and the standardized inoculum of target micro-organism. In addition, each test should without exception include a growth control and a negative (non-inoculated) control. The inoculated microdilution trays are usually incubated at 35 ^{o}C for 16 to 20 hours before reading the results. The results are mostly detected by unaided eye but viewing devices can be used to facilitate the interpretation of microdilution tests since results may otherwise be impossible to read. Accordingly, the growth of target organisms in trays is mostly followed by spectrophotometric means or using various microscopes. However, these techniques can be exploited for the interpretation of test results only if turbidity of culture is high enough, that is a possible change in the cell number may remain unobservable if the comparable cell concentration is too low. The results of susceptibility testing are in most cases reported as the lowest concentration of antimicrobial agents found to inhibit the growth of target micro-organism (MIC), yet the true MIC mostly locates somewhere between the lowest concentration found and the next lower concentration. Macrodilution broth method is in outlines equal with microdilution method (NCCLS, M7-A6. 2003). The only differences lie in facts that macrodilution involves larger volume of broth (a minimum final volume of 1 ml) and the broth containing dilutions of antimicrobial agent and inoculated bacteria are not dispensed into the microtiter trays but pipetted to the test

tubes with size of approximately at 13 x 100 mm. Macrodilution method is typically used for various antifungal susceptibility testing (Barros & Hamdan, 2005; Dogruman Al *et al.*, 2003; Perea *et al.*, 2001). The results of macrodilution are mostly detected by unaided eye, simply by comparing the amount of growth in the tubes containing antimicrobial with the growth in the control tube (NCCLS, M7-A6. 2003). However, macrodilution is a very labour-intensive method and fits poorly for routine work. Moreover, it cannot be applied for screening large amounts of samples.

2.5.2 Agar dilution and disk diffusion methods

The agar dilution and disk diffusion (DD) methods are also well-established and widely used techniques for assessing the susceptibility of various micro-organisms (Jorgensen *et al.*, 1999; NCCLS, M2-A8. 2004; NCCLS, M7-A6. 2003; Washington & Sutter, 1980). The agar dilution method is very similar to the micro- and macrodilution methods. The plates containing variable concentrations of antimicrobial agent are inoculated with the standardized amount (10^4 CFU/spot) of target micro-organism. The inoculated plates are allowed to dry at room temperature and mostly incubated at 35 ^{o}C for approximately 16 to 20 hours before reading the results. To facilitate colony counting, the plates should be placed on a dark and non-reflecting surface (NCCLS, M2-A8. 2004). Notably, several automated systems have recently been developed both for the inoculation and for the colony counting, which substantially diminishes the workload and improves the reliability of results. Disk diffusion method saves time compared to the agar dilution, thereby being relatively practical method if rapidly growing organisms such as *Staphylococcus* or *Pseudomonas* are going to be tested. The principles behind this method are simple and very similar to agar dilution. It involves tablets or disks impregnated with antimicrobial agent that are placed on plates inoculated with target bacteria. Tablets pick up moisture resulting a radial diffusion of antimicrobial outward on agar, which produces an antimicrobial concentration gradient. Since the concentration of antimicrobial is high near the tablet, it affects even minimally susceptible organisms, whilst only more susceptible organisms are harmed as the distance from the tablet increases. Consequently, a clear zone is present around the tablet if the antimicrobial inhibits the growth of target bacteria. The wider a clear zone surrounding the tablet, the more susceptible the target organism is against antimicrobial. However, if two or more different antimicrobials are tested simultaneously, the width of clear zone is not necessarily comparable to the effectiveness of compounds as the initial concentration, the solubility and the diffusion rate on agar usually varies between different antimicrobials (Prescott *et al.*, 2004). Despite the fact that broth and agar dilution methods as well as disk diffusion method are listed by CLSI as standard methods for testing susceptibility of various micro-organisms against different antimicrobials, there are only few studies where the comparability of these methods are examined. Recent findings show that depending on organism and antimicrobial to be tested, there might be remarkably discrepancies between different methods (Luangtongkum *et al.*, 2006; Swenson *et al.*, 2004), thus clinical laboratories which perform routine testing should exercise extreme caution when determining susceptibility in order reproducible results could be provided.

2.6 Importance of kinetic analysis in microbiology

Dynamics and complexity of bacterial cell cultures are well evident, yet the main feature of most assays used to detect viability and killing of micro-organisms is endpoint reading of results. The simple one-step measurement is often fundamental criteria to keep various measurements reliable, comparable and easy to repeat. However, it is not necessarily expedient to situations in which much more detailed information about the effects of various external and internal factors on different bacteria are required. Moreover, it might be notably troublesome to control the growth and viability of various bacteria if the results are revealed unacceptably late. Moreover, the effect of antimicrobial agents varies considerably depending on factors such as incubation time, concentration of drug, size of inoculum and nature of target micro-organism. Also environmental factors such as pH, temperature and medium composition affect viability and killing of target organisms (Hartzen *et al.*, 1997; Kenny & Cartwright, 1993; Tornatore *et al.*, 1997). Therefore, it is predictable that non-kinetic measurements can only produce a rough estimation of bacterial viability since possible transitions in viability cannot be followed. On the other hand, the kinetic approach overcomes typical limitations of the endpoint assays by offering multiple data about the viability of target bacteria. It also reveals the antibacterial efficacy of various antimicrobial agents as a function of time, which significantly improves and accelerates the assessment of bacterial viability and killing. Kinetic measurements have been used to detect the activity of various antimicrobial agents using instruments such as tube luminometers (Salisbury *et al.*, 1999; Virta *et al.*, 1994; Virta *et al.*, 1997). More recently miniaturized kinetic assay formats are applied for the assessment of viability and killing of various gram-positive and gram-negative bacteria. These assay systems can measure parameters such as optical density (Holowachuk *et al.*, 2003; Koutny & Zaoralkova, 2005) and bioluminescence (Beard *et al.*, 2002; Deryabin & Polyakov, 2006) or their combination (Qazi *et al.*, 2004). Specifically, miniaturized assays are easily used on a real-time basis, which offers a rapid and robust tool to assess viability and killing of different micro-organisms. Moreover, a kinetic procedure allows the determination of viability parameter at any desired time-point of the incubation period. It also enables the evaluation of the time interval from exposure to visible effect for each antimicrobial agent to be tested, which helps to restrict the growth of infectious organisms more precisely.

2.7 Use of luminescent reporter proteins for monitoring bacterial viability and killing

Luminescent micro-organisms are well abundant, and thus widely exploited in diverse microbial analysis. Accordingly, numerous clinical and research laboratories throughout the world utilize more and more different luminescence based techniques for the assessment of viability of various micro-organisms. Specifically, different reporter proteins originated from various naturally luminescent organisms have lately been used as specific indicators of viability. Ideal reporter protein should produce an easily measured signal, which is proportional to the parameter of interest. Moreover, each reporter should satisfy some other criteria of particular importance such as specificity, sensitivity, detectability and ease of use

(Mason, 1999). These requirements are easily fulfilled by particular molecules from two important categories of highly luminescent reporter proteins. Proteins from thr first category are enzymes that produce bioluminescence in their catalyzed reactions, whilst proteins from second category are naturally brightly fluorescent. Specifically, proteins falling into the first category are called luciferases. These proteins can be found from various different bioluminescent organisms from bacteria to more complex multicellular organisms (Greer & Szalay, 2002), and similar diversity is evident in components responsible for light producing reactions indicating that different bioluminescence systems have been originated independently during evolution (Hastings & Johnson, 2003; Wilson & Hastings, 1998). Yet, there is one common feature in almost all different bioluminescence systems that matches them tightly together, light is produced as a by-product in luciferase-catalyzed reactions in which oxidation of different substrates called luciferins takes place in the presence of molecular oxygen. In all probability all reactions involve the formation and breakdown of luciferase-bound intermediate, which is in an electrically excitated state and hereby finally emits a photon. It is worth noting that luciferase and luciferin are only generic terms reflecting on enzymes and substrates found from different bioluminescent organisms.

In contrast to different luciferases, reporter proteins from the second category are directly luminescent since no enzymes or co-factors are needed for light production but bright fluorescence is exhibited once a mature protein is formed and exposed to suitable excitation light. Specifically, these proteins can be further divided into two groups of fluorescent proteins classified as green fluorescent proteins (GFP) originally isolated from jellyfish *Aequorea victoria* (synonyms *A. aequorea*, *A. forskalea*) and other GFP-like proteins first isolated from some Anthozoa species. All GFP molecules and GFP-like proteins contain a similar chromophore, which in its mature form is highly fluorescent and gives a distinct coloured fluorescence to different organisms expressing given protein (Chalfie & Kain, 2006). The use of different luciferases and GFP, with all mutational variants, in microbiological analytics has increased enormously during past decades. Consequently, luminescent reporter proteins have been exploited for the assessment of viability related parameters such as cellular metabolic activity (Duncan *et al.*, 1994; Lowder & Oliver, 2001; Parveen *et al.*, 2001; Rasanen *et al.*, 2001), biomass, which consists of viable or total cell counts (Herzberg *et al.*, 2006; Lowder *et al.*, 2000; Parveen *et al.*, 2001; Wiles *et al.*, 2005) and viable but non-culturable cells (Cho & Kim, 1999a; Cho & Kim, 1999b; Duncan *et al.*, 1994; Lowder *et al.*, 2000). Luciferase and GFP are mostly used in separate assays but also applications based on their combination are reported (Elvang *et al.*, 2001; Errampalli *et al.*, 1998; Maraha *et al.*, 2004; Qazi *et al.*, 2004; Unge & Jansson, 2001; Unge *et al.*, 1999). Overall, these reporter proteins enable the assessment of bacterial viability with simple, inexpensive and rapid way. Assays are also easily automated, which makes luciferase and GFP based measurements as one of the most reliable way to determine the viability of various micro-organisms.

