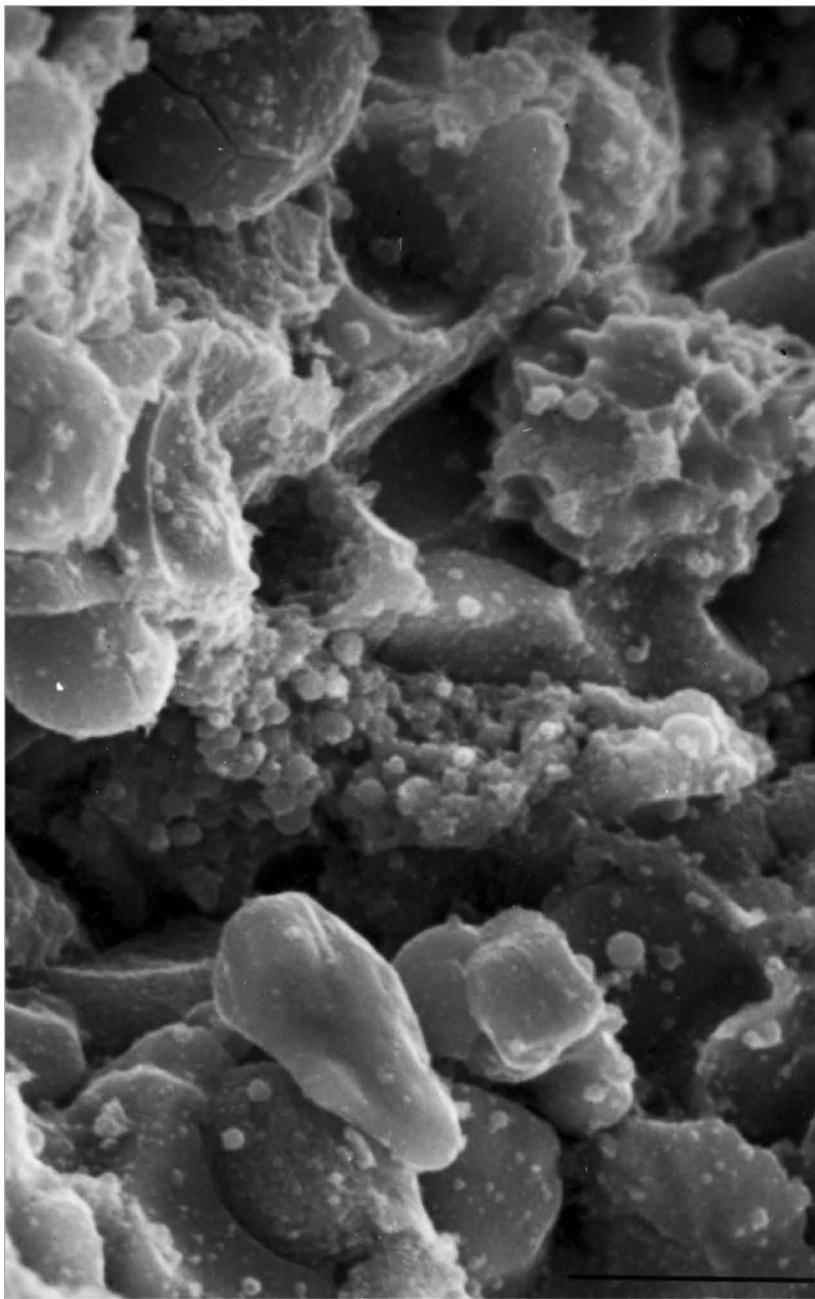


Virulence

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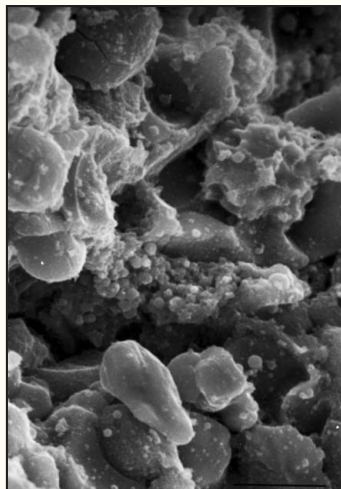
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Scanning electron microscopy micrograph of bone matrices in a host suffering from osteomyelitis. The micrograph shows the adherent population of cocci (*Staphylococcus aureus*) where one can see the collapsed glycocalyx material that is normally associated with bacterial biofilms of this microbial species.

