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Review

In vitro and *in vivo* model systems to study microbial biofilm formation

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ABSTRACT

Biofilm formation is often considered the underlying reason why treatment with an antimicrobial agent fails and as an estimated 65–80% of all human infections is thought to be biofilm-related, this presents a serious challenge. Biofilm model systems are essential to gain a better understanding of the mechanisms involved in biofilm formation and resistance. In this review a comprehensive overview of various *in vitro* and *in vivo* systems is presented, and their advantages and disadvantages are discussed.

Contents

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1. Introduction

Since 1943, when marine microbiologist Claude ZoBell described the so-called "bottle effect" (referring to the phenomenon that the number of free-living microorganisms in fresh sea water gradually declines when the water is kept in a glass bottle, while the number of attached microorganisms increases) (ZoBell, 1943) we have been aware of the fact that microorganisms are capable of living their life attached to a surface. However, it then took more than 30 years (and the paradigm-changing work of Bill Costerton and colleagues) to accept that for microorganisms (both bacteria and fungi) the biofilm mode of life is the rule rather than the exception (Costerton et al., 1978, 1999). Biofilms are defined as consortia of microorganisms that are attached to a biotic or abiotic surface. Biofilm formation is a multi-stage process in which microbial cells adhere to the surface (initial reversible attachment), while the subsequent production of an extracellular matrix (containing polysaccharides, proteins and DNA) results in a firmer attachment (Sauer, 2003; Stoodley et al., 2002). Cells embedded in this matrix communicate with each other and show a coordinated group behaviour mediated by a process called quorum sensing (QS) (Zhang and Dong, 2004). Sessile (biofilm-associated) cells are phenotypically and physiologically different from non-adhered (planktonic) cells and one of the typical properties of sessile cells is their increased resistance to antimicrobial agents (Donlan and Costerton, 2002; Mah and O'Toole, 2001; Stewart and Costerton, 2001). Biofilm formation is often considered the underlying reason why treatment with an antimicrobial agent fails and as an estimated 65–80% of all infections is thought to be biofilm-related, this presents a serious challenge (Costerton et al., 1999; Hall-Stoodley et al., 2004; Parsek and Singh, 2003). Biofilm formation can also have detrimental effects in industrial systems. Biofouling is especially problematic in systems in which materials come into contact with water, including heat exchangers, ship hulls and (marine) fish cages (Braithwaite and McEvoy, 2005; Coetser and Cloete, 2005; Flemming, 2002). Of particular relevance to human health is biofilm formation in drinking water reservoirs and distribution systems as these biofilms hinder the efficient operation of these systems. In addition, they may also pose a health risk to the users, providing a habitat for pathogenic miroorganisms like *Legionella pneumophila* and *Escherichia coli* (Flemming, 2002; Juhna et al., 2007). On the other hand, there are many (potential) applications of microbial biofilms, in processes as diverse as bioremediation (Singh et al., 2006), production of fine chemicals (Li et al., 2006), fermentation (Kunduru and Pometto, 1996), biofiltration (Cohen, 2001), wastewater treatment (Nicolella et al., 2000), biofuel production (Wang and Chen, 2009) and generation of electricity in microbial fuel cells (Rabaey et al., 2007).

In order to increase our knowledge concerning biofilm biology, biofilm model systems to be used for the study of the often complex communities under controlled conditions are indispensable (Doyle, 1999; Hamilton et al., 2003; Wolfaardt et al., 2007). In this review we present an overview of *in vitro* and *in vivo* model systems and discuss their advantages and disadvantages. The focus of this review is on tools to study medically-relevant biofilms, but many of the models can of course also be used to mimick biofilm formation in other settings.

2. *In vitro* biofilm model systems

2.1. Microtiter plate-based model systems

Microtiter plate (MTP)-based systems are among the most-frequently-used biofilm model systems (see for example Cerca et al., 2005; Christensen et al., 1985; Coenye et al., 2007; De Prijck et al., 2007; Gabrielson et al., 2002; Krom et al., 2007; Miyake et al. 1992; Peeters et al., 2008a,b,c; Pettit et al., 2005; Pitts et al., 2003; Ramage et al., 2001; Shakeri et al., 2007; Stepanovic et al., 2000; Toté et al., 2008; Silva et al., 2010; Uppuluri et al., 2009b; Walker and Sedlacek, 2007). In these systems, biofilms are either grown on the bottom and the walls of the microtiter plate (most commonly a 96-well plate) or they are grown on the surface of a coupon placed in the wells of the microtiter plate (most commonly a 6, 12 or 24-well plate). MTP-based systems are closed (batch reactor-like) systems (Fig. 1), in which there is no flow into or out of the reactor during the experiment (Heersink and Goeres, 2003). As a consequence, the environment in the well of a MTP will change during the experiment (e.g. nutrients become depleted, signalling molecules accumulate, etc), unless the fluid is regularly replaced.

The multitude of advantages offered by these straightforward and (generally) user-friendly systems explains their widespread use. Firstly, MTP-based assays are fairly cheap as only small volumes of reagents are required, they provide the opportunity to perform a large number of tests simultaneously and this system is ideal for screening purposes (Niu and Gilbert, 2004). MTP-based model systems have been used to distinguish biofilm-deficient mutants from biofilm-forming wild type strains (Heilmann et al, 1996; O'Toole and Kolter, 1998) and to screen for the antimicrobial and anti-biofilm effects of various antibiotics, disinfectants, chemicals (including quorum sensing inhibitors) and plant extracts (Ali et al., 2006; Amorena et al., 1999; Pitts et al., 2003; Quave et al., 2008; Ramage et al, 2001; Shakeri et al., 2007; Peeters et al., 2008b, 2008c; Brackman et al., 2009; Vandenbosch et al., 2010). Secondly, a profound examination of the effects of modification, coating or impregnation of materials on various stages of biofilm development can easily be performed in microtiter plate model systems (Chandra et al., 2001; De Prijck et al., 2007, 2010b; Imamura et al. 2008; Mowat et al., 2007). Thirdly, this system also allows researchers to easily vary multiple parameters including the composition of growth media, incubation temperatures, humidity, presence or absence of shear stress and O_2 and CO_2 concentrations (Krom et al., 2007; Stepanovic et al., 2003).

Ceri et al. (1999) developed a variation of the traditional MTP model system. The "Calgary Biofilm Device" was introduced as a rapid technology to determine the antibiotic susceptibility of biofilms and it has been commercialized as the MBEC Assay ("Minimal biofilm eradication concentration" assay) by Innovotech. In this system, pegs are attached to the top lid of a microtiter plate and by closing the microtiter plate, these pegs will be immersed in the media present in the wells of the 96-well MTP. Following biofilm growth, the lid can be transferred to a second plate, which contains various (antibiotic) solutions. After the treatment, the top lid can either be transferred to a new microtiter plate containing media to allow regrowth, or the pegs can be clipped from the top lid and the biofilm biomass or the number of sessile cells present in the biofilm can be quantified using traditional viable plate counting or microscopic techniques. This rapid and miniaturized biofilm assay is mostly applied to evaluate the effects of various antimicrobial agents on biofilm eradication (see for example Aaron et al., 2002; Bardouniotis et al., 2001; Ceri et al., 1999; De Kievit et al., 2001; Finelli et al., 2003; Hill et al., 2005; Arias-Moliz et al., 2010; Melchior et al., 2007; Harrison et al., 2005), but it has also been used to assess the influence of quorum sensing on biofilm formation (Tomlin et al., 2005).

Another MTP-based commercially available method is the Biofilm Ring Test (BioFilm Control SAS) (Chavant et al., 2007). With this technology, the immobilisation of inert paramagnetic beads included in the culture medium during the formation of the biofilm is measured. A magnet is used to collect the non-immobilised beads into a single spot which is then quantified through specialised image algorithms. This technology has been used to study the kinetics of biofilm formation of *Listeria monocytogenes*, *E. coli*, *Staphylococcus carnosus* and *Staphylococcus xylosus* (Chavant et al., 2007), to determine the influence of matrix components on *Leuconostoc mesenteroides* biofilm formation (Badel et al., 2008), to confirm that AI-2 based quorum sensing affects biofilm formation in *Streptococcus mutans* (Huang et al., 2009), to evaluate the effect of co-administration of antibiotics on *Pseudomonas aeruginosa* biofilms (Tré-Hardy et al., 2009), to compare biofilm formation between