2.7.1 Bacterial and beetle luciferases

Bacterial and beetle luciferases are probably the most studied and exploited enzymes in the world of bioluminescence. Both type of luciferases are responsible for light producing reactions but the mechanisms used to control the intensity and kinetics of light

emission varies. Bacterial luciferases (luxAB) are found mainly from marine bacteria, and most bioluminous bacteria belongs to three genera: *Vibrio*, *Photobacterium* and *Photorhabdus* (Mason, 1999). Some but not all species of luminous bacteria grow as symbionts in photogenic organs of many hosts, notably fish and squid, thereby providing the light source for the host. All luminous bacteria emit light continuously, and a single bacterium may emit as much as 10^4-10^5 photons per second. Although luciferases found from different bacteria differ more or less from each other as an amino acid sequence, they all catalyze the mixed function oxidation of a long-chain aldehyde and reduced flavin mononucleotide ($FMNH_2$) with the emission maximum at 490 nm (Hastings & Johnson, 2003; Wilson & Hastings, 1998). The reaction catalyzed by different bacterial luciferases is illustrated in outline as follows (Mason, 1999):

$$FMNH_2 + RCHO + O_2 \longrightarrow FMN + RCO_2H + H_2O + light$$

Pathway itself constitutes a shunt of cellular electron transport at the level of flavin ($FMNH_2$) that is actually called the luciferin because it gives rise to the emitter, luciferase-bound 4a-hydroxyflavin. Reaction proceeds through several intermediates, with the quantum yield ca. 0.2 - 0.3 emitted photon per reacting $FMNH_2$ molecule (Hastings & Johnson, 2003; Wilson & Hastings, 1998). All luciferases from different bioluminescent bacteria studied, are found to be chimeric proteins of two non-identical α (~40-kDa) and β (~35-kDa) subunits, which are coded by genes called *luxA* and *luxB*, respectively. Both genes are adjacent in the lux operon in which among others three other genes (*luxC,D*, and *E*), encoding proteins that make up the fatty acid reductase complex (for aldehyde synthesis), are included (Hastings & Johnson, 2003; Mason, 1999; Wilson & Hastings, 1998). Note that the expression of whole luciferase operon (*luxABCDE*) produces a self-luminous cell without any addition of substrate(s), whilst the expression of *luxAB* is not sufficient to start the light producing reaction but the addition of aldehyde must be provided to generate the light production.

Most bioluminescent insects are beetles, and presumably all beetle luciferases catalyze similar oxidative decarboxylation of benzothiazole luciferin in the presence of Mg-ATP. The best characterized and first cloned beetle luciferase is that from firefly *Photinus pyralis*, but several other beetle luciferases such as that from click beetle *Pyrophorus plagiophthalamus* have also been cloned and expressed in many organisms from bacteria to eukaryotes (Hastings & Johnson, 2003; Mason, 1999; Wilson & Hastings, 1998). Each luciferase has a unique capability to produce bioluminescence of distinct colour during the catalysis (firefly luciferase, for instance, emits light with a peak at 561 nm under optimal conditions), and some are even capable to yield simultaneous emission of light of several different colours (Mason, 1999; Wood *et al.*, 1989). Firefly luciferase is a 62kDa protein encoded by *lucFF* gene, and there is 40-50 % sequence homology at the amino acid level between different beetle luciferases (Hastings & Johnson, 2003). Luciferase acts as mono-oxygenase, which apparently is active as monomer. It catalyzes the conversion of cellular energy into light by a two-step process in which ATP, O_2 and luciferin are utilized as illustrated below (Hastings & Johnson, 2003; Wilson & Hastings, 1998; Wood *et al.*, 1989):

D-luciferin + ATP-Mg $\longrightarrow$ luciferin adenylate + Mg-Ppi
luciferin adenylate + O_2 $\longrightarrow$ oxyluciferin + CO_2 + AMP + light

In the first step, the enzyme catalyzes the condensation of D-luciferin (a benzothiazoylthiazole) with ATP in the presence of Mg^{2+} to form the luciferyl adenylate, with ATP providing AMP as a good leaving group. In the second step, the luciferyl adenylate is oxidized by molecular oxygen to yield oxyluciferin, AMP, carbon dioxide and light. Note that the oxidation of luciferyl adenylate to oxyluciferin proceedes through the cyclization of the peroxide in order to create energy-rich dioxetanone intermediate. The breakdown of intermediate provides energy, thereby forming the excitated oxyluciferin which, upon transition to the ground state, emits the photon. The energy of excited state, hence the colour of the emission, probably depends on the tertiary structure at the catalytic site. The overall quantum yield for reactions catalyzed by different beetle luciferases is ca. 0.9 emitted photon per oxidized luciferin, which is the highest yield reported for any luminescent reaction (Hastings & Johnson, 2003; Wilson & Hastings, 1998).

Overall, bacterial and beetle luciferases are widely used proteins for the detection of viability of various micro-organisms, which express these enzymes. However, luciferase catalyzed reaction requires energy from cell metabolism, that is the metabolic state of microbial cells affects to the level of bioluminescence (Hakkila *et al.*, 2002). Thus, bioluminescence actually reveals cellular metabolic activity, not the actual viability. Fortunately, metabolic activity in most cases equals viability, as discussed in previous sections, which makes it possible to assess viability using either bacterial or beetle luciferase system. Furthermore, light can be detected even in few cells in assay, which makes systems consisting of bacterial or beetle luciferases as one of the most sensitive method to detect metabolically active, viable cells. Besides, different luciferases are nowadays actually more accessible than ever before, which has further increased the proportion of luciferase based applications in diverse microbial research.

2.7.2 Green fluorescent protein (GFP) and other related fluorescent proteins

Ever since the gene coding for the green fluorescent protein (GFP) was cloned (Prasher *et al.*, 1992), followed by the first demonstrations that the expression of gene in bacteria produced distinct fluorescence (Chalfie *et al.*, 1994; Inouye & Tsuji, 1994), the monitoring of physiological status of various micro-organisms has become more and more straightforward. As a reporter protein, GFP is versatile choice. The availability of different GFP variants with altered fluorescent properties as well as the discovery of other GFP-like proteins has ever expanded the use of fluorescent proteins in various applications in the field of microbial analysis. GFP cloned from jellyfish *Aequorea victoria* is an extremely stable protein that exhibits bright fluorescence in the absence of any enzymes and cofactors (Chalfie & Kain, 2006). It is worth noting that there are nowadays several different organisms found from various genera, such as jellyfish *Phialidium*, jellyfish *Mitrocoma*, hydroid *Obelia* from class of Hydrozoa and sea pansy *Renilla*, sea cactus *Cavernularia*, reef coral *Discosoma*, star coral *Montastraea* from class of Anthozoa, which all contain GFP or GFP-like proteins. GFP is about 27kDa monomeric protein composed from 238 amino acids, which form a compact barrel like

symmetrical structure. The part of molecule being responsible for fluorescence properties of GFP is located inside of the barrel that provides the proper environment for the chromophore to fluoresce, thereby explaining the unusual stability of protein fluorescence. The actual chromophore is composed from three amino acids, residues 65-67 that are Ser-Tyr-Gly, which form an imidazolone structure essential for fluorescence (Heim *et al.*, 1994). Note that nascent protein is not fluorescent since chromophore formation occurs post-translationally (Heim & Tsien, 1996). The formation of fully fluorescent protein is a multistep process in which GFP first folds into a nearly native conformation. In second step the imidazolone ring is formed by cyclization of residues 65 and 67 followed by dehydration. Finally molecular oxygen oxidizes cyclized intermediate at residue 66 to form strongly fluorescent structure (Figure 4). Each step of chromophore formation is either autocatalytic or uses factors that are ubiquitous, which enables the expression of fluorescent GFP in broad range of various organisms. The entire structure of various GFP molecules and their chromophores is well evident, and fundamental reviews about the topic can be found from Chalfie & Kain (2006); Kay & Sullivan (1999); Tsien (1998).