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Reports from the COST workshop on biofilms

Biofilms: Friend or foe?

Meeting report; June 2011

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COST is one of the longest-running European instruments supporting cooperation among scientists and researchers across Europe. COST is also the first and widest European inter-governmental network for coordination of nationally funded research activities. COST mission is to strengthen Europe in scientific and technical research through the support of European cooperation and interaction between European Researchers via the formation of networks called COST Actions.

Biofilms were first observed and described in 1684 by Anthony van Leewenhoek, but named centuries afterwards. In a report to the Royal Society of London he remarked on the vast accumulation of microorganisms he was able to see in dental plaque: "The number of these animalcules in the scurf of a man's teeth are so many that I believe they exceed the number of men in a kingdom." Only many years afterwards the significance of microbial activity and the economic costs associated with biofilms was realized.

Biofilms cost Europe billions of euros each year in medical infections, equipment damage, energy losses and product contamination. Biofilm is the preferred mode of existence of microbes in a range of habitats allowing bacteria to survive in hostile environments and to colonize new niches by various dispersal mechanisms. Being in a community has a lot of advantages in comparison to a planktonic life.

However, biofilms also have favorable properties; for instance biofilms decompose and recycle organic material and help keep nutrients circulating in the marine food chain. Well-controlled biofilms have potential application on biofiltration of industrial waste water, bioremediation, formation of natural biological barriers for soil and groundwater protection from contamination and others.

The EU has already started strategic initiatives focusing on biofilms, with topics in the Framework Programme 7. The European Technology Platform on Plants for the Future has in its Strategic Research Agenda 2025

(Part II) the development of biofilms to protect plants against disease as one of the ways to achieve the goal of reduction and optimization of the environmental impact of agriculture. These initiatives are examples of EU efforts to promote activities that can provide the means of addressing problems caused by biofilms or where biofilms could have a role in ameliorating undesirable conditions.

As biofilms is a very diverse subject ranging from medical implant development to bioremediation there is an urgent need to cross the boundaries of isolated efforts and set up a coordinated interdisciplinary network able to address the current challenges in a more effective way. This will be achieved by exchange of knowledge, concepts and work and by enhancement of creativity. This need for stronger cooperation is acknowledged by the recent establishment of well-known research centers of biofilms in Europe and by the number of conferences organized on biofilms. Nevertheless, there is still no fora to bring the relevant scientists together in a more systematic and effective way.

In order to stimulate the formation of such an interdisciplinary forum able to tackle current and future challenges on biofilms in an increasingly globalized world, COST (European Cooperation in Science and Technology; www.cost.eu) organized a multidisciplinary two-day Exploratory Workshop on "Biofilms: Friend or foe?" in June 2011.

The two-day invitation-only workshop took place in Berlin, Germany and attracted around 50 participants from 18 countries, mostly from Europe but also from the US, Australia, New Zealand, South Africa, Sri Lanka and others. The workshop was divided in four sessions focusing on cell signaling (within a biofilm and cross-kingdom interactions), novel biotech and bioengineering applications (environmental, agricultural and medical), industry priorities and future perspectives. A poster session also allowed scientists to exchange views and ideas during the walking lunches.

The first day started with a welcome address by the Scientific Organisers from COST Office, Lucia Forzi (Science Officer, Chemistry and Molecular Sciences and Technologies) and Ioanna Stavridou (Science Officer, Food and Agriculture), followed by an opening address by Henk van Liempt of the German Federal Ministry of Education and Research (BMBF). The COST framework, its mission and goals were outlined in the presentation given by Ioanna Stavridou. Then Philip Stewart (Director of the Center for Biofilm Engineering at Montana State University) gave an overview on the concept of biofilms and their antimicrobial tolerance illustrating different phenomena such as diffusion of antibiotics into biofilm, hydrodynamics around the biofilm, dormancy (persister cells that can reseed community after catastrophe) and physiological heterogeneity in biofilm cell populations.