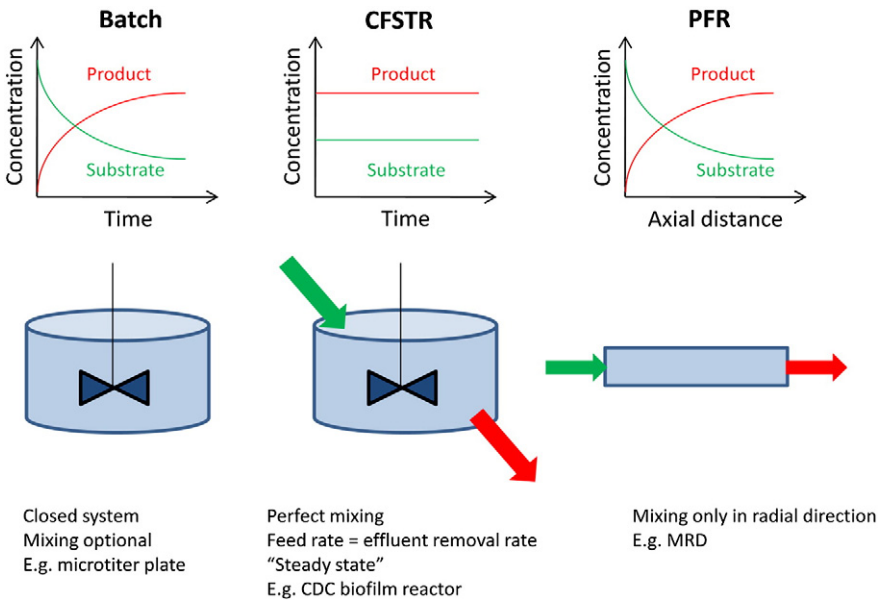

Fig. 1. Important conceptual differences between batch systems, "continuous flow stirred tank reactor" (CFSTR) systems and "plug flow reactor" (PFR) systems.

Campylobacter coli and *Campylobacter jejuni* (Sulaeman et al., 2010) and to study early phases of *P. aeruginosa* biofilm formation (Nagant et al., 2010).

2.2. Flow displacement biofilm model systems

In contrast to MTP-based systems, flow displacement systems are "open" systems in which growth medium with nutrients is (semi-) continuously added and waste-products are (semi-)continuously removed (Heersink and Goeres, 2003; Busscher and van der Mei, 2006). These systems can be subdivided into two broad groups, either following the "continuous flow stirred tank reactor" (CFSTR) approach or the "plug flow reactor" (PFR) approach (Heersink and Goeres, 2003) (Fig. 1). In CFSTR systems there is perfect mixing and the "feed rate" (i.e. the rate at which growth medium is added to the reactor) is identical to the effluent removal rate. As such a CFSTR system operates at steady state, i.e. without changes in concentration over time. When the dilution rate is higher than the doubling time of the microorganism(s) present in the reactor, planktonic cells are washed out of the reactor and only the sessile cells attached to a surface will remain and will be able to multiply. In PFR systems, the influent moves as a single "plug" in the direction of the flow (axial direction), with mixing (through diffusion) only occurring in the radial direction. While in CFSTR systems conditions are identical throughout the reactor, this is not the case for PFR systems, as environmental conditions change progressively through the reactor (Heersink and Goeres, 2003) (Fig. 1).

2.2.1. Modified Robbins device (MRD)

The modified Robbins device (MRD) was developed by Jim Robbins at the University of Calgary to allow the reproducible and simultaneous formation of biofilms exposed to a fluid flow (McCoy et al., 1981; Nickel et al., 1985; Jass et al., 1995). It is commercially available from several companies (including BioSurface Technologies and Tyler), but many research groups use homemade MRDs. A MRD can be constructed from plastic or stainless steel and contains a number of individual ports in a linear array along a channel of rectangular cross-section. Each port accepts a press-fit plug holding a disc on which a biofilm is formed (Fig. 2) (Honraet and Nelis, 2006; Krom et al., 2009). In a typical experiment, the MRD is filled with a suspension of microorganisms and is flipped over to improve the adhesion of the planktonic cells to the discs. Once the devices are filled with the inoculum suspensions, the tubing at the inlet and outlet side is clamped off and the remaining cell suspension in the tubing at the inlet side is flushed out through the bypass. After the adhesion phase, the devices are flipped back, the clamps are loosened and the pump is started to allow a continuous flow of the growth medium and biofilm development on the discs. Variations in this setup are possible, i.e. the MRD can be attached to the effluent of a chemostat (Jass et al., 1995) or following the initial adhesion biofilms are allowed to mature in the absence of flow and the fluid flow is only switched on once mature biofilms are formed (Krom et al., 2009). The MRD was found particularly useful to evaluate the effect of modified materials (i.e. surface-modified materials or materials impregnated with antimicrobial agents) under flow conditions (Nava-Ortiz et al., 2010; De Prijck et al., 2010a,b,c; Coenye et al., 2008; Ruiz et al., 2008; Honraet and Nelis, 2006; van de Belt et al., 2001) and has extensively been used as an "artificial throat" to study biofilm formation on laryngeal shunt prostheses (Leunisse et al., 1999; Schwandt et al., 2005; Oosterhof et al., 2003, 2006; Krom et al., 2009). In addition, the MRD has found application to assess the potential of antibiotic lock therapy for biofilm removal from colonised surfaces (Curtin et al., 2003; Raad et al., 2007).

2.2.2. Other PFR systems

PFR devices other than the MRD have been used in numerous studies on microbial biofilms. Many of these devices are custom-made and typically consist of a pump required to circulate the growth medium and/or cell suspension, a vessel with fresh growth medium connected by tubing to a chamber containing the material on which the biofilms are formed, and a second vessel for waste collection (e.g. Foster and Kolenbrander, 2004; Percival et al., 2005; Uppuluri et al. 2009a; Seidler et al., 2010).

A special type of plug flow reactor systems is the so-called "flow cell". These commercially available devices (e.g. Stovall, BioSurface Technologies) with glass chambers are particularly well-suited for real-time non-destructive microscopic analyses of biofilms. Flow cells were first used in the 1990s to study environmental biofilm communities capable of biodegrading various recalcitrant xenobiotics (Wolfaardt et al., 1994; Moller et al., 1997) and have since been used to study a wide range of microorganisms, including clinically relevant organisms like *Neisseria meningitidis* (Lappann et al., 2006), *L. pneumophila* (Mampel et al., 2006), *Staphylococcus epidermidis* (Qin et al., 2007) and *P. aeruginosa* (Haagensen et al., 2007) and to study oral biofilms (see for example Foster and Kolenbrander, 2004; Foster et al., 2004 and Palmer and Caldwell, 1995).

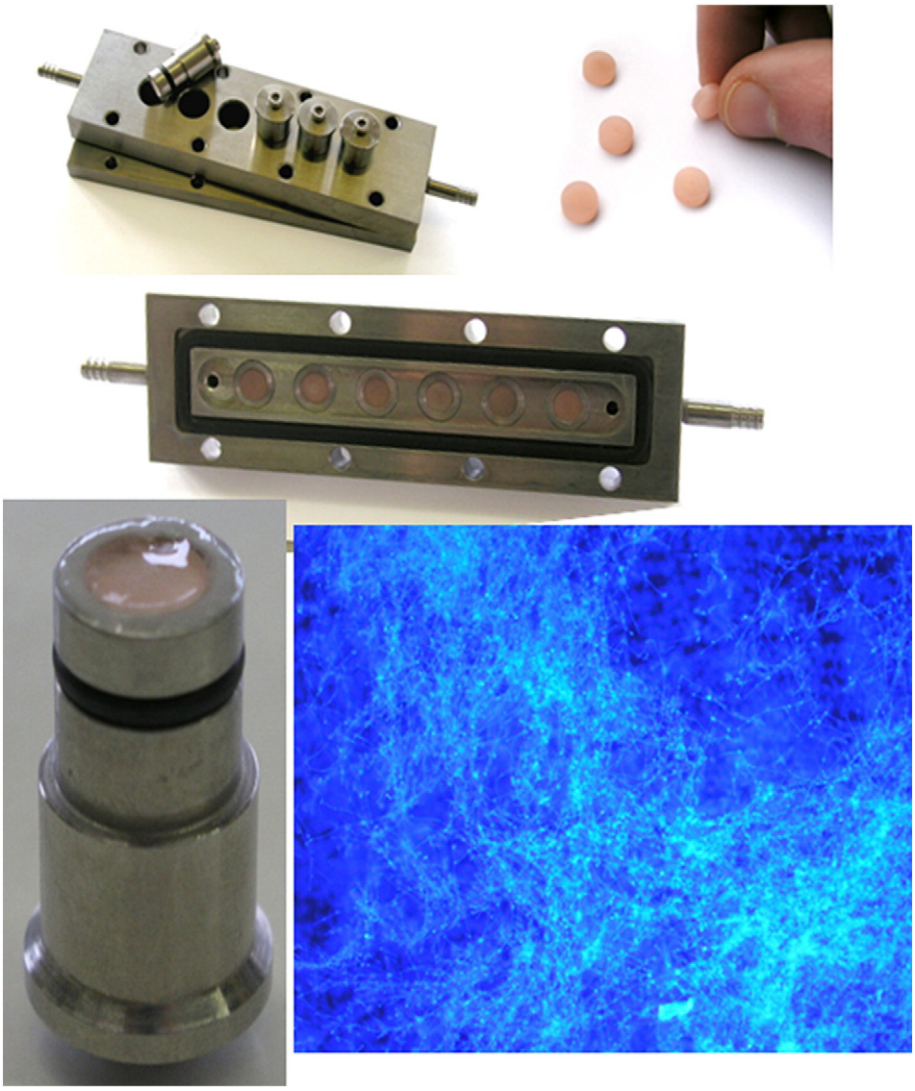

Fig. 2. Setup of the MRD. Top left: single MRD unit. Top right: poly methyl-methacrylate (PMMA) discs ready to be used in the MRD. Middle: PMMA disc loaded in MRD (view of luminal side). Bottom left: PMMA disc in plug, covered with *C. albicans* biofilm. Bottom right: image of a *C. albicans* biofilm obtained on PMMA in the MRD (stained with calcofluor white).