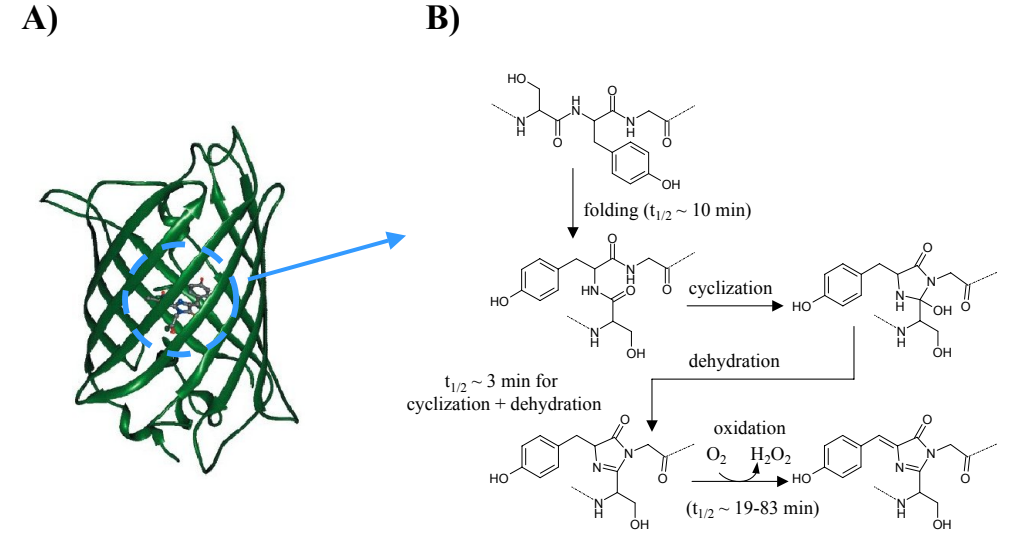

Figure 4. A) The three-dimensional structure of GFP. The chromophore is shown as a ball-and-stick representation inside the barrel. B) Proposed mechanism for the intramolecular biosynthesis of the GFP chromophore. The figures have been modified from Tsien (1998).

The wild type GFP has a large excitation maximum at 395 nm and another minor peak at 475 nm. The excitation of protein at 395 nm results an emission maximum at 508 nm, whereas excitation at 475 nm gives a maximum at 503 nm, respectively. On the other hand, GFPs with mutational substitutions have generated a wide variety of proteins, such as enhanced blue (EBFP), cyan (ECFP), green (EGFP) and yellow fluorescent protein (EYFP), with altered spectral properties compared to wtGFP (Chalfie & Kain, 2006). The most commonly used GFP variants are perhaps GFPmut1 and EGFP in both of which serine at position 65 is replaced by threonine (S65T) and phenylalanine at position 64 by leucine (F64L) (Cormack *et al.*, 1996; Heim *et al.*,

1995). Both proteins have identical amino acid sequence but the coding sequence of EGFP has been further modified with several silent base changes in order to humanize the codon usage. Mutations at positions 64-65 result the latter peak of wtGFP to be enhanced five- to sixfold and shifted to 489-490 nm, whilst the wild type 395 nm excitation peak is suppressed. Emission maximum for GFPmut1 and EGFP is observed at 508-509 nm. The fluorescence quantum yield for wtGFP molecule is about 0.8 photons emitted to the number of photons absorbed, which is in most cases higher than those of different mutational variants (Chalfie & Kain, 2006; Tsien, 1998). However, the folding of protein in its native form and the formation of mature chromophore at higher temperatures is considerably faster and more efficient in mutants than in the wild type. Furthermore, both the excitation and emission wavelengths of mutational variants encompass better the wavelengths of commonly used fluorescence filter sets than wild type protein. Additionally, different mutants exhibit stronger fluorescence intensity and greater stability to various environmental factors, besides they display much reduced rates of photobleaching (Chalfie & Kain, 2006; Kay & Sullivan, 1999).

The use of GFP-like proteins in microbiology is notably increased ever since first fluorescent homologs of GFPs were cloned from the class of Anthozoa including genera such as *Discosoma*, *Zoanthus*, *Anemonia* and *Clavularia* (Matz *et al.*, 1999). Today GFP-like proteins from the classes of Anthozoa and Hydrozoa includes over 100 members, and in addition, few more GFP homologs from the class Crustacea are currently known (Shagin *et al.*, 2004). The color variety of these proteins is interesting consisting of four different group: green, yellow, and red fluorescent proteins and nonfluorescent chromoproteins of different hues, from orange to blue (Shaner *et al.*, 2004; Verkhusha & Lukyanov, 2004). The most commonly exploited GFP-like protein is probably an Anthozoan red fluorescent protein known as dsRed. It is originated from *Discosoma sp.* of reef coral (Campbell *et al.*, 2002; Miyawaki, 2002; Shrestha & Deo, 2006). The chromophores of GFP-like proteins resemble the chromophore structure of GFP in which an imidazolone structure forms a basis for fluorescence properties (Pakhomov *et al.*, 2006). Both dsRed and most other Anthozoa GFP-like proteins appear as tetramer in their natural state (Baird *et al.*, 2000), whilst all GFP molecules exist as monomer (*Aequorea* GFP and all its mutated forms) or dimer form (other GFP molecules such as *Renilla* GFP) (Chalfie & Kain, 2006).

Unlike more conventional reporter proteins such as β-galactosidase, luciferase or bacterial chloramphenicol acetyltransferase (cat), GFP is not an enzyme. Therefore, there is no signal amplification, which denotes that GFP fluorescence is directly proportional to the number of bacterial cells. On the other hand, there are elements such as the stability of GFP and the rate of synthesis of fully fluorescent protein, which affect fluorescence. It is obvious that total fluorescence is consisted of an increase both in the cell number and in the number of fluorescent molecules per cell. Note that GFP is not an actual marker of viability, though the level of GFP synthesis roughly correlates with the level of overall protein synthesis. However, GFP molecules are unique proteins for diverse bacterial viability and killing analyses, and there are several studies in which bacterial localization, association and multiplication in various enviroments is monitored by GFP fluorescence both temporally and spatially (Finer & Finer, 2000; Grall & Manceau, 2003; Rice *et al.*, 2003; Valdivia *et al.*, 1996; Wu *et al.*, 2004).

3. AIMS OF THE PRESENT STUDY

The aim of this study was to develop a kinetic method for the simultaneous detection of bacterial viability and killing on a real-time basis, with a view to improve the assessment of bacterial growth, metabolic activity and total cell number as well as to prove the suitability of the multiparameter assay system for the establishment of antimicrobial drug susceptibility of bacteria.

The more specific aims were:

1. To construct luminescent sensor bacteria by exploiting green fluorescent protein (GFP) as well as bacterial and beetle luciferases as reporter proteins.

2. To test whether bioluminescence and fluorescence emitted from bacteria expressing *gfp*, *luxABCED* and *lucFF* genes can be used in kinetic measurements for the assessment of bacterial viability and killing using flow cytometry or automated multi-counter device.

3. To examine on a real-time basis the effects of various antimicrobial agents on bacteria as changes in cell activity (bioluminescence) and in cell number (fluorescence).

4. To compare the speed, sensitivity and feasibility of developed methods with those of more traditional microbiological measuring techniques.

5. To evaluate the suitability of the multiparameter microplate assay system for diverse high throughput screening applications in various microbial analyses such as drug screening and susceptibility assays.

4. SUMMARY OF MATERIALS AND METHODS

The more detailed information concerning the materials and methods used in this study can be found from original publications **I** – **IV.**

4.1 Plasmids and bacterial strains

Plasmids and bacterial strains used are presented in Table 3. Briefly, *Escherichia coli* MC1061 and *Escherichia coli* K-12 were used as host strains throughout all experiments. Plasmids used in this study were constructed by standard molecular biology techniques as described in Sambrook *et al.* (1989). More detailed cloning procedures are given in original publications (**I&II**). All plasmids were transformed to host bacteria by electroporation (Dower *et al.*, 1988) using the Bio-Rad Gene Pulser system (Bio-Rad Laboratories, Richmond, CA).

Table 3. Bacterial strains and plasmids used in this study.

Strain or plasmid	Description	Reference or source
Strain		
E. coli MC1061	cI^+ Δ(*ara leu*)7697 Δ*lacX*74 *galU galK hsr hsm* $^+$*rpsL araD*139	(Casadaban & Cohen, 1980)
E. coli K-12 M72	Sm^R*lacZ*(Am) Δ*bio–uvrB*Δ*trpE*42[λ*N7*(Am)-*N*53(Am)*c*1857Δ*H*1]	(Bernard *et al.*, 1979)
Plasmid		
pEGFPlucAmp	Contains enhanced green fluorescent protein (*egfp*) and firefly luciferase (*lucFF*) genes, ampicillin resistance	**I** & **III**
pEGFPlucTet	Same as above, except tetracycline resistance	**II** – **IV**
plucAmp	Contains firefly luciferase (*lucFF*) gene, ampicillin resistance	**I**
plucTet	Same as above, except tetracycline resistance	**II**
pEGFPluxABCDEamp	Contains *egfp* gene and bacterial luciferase (*luxABCDE*) operon, ampicillin resistance	(unpublished)

4.2 Culture conditions

For all experiments, bacterial cells were cultivated in Luria-Bertani broth (10 g tryptone, 5 g yeast extract, 5 g NaCl per liter, pH 7.0) containing appropriate antibiotic for selection pressure, either 100 µg/ml of ampicillin or 5 µg/ml of tetracycline, in a shaker (280 rpm) at 37°C. The overnight culture of bacteria was diluted (1:100) with fresh LB-broth containing antibiotic and further cultivated to mid-logarithmic phase. Cells were harvested by centrifugation (10 min, 3000 rpm) and resuspended in fresh LB-broth to obtain an appropriate optical density measured at 600 nm with a UV-1601 Shimadzu spectrophotometer (Shimadzu corp., Tokyo, Japan).