The first two sessions of the workshop focused on cell signaling. Since biofilms are extremely relevant in medicine and in biotechnology, it is fundamental to study how bacteria switch from a planktonic, individualized lifestyle, to biofilms. The sessions that focused on signaling within a biofilm highlighted the importance of intra- and inter-bacterial communication and information exchange. Various topics were addressed going from the production of interference molecules involved in competition interactions within multispecies biofilm communities, to the production, release and response to signal molecules in quorum sensing signaling and to the role of the signaling molecule cyclic-di-GMP on biofilm formation. The second session on cross-kingdom interactions was dedicated to signaling and interaction between organisms of different kingdoms and on potential applications to biofilm regulation or control. Interactions between biofilm bacteria and (1) plants (specific factors for adhesion and biofilm formation and relevant application for biocontrol to contain plant infections), (2) barnacles and macro-algae

(use of quorum sensing molecules produced by marine bacterial biofilms as cues to locate surface for settlement thereby influencing the development and functioning of biofouling communities), (3) fungi (the nematode *Caenorhabditis elegans* as model host to identify genes and functions of fundamental relevance for pathogenesis and to screen in vivo for anti-fungal compounds) and (4) humans (quorum sensing inhibition as a strategy against pathogenic bacteria biofilms) were presented.

The first session of novel biotech and bioengineering applications addressed different technologies with important environmental applications. It was opened by addressing the important role of biofilm monitoring, presenting an overview of the most advanced examples of monitoring devices used in drinking water networks and to evaluate cleaning measures. In the following presentations different beneficial biofilm innovative technologies were presented. The various application areas included waste water treatment plants (i.e., granular sludge), degradation of persistent pollutants (i.e., PCB), controlled antifouling with conductive paints supported by growth inhibiting nanoparticles, and biofuel cells using electroactive biofilms (relevant to bioremediation, bioelectricity production and environmental sensing). To conclude, alternative nanotechnology methods were presented focusing on the use of nanofibers, produced by electrospinning, with antimicrobial and antifouling properties. The second

day started with a presentation by Tomasz Calikowski (European Commission) where he gave an overview of the FP7 Theme 2 and call proposals for 2012. This was followed by the session on Novel Biotech and Bioengineering Applications in Agriculture. During this session different issues were addressed including positive applications of biofilms, i.e., as biofertilizers, that could increase crop yields, reduce inputs of chemical fertilizers, pesticides and reduce harvest loss. The biofilms as a friend concept was then followed by two presentations dealing with the negative aspects of biofilms, one in inflammations in dairy cows and another in food processing, and innovative technologies to control their detrimental effects. For instance, vaccination of cows with strong biofilm-producing bacteria expressing the Slime Associated Antigenic Complex (SAAC) confers protection against infection and mastitis, whereas natural-derived products (e.g., essential oil and hydrosol) may be equally effective for sanitation purposes as the currently used chemical ones. This session was closed by Winy Messens (EFSA) presenting the principles of risk analysis in food and feed safety and in particular the activities of the Biological Hazards (BIOHAZ) Panel that focuses among other topics on antimicrobial resistance.

In the session of Novel Biotech and Bioengineering Applications in Medicine the opening lecture highlighted the relevance of biofilms in clinical microbiology and pathogenesis of biofilm-related infections,

whereas the presentations that followed were focused on potential strategies to control biofilms, with the use of phages, enzymes that degrade the extracellular polymeric substances composing the biofilm matrix, essential oils and terpenes and nanoparticles as function of biocidal, anti-adhesive and carriage capabilities.

The third session on biofilms-related priorities in industry included an overview on the development of new agents for prevention, treatment or management of biofilm-associated infections and of biofilm development on abiotic surfaces. These new agents included among others antibiotics, microbicides, enzymes and even bacterial strains that form thin or no biofilms but can inhibit the growth of more harmful biofilms. This session was of high value because industry representatives had the opportunity to discuss with highly qualified researchers the need for diagnostic and monitoring technologies for detecting the presence of biofilms and possible strategies for identifying new solutions to problems caused by biofilms.

The last session of the workshop consisted of a presentation by Lucia Forzi focusing on the fact that COST provides a platform for European scientists to cooperate on a particular project, an Action, and which steps researchers who wish to launch a new Action have to follow. The workshop ended with a round table discussion on possible future cooperations among the researchers and industry representatives present.