2.2.3. Centers for Disease Control (CDC) biofilm reactor

The CDC biofilm reactor (commercially available from BioSurface Technologies) consists of a glass vessel with a polyethylene top supporting eight removable polypropylene rods. Each polypropylene rod can hold three removable coupons on which biofilms can form (Donlan et al., 2004; Goeres et al., 2005) (Fig. 3) and is oriented in such a way that the coupon is perpendicular to the rotating baffle (Buckingham-Meyer et al., 2007). In this reactor, the magnetic stirrer in the center of the vessel provides a

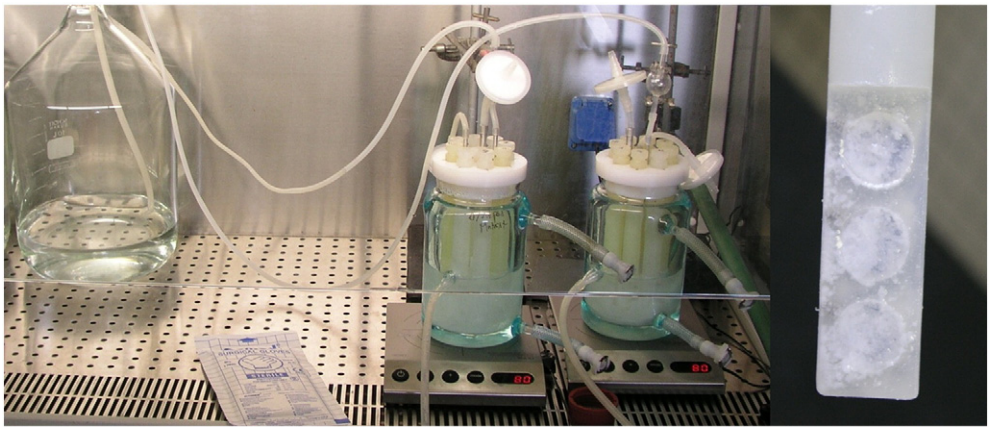

Fig. 3. Setup of the CDC biofilm reactor. Left: two reactor vessels coupled to a bottle with fresh medium. Right: poypropylene rod holding three medical grade silicone discs covered with a *C. albicans* biofilm.

continuous flow of nutrients (introduced in the reactor by means of a peristaltic pump) over the colonised surfaces (Goeres et al., 2005). Thorough statistical analysis has indicated that the CDC biofilm reactor is a reliable experimental tool to study biofilm formation by a wide range of organisms (Goeres et al., 2005; Honraet et al., 2005). The CDC biofilm reactor is recognised as a suitable tool to grow *P. aeruginosa* biofilms under high shear and continuous flow (American Society for Testing and Materials [ASTM] standard method E5262-07: Standard Test Method for Quantification of *Pseudomonas aeruginosa* Biofilm Grown with High Shear and Continuous Flow using CDC Biofilm Reactor). In the CDC biofilm reactor, 24 identical biofilms can be formed simultaneously and as the reactor setup allows for the easy removal of discs during the experiment, this model system is particularly well-suited to study biofilm formation over time (e.g. Honraet et al., 2005; Nailis et al., 2009). Similarly, this model system allows the formation of a large number of identical coupon-attached biofilms for subsequent testing of disinfection and cleaning procedures ("off-line testing") (Buckingham-Meyer et al., 2007; Hadi et al., 2010). The relatively large reactor volume (500–1000 ml) makes the CDC reactor less-suitable to assess the effect of antimicrobial agents in the reactor itself, as large amounts of reagents would be required to do so, although this model system was recently used to evaluate the activity of high dose daptomycin, vancomycin and moxifloxacin (alone or in combination with other antibiotics) against *S. aureus* biofilms (Parra-Ruiz et al., 2010). Other applications include the testing of materials coated with antimicrobial agents (Agostinho et al., 2009) and the simulation of encrustation of urinary catheters (Gilmore et al., 2010).

2.2.4. Other flow displacement systems

In drip flow reactors, biofilms are grown on angled slides continuously irrigated with small volumes of (inoculated) media (Buckingham-Meyer et al., 2003; Goeres et al., 2009), thereby providing a low shear environment with dispersive mixing (Stewart et al., 2001; Buckingham-Meyer et al., 2007). Drip flow reactor-grown biofilms have been used to study spatial heterogeneity in bacterial biofilms (Huang et al., 1998; Xu et al., 1998; Hu et al., 2005), to evaluate the effect of powered brushing on the removal of biofilm plaque (Adams et al., 2002), to assess reduction of *S. epidermidis* biofilm formation by bacteriophages (Curtin and Donlan, 2006) and to evaluate the efficacy of various disinfectants (Stewart et al., 2001; Buckingham-Meyer et al., 2007). The drip flow reactor is recognised as a suitable tool to grow *P. aeruginosa* biofilms and is included in ASTM standard method E2647-08 (Standard Test Method for Quantification of a *Pseudomonas aeruginosa* Biofilm Grown Using a Drip Flow Biofilm Reactor with Low Shear and Continuous Flow).

The rotating disc reactor consists of a teflon disc designed to hold six 12.7 mm diameter coupons. The bottom of the rotating disc contains a magnetic stirring bar, which allows rotation of the disc and consequently creates liquid surface shear across the coupons. The disc containing the coupons is placed in a reactor vessel and liquid growth medium (with or without antimicrobial agents) is circulated through the vessel while the disc is rotating (Buckingham-Meyer et al., 2003). Rotating disc reactors have been used to study various aspects (including resistance) of *Staphylococcus aureus* and *P. aeruginosa* biofilms (Cotter et al., 2009; Hentzer et al., 2001; Teitzel and Parsek, 2003), interactions in multispecies biofilms (Komlos et al., 2005) as well as to evaluate particular treatments for eradication of microbial biofilms (e.g. Lee et al., 2004). Just like the drip flow reactor and the CDC biofilm reactor, the rotating disc reactor is recognised as a suitable tool to grow *P. aeruginosa* biofilms and is included in an ASTM standard method (E2196-07 Standard Test Method for Quantification of a *Pseudomonas aeruginosa* Biofilm Grown with Shear and Continuous Flow Using a Rotating Disc Reactor).

A flow displacement model often used in (but not limited to) the study of oral biofilms (especially dental plaque) is the constant depth film fermenter (CDFF). In this system, the development of a biofilm on a surface is limited to a predetermined depth by mechanically removing excess biofilm, a situation mimicking the movement of the tongue over the teeth (Peters and Wimpenny, 1988; Pratten, 2007). The CDFF has been used to study the effect of antimicrobial agents added to the reactor or released from the substrate of the biofilm (Lamfon et al., 2004; Hope and Wilson, 2004; Leung et al., 2005) and to study the influence of surface characteristics (e.g. roughness) on biofilm formation (Morgan and Wilson, 2001).

The final type of flow displacement biofilm model system to be mentioned here is the annular biofilm reactor (Lawrence et al., 2000). This system consists of an inner rotating cylinder on which a number of slides are mounted. A motor drives the inner cylinder, providing liquid/surface shear. This type of model system has mostly been used in studies pertaining to disinfection of drinking water systems (see for example Butterfield et al., 2002; Declerck et al., 2009; Szabo et al., 2007; Williams et al., 2005).