4.3 Instrumental analysis of cell number, bacterial viability and cell growth

4.3.1 Fluoro-luminometric analyses

Bacterial culture (50 or 100 µl) was mixed with a 50-100 µl solution of disinfectant such as ethanol (**I&II**), antibiotics such as ampicillin, erythromycin, nalidixic acid, penicillin G, polymyxin B, tetracycline, and trimethoprim (**III&IV**) or tricyclic cationic

neuroleptics such as promethazine (**IV**, unpublished) in the wells of a 96-well plate (white Cliniplate, Thermo Labsystems, Helsinki, Finland). The Fluoroskan Ascent FL fluoro-luminometer (Thermo Labsystems) controlled by Ascent software™ for Fluoroskan Ascent FL was programmed to perform measurements at 23°C (**III**&**IV**) and 25°C (**I**) for appropriate period of time. In addition, comparable multi-counteres such as Plate Chameleon (Hidex, Turku, Finland), EnVision 2100 (Perkin Elmer, Turku, Finland) and Mithras LB 940 (Berthold Technologies, D-Bad Wildbad, Germany) were applied to some measurements (unpublished). The Labsystems Luminoskan RT (Thermo Labsystems) was used for luminometric measurements (**II**). At selected time intervals, the fluorescence of selected wells of the plate were read with the following settings: 0.1 s per well using 485 nm excitation and 510 nm emission filter sets. Following fluorescence measurements in particular wells, 100 µl of 0.5 mM D-luciferin solution (Bio-Orbit, Turku, Finland or BioThema, Dalarö, Sweden) in 100 mM sodium citrate buffer, pH 5.0 was automatically dispensed into the wells, and bioluminescence was measured for 1 s per well.

4.3.2 Flow cytometric analyses

4.3.2.1 Flow cytometric cell number and green fluorescence analyses

For flow cytometric cell number analyses (**I-III**), $4.4x10^5$ fluoresbrite beads (Prolabo) per ml or $7.5x10^5$ fluoresbrite beads (Polysciences) per ml were added in appropriately diluted cell suspension. For flow cytometric green fluorescence analysis, tubes containing 500 µl of *Escherichia coli* with pEGFPlucAmp and 500 µl of various ethanol dilutions were incubated at 25°C. At specific time-points of incubation, samples of 10 µl were taken and immediately diluted 100-fold with 150 mM NaCl containing fluoresbrite beads (Prolabo) for internal calibration (**I**). All samples were analyzed in duplicate with an Epics XL flow cytometer (Coulter Corporation, Miami, Fla.) by illuminating with a 15 mV air-cooled argon ion laser (488 nm). The fluorescence of GFP-producing cells was detected via 525 ±10 nm (green) bandpass filter. Signals were amplified with the logarithmic mode for side scattering, forward scattering, and fluorescence. To exclude debris, the discriminant was set to the forward scatter channel. Flow cytometric data were analyzed with the WinMDI 2.8 program (Joseph Trotter, The Scripps Research Institute, http://facs.scripps.edu/). In dot plots of light scatter properties, bacterial cells and fluoresbrite beads were gated from irrelevant counts for fluorescence and cell number analyses.

4.3.2.2 Flow cytometric viability analysis using SYTO9-PI or GFP-PI combination

For SYTO9-PI based flow cytometric viability analysis, tubes containing 500 µl of *Escherichia coli* cells with plucAmp (**I**) or plucTet (**II**) and 500 µl of various ethanol dilutions were incubated at 25°C. At specific time-points of incubation, 1.5 µl of each of the DNA-binding stains of the LIVE/DEAD kit, (SYTO9 and PI) were added to the tubes, mixed, and further incubated in the dark for 15 minutes. Immediately following incubation, samples were diluted and analyzed with flow cytometer as described above with exception that fluorescence was detected both via 525 ±10 nm (green) and 620 ±10 nm (red) bandpass filters. For GFP-PI based flow cytometric viability analysis,

tubes containing 500 µl of *Escherichia coli* cells with pEGFPlucTet (**II**) and 500 µl of various ethanol dilutions were incubated at 25°C. At specific time-points of incubation, 1.5 µl of propidium iodide (PI) of the LIVE/DEAD kit was added to the tubes, mixed, and immediately diluted and analyzed by flow cytometry. Bacterial cells were gated in the dot plots of light scatter properties from irrelevant counts for fluorescence analyses. Similarly, in dot plots of fluorescence, different bacterial populations were gated according to the viability, specifically living, compromised and dead cells.

4.3.3 Optical density (OD) measurements

Bacterial culture (50 or 100 µl) was mixed with a 50-100 µl solution of ethanol (**I**) or specific antibiotic (**III**) in the wells of a 96-well plate (white well matrix with clear styrene base, optiplate I, Thermo Labsystems). The iEMS platecounter reader MF (Thermo Labsystems) controlled by Ascent software^{TM} for iEMS Reader MF was programmed to obtain measurements at 23°C (**III**) and 25°C (**I**) for appropriate period of time. Optical density of cell cultures containing either ethanol or antibiotic was read using a 620 nm emission filter at selected time intervals.

4.3.4 Colony forming units (CFU) measurements

Bacterial culture of 500 µl was mixed with 500 µl of various ethanol dilutions (**I-II**) or specific antibiotic (**III**) in test tubes, and incubated at 23°C (**III**) and 25°C (**I&II**). Samples (100 µl) were removed at desired time points of incubation, diluted up to 10^7-fold with 150 mM NaCl, and plated in duplicate onto LB-agar plates containing appropriate antibiotic. Colonies were counted after overnight incubation at 37°C.

4.4 Purification of GFP and fluorescence properties of purified protein

GFP was extracted from bacteria and purified using three-phase-partition (TPP) technique as described by Thomson & Ward (2002). Briefly, cells containing GFP were suspended in ammonium sulfate solution following the addition of tert-butanol, and mixed thoroughly. The suspension was further centrifuged and lower phase containing fluorescent protein was removed into a clean tube. Fresh tert-butanol was added to recovered solution, mixed thoroughly following recentrifugation of the solution. Precipitate protein layer between two liquid phases was separared and suspended in appropriate buffer for further use. The fluorescence properties of purified protein was defined in the presence of 150 mM NaCl and in various ethanol dilutions (**I**).

4.5 Spectrum analyses

4.5.1 Fluorescence spectrum of purified GFP

In order to measure the excitation and emission maximum values of purified GFP-molecules and to compare measured values with the values reported in literature, the purified protein in 50 mM Tris-HCl, pH 7,5 was further diluted either in distilled water or in 25% ethanol and spectrums were measured at wavelengths between 510 – 350 nm for excitation and at wavelengths between 650 – 495 nm for emission with Perkin

Elmer LS-5 Luminescence spectrometer using Spectre software (version 1.2). (unpublished)

4.5.2 Absorbance spectrums of various antimicrobial agents

In order to figure out if tricyclic cationic neuroleptics such as promethazine directly interact with a specific antibiotic forming a charge-transfer complex, drugs were dissolved in distilled water for spectrum analysis (**IV**). Solutions of drugs (100 μM ampicillin, 100 μM penicillin G, 50 μM promethazine) were further pipetted to quartz cuvettes and spectrums were measured at wavelengths between 375 – 190 nm with Perkin Elmer UV/VIS Spectrometer Lambda 2 (Perkin Elmer corporation, USA) using UV WinLab software (version 2.80.03).

5. SUMMARY OF RESULTS AND DISCUSSION

5.1 Construction of plasmid systems for the production of green fluorescent protein (GFP) and luciferase in bacterial cells

Five plasmids, specifically named as pEGFPlucAmp (**I**, **III**), pEGFPlucTet (**II-IV**), plucAmp (**I**), plucTet (**II**) and pEGFPluxABCDEamp (unpublished), were constructed for the assessment of bacterial viability and killing with GFP fluorescence and luciferase bioluminescence (Table 3). The gene e*gfp* encoding enhanced green fluorescent protein (EGFP) enabled the estimation of cell growth and cell number throughout the experiment, whilst the genes *lucFF* and *luxAB* encoding firefly (*Photinus phyralis*) luciferase and bacterial luciferase of *Photorhabdus luminescencens* were the markers of the cellular metabolic activity thus enabling the estimation of cell viability.

5.2 Use of GFP fluorescence and luciferase bioluminescence as markers of bacterial viability and killing

5.2.1 Assessment of metabolic activity by luciferase bioluminescence (I-IV, unpublished)

Numerous different methods are currently available for the assessment of bacterial viability, and in general each method yields slightly different information compared to others. Bioluminescence produced in luciferase catalyzed reactions has shown to reveal metabolic activity of the target cells, which in most cases equals to viability (Virta *et al.*, 1994). Bioluminescence responds rapidly to changes in metabolic activity, thereby its suitability for revealing viability on a real-time basis was tested here using kinetic microplate assay. Both beetle luciferase (**I-IV**) and bacterial luciferase (unpublished) were tested using different incubation times (240 min in **I**&**II**, unpublished, and 10 hours in **III** and 20 hours in **IV**) and temperatures (25 ^{o}C in **I**&**II**, unpublished, and 23 ^{o}C in **III**&**IV**). Results obtained clearly demonstrated that bioluminescence increased as a function of both incubation time and temperature, which refers to the growth and replication of bacterial cells, thereby being a direct evidence of viability. On the other hand, a decrease in bacterial viability was observed when various antimicrobials were introduced into the bacterial culture, that is bioluminescence was consistently the lower the more antimicrobial was present. This was obviously due to cessation of the growth, decreased metabolic activity, death of bacteria or all three of them. Note that the expression of the whole bacterial luciferase operon generates continuous light production without the addition of substrates, whilst light production by beetle luciferases requires addition of luciferin, which impedes the assessment of kinetic measurements. In addition, the production of long-chain aldehydes likely responds more closely to the changes in the metabolic state of bacteria than ATP whose cellular concentration is usually more constant. Yet there are situations in which metabolic activity is not necessarily tightly connected to the current viability status of bacteria

(Nystrom, 2001). This effect was clearly seen in Figure 6 (**I**) as ethanol notably decreased bioluminescence, yet the culturability of bacteria was not affected according to the plate counting.