Enzymatic removal of biofilms

A report

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Biofilms are recognized to be a critical and hard to remove contamination source. The presence of a biofilm results in a number of dangers for human health or industrial activities: hygiene and food safety in the food industry, nosocomial diseases in hospitals, clogging and contaminations in water systems including cooling systems, pipes corrosion, boat-hulls fouling, etc. Many branches of industry or healthcare services attempt to control biofilm by chemical shocks (acid, base and disinfectant). But this has been proved to be inefficient in removing entirely the biofilm, presenting furthermore a high aggressiveness

for materials. Following a two-year research project, REALCO and the INRA (French National Institute for Agricultural Research) developed an enzyme-based process to remove biofilms from surfaces in numerous applications. That process, involving a specific enzyme cocktail and a detergent phase, showed high efficiency at both lab and pilot scale on different biofilms, before it now reaches proved success in industrial applications.

Biofilms

A biofilm is a viscous slime layer that develops on almost every surface in contact with water and microorganisms. It is composed

of bacteria and organic polymers called EPS (Extracellular Polymeric Substances, mainly polysaccharides and proteins) that promote the irreversible adhesion of microorganisms and create a protective and evolving structure around them. The biofilm is thus a reservoir of microorganisms that protects them from external aggressions, among which are cleaning and disinfection.¹

Research Program

REALCO and its partner INRA realized a two year project, starting October 2006, sponsored by the EURAKA agency. The first step of that project focused on the selection of

microorganisms developing rapidly a biofilm colony. All microorganisms were originated from different food industries. These biofilms were then tested for their resistance to soft cleaning procedures and mechanical removal, with the goal of selecting the toughest biofilm on which to work. For this purpose, a specific screening method was developed that ended with the selection of six bacterial strains from different bacterial families. The second step of the project targeted the selection of enzymes activities for their ability to degrade EPS and biofilms. The composition of a detergent phase has also been screened in order to create a medium (i.e., cleaning solution) increasing the efficiency of enzymes. Biofilms were developed on stainless steel slides and enzymatic solutions were used to remove biofilms in a Cleaning-In-Place procedure, first at lab scale and then in an industrial pilot installation. Results showed that different enzyme activities were more or less efficient on different bacteria strains originated biofilms.² This research program ended with the selection of a detergent phase and a multi-activity enzyme cocktail able to degrade a broad variety of biofilms.

The Enzyme-Based Process

The interest of an enzyme-based solution for biofilm removal relies on the action the selected enzymes have on biofilm. The selected cocktail targets the degradation of the biofilm EPS matrix. No biocide effect is sought on biofilm microorganisms. Indeed, what makes a biofilm resistant to cleaning and disinfection procedures, originating biofilm-linked concerns, is precisely the EPS matrix. As this matrix is composed of organic macromolecules, the right enzymes activities are able to degrade those structural components and to weaken the biofilm structure. Once partly degraded, the biofilm matrix is on one hand more easily removed by mechanical effect (flow, pressure, brushing) and on the other hand, is less protective for microorganisms that are then more efficiently attacked by a disinfection procedure.

These two actions result in the complete removal of the biofilm from the surface. **Figure 1** illustrates this.

A biofilm (*Bacillus cereus*) has been developed on the faces of a stainless steel ring that is in a second step introduced in a pipe showing a broader section than the ring. The sudden section change encountered by the flow when reaching the ring creates a perturbation

zone, reducing mechanical forces and cleaning agent efficiency, and so usually favorable to the development of biofilm in closed installations. Different cleaning solutions have been circulated in the pipe and the biofilm removal efficiency followed by microorganisms enumeration on the ring surface and by fluorescence microscopy analyses. **Figure 1A** shows the biofilm structure on the stainless

steel ring after incubation ($5.34E+06$ CFU). The EPS matrix is visible. **Figure 1B** shows the ring surface after a cleaning procedure with caustic soda (0.5%, 30 min at 45°C). A part of the biofilm has been removed ($5.2E+04$ CFU) but what is left on the surface is still the entire biofilm structure. So the EPS matrix is still protecting microorganisms. **Figure 1C** shows the ring surface after a cleaning procedure with