2.3. Cell-culture-based model systems

Biofilms not only are formed on abiotic surfaces but also on biotic surfaces. Human cell lines that mimic the *in vivo* situation *in vitro* can be used for this purpose. Well-studied examples of mucosal biofilms include those formed by *Candida albicans* on oral and vaginal tissues (Dongari-Bagtzoglou, 2009; Dongari-Bagtzoglou et al., 2009). Models of oral and vaginal candidiasis, based on reconstituted human epithelia (RHE) have been established and are commercially available (Schaller et al., 2006). *C. albicans* inoculated on RHE forms a biofilm-like structure on top of the epithelial layer, indicating that these models can serve as *in vitro* biofilm models systems (Green et al., 2004). The RHE model is very suitable to study the interaction between sessile *C. albicans* cells and epithelial tissue (Green et al., 2004; Jayatilake et al., 2005; Malic et al., 2007; Nailis et al., 2010; Schaller et al. 2004; Zakikhany et al. 2007). Nevertheless, this model also has its limitations, as the commensal flora and normal humoral and cell-mediated immune responses are not included. Other *C. albicans* superficial tissue infection models have recently been reviewed by Jayatilake and Samaranayake (2010). *C. albicans* can also cause more serious systemic infections (Grubb et al., 2008) and to investigate the abilities of the different morphological forms of *C. albicans* to adhere to the endothelium under conditions of flow, an *in vitro* flow adhesion assay that mimics the conditions found within blood vessels was developed (Grubb et al., 2009). In this assay, immortalized human microvascular endothelial cells (HMEC-1 cell line) are coated on glass slides mounted in a parallel plate flow chamber; subsequently the flow chamber is perfused with a *C. albicans* suspension. Using this model it was found that yeast cells adhered to endothelial cells in significantly higher numbers than did pseudohyphal and hyphal forms, which is in contrast with observations obtained in previous assays under static conditions. These differences highlight the importance of selecting the most appropriate assay conditions. In addition, many *in vitro* cell-culture-based models have been described for the study of the interaction of human cells with bacterial biofilms. Examples are *P. aeruginosa* biofilms on airway epithelial cells (Woodworth et al., 2008), *Streptococcus gallolyticus* biofilms grown on EA.hy926 endothelial cells and primary human umbilical vein endothelial cells (Vollmer et al., 2010), biofilms of enterohemorrhagic *E. coli* on HeLa cells (Kim et al., 2010a, b) and *Stenotrophomonas maltophilia* biofilms formed on cystic fibrosis derived IB3-1 bronchial cells (Pompilio et al., 2010).

These models not only allow to monitor microbiological biofilm formation, but also to assess the damage inflicted upon the human cells by this process. For example, internalisation of microbial cells and damage to human cells can be determined by microscopy (e.g. Jayatilake et al., 2005; Zakikhany et al., 2007). Other assays (including those assessing metabolic activity with XTT or measuring the release of lactate dehydrogenase) also allow to obtain a picture of the inflicted tissue damage (e.g. Zakikhany et al., 2007; Pompilio et al., 2010; Jayatilake et al., 2005).

2.4. Microfluidic devices

The use of microfluidic devices to study biofilm formation and eradication is rapidly gaining interest. These devices offer the possibility of developing biofilms under physiologically relevant conditions, including physiological flow velocities and low fluid-to-cell volume ratios, while the small size of the chambers allows the microscopic analysis of the biofilm at single-cell resolution. In addition, the environmental conditions can be controlled very precisely (De La Fuente et al., 2007; Richter et al., 2007). Fabrication of microfluidic devices for biofilm formation often requires photolithography, a process in which a pattern is transferred from a mask onto a thin layer of photosensitive polymer and then onto the surface of a substrate (Weibel et al., 2007), although other approaches have also been reported (e.g. Richter et al., 2007). The size of the channels in these devices are model-dependent, but is typically in the range of 50–500 μm wide and 30–250 μm deep, while the length can vary from 5 to 40 mm. Flow rates are typically very low (0.1–50 μl/min), resulting in very low Reynolds numbers and highly laminar flows (De La Fuente et al., 2007; Lee et al. 2008; Eun and Weibel, 2009; Janakiraman et al., 2009). Recently a "well plate microfluidic" device was described that allows high-throughput screening of biofilms in a microfluidic device (Benoit et al., 2010; Conant et al., 2010; Ding et al., 2010). This system consists of microchannels (370 μm wide and 70 μm deep) integrated into a MTP. Pneumatic pressure pushes fresh medium through the microchannel, from an inlet well (containing fresh medium) to an outlet well (containing spent medium). This BioFlux system (commercially available through Fluxion Biosciences) allows the simultaneous analysis of 96 biofilms. Kim et al. (2010a,b) recently reported on the development of a microfluidic co-culture model, in which a microbial biofilm can be developed in the presence of an epithelial cell monolayer. With this model it was possible to develop an *E. coli* biofilm on HeLa cells under conditions mimicking gastrointestinal tract infections.

3. *In vivo* biofilm model systems

3.1. Caenorhabditis elegans model

Studies using the *C. elegans* model system usually focus on virulence as such (i.e. determining whether infection results in reduced survival of the worms) and/or on the effect of particular chemical compounds on this survival. However, a number of studies employing *C. elegans* have specifically dealt with microbial biofilm formation. The first indication that bacteria can form biofilms in *C. elegans* came from the study by Darby et al. (2002) with *Yersinia pestis*. In this study it was shown that *Y. pestis* biofilm formation in the mouth and on the head prevented *C. elegans* feeding. Furthermore, by screening a transposon-insertion mutant bank it was shown that *Y. pestis* genes involved in the synthesis of the polysaccharide matrix are required for this biofilm formation. Additional studies confirmed that the infection phenotype is the result of biofilm formation and identified genes involved in haemin storage and lipopolysaccharide biosynthesis as important for biofilm formation (Joshua et al., 2003). This situation has some analogy with that in the proventriculus of fleas infected with *Y. pestis*, where the presence of a bacterial biofilm results in blockage of the gut and increased transmission of the pathogen (Darby, 2008). Other microorganisms tested in the *C. elegans* model include staphylococci (*S. epidermidis* and *S. aureus*) (Begun et al., 2007) and *Xenorhabdus nematophila* (Drace and Darby, 2008). In both studies the importance of the extracellular matrix for biofilm formation on and/or in *C. elegans* was demonstrated, and it was suggested that this matrix may play an immunoprotective role during infection (Begun et al., 2007).

The *C. elegans* model system also allows to identify host genes required for bacterial adhesion. An interesting *C. elegans* gene identified this way is *srf-3*, encoding a nucleotide sugar transporter of the Golgi apparatus. The deletion of *srf-3* results in reduced levels of O- and N-linked glycoconjugates on the nematode's cell surface and consequently less bacteria will adhere to the surface of this mutant (Cipollo et al., 2004; Darby et al., 2007; Joshua et al., 2003; Höflich et al., 2004).

3.2. Vertebrate animal models

3.2.1. Central venous catheter models

Microbial biofilm formation on central venous catheters (CVC) can result in considerable morbidity and mortality (Donlan, 2008). Several animal models have been developed to study biofilm formation on CVC *in vivo*. *In vivo* CVC models not only allow to study the efficacy of various antimicrobial agents (including antimicrobial lock therapy) and/or fundamental aspects of microbial biofilm formation in a realistic setting, but also to investigate the dissemination of the microorganism to various organs.

A first animal model developed was a rat model designed to study biofilm formation by *S. aureus* and *S. epidermidis* (Ulphani and Rupp, 1999; Rupp et al., 1999b). In this model, Silastic lumen-within-lumen catheters are inserted in the external jugular vein of rats and advanced into the superior vena cava. Following insertion, the CVC are contaminated with bacteria. This model system has extensively been used to evaluate the effect of various antimicrobial agents against *S. aureus* biofilms (including linezolid, vancomycin, ciprofloxacin, the antimicrobial peptides citropin 1.1, disinctin and protegrin IB-367, the RNAIII-inhibiting peptide and the cathelicidin BMAP-28) (Cirrioni et al., 2006a,b,c; Giacometti et al., 2005, 2007; Ghiselli et al., 2007) and also to evaluate the effect of the semi-synthetic glycopeptide LY333328 against *Enterococcus faecium* biofilms (Rupp et al., 2001). This rat model was also used to study factors important for *in vivo* biofilm formation and experiments with the rat CVC model confirmed the importance of polysaccharide intercellular adhesin/hemagglutinin and the *ica* locus in *S. epidermidis* (Li et al., 2005; Rupp et al., 1999b). Alternative animal models to study bacterial adhesion to CVC include mouse models (*S. aureus*, *P. aeruginosa*) (Kadurugamuwa et al., 2003; Kokai-Kun et al., 2009; Lorenz et al., 2008) and a rabbit model (*S. aureus*) (Fernández-Hidalgo et al., 2010). *C. albicans* is also an important causative agent of CVC-related infections (Donlan, 2008) and several animal models were developed to study biofilm formation on CVC *in vivo*. The most-frequently-used models are the rabbit CVC model (Schinabeck et al., 2004) and the rat CVC model (Andes et al., 2004). These models have been used to study *in vivo* susceptibility and molecular response to fluconazole treatment (Andes et al., 2004), the effect of liposomal amphotericin B lock therapy (Schinabeck et al., 2004), and the effect of various antifungal compounds (including caspofungin, amphotericin B and chitosan) against *C. albicans* biofilms (Martinez et al., 2010; Mukherjee et al., 2009; Shuford et al., 2006). In addition, they have been used to increase our understanding of the molecular basis of *C. albicans* biofilm formation, and helped to increase our knowledge regarding the role of specific adhesins in biofilm formation (Li et al., 2007) as well as to obtain a better picture of changes in gene expression in *in vivo* biofilms over time (Nett et al., 2009). Recently, Lazzell et al. (2009) developed a mouse model for *C. albicans* CVC infection. Although the catheterisation in these smaller animals is more challenging, the use of mice also has considerable advantages, including the possibility to compare the data with data obtained in the widely used mouse model for haematogenously disseminated candidiasis (tail vein infections). This model has been used to demonstrate the efficacy of caspofungin both for the prevention and the eradication of *C. albicans* biofilms on CVC (Lazzell et al., 2009).