5.2.2 Determination of cell growth and number by GFP fluorescence (I, III&IV)

Whilst bioluminescence is a good indicator of cell viability, it does not reveal the actual cell number. Thus alternative markers are needed to follow an increase in the total cell number. Green fluorescent protein (GFP) is an extremely stable protein that accumulates in cells during growth making the measured fluorescence signal proportional to the total number of bacteria. In this study cell number was followed as GFP fluorescence using kinetic microplate assay. It was found that fluorescence inreased throughout the experiments indicating an increase in the total cell content (**I**, **III&IV**). However, it is obvious that an increase in fluorescence consisted of an increase both in cell number and in the number of GFP molecules per cell. Therefore, GFP fluorescence may easily overestimate the bacterial population size as the concentration of GFP in individual cells is increased during population growth. In order to calculate the increase in the number of bacteria, the total fluorescence signal need to be divided by correction factor (CF), which can be settled with the aid of mean fluorescence intensity (MFI) of bacteria. An increase in MFI as a function of time was found to be linear, evidently due to constant growth of cell culture (Figure 5). The detailed calculations of CFs as well as an example of flow cytometric MFI-measurements are presented in **I**. Note that the stability of GFP does not only affect the total number of GFP molecules in cells but it may also complicate the quantification of possible cell lysis, since the detectable fluorescence signals do not decline during the lysis, that is, intact GFP molecules are released into medium in which they normally retain their fluorescence capability even if cells are lyzed. On the other hand, cell lysis can be observed using specific instruments such as FCM, which detects fluorescence of cell-bound GFP, whilst fluorescence emitted by free GFP molecules is not recorded (**I**). Alternatively, possible cell lysis can easily be observed by simply measuring optical density of cell culture instead of fluorescence (**I&III**). Moreover, numerous different GFP mutants with short half-lives are developed (Andersen *et al.*, 1998; Blokpoel *et al.*, 2003; Deichsel *et al.*, 1999; Lowder & Oliver, 2001). These proteins enable the fluorometric detection of possible cell lysis. Furthermore, it is worth noting that wtGFP folds fairly efficiently when expressed at or below room temperature (Tsien, 1998). Similarly GFP variant (EGFP) used in this study exhibits strong fluorescence at temperatures of 23 °C (**III&IV**) and 25°C (**I**). However, the increase in GFP fluorescence at 25°C (Figure 5 in **I**) was notably higher than at 23°C (Figures 1-3 in **III** and figures 1-2 in **IV**). This probably derives from mutational substitutions that improve folding and maturation efficiency of EGFP variant when the temperature is raised. Furthermore, the excitation and emission spectra of EGFP were measured both in water and in ethanol (Figure 6). Observed exc_{max} and em_{max} were 488nm/509nm in water and 490nm/509nm in ethanol. These values were consistent with earlier reports of exc_{max} and em_{max} for related GFP variant (Tsien, 1998).

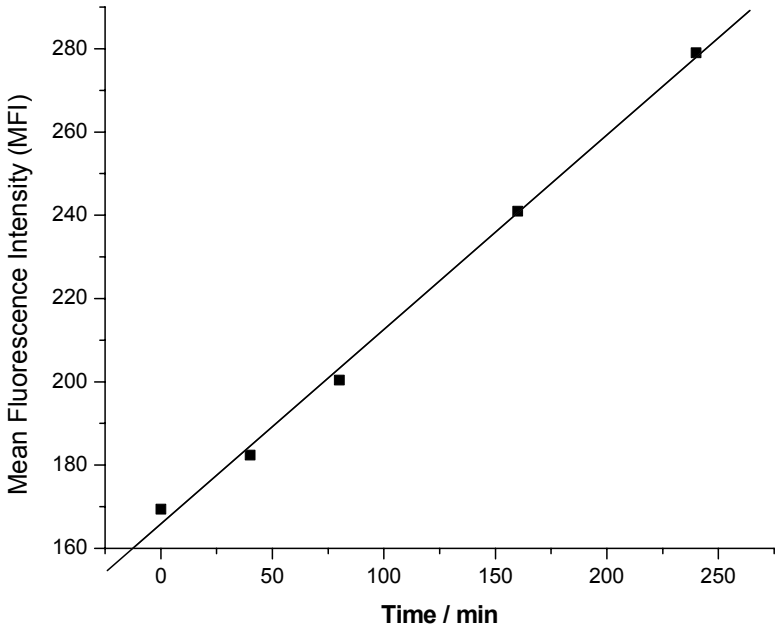

Figure 5. An increase in mean fluorescence intensity (MFI) of *E. coli* MC1061 cells as a function of time. Bacterial cells were expressing green fluorescent protein (GFP), and MFI was measured using Coulter EPICS XL flow cytometry, respectively.

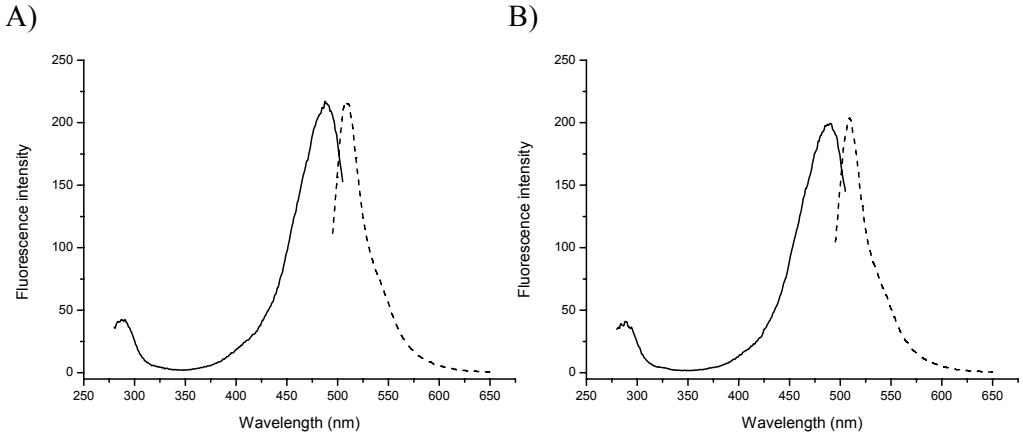

Figure 6. A) The excitation (straight line) and emission (dotted line) spectra for EGFP produced by *E. coli* cells used in this study. Purified protein was dissolved in water and spectra were recorded using Perkin Elmer LS-5 Luminescence spectrometer. The corresponding exc_{max} and em_{max} were found to be 488nm and 509nm. B) The excitation (straight line) and emission (dotted line) spectra for EGFP produced by cells used in this study. Purified protein was dissolved in ethanol and spectra were recorded as above. The corresponding exc_{max} and em_{max} were found to be 490nm and 509nm.

5.2.3 Evaluation of the current vital stage of bacteria using GFP-PI combination (II)

Several staining protocols have been developed for the quantification of different vital stages (viable, dormant or dead) of a diverse array of bacterial genera. The majority of

staining techniques are based on fluorescent dyes, which allow rapid and simple cell separation according to the fluorescence emission (Davey & Kell, 1996). The most simple way is to use a single staining but a dual staining technique in which two dyes have distinct binding properties toward bacteria with different physiological states, is a more preferable choice. One particularly effective method for the assessment of bacterial viability is dual staining with SYTO dyes such as SYTO 9, SYTO 13 or SYTO BC in combination with propidium iodide (PI) (Ben-Amor *et al.*, 2005; Stocks, 2004; Virta *et al.*, 1998). These dyes have affinity to DNA but the diffusion of PI into bacteria is mostly hindered by intact cell membrane. This enables the assessment of current vital stage of bacteria according to the proportion of bound stains. The binding into DNA causes a significant increase in fluorescence compared to fluorescence of unbound dye, which refers to the virtually nonfluorescent background in the absence of binding. However, number of unfavorable properties limit the use of SYTO-PI combination. Therefore, the possibility for replacing SYTO dye with green fluorescent protein (GFP) in bacterial viability analyses was studied here, with a view to overcome the current disadvantages of SYTO-PI combination and to achieve improved data reliability (**II**). Specifically, SYTO9-PI combination revealed three different cell populations: living, compromised and dead cells (Figure 3A in **II**). However, the substitution of SYTO 9 with GFP provided more distinct cell populations, thereby notably improving the reliability of the assessment of viability (Figure 3B in **II**). GFP turned to be also more cost-effective choice than SYTO 9. Moreover, the overall procedure was more rapid because no incubation was required. As a conclusion it can be stated that GFP-PI combination is a good option for the evaluation of bacterial viability using green (GFP) and red (PI) fluorescence. The procedure can also be used in parallel with other methods such as luciferase assay and plate counting (Tables 1 and 2 vs. Table 3 in **II**), thereby improving the accuracy of results.