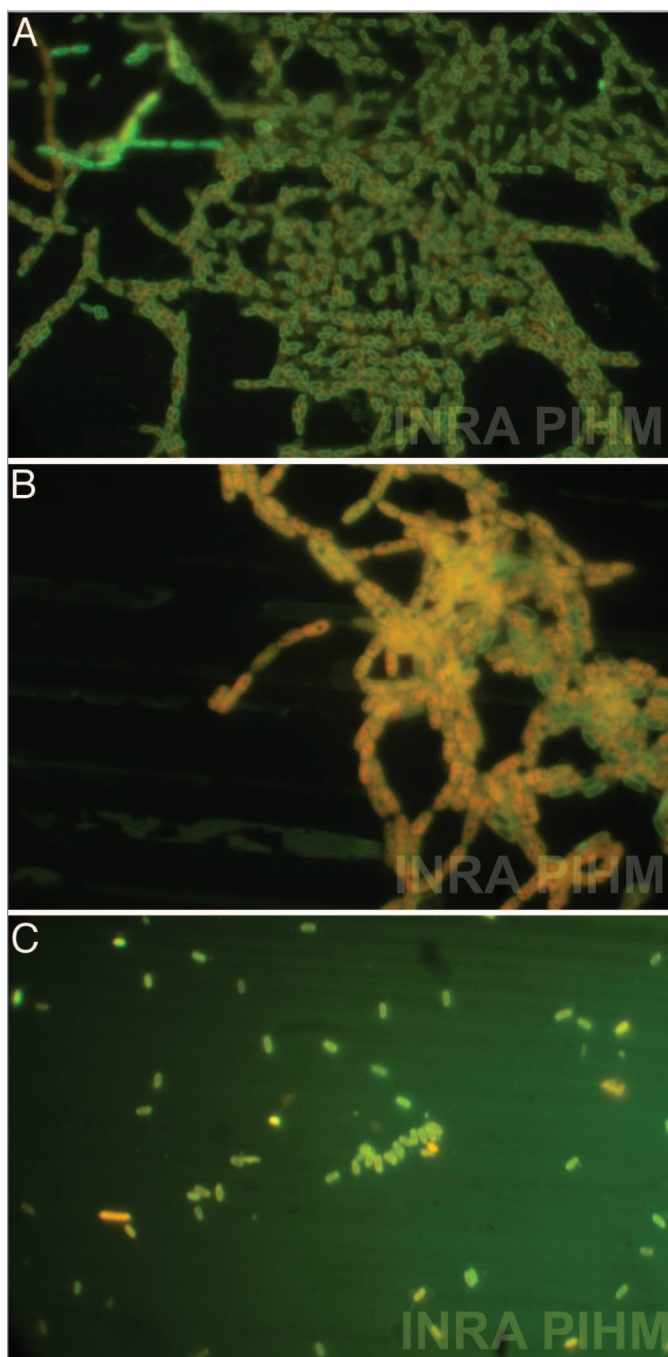


Figure 1. *Bacillus cereus* biofilm developed on a stainless steel surface, after 48 h incubation (A) and after subsequent application of caustic soda (B) and enzymatic (C) cleaning procedures. [INRA-UR638, Y. Lequette, C. Faille (contrat NETZYM)].

the enzyme-based solution (detergent phase 0.5%, enzyme cocktail 0.05%, 30 min at 45°C). The biofilm matrix has been entirely removed and only bacteria's cells are visible (1.32E+03 CFU). The first conclusion is the much better efficiency of enzymes to remove biofilm EPS. The second conclusion is the understanding of the different efficiency levels that a disinfection step, realized with any classical disinfection means, will reach after one or the other cleaning step. A disinfectant media applied on the surface treated with caustic soda will face the EPS structure that will prevent it reaching bacteria's. It will then be inefficient and these microorganisms contained in the biofilm will be able to continue their growth. On the other hand, a disinfection step applied on the surface cleaned with enzymes will directly reach bacteria cells that have no protection means anymore. The efficiency will be much greater on cell destruction, leading to a much cleaner and less contaminated surface. The biofilm concern would then be solved.

Field Application

This enzyme-based solution is now applied in different industries, in various application fields (water, catering, food processing, heat exchangers). Most frequent demands for biofilm removal come from the food industry, where biofilm presence leads to finished product contamination and important production losses due to contaminated finished product destruction. Another field for which biofilm removal has a crucial importance is hospitals, where about 65% of the nosocomial diseases are linked with biofilms.