3.2.2. Subcutaneous foreign body infection models

Subcutaneous foreign body infection models have been developed in guinea pigs, hamsters, mice, ponies, rabbits and rats (Table 1, Fig. 4). In

Table 1
Selection of subcutaneous foreign body infection models.

Animal	Reference	Implantation of[a]	Micro-organism
Guinea pig	Zimmerli et al. (1982)	Tissue cage (teflon)	*S. aureus*
	Fluckiger et al. (2005)	Tissue cage (teflon)	*S. epidermidis*, *S. aureus* (WT, *ica*$^-$mutant)
Hamster	Chang and Merritt (1994)	Stainless steel, polymethylmethacrylate, titanium (both sterile and colonised)	*S. epidermidis*
	Nakamoto et al. (1995)	Steel wire	*S. epidermidis*
Mouse	Christensen et al. (1983)	Plastic catheter	*S. epidermidis*
	Patrick et al. (1992)	Teflon catheter	*S. epidermidis*
	Roehrborn et al. (1995)	Teflon tubes	*S. aureus*
	Rupp et al. (1999a,b)	Teflon catheter	*S. epidermidis* (WT, PIA/HA mutant)
	Kristian et al. (2003)	Tissue cage (teflon) (immunosuppression)	*S. aureus* (WT, *dltA*$^-$mutant)
	Kristian et al. (2004)	Tissue cage (teflon)	*S. aureus* (WT, *ica*$^-$mutant)
	Fluckiger et al. (2005)	Tissue cage (teflon)	*S. epidermidis*, *S. aureus* (WT, *ica*$^-$mutant)
	Nejadnik et al. (2008)	Colonised silicone discs	*S. aureus*
	Engelsman et al. (2009)	Colonised surgical meshes	*S. aureus*
Pony	Voermans et al. (2006)	Tissue cage	*S. aureus*
Rabbit	Rediske et al. (1999)	Infected polyethylene discs	*E. coli*
Rat	Yasuda et al. (1993)	Carboxymethyl cellulose pouch	*P. aeruginosa*
	Van Wijngaerden et al. (1999)	Infected polyurethane catheters	*S. epidermidis*, *S. aureus*
	Yoshikawa et al., (2004), Morikawa et al., (2005)	Carboxymethyl cellulose pouch	*S. aureus*
	Nair et al. (2008)	Tissue cage (teflon)	*A. radicidentis*
	Ricicová et al., (2010), Kucharikova et al. (2010)	Colonised polyurethane catheters (immunosuppression)	*C. albicans* (WT, various mutants)

[a] Inserted material is sterile prior to insertion unless otherwise mentioned; no immunosuppression was used unless otherwise mentioned.

these models, a foreign body is inserted in subcutaneous pockets and a biofilm is allowed to develop on the implanted material. Most studies have been carried out with *S. aureus* and/or *S. epidermidis*, but other microorganisms (including *E. coli*, *Actinomyces radicidentis*, *P. aeruginosa* and *C. albicans*) have also been included. While studies with relatively virulent organisms (including *S. aureus*) may not require immunosuppression, the inflammatory response associated with surgery may inhibit biofilm formation in less virulent organisms (e.g. *C. albicans*) and for these microorganisms the use of immunosuppressive drugs (e.g. dexamethasone) may be recommended (Ricicová et al., 2010). Materials can either be contaminated prior to implantation (Chang and Merritt 1994; Van Wijngaerden et al., 1999; Ricicová et al., 2010) or the animals can be infected post-implantation (Table 1). In the latter case, animals are injected with microorganisms several days, weeks or even months after implantation of the foreign body (Christensen et al., 1983; Fluckiger et al., 2005; Kristian et al., 2003, 2004; Nair et al., 2008; Patrick et al., 1992; Roehrborn et al., 1995; Rupp et al., 1999a,b; Voermans et al., 2006) and hence these models do not mimic perioperative infections (Ricicová et al., 2010; Van Wijngaerden et al., 1999). Subcutaneous foreign body infection model systems are particularly well-suited to study the effect of (modified) substrates on biofilm formation (Nakamoto et al., 1995; Nejadnik et al., 2008; Engelsman et al., 2009).

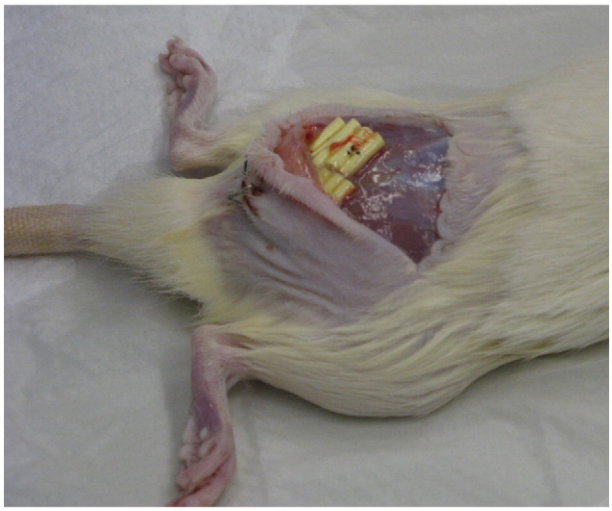

Fig. 4. Subcutaneous implantation of polyurethane catheter segments colonised with *C. albicans* according to the model described by Ricicová et al. (2010).

The tissue cage model is based on the subcutaneous implantation of so-called tissue cages. These perforated cylinders (often made out of teflon) contain glass beads or other materials to increase the available surface area for biofilm formation (Kristian et al., 2004). Following implantation, the animals are allowed to recover from the surgery and subsequently bacterial suspensions can be injected in the tissue cage. A major advantage of this model is that microbial cells can easily be recovered from the fluid inside the tissue cage without the need for explantation (Handke and Rupp, 2006). The tissue cage model has been used to study immune responses from the host (Zimmerli et al., 1982), *in vivo* gene expression (Goerke et al., 2001), the efficacy of particular antimicrobial agents (Murillo et al., 2006; Voermans et al., 2006), and to determine the role of particular genes in establishing biofilm-associated infections (Kristian et al., 2003, 2004; Fluckiger et al., 2005).

In the carboxymethylcellulose (CMC) pouch infection model, an air pouch is created on the back of an animal (typically a rat) by the subcutaneous injection of air, followed by the simultaneous injection of CMC and infecting organisms (Morikawa et al., 2005; Yasuda et al., 1993; Yoshikawa et al., 2004). This model has been used to study the effects of antibiotics (ofloxacin and clarithromycin) on *P. aeruginosa* biofilms (Yasuda et al., 1993) as well as the effect of arbekacin (alone or in combination with fosfomycin) on *S. aureus* biofilms (Morikawa et al., 2005; Yoshikawa et al., 2004).

3.2.3. Intraperitoneal foreign body infection models

In many *in vivo* studies, biofilms are formed on biomaterials inserted in the peritoneal cavity of rabbits or mice. In these model systems, materials can be precolonised or microorganisms can be injected intraperitoneally following implantation (Buret et al., 1991; Gallimore et al., 1991). An advantage of these model systems is that they allow to establish a chronic infection which can be followed for longer periods (up to 6 months) (Gallimore et al., 1991; Gagnon and Richards, 1993). Intraperitoneal foreign body infection models have been used to study the effect of antibiotics against bacterial biofilms (*S. aureus*, *S. epidermidis*, *P. aeruginosa*) (Carsenti-Etesse et al., 1992; Gagnon and Richards, 1993; Espersen et al., 1994; Owusu-Ababio et al., 1995) as well as to evaluate the importance of quorum sensing in *P. aeruginosa* biofilm persistence (by using mutant strains and quorum sensing inhibitors)

(Christensen et al., 2007). Finally, these infection models are particularly well-suited to study the effect of modified peritoneal dialysis catheters (e.g. Finelli et al., 2002, 2003; Kim et al., 2001).