5.2.4 Comparison of GFP-luciferase method with plate counting (I-III), optical density measurements (I&III) and flow cytometric analyses (I&II) in the assessment of bacterial viability and killing

Fluoro-luminometric real-time assay was used to assess the changes in cell number and in cell viability using GFP fluorescence and bioluminescence produced in luciferase catalyzed reactions. The results were compared to those obtained by plate counting, optical density measurements and flow cytometric analyses. Accordingly, the detection limits for different methods employed in this study are depicted in Table 4. It is obvious that luciferase based bioluminescence assay and plate counting are most sensitive alternatives for the assessment of viability, yet plate counting is sensitive to errors resulting from multiple dilutions. Moreover, it does not detect viable but non-culturable cells. Similar or even better accuracy could be achieved with molecular methods and with direct ATP-detecting assays, yet these methods were not used in this study. Specifically, molecular methods are able to detect even one single molecule representing a viable cell (Simpkins *et al.*, 2000; Tsai & Olson, 1992), and the most sensitive ATP assays can detect ATP with attomolar sensitivity, which enables also the detection of a single cell (Lundin, 2000). However, the actual detection limit for these methods is at best set around some dozen cells. This is a consequence of multiple

Table 4. The detection limits for methods used in this study for revealing bacterial viability and killing.

Method	detection limit (bacteria per ml of sample)	Reference
Bioluminescence assay[a]	~10^2 to 10^4	(Billard & DuBow, 1998), and (**I**) in this study
Plate counting[b]	~10^2 to 10^3	(Fuller *et al.*, 2000)
Fluorometry[c]	≥10^4	(Martens-Habbena & Sass, 2006)
Flow cytometry[d]	≥10^4	(Malacrino *et al.*, 2001; Gunasekera *et al.*, 2000)
Optical density measurement[e]	~10^6 to 10^7	(Prescott *et al.*, 2004)

[a]The sensitivity of luciferase based assay can be affected by the origin of luciferase, that is bacterial luciferase based assays may have lower detection capability compared to beetle luciferase based assays. This might arise from the fact that bacteria need to communicate with each other for growth (Kaprelyants & Kell, 1996), and therefore light from *lux* which originates from bacteria can stall easier than light from eukaryotic originated *luc*
[b]The sensitivity of plating methods is notably diminished if nongrowing i.e. nonculturable cells exist
[c]Background fluorescence greatly diminishes the sensitivity of fluorometric detection
[d]Detection limits of flow cytometric measurements are diversed. FS and SS properties are used for direct counting of bacteria which results lower detection capability compared to fluorescent staining which allows also the analysis of functional cell parameters thus enabling better discrimination between cells and particles of a size with bacterial cells
[e]Poor sensitivity of turbidimetric measurements is further diminished by precipitates and cell aggregate formation

reasons such as the inhibition of enzymes during sample preparation, which greatly limits the sensitivity of the assays. Flow cytometric viability analyses, optical density measurements and fluorometric detection of various fluorochromes such as GFP are orders of less sensitive methods than bioluminescence assays and plating techniques. However, these methods are rapid and easy to perform, thereby being well applicable to situations in which very low detection limit is not critical. Furthermore, fluorescence microscopy (Auty *et al.*, 2001) and scintillation counting could reveal cell viability and cell number with rather similar accuracy than flow cytometric and fluorometric measurements. However, these methods were not tested here. Notably, the effect of any factor on cell viability is more or less dependent on incubation time and concentration as already discussed in section 2.6. It is therefore predictable that the longer incubation time or higher drug dosage, the more target cells are affected. However, during long incubations the drug effect may also be reversed. Specifically, under antimicrobial pressure bacterial subpopulations with reduced susceptibilities to antimicrobial agents have a survival advantage, proliferating preferentially (Tam *et al.*, 2005). Alternatively drug may became simply inactivated or metabolized during long incubation period. Therefore, rapid methods such as multiparameter microplate assays revealing the effect of various antimicrobial agents against target bacteria within some hours are generally most preferable choices. Consequently, fluoro-luminometric measurement used in this study revealed the total number of bacterial cells and cell viability with good accuracy, and significantly increased the speed of assessing the effects of various antimicrobials on bacteria. Plate counting and flow cytometric LIVE/DEAD analysis were used as reference assays. The results obtained with these methods appeared to be surprisingly coincident with fluoro-luminometric analysis. However, the results obtained by plate counting showed slight difference compared to that of bioluminescence because plates were not read until the overnight incubation. Results were also affected by the antimicrobial agents used (ethanol in **I&II** or various antibiotics in **III**) and by the time of exposure (from 4 to 10 hours) before plating samples. Specifically, ethanol directly affected cell membrane but it could not accumulate into cells similarly as conventional antibiotics. Therefore, it was obvious that plate counting revealed somewhat higher

viability than luciferase assay (Figure 6 in **I** and Table 3 in **II**). It is obvious that some cells, which were not dead but whose metabolic activity was significantly decreased due to ethanol treatment, recovered during long incubation on plates and hereby CFUs were increased. Contrary to ethanol, conventional antibiotics mostly accumulated into cells and their toxic effects were mediated to the cells throughout the following incubation on plates, which likely resulted somewhat lower viability than assessed by bioluminescence (Figures 1-3 in **III**). Flow cytometric viability measurements revealed the current vitality stage of bacterial cells (Figure 4 in **I** and Figure 2 in **II**). The proportion of viable cells were according to flow cytometric analysis somewhat higher in the presence of various ethanol concentrations compared to that of measured by luciferase assay (**I**). This probably resulted from inaccuracy in setting gates for the bacterial populations according to the current vitality stage. Overall, when comparing the results obtained with different methods, it should be remembered that it might be almost impossible to provide completely comparable circumstances. This greatly impedes the direct comparison.

GFP fluorescence was used here to reveal the cell growth and killing. Fluorescence was measured using fluoro-luminometric microplate assay, and the results were compared to those of optical density measurements (**I&III**) and flow cytometric analysis (**I**). All methods produced similar results despite the fact that the assays were not done in the same culture batches. Note that the increase in fluorescence was significantly higher than increase in optical density. This was due to increase both in cell number and in the number of GFP molecules per cell, and can readily be corrected as discussed in previous sections. On the other hand, it was not possible to detect possible cell lysis using GFP fluorescence. However, using plate reader capable to measure optical density in parallel with fluorescence measurements this limitation was compensated as seen in Figure 1 (**III**), which shows the effects of some bacteriolytic agents on *E.coli*. Flow cytometric cell number analysis was used to assess the real number of bacteria in a sample. The flow cytometric data was found to be consistent with results from optical density measurements as well as with fluorescence analysis of GFP (Table 2 and Figure 5 in **I**) supporting the assumption that the growth of bacterial cells can be followed using GFP fluorescence.

5.3 Effects of various antimicrobial agents on bacteria evaluated by dual reporter (GFP-luciferase) system, cultural methods and flow cytometrically

5.3.1 Effects of ethanol (I&II)

The effects of alcohols such as ethanol on the physiology of bacteria have been far established (Ingram & Buttke, 1984). Ethanol affects several physical and chemical properties of *E.coli*, yet the most pronounced effect is the increase in permeability of the plasma membrane (Dombek & Ingram, 1984; Ingram, 1990). In this study the effect of ethanol on viability and killing of bacteria was found to be bacteriolytic as a consequence of decreased membrane rigidity (**I&II**). Lysis was more prevalent over longer periods of ethanol incubation, and most of the remaining cells in the suspension at higher ethanol concentrations were classified as dead or dormant according to

LIVE/DEAD staining (Figure 4 & Table 3 in **I** and Figure 2 in **II**). Accordingly, the mean fluorescence intensity of GFP-containing cells detected by FCM decreased during ethanol incubation, indicating that ethanol strongly increased the permeability of plasma membrane and caused leaking of GFP into the surrounding medium (Figure 3 & Table 2 in **I** and Table 1 in **II**). Moreover, at the beginning of incubation, optical density of cell culture containing ethanol was notably lower as compared to control culture (Figure 5 in **I**), which probably resulted from cell lysis. Lysis was also suggested by the flow cytometric analysis, which showed that the relative number of bacterial cells was significantly reduced in the presence of high ethanol concentrations (Table 2 in **I** and Table 2 in **II**). These findings were consistent with the analyses of metabolic activity measured as bioluminescence and cell number measured as colony forming units, respectively (Figure 6 in **I** and Table 3 in **II**). However, low ethanol concentrations were shown to have a reversed effect on bacteria, since the growth and metabolic activity were increased at the beginning of incubation (Figure 6 & Table 2 in **I** and Table 3 in **II**). This suggests that low ethanol concentrations can actually increase bacterial growth by promoting cell metabolism. It is noteworthy that GFP fluorescence was consistently the higher the more ethanol was present (Figure 7 in **I**). The increase in GFP fluorescence, on the other hand, was constant during the first 75 minutes of incubation, which obviously indicates the maturation of the preformed apoprotein into its fully fluorescent form irrespective of bacterial growth (Cubitt *et al.*, 1995).