Biofilm removal on site requires different steps and expertise:

Biofilm detection. A biofilm has to be clearly identified as the contamination source and must be located in order to realize a targeted treatment. Various methods exist to detect a biofilm in closed installations, all requiring experience for results analyses. No method was available for biofilm detection on open surfaces. REALCO developed a specific coloration method to answer portability, rapidity, accuracy and specificity requirements for biofilm detection at industrial scale.

Cleaning protocol. A complete protocol, adapted to the installation/equipment specificity and materials encountered has to be set up. Usual sequence is: previous classic cleaning procedure/rinsing/biofilm removal treatment/rinsing/disinfection step/rinsing.

Enzymatic biofilm removal processes exist for both open plant cleaning and cleaning-in-place procedures.

Proof of biofilm removal. Another biofilm detection step is required to prove the effective biofilm elimination.

Prevention treatment set-up. As biofilm development cannot be avoided, a regular application of the anti-biofilm specific process has to be applied to secure the installation against biofilm recontamination. The frequency of this treatment is evaluated for each case depending on the biofilm development rapidity, which depends on contamination source, available nutrients, medium conditions and cleaning procedures.

The results and benefits achievable at industrial scale are mainly measurable on productivity and related profitability. Lower contamination levels in finished products allow important savings by reducing the need of contaminated finished product destruction.

A global improvement of hygiene results has also been measured in different industries. Product quality is also improved when undesired contaminations lead to product organoleptic quality modification, even not being a danger for health. Finally, reducing the risk of contaminations represents an important benefit regarding food safety and human health.

Conclusions

In the healthcare sector and/or in many industrial fields, biofilms represent a threat for human health, and to another level, for profitability. This threat is directly represented by the bacteria's reservoir biofilms are. But what creates the threat and makes it long-lasting and always pending is the resistance of biofilm to cleaning and disinfection procedure. A specific enzyme-based treatment offers a reliable and sustainable solution. By targeting the EPS matrix, adhesive armorplate protecting the biofilm, enzymes achieve to weaken biofilm structure and promote its entire removal from contaminated surfaces.

Acknowledgments

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Biofilms for environmental biotechnology in support of sustainable development

A report

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The world is facing formidable challenges in meeting the rising demands of potable water and consumer products. The health and welfare of people are closely connected to the availability of adequate, safe and affordable water supplies. Also cost-effective recovery methods of chemicals and resources from waste and wastewater play an increasing role in sustaining human civilization on Earth. Many of these processes are based on microbial

biofilms.¹

One of the major hazards humanity is facing is existing and new environmental pollution of our foundations and primary resources of life—air, water and soil—by (eco)toxic chemicals. There is now overwhelming evidence that human health is directly dependent on the state of our environment. Chemical pollutants from the environment, apart from having other effects, are the cause of or provoke

many human diseases. Consequently, we need a better balance between disease-centered (therapy) and environment-centered (prevention) research. Of particular concern is the pollution of the aqueous environment with, e.g., carcinogens, hormones or hormone-like substances, pesticides and antibiotics.² This is a major contribution to the increasing loss of the already inadequate supply of safe drinking water in most countries and results—at the

best, for those who can pay—in the necessity of expensive treatment. We can never approach sustainable development, a goal constantly reiterated by almost all governments, if we do not find ways and means of eliminating existing toxic chemicals from the environment and also prevent new chemicals entering it and control their use at the source.³

Environmental biotechnology, which was restricted in the early 80s to the traditional biological wastewater treatment processes, has experienced a tremendous development in the last two decades. Impressive progress was made particularly in biodegradation and bioremediation research. In many cases it was shown that this technology is able to permanently eliminate pollutants inexpensively, especially the highly problematic pollutants present in low but toxicologically relevant concentrations.⁴ Probably the best example for this is the striking commercial success of air and off-gas cleaning by biological filters, a field in which many companies have established and the technology has proven to be cheap and reliable when compared with the alternative incineration technology.^{5,6} Many other bioremediation processes are now at the level of scaling up and *Nature Biotechnology* has published in 2003 that bioremediation is the fastest growing sector in the hazardous waste industry in the US (a \$7–8 billion turnover annually).