3.2.4. Urinary tract infection models

Both surgical and non-surgical urinary tract infection models have been developed in rats, rabbits and mice. A first model was described by Satoh et al. (1984) in which a zinc disc was implanted in the bladder of rats, followed by transvesical inoculation with *Proteus mirabilis*. This model was subsequently used to demonstrate the importance of biofilm formation and matrix production in the development of urinary tract infections (Nickel et al., 1987). To study catheter-associated urinary tract infections, a catheterized rabbit model was developed in Bill Costerton's group and subsequently used to evaluate the effect of various antibiotics on *E. coli* biofilms developing on these catheters and on adjacent tissues (Morck et al., 1993, 1994; Nickel et al., 1991; Olson et al., 1989). Haraoka et al. (1995) used a rat model of renal infection in which glass beads covered with a bacterial biofilm were inserted in the bladder, followed by clamping of the urethra. More recently a non-surgical approach in rats and mice was developed, in which a polyethylene tube is placed in the bladder transurethrally (without surgical manipulation) (Kadurugamuwa et al., 2005; Kurosaka et al., 2001; Mikuniya et al., 2007). This polethylene tube can be colonised with pathogens before implantation, or a sterile segment can be implanted, followed by inoculation of the bladder with a defined number of microorganisms (Kadurugamuwa et al., 2005). A similar model was developed in rabbits (Fung et al., 2003). To evaluate the effect of coating urinary stents with RNAIII-inhibiting peptide (RIP) against *S. aureus* infections, coated (either with RIP alone or with RIP combined with teicoplanin) and uncoated stents were surgically implanted in the bladder of rats, followed by inoculation of *S. aureus* in the bladder (Cirioni et al., 2007). Results from this study clearly indicate that RIP increases the efficacy of teicoplanin against surface-associated *S. aureus* infections. *In vivo* models have also been used to study the efficacy of other coated catheters in preventing urinary tract infections (see for example Darouiche et al., 2008; Hachem et al., 2009; Orlando et al., 2008).

3.2.5. Ear, nose and throat infection models

As biofilms are increasingly recognised as important in ear, nose and throat (ENT) infections (Vlastarakos et al., 2007), several animal models have been developed to study these biofilm-associated infections. Most attention has been paid to otitis media (middle-ear infection) and the chinchilla has emerged as the animal of choice to investigate this biofilm-associated infection (Bakaletz, 2009). Developed in the mid 1970s (Giebink et al., 1976; Juhn et al., 1977), use of the chinchilla model led to an unambiguous establishment of a causal relationship between otitis media and biofilm, by allowing direct visualisation of the biofilm on the middle-ear mucosa (using SEM and CLSM) following transbullar injection with *Haemophilus influenzae* (Ehrlich et al., 2002; Post, 2001). The same model was later also used to demonstrate the ability of *Streptococcus pneumoniae* to form nasopharyngeal and middle-ear mucosal biofilms following transbullar inoculation (Hoa et al., 2009; Reid et al., 2009). Dohar et al. (2005) developed a similar model in cynomolgus monkeys for the study of biofilm formation by *P. aeruginosa*.

A second biofilm-associated ENT disease is chronic (rhino-)sinusitis (Cohen et al., 2009; Harvey and Lund, 2007; Vlastarakos et al., 2007). Two animal models were used to study the involvement of bacterial biofilms in this disease. In the rabbit model of acute sinusitis (Johansson et al., 1988), instillation of *P. aeruginosa* in the maxillary sinus allowed for the visualisation of biofilms with a pronounced three-dimensional structure from the sinus epithelium (Perloff and Palmer, 2005; Palmer 2006). This model has also been used to study the *in vivo* efficacy of topical tobramycin (Chiu et al., 2007) and the effect of an antimicrobial peptide derived from the innate immunity protein LL-37 (Chennupati et al., 2009). While high concentrations of these compounds result in the eradication of *P. aeruginosa* biofilms in this model, proinflammatory and ciliotoxic effects on sinus mucosa may limit their application (Chennupati et al., 2009). More recently a sheep model was developed to study the role of bacterial biofilms (*S. aureus*) in rhinosinusitis (Ha et al., 2007) and to assess the efficacy of topical antimicrobial agents (Le et al., 2008). Data from this experiment showed that regular treatment with mupirocin resulted in maximal reduction in biofilm surface coverage with sustained effects during the 8 day follow-up period (Le et al., 2008).

3.2.6. Respiratory tract infection models

The observations by Singh et al. (2000) that *P. aeruginosa* cells recovered from sputum of cystic fibrosis (CF) patients are in a biofilm-like structure (i.e. microcolonies embedded in matrix material) and that CF sputum contains *P. aeruginosa* quorum sensing molecules in the same ratios found in *in vitro* grown biofilms, confirmed the hypothesis that respiratory tract infections in CF patients are biofilm-related (Costerton et al., 1999). Several animal models for chronic respiratory tract infections have been developed, and these can roughly be divided into three groups.

In a first set of models, bacteria embedded in agar or agarose beads are used to establish pulmonary infections in laboratory animals. The use of these encased bacteria results in proliferation and allows for the establishment of a chronic infection with histological damage similar to that observed in patients with cystic fibrosis or chronic obstructive pulmonary diseases. In contrast, inoculation through the nasal or intratracheal route often only results in a transient pulmonary colonisation in which bacteria are rapidly cleared (O'Reilly, 1995; Sokol, 2006). In addition, in agar-bead based animal infection models, infections are characterised by consistent numbers of organisms and a relatively constant immunological response (Sokol, 2006). This model was originally developed using rats (Cash et al., 1979), but in later studies guinea pigs (Pennington et al., 1981), cats (Winnie et al., 1982), mice (Starke et al., 1987) and rhesus monkeys (Cheung et al., 1992, 1993) were used. This model has mainly been used to study microorganisms involved in respiratory tract infections in CF patients, and the majority of studies were carried out with *P. aeruginosa* and/or *Burkholderia cepacia* complex organisms (Bernier et al., 2003; Cash et al., 1979; Cheung et al., 1992, 1993; Cieri et al., 2002; Grimwood et al., 1989; Kukavica-Ibrulj et al., 2008; Meers et al., 2008; Pennington et al., 1981; Sokol, 2006; Starke et al., 1987; Winnie et al., 1982). A modified agar-bead model was developed by Sawai et al. (1997). In this model, rats were intravenously injected with agar beads (appr. 200 μm in diameter) containing various bacterial pathogens (including *S. aureus*). These agar beads were subsequently transported into the pulmonary circulation to become entrapped in the pulmonary microvasculature. This system has been used to evaluate the efficacy of antibiotics (including linezolid, quinolones and novel carbapenems) (Kihara et al., 2008; Yanagihara et al., 2006, 2008, 2009).

A second type of model is a murine model of chronic *P. aeruginosa* respiratory tract infection, developed to mimick infections observed in diffuse panbronchiolitis (Yanagihara et al., 1997). In this model, a plastic tube (e.g. a piece of a plastic intravenous catheter) is precoated with *P. aeruginosa* and subsequently inserted in the trachea (through the mouth) (Nagata et al., 2004; Yanagihara et al., 1997, 2000). With this approach, infection is restricted to the lungs and this model has been used to study the efficacy of various (combinations of) antibiotics (including clarithromycin, levofloxacin and erythromycin) against *in vivo P. aeruginosa* biofilms.

A final model is the pulmonary infection model without artificial embedding (Hoffmann et al., 2005). In this model, animals are infected intratracheally with a small volume of a planktonic culture of an alginate-hyperproducing *P. aeruginosa* strain. To this end either wild type mice (e.g. BALB/c) or "CF mice" ($CFTR^{-/-}$) can be used (Hoffmann et al., 2005, 2007), or the experiments can be carried out

using wild type rats (Song et al., 2005). This model has been used to evaluate the efficacy of the octadecapeptide novispirin G10 (Song et al., 2005) as well as to demonstrate the potential usefulness of azithromycin to attenuate *P. aeruginosa* virulence (Hoffmann et al., 2007).

3.2.7. Osteomyelitis infection models

Biofilm infections associated with orthopedic prosthetic devices are a major complication of orthopedic surgery and have a considerable impact on patient morbidity and mortality as well as on cost (An et al., 2006; Brady et al., 2008; Campoccia et al., 2006; Costerton, 2005). Treatment of these infections is particularly difficult and surgical interventions are often required (Brady et al., 2008). To study the efficacy of conventional antibiotic treatment as well as of the potential beneficial effect of antibiotics delivered *in situ* in high concentrations, several animal models were developed (Crémieux and Carbon 1997). The microorganism included in the vast majority of these studies is *S. aureus*.