5.3.2 Effects of some conventional antibiotics (III&IV)

Kinetic measurements of the bacteriostatic, bactericidal and bacteriolytic effects of various antibiotics against *E. coli* were performed using bioluminescence, fluorescence and optical density based real-time assay. Additionally, plate counting was used as a control assay. The antibiotics tested in this study were ampicillin (**III&IV**), erythromycin (**III**), nalidixic acid (**III**), penicillin G (**IV**), polymyxin B (**III**), tetracycline (**III**) and trimethoprim (**III**). In order to determine the susceptibility of bacteria to antimicrobial agents, a variety of methods can be employed, and in general each method is most suitable for a given application. Microbroth dilution method is the conventional technique for measuring the susceptibility of bacterial cells to various antimicrobial agents (Amsterdam, 1996), whilst the checkerboard method (Eliopoulos & Moellering, 1996) is most frequently used for simultaneous detection of the synergistic/antagonistic action of two drugs against a single micro-organism. However, these techniques do not display results in real-time. Using kinetic procedures, such as fluoro-luminometric and optical density measurements, the effects of antibiotics on target bacteria are easily measured at any desired time-point of the incubation period. Moreover, the time interval from exposure to visible effect can readily be determined (Figures 1-3 in **III** and Figure 1 in **IV)** and antimicrobial efficacies for tested antibiotics are easily evaluated with kinetic methods (Table 1 and Figure 4 in **III**). All tested antibiotics, except penicillin G, decreased viability of *E. coli* cells. The effect of some antibiotics was found to be clearly bactericidal, whilst some only stopped the growth of bacteria, thereby being bacteriostatic drugs. Specifically, ampicillin and polymyxin B had a distinct effect on the integrity of the cell wall or cell membrane, hereby leading to cell lysis, which evidenced bacteriolytic character of these drugs

(Figure 1 in **III**). Nalidixic acid affects DNA synthesis, whilst tetracycline inhibits protein synthesis. Consequently, the effects of these drugs were shown to be bactericidal in this study (Figure 2 in **III**). Erythromycin inhibits also protein synthesis, whilst trimethoprim inhibits bacterial dihydrofolate reductase. Yet, the effects of these drugs were found to be only bacteriostatic (Figure 3 in **III**). Note that *E. coli* was found to be resistant to Penicillin G (Figure 1 in **IV**), whilst penicillins (such as ampicillin) with slightly modified molecular structure compared to penicillin G displayed a clearly bacteriolytic effect (Figure 1 in **III** and **IV**). The time interval from exposure to visible effects varied between the antibiotics, mainly due to their different mechanisms of action. The observed time-course here was between 2 and 6 h for erythromycin, nalidixic acid, tetracycline and trimethoprim (Figures 2 and 3 in **III**), and between 1 and 2 h for ampicillin (Figure 1 in **III** and **IV**). The influence of polymyxin B on target bacteria was very rapid, the effects being observed within 20 min of exposure (Figure 1 in **III**). Yet, the effect of any antibiotic on bacteria is dependent on drug concentration and incubation time as already mentioned. This was readily seen as low drug concentrations caused only a bacteriostatic effect, whilst a bactericidal effect was evident at high drug concentrations (Figures 1-2 in **III** and Figure 1 in **IV**).

5.3.3 Effects of non-antibiotic drugs such as promethazine (IV, unpublished)

It has been long evidenced that tricyclic neuroleptics, especially phenothiazines such as promethazine, employed for treatment of psychosis exhibit direct antimicrobial activity against various micro-organisms as well as enhance the activity of conventional antibiotics (Kristiansen, 1992). In this study the capability of promethazine to increase the activity of some antimicrobial compounds or even to render bacteria susceptible to antimicrobial agents, to which resistance was evident, was examined (**IV**, unpublished). *E. coli* strain used in this study was found to be susceptible to promethazine at concentrations ≥64 μg/ml as observed from fluorescence and bioluminescence signals (Figure 1 in **IV**). Note that ampicillin notably decreased viability of bacteria, whilst penicillin G had no effect on bacteria as discussed in previous section. However, promethazine in combination with penicillin G produced a significant synergistic activity against *E. coli* cells (Figures 2-3 and Table 1 in **IV**), yet the efficacy of ampicillin, interestingly, was not altered in the presence of promethazine (Figure 2 in **IV**). Specifically, the combination of promethazine and penicillin G suppressed the growth and the viability of bacteria, thereby converting penicillin G resistant cells susceptible to this antibiotic. It is obvious that the mechanism by which promethazine affects bacteria, allows penicillin G to transmit its antimicrobial effects against *E. coli* but at the same time the mechanism of action of ampicillin is not affected. Accordingly, the finding that penicillin G, in opposite to ampicillin, does not pass the outer membrane of most gram-negative bacteria such as *E. coli* (Nikaido, 1994) as well as the fact that many cationic compounds such as phenothiazines possess a direct action on the membrane permeability in gram-negative bacteria (Kristiansen, 1992) suggest that promethazine may directly facilitate the penetration of penicillin G into the cells. On the other hand, the expression of efflux pumps occurs in many bacteria and is associated with reduced drug accumulation that causes increased resistance to the antibiotics in bacteria. Promethazine has been shown

to reverse the resistance of many gram-negative bacteria including *E. coli* by inhibiting these efflux pumps (Molnar *et al.*, 1997). Therefore, the synergy between promethazine and penicillin G may also result from the ability of promethazine to inhibit efflux pump systems. Chemical interaction between antibiotics and promethazine, that is the formation a complex with increased antimicrobial activity compared with a single drug, was not observed here (Figure 4 in **IV**). This suggests that synergy was not caused by formation of any complex. On the other hand, promethazine may also inhibit the action of some antimicrobial agents. This effect was readily seen with serum complement assays in which the bacteriolytic activity of human complement system against *E. coli* decreased notably in the presence of promethazine (unpublished). This finding will be discussed in more details in the next section.

5.3.4 Effects of cellular defense mechanisms such as complement system (unpublished)

The effect of complement system against *E. coli* cells was measured here as bioluminescence that was produced in reactions catalyzed by bacterial luciferase. The human complement system, a key component of the innate immune system, plays an important role both in killing and neutralization of micro-organisms. It refers to a series of proteins, which circulate in an inactive form in the blood and other body fluids, but in response to the recognition of micro-organism, they become activated which initiates specific complement pathway(s) for destroying the foreing factor. The complement system consists of three different activation pathways, namely the classical pathway, the lectin pathway and the alternatively pathway (Kindt *et al.*, 2006). In this study the kinetics of total complement activity against target bacteria was measured, and as expected, it was found that the viability of *E. coli* cells decreased as a function of increasing serum concentration (Figure 7). However, the addition of promethazine into reactions strongly inhibited the action of complement system (Figure 8). This was consistent with the earlier observations that cationic compounds inhibit the complement action (Taylor, 1983). The inhibition caused by promethazine probably indicates that the binding of drug into the cell membrane simply hinders either the attachment of initial complement components to the cell membrane or the formation of terminal membrane attack complex (MAC), the structure finally responsible for cell death and lysis. Note that the strength of inhibition was strongly dependent on the amount both of serum and promethazine. Specifically, the inhibition effect was directly proportional to the amount of promethazine present. However, the high promethazine concentrations not only inhibited complement activity but at the same time killed target bacteria. Thus, viability of bacteria is actually decreased at high promethazine concentrations, whilst the activity of complement system is mostly inhibited. Also the effect of penicillin G in the presence of complement system was studied. It was found that an increasing penicillin G concentration, up to ca. 150 μg/ml, decreased cell viability at a constant (1:80) serum dilution (Figure 9). On the other hand, when the amount of active complement system was increased, penicillin G showed no effect on the viability of bacteria (data not shown). Taken together, the activation of complement system occurs at cell membrane. The activation leads to the formation of MAC, thereby increasing the permeability of cell membrane. This effect

can be altered with promethazine and penicillin G as shown. Therefore, it is probable that the direct membrane effect is the actual mechanism how promethazine renders *E. coli* susceptible to penicillin G. This assumption means that the ability of promethazine to inhibit the function of efflux pumps is probably not the case here.

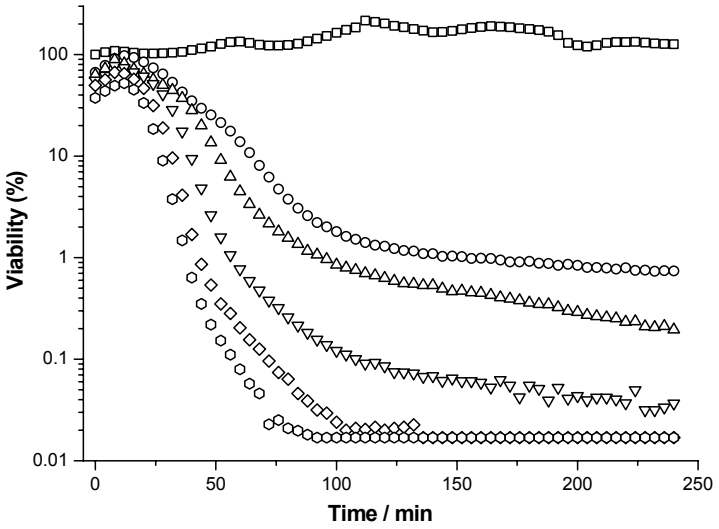

Figure 7. The percentage viability of *E. coli* (pEGFPluxABCDEamp) measured as a function of time in various serum dilutions: open square (0), open circle (1:80), open triangle (1:40), open down triangle (1:20), open diamond (1:10) and open pentagon (1:5). Viability (%) was calculated from measured bioluminescence signals. Reference value with no added serum at the beginning of incubation was set as 100.