Energy and feedstock materials for the industry are in increasing demand. With constraints related to availability and use of oil, the energy and chemical industry is undergoing considerable changes. The need for the use of cheaper and widely available feedstocks, and the development of sustainable and environmentally friendly chemical processes is rapidly growing under both economical and public

pressure.⁷ Therefore, treatment of wastewaters, waste gas and solid waste need to be gradually integrated into process design. Instead of discharging their waste into the environment, industries need to become increasingly self-sufficient and recover compounds from their own wastestreams or use (upgraded) wastestreams of neighboring industries as raw material. Many biofilm based environmental biotechnological processes support these developments.

Sustainable waste treatment concepts are under development with environmental biotechnology as key component, and can lead to the recovery of useful by-products like chemicals in the form of e.g., fertilizers (ammonia, phosphates), raw materials (elemental sulfur and selenium, sulfuric acid,...) or energy (biogas, hydrogen or electricity). Obviously, adding value to waste by upgrading the recovered compounds will only be a reality if it is demonstrated that there is a fundamental basis and a tangible advantage in using these recovered compounds rather than buying raw materials and feedstock.⁸

It is common knowledge that environmental protection is simply and purely legislation driven. Ergo, the main barriers for development, acceptance and penetration of existing and new technologies are the lack of appropriate environmental legislation. Establishing and enforcing appropriate environmental legislation, together with a good strategy for financial incentives for start-up activities, would provide the economic basis for such technology, and hence, would also create jobs in this area. If there is no legislation, the environment is 'exploited' and used in a non-sustainable way. An important aspect of this legislation would include an internalization of environmental costs in prices of goods produced and,

consequently, also the taxing of (produced and imported) products in which these costs are not included.

Policy should at this moment draw the attention of the public to the dangers of having insufficient environmental technologies. For instances, we seem to have no answer to the increase of CO₂, to the presence of endocrine disrupters at ng/L everywhere in the environment, and so on. Hence, we need to continue our search so that we in the future have tools to deal with these environmental challenges also. One of the most pressing areas to support sustainable development is the generation and use of renewable energy from solar, wind or wave energy, an area linked tightly with global warming and CO₂ sequestration. Several of the existing or prospective technologies involve biotechnology, e.g., microbial fuel cells, biogas or biohydrogen production from (waste) biomass.³ Focused support of these technologies will link the rapidly developing biotechnology R&D to the conservative transport and energy sector.

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Zinc oxide and indium tin oxide thin films for the growth and characterization of *Shewanella loihica* PV-4 electroactive biofilms

A report

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Transition metal oxides (TMO) electrodes provide a suitable platform for spectroscopy- and microscopy-based investigation of viable electroactive biofilms (EABs). Commercially available TMOs, such as indium titanium oxide

(ITO), are produced from rare and expensive elements. Thus, there is a significant need to explore alternative technology to meet the demand. ZnO-based TMOs are low-cost and have excellent electrochemical properties. In

this study, we report for the first time a model EAB of *Shewanella loihica* PV-4 on ZnO films deposited on perspex substrates via pulsed laser deposition (PLD). Commercially available ITO electrodes were also used for comparison.

EAB electroactivity was characterized through microbial biofilm voltammetry (MBV). ITO and ZnO films show flat surfaces and amorphous structure, and have 60 and $10^5 \Omega/\text{sq}$ sheet resistance, respectively. *S. loihica* PV-4 biofilms on ITO and ZnO produced maximum current density of 3.38 and $0.25 \mu\text{A}/\text{cm}^2$, respectively. Our results suggest that ZnO has potential for the growth and characterization of EABs.