In a first group of models, osteomyelitis is induced by instilling a bacterial suspension followed by implanting a foreign body in the bone or by the direct implantation of a contaminated foreign body. Osteomyelitis is a chronic disease, and to mimic this in the animal models, implants are left in place for longer periods (weeks to months) (Handke and Rupp, 2006). Models were developed in dogs (Fitzgerald, 1983; Petty et al., 1985), rats (Gerhart et al., 1993; Gracia et al., 1998; Monzón et al., 2001, 2002), rabbits (Eerenberg et al., 1994) and mice (Li et al; 2008).

In a second group of osteomyelitis infection models, planktonic cells are administered in the intramedullar cavity of the tibia of rabbits, without implanting a foreign body (Mader and Wilson 1983; Shirtliff et al., 2002a,b; Del Pozo et al., 2009).

3.2.8. Other models

Nett et al. (2010) recently described an *in vivo C. albicans* biofilm denture model. In this model, an orthodontic wire was threaded across the hard palate of immunosupressed rats and then secured between the cheek teeth. Subsequently, acrylic denture material was applied over the palate and, after solidification of the device, the hard palate was inoculated with 10^7 *C. albicans* cells. Histopathological examination of the rat oral mucosa following infection indicated that the model accurately mimics acute human denture stomatitis (including the presence of fungal invasion and neutrophil infiltration).

Two recent studies report on the development of animal models for vaginal infections. Carrara et al. (2010) used rats in which pseudo-estrus was induced by estradiol hexa-hydrobenzoate (1 week before inoculation). Following inoculation, the infection was monitored for up to 3 weeks. Harriott et al. (in press) developed a murine model for vaginal candidiasis: following administration of 17-β-estradiol (3 days prior to inoculation) mice were intravaginally inoculated with *C. albicans* and after a certain period the vagina was excised and investigated. Microscopic analyses (electron microscopy and CLSM) clearly indicated the presence of *C. albicans* biofilms on vaginal epithelial cells (Carrara et al., 2010; Damke et al., 2010; Harriott et al., in press).

4. Wound biofilm models

4.1. In vitro models

Recent evidence has shown that also in chronic wounds (e.g. diabetic foot ulcers, pressure ulcers) microbial biofilms (often polymicrobial) can be found. It has been hypothesised that these biofilms and the lack of their elimination by leukocytes are responsible for the chronic nature of the infection (Bjarnsholt et al. 2008; James et al., 2008). The first chronic wound biofilm model was developed at the Medical Biofilm Research Institute in Lubbock (Texas, USA) and was aptly named the Lubbock chronic wound biofilm model (Sun et al., 2008). This model allowed to rapidly (24 h) grow multispecies biofilms (containing the three bacteria typically observed together in chronic wounds: *P. aeruginosa*, *Enterococcus faecalis* and *S. aureus*) in a compact and relatively inexpensive model. The medium used in this model (Bolton broth with 50% heparinised bovine plasma and 5% freeze–thaw laked horse red blood cells) was selected to contain the major host factors (damaged tissue, red blood cells and plasma) found in the wound bed (Sun et al., 2008). Although *in se* an aerobic model, it also allows to study anaerobes (which can constitute a considerable part of the population in chronic wounds) (Sun et al., 2009). The model has been used to study the effect of treatment of wounds with antimicrobial agents and it was shown that microorganisms grown in this model are much more resistant than planktonic cells (Sun et al., 2008; Dowd et al., 2009). A somewhat similar model was described by Werthén et al. (2010) who developed a model in which biofilms can develop in the absence of a solid surface but in the presence of simulated wound fluid (containing 50% fetal calf serum and 0.1% peptone) and a collagen matrix. Biofilms formed in this model had a structure very similar to biofilm structures observed *in vivo*, indicating the presence of a "wound-like" environment. The most advanced *in vitro* wound model used to study biofilms is based on Graftskin, a tissue engineered skin equivalent (Charles et al., 2009). Graftskin contains human neonatal dermal fibroblast in a collagen matrix over which a cornified epidermis (derived from neonatal keratocytes) is produced. On the Graftskin specimens full-thickness wounds can be made and these can be inoculated with typical wound pathogens like *S. aureus* or *P. aeruginosa*. This model has the advantage that it is histologically similar to human skin and provides a controlled environment similar to the one encountered in *in vivo* wounds (Charles et al. 2009).

Other *in vitro* models have also been reported. Thorn et al. (2007) and Thorn and Greenman (2009) described model systems (including a flat-bed perfusion growth chamber) in which *P. aeruginosa* or *S. aureus* biofilms were grown on cellulose support matrices. Hill et al. (2010) used a constant depth film fermentor to form multispecies biofilms consisting of wound isolates. Lipp et al. (2010) used a colony-drip flow reactor model to grow *P. aeruginosa* or *S. aureus* biofilms underneath various wound dressings to evaluate their effect. At present it is unclear whether these systems – in which a fluid flow is present and/or marked shear forces are applied – have an added value over more conventional *in vitro* wound biofilm models.

4.2. In vivo models

As host-derived factors play an important role in chronic wound infections, it should not come as a surpise that several *in vivo* wound models were developed. However, as the role of biofilms in these chronic infections has only recently begun to be understood (Bjarnsholt et al. 2008; James et al., 2008), the presence of biofilms has only been confirmed in a few *in vivo* models.

Experimental inoculation of partial thickness wounds in pigs with *S. aureus* resulted in the formation of biofilm-like structures 48 h post-inoculation (Davis et al., 2008). Treatment with mupirocin or a triple antibiotic ointment had limited effect on these biofilms. Nakagami et al. (2008) created pressure-induced ischemic wounds in rats and inoculated these with *P. aeruginosa*. Subsequently the authors were able to demonstrate the presence of quorum sensing molecules in the infected wounds 3 and 7 days post wounding. The role of quorum sensing in *S. aureus* wound infections was investigated in a mouse model (full-thickness wound) by treating infected wounds with locally administered RIP (either as such or in a soaked wound dressing). Treatment with RIP-soaked wound dressing significantly reduced the bacterial load in the wounds, and this effect was even more pronounced when the treatment was combined with intraperitoneal injections with

teicoplanin (Simonetti et al., 2008). Using a similar model, Schierle et al. (2009) demonstrated that treatment of *S. aureus* or *S. epidermidis* infected wounds with RIP speeds up wound healing and restores reepithelialisation to levels observed in uninfected wounds. They also showed that wounds infected with biofilm-deficient *S. aureus* mutants resulted in improved wound healing kinetics. Kanno et al. (2010) described a rat skin model (full-thickness wounds) for *P. aeruginosa* infected acute wounds. While biofilms could be observed from 8 h post wounding until 7 days, infection did not appear to delay wound healing in this model.

5. Quantification and visualisation of biofilms grown in various model systems

Following biofilm growth in an *in vitro* or *in vivo* model system, the extent of biofilm formation can be measured in a variety of ways. Before providing a brief overview of the different approaches available, we want to stress the importance of standardising the techniques used to recover biofilm-grown cells from the surface. For example, it has been shown that the passage of a liquid–air interface (e.g. an air bubble) can result in considerable detachment and often-used procedures like dipping and rinsing to remove loosely-adhered microorganisms may consequently result in artifacts (Gomez-Suarez et al., 2001; Pitt et al., 1993; Sharma et al., 2005). As the extent of the detachment depends on the surface, the speed of the air bubble, and the strain being studied, the influence of detachment artifacts introduced by rinsing and dipping on the final result is unpredictable, highlighting the need for standardised procedures (Gomez-Suarez et al., 2001). Hamilton et al. (2009) recently described methods that can be used to check the validity of harvesting and disaggregating biofilm cells from surfaces. An important conclusion from the latter study was that, in order to allow the comparison of biofilms grown under different conditions and/or following different treatments, it is critical that efficiencies of harvesting and disaggregation are similar for the different biofilms; and that this is much more important than obtaining high efficiencies for control biofilms.

The number of culturable cells can be determined using conventional plate count methods and to this end, sessile cells can be removed from the surface by scraping and/or sonication (e.g. when biofilms are grown on the bottom and the walls of the microtiter plate) or they can be detached from the surface by applying cycles of sonication and vortexing (e.g. when biofilms are grown on discs or pegs placed in the wells of the microtiter plate) (Heersink, 2003). Subsequently, the suspended cells can be diluted and plated. However, conventional plating is labour-intensive and time-consuming and does not allow to recover viable but non-culturable organisms. Various alternative techniques have been developed for the quantification of biofilms grown in microtiter plates, including techniques to determine the total biofilm biomass (i.e. matrix and both living and dead cells, e.g. crystal violet staining, Syto9 staining), the number of viable sessile cells only (e.g. resazurin staining) or the amount of extracellular polymers in the biofilm matrix (e.g. staining with dimethylmethylene blue). These non-culture-based methods have been discussed in detail by Honraet et al. (2005), Peeters et al. (2008a) and by Toté et al. (2009, 2010).