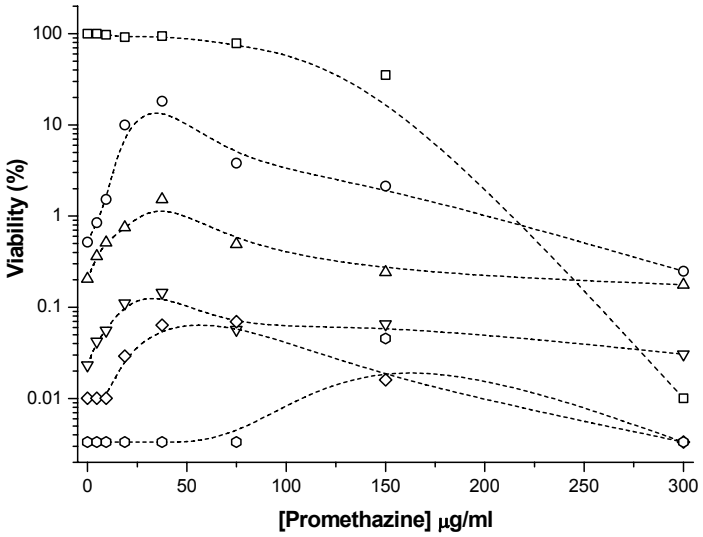

Figure 8. The percentage viability of *E. coli* (pEGFPluxABCDEamp) measured as a function of increasing promethazine concentration in various serum dilutions: symbols refer to the same serum dilutions as shown in Figure 7. Viability (%) was calculated from measured bioluminescence signals. Reference value with no added serum and promethazine was set as 100.

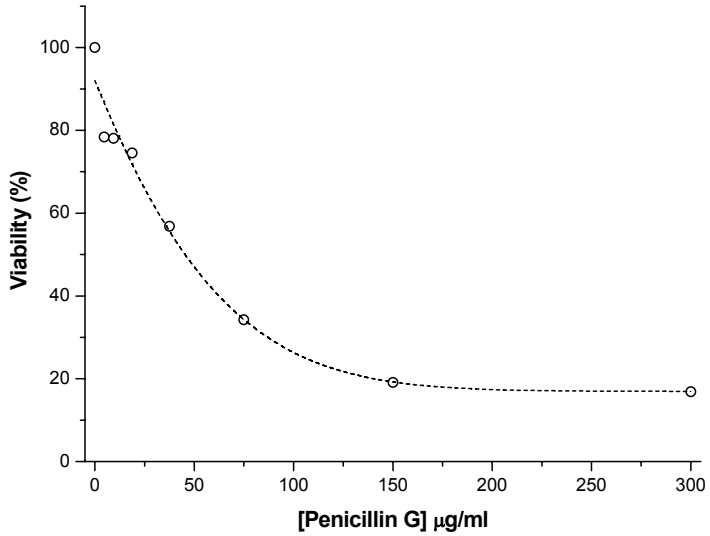

Figure 9. The percentage viability of *E. coli* (pEGFPluxABCDEamp) measured as a function of increasing penicillin G concentration at a constant serum dilution of 1:80. Viability (%) was calculated from measured bioluminescence signals. Reference value with no added penicillin G was set as 100.

5.4 Suitability of GFP-luciferase based multiparameter assay for kinetic screening applications

The action of antimicrobial agents on bacteria may emerge within from minutes to some hours, and the information on this action cannot be achieved by non-kinetic methods. Kinetic measurements, however, enable the real-time detection, thereby greatly improving the accuracy of various viability assays. Note that most conventional methods are simple end-point assays, which mostly give information about one measurable parameter at a time. Accordingly, the actual viability of target bacteria is more reliably measured using multiparameter assays on a real-time basis. In the present work, the effects of various antimicrobial agents on viability and killing of *E. coli* were examined in real-time using GFP-luciferase assay (**I**, **III&IV**, unpublished). It was found that the effect of ethanol on bacteria occurred instantly (Figures 5 and 6 in **I**). The effects of polymyxin B and complement appeared within 20 minutes of exposure (Figure 1 in **III**, figure 7), whilst the time interval from exposure to visible effects varied from one hour to some hours for other antimicrobial agents tested (**III&IV**, unpublished). Interestingly, bactericidal effects of some drugs appeared during the first hours of incubation, yet the effects of these drugs were only bacteriostatic henceforth as seen in Figure 3 (**IV**). Overall, kinetic measurements allow rapid and accurate measurements that reveal bacterial viability on a real-time basis. Moreover, assays are suitable for monitoring the time interval from exposure to visible effect for any antimicrobial agent to be tested. Also the antimicrobial efficacy (such as MIC) is easily evaluated. In addition, multiparameter assays reveal more diverse information about bacterial viability compared conventional single-technology assays. On the other hand, measurements utilizing GFP and luciferase are limited by the fact that target cells for

the antimicrobial agents to be evaluated need to be transformed with genes responsible for fluorescence and bioluminescence. Despite this limitation, the fluoro-luminometric kinetic assay has substantial advantages over traditional viability and susceptibility assays such as plate counting and microdilution techniques. The assay system significantly increases the speed of the assessment of the bacteriostatic and bactericidal effects of various antimicrobial agents. Furthermore, this reliable and fully automated measurement with high sample capacity offers new possibilities for real-time detection, which makes the assay suitable for diverse high throughput screening (HTS) applications.

6. CONCLUSIONS AND FUTURE PROSPECTS

The present study describes alternative methods to measure whether a microbial cell is alive or not. This issue is one of the most basic questions in the field of microbiology. However, it is not always a question that can easily be answered, and therefore new methods to reveal viability of microbial cells are developed. This thesis provides new insights into the assessment of bacterial viability and killing that may help to find answers to this key question. The methods developed in this thesis provide an efficient way to control and follow the growth of microbial cells as well as to determine the effects of various antimicrobial agents on bacteria. Bacterial (lux) and beetle (luc) luciferases as well as green fluorescent protein (GFP) were utilized as measurable reporters in assay systems developed in this study. These proteins proved to be good markers of bacterial growth and viability, enabling the subsequent analysis of target microbial populations. The developed flow cytometric assay and fluoro-luminometric microplate assay revealed several significant improvements in the assessment of bacterial viability and killing. Specifically, data reliability was notably improved and the overall assay procedures were accelerated. A kinetic approach allowed the monitoring of time interval from exposure to visible effect in real-time. Furthermore, a multiparametric assay protocol increased the accuracy of the results compared to those of simple one-technology measurements.

The most important findings of present study can be summarized as follows: indicator strains of bacteria expressing luciferase and green fluorescent protein were produced by genetic manipulation to reveal the viability of the target population and, by extension, the effectiveness of antimicrobial agents or other methods of killing the bacteria. Flow cytometric method and fluoro-luminometric microplate assay system were developed in this study, and luminescent bacteria were used to demonstrate the ease of use and accuracy of these methods. The methods were compared to existing methods to produce comparable data between different methods used in the assessment of bacterial viability and killing. Moreover, methods developed were used to measure the possible synergistic interaction between various antibiotics and non-antibiotic drugs which may lead to clinical applications in the future. The methods were also applied to the testing whether a wide range of different antimicrobial agents possess bacteriostatic, bactericidal or bacteriolytic activities. The measurements were possible in real-time and thus provided information that could not be obtained by conventional end-point techniques. However, the fact that target bacteria used in the assay systems need to be transformed with genes responsible for the production of bioluminescence and fluorescence greatly diminished the suitability of the methods in the field of clinical microbiology. On the other hand, the main application of the methods is to monitor the effects of various antimicrobial agents and thus that the use of a few transformed bacterial strains as model organisms is appropriate. Naturally, the expression of the luciferase-GFP marker system in any bacteria of interest would largely expand the importance of these methods by providing information that may be helpful in informing clinical decisions regarding choice of antimicrobial agent. However, notwithstanding this possible limitation of the techniques, the ability to

measure the effects of various bacteriostatic and bactericidal agents against given micro-organisms with fast, simple and reliable manner gave substantial advantages over more traditional detection methods. Particularly, the kinetic assay system, with the ability for simultaneous detection of fluorescence and bioluminescence, was found to be suitable for diverse HTS applications. Also the interaction between different antimicrobial agents and the effects of these combinations on bacteria were readily assessed.

To date, there are several different methods that can be exploited in the field of diverse microbiological analysis. In the present study alternative methods were developed. New and powerful methods are fundamental to further research on the role of different factors in controlling viability and death as well as to ascertaining the effectiviness of various antimicrobial agents or their combinations for killing micro-organisms. The key advance of methods developed here centers on the availability of real-time data concerning bacterial viability. Real-time data provides more accurate estimation of the bacterial viability, and may help to explain the difference between findings of different researchers as a result of varying experimental approaches. The real-time approach is extremely elegant and provides a valuable tool to future researchers wishing to elucidate mechanisms of cell death in response to different antimicrobials or environmental stress factors. In near future the effects of combinations between conventional antibiotics and non-antibiotics drugs in the presence of natural cell defence systems such as complement and antibodies should be studied in more details. This subject is of particular importance since there are ever increasing interest to understand how the efficacy of antimicrobial therapy could be improved and, at the same time, to reverse the resistance caused by inappropriate use of antibiotics. Also bacterial luciferase system containing whole operon should be preferably exploited over beetle luciferase system as it overcomes the need of substrate addition, thereby enabling more simple measurements. Finally, some other organisms, in addition to *Escherichia coli*, should be made bioluminescent and fluorescent in order to increase the suitability of developed methods for revealing viability of different gram-negative and gram-positive bacteria.

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