Visualisation techniques and advanced imaging techniques have recently been surveyed by Neu et al. (2010) and Hannig et al. (2010). In recent years, particular attention has been paid to the development of non-destructive "real-time" monitoring of biofilm development in various biofilm model systems. The combined use of flow-cell technology and confocal laser scanning microscopy (CLSM) has been very useful to study various aspects of microbial biofilm formation, development and resistance *in vitro* and was recently reviewed by Pamp et al. (2009). In cases where real-time monitoring is not possible (either due to the incompatibility of the biofilm model system with the microscope and/or practical problems associated with the use of the visualisation equipment for longer periods of time), "offline" visualisation is used in which substrates containing the biofilm are removed from the biofilm reactor before being studied under the microscope. When studying "fragile" biofilms that dissociate easily from the surface during handling, this may require stabilisation (e.g. by using low-melting agarose) prior to further investigations (Pittman et al., 2010). The use of dedicated software (e.g. COMSTAT, developed by Heydorn et al. (2000a, b)) to quantify three-dimensional biofilm image stacks has been of tremendous value in quantitative biofilm research. Features calculated by COMSTAT include thickness distribution and mean thickness, roughness, substratum coverage and surface to volume ratio (Heydorn et al., 2000b).

The development of strains engineered to be constitutively bioluminescent (mostly by insertion of the *Photorhabdus luminescens luxCDABE* operon), combined with the development of highly sensitive imaging procedures, allows for the direct continuous monitoring of biofilm infections *in vivo*, e.g. by *P. aeruginosa* and *S. aureus* on subcutaneously implanted teflon catheters in mice (Kadurugamuwa et al., 2003), by *P. aeruginosa* and *P. mirabilis* on polyethylene tubes implanted in the bladder of mice (Kadurugamuwa et al., 2005), by *S. aureus* on cardiac tissue in rats (Xiong et al., 2005), by *S. aureus* on subcutaneously implanted surgical meshes (Engelsman et al., 2009), by *S. aureus* in an osteomyelitis infection model in mice (Li et al., 2008), by *S. epidermidis* on subcutaneously implanted catheters in mice (Vuong et al., 2008) and by *E. coli* (in combination with non-luminescent *Bacteroides fragilis*) in localised bacterial peritonitis in rats (Sharma et al., 2010). For a recent review on this topic, the reader is referred to Sjollema et al. (2010).

Finally, the technical developments in and increased use of non-destructive chemical analytical techniques, including Raman spectroscopy (Ivleva et al. 2008, 2009; Pätzold et al., 2006; Sandt et al., 2007), FTIR spectromicroscopy (Holman et al., 2009) and magnetic resonance imaging (Neu et al., 2010; Ramanan et al., 2010) are likely to futher increase our knowledge of biofilm biology in various model systems.

6. Which model system to choose?

The goal of this review was to present an overview of commonly used *in vitro* and *in vivo* biofilm model systems, their potential applications, and their advantages and disadvantages.

It is obvious that MTP-based systems permit a higher throughput, are generally less labour-intensive, do not require specialised equipment and are cheaper. These systems allow "multiplexing" (i.e. multiple organisms and/or treatments can be included in a single run) and as such are very well-suited for screening purposes (especially when they are combined with rapid methods to quantify biofilm formation). However, they are closed systems and the conditions in which biofilms are formed and/or tested are more often than not very different from the *in vivo* situation. *In vitro* "flow" systems have a lower throughput, are more labour-intensive and require specialised equipment and technical skills. In addition, they often require larger volumes of media and/or other reagents. While these systems have the advantage of incorporating the important aspect of fluid flow in the setup, other aspects encountered in the *in vivo* situation (e.g. immunological factors) are missing. In most flow displacement biofilm model systems biofilms are formed on multiple coupons in the same reactor vessel and as a consequence only a single organism (or a single community) can be tested per run. The same is true for "online" treatments in the reactor vessel: only treatment with a single product or a single combination of products is possible and only a single type of modified material can be tested per run. Cell-culture-based model systems often offer an elegant solution to some of the shortcomings of the other *in vitro* systems, but few of these standardised systems are commercially available and developing and maintaining these model systems is not always straightforward.

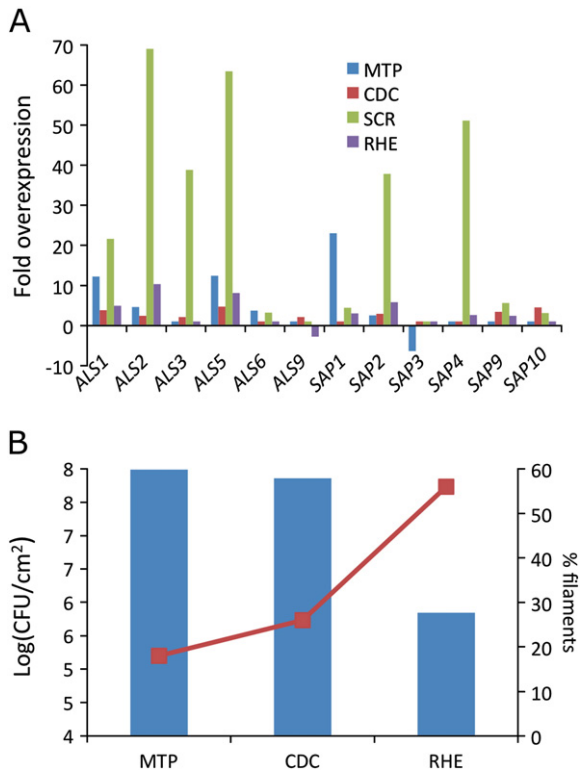

Fig. 5. A. Changes in gene expression in 48 h old *C. albicans* biofilms grown in different model systems. Shown are the fold changes compared to planktonic cultures. B. Number of colony forming units (blue bars, left axis) and fraction of filaments (red line, right axis) in 48 h old *C. albicans* biofilms grown in different model systems. MTP: microtiter plate, CDC: CDC biofilm reactor, SCR: subcutaneous rat model; RHE: reconstituted human epithelial cell model. Data are from Nailis et al. (2010).

Non-mammalian animal models are increasingly used to study various aspects of infectious diseases and an animal frequently used for this purpose is the nematode *C. elegans* (Mylonakis et al., 2007). This biofilm model offers considerable advantages in terms of logistics, budget, throughput and ethics, but it is more distantly related to the natural pathology (O'Callaghan and Vergunst, 2010). When compared to non-mammalian *in vivo* models, vertebrate *in vivo* biofilm models better take into account the host immune system and are indispensable for a better understanding of medical-device related infections. However, it is clear that vertebrate animal models should only be used if *in vitro* models or *in vivo* models with lower animals are not suitable to address the specific research question. Furthermore, the number of animals to be used should be kept to a minimum and considerable attention should be paid to minimize potential pain by using alternatives to painful procedures and/or appropriate anaesthesia (An et al., 2003; Handke and Rupp, 2006). While better mimicking the actual situation in a human host, the use of larger animals (sheep, goats, dogs) is associated with high costs, especially when a relatively high number of animals is required (An et al., 2003). For that reason, small animals which are easy to infect and are relatively economical, including mice, rats, rabbits, guinea pigs and hamsters are preferred in most studies (An et al., 2003; Handke and Rupp, 2006).

Which biofilm model system is selected not only depends on the preferences of the investigators and the resources available, but most importantly on the questions being addressed. There are only a handful of studies in which a meaningful number of biofilm model systems were systematically compared, but these studies clearly indicate that the selection of the model system can have a profound influence on the results. A first example is the comparative evaluation of biofilm disinfectant tests reported by Buckingham-Meyer et al. (2007). In this study, the efficacy of three disinfectants against *P. aeruginosa* and *S. aureus* biofilms grown either in the CDC biofilm reactor, a drip flow reactor or in a static biofilm, were compared. Overall, reductions were lowest in the CDC biofilm reactor, while the highest reductions were obtained in the static biofilm model. Reductions observed for biofilms grown in the drip flow reactor were intermediate. The authors strongly recommend that efficacy of disinfectants against biofilms should be tested with a laboratory method producing a biofilm under conditions similar to the environment where the disinfectant will be applied. A second example is the study by Nailis et al. (2010) in which morphological, phenotypic and genotypic characteristics of sessile *C. albicans* cells grown on silicone discs in MTPs and in a CDC biofilm reactor, on RHE cells and on polyurethane catheter segments implanted subcutaneously in rats were compared. While the overall number of culturable cells recovered from these biofilms was similar, there were considerable differences in filamentation patterns, extracellular lipase activity and expression levels of genes encoding factors involved in adhesion and virulence (Fig. 5). Interestingly, while the expression of some virulence genes appeared largely model system independent, the expression of other virulence genes was strongly influenced by the biofilm model system (Fig. 5). These examples clearly illustrate that data obtained in one model system cannot be extrapolated to another one and highlight the importance of the selection of an appropriate model system that most closely resembles the real-life situation the researcher wants to mimick.

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