Introduction

Electroactive biofilms (EABs) conserve energy through extracellular electron transfer (EET) to insoluble electron acceptors such as metal oxides and electrodes.¹ Depending on the electrochemical potential and the availability of soluble electron donor and acceptor, the EET may occur from the EAB to the electrode or vice versa; the former are often termed cathodic EABs and the latter anodic EABs, respectively. In this study, we focused on anodic EABs, due to their relevance to microbial fuel cells, biosensors, wastewater treatment and biosynthesis of bulk chemicals.²⁻⁵

Well-known EABs-forming microorganisms include Gram-negative *Geobacter* and *Shewanella* genus. EABs are usually grown at the working electrode of potentiostat-controlled electrochemical cells (ECs), where the potential can be set with great accuracy and the electron flow at the interface EAB/electrode can be measured with high precision.⁶ The thermodynamic and the kinetics of the EET process are studied by direct electrochemistry methods, such as chronoamperometry (CA), cyclic voltammetry (CV), and electrochemical impedance spectroscopy (EIS).⁷

However, electrochemical methods alone cannot reveal the nature of EET agents. Spectroscopy is needed for this purpose. Such analysis is best performed when the EABs are grown on an optically transparent conductive substrate,^{5,8} among which only ITO has been used to grow EABs.⁵ Thus, research into economically viable and environmentally friendly materials for the routine investigation of EABs is needed. In this work, we characterize a model anodic EAB of *Shewanella loihica* PV-4 on ZnO and commercial ITO thin films. ZnO thin films were grown using PLD on acrylic glass.⁹ We characterized the electroactivity of *Shewanella loihica* PV-4 biofilms through MBV.⁷ The surface topography, crystallinity and electrical properties of both ZnO and ITO thin films were determined using atomic force microscopy (AFM), x-ray diffraction (XRD), and four-point probe I-V. The suitability of ZnO and

ITO films as substrate materials for the characterization of anodic EABs is discussed.

Experimental Details

ZnO and ITO thin films. ITO thin films deposited on polyethylene terephthalate (PET), with sheet resistance of $60 \Omega/\text{sq}$ and film thickness of $150 \pm 25 \text{ nm}$ were purchased from Sigma Aldrich (CAS 50926-11-9). The ZnO thin films were prepared in our laboratory on 20 mm x 5 mm perspex substrates using a conventional PLD system.⁹ The cleaned substrates were placed in a vacuum chamber filled with pure oxygen at 3×10^{-8} mbar and at 5 cm from the surface of a high-purity ZnO target. A short-pulse (6 ns) of UV laser light (266 nm) from a frequency-quadrupled Nd:YAG laser was focused onto the ZnO target with a fluence of $2.0 \text{ J}/\text{cm}^2$ to produce the expanding ZnO plasma which recondensed on to the perspex substrate forming the ZnO thin film. The laser was operated at a repetition rate of 10 Hz and all the depositions were performed at room temperature in varying ambient pressures ranging from 0.1 to 0.4 mbar. A calibration curve of the film thickness as a function of the number of laser shots was constructed. The growth rate in the above conditions was found to be linear over the range 150–2,000 nm and equal to 0.03 nm/shot.

ZnO and ITO thin film characterization.

The surface morphology, roughness, sheet resistance, crystallinity and hydrophobicity of ITO and ZnO were studied using the following techniques/methods: AFM in tapping mode, four-point probe (van der Pauw) current-voltage measurements, XRD (θ - 2θ) scans with the $\text{Cu K}\alpha$ wavelength of 0.154 nm, and water contact angle with a commercial goniometer, respectively.

Biofilms formation and characterization.

Shewanella loihica PV-4 strain (DSM 17748) was subcultured¹⁰ and a three-electrode EC used⁴ as previously described. The working electrode (ZnO or ITO coated) was connected to the current collector using platinum wire and miniature nylon screws. Platinum wire was used as a counter electrode and a SCE was connected to the EC via a salt bridge. The assembled ECs were autoclaved prior to the insertion of the working electrode. All ECs were operated under a constant flow of sterile humidified N_2 . The ECs were maintained at 30°C and mixed via a magnetic stirrer, which was activated during CA and inactivated during CV. After inoculation, the ECs were covered with aluminum foil to prevent exposure to

ambient UV light and the consequent formation of reactive oxygen species (ROS) on ZnO surface. In all experiments, lactate was added as electron donor to the non-limiting concentration of 20 mM.

The ECs were connected to a multichannel potentiostat (Bio-Logic, France). Both ITO and ZnO electrodes were tested under identical experimental conditions. The parameters for each method were: (a) CV: scan rate = 1 mV/s, $E_i = -0.8 \text{ V}$ vs. standard calomel electrode (SCE), $E_f = 0 \text{ V}$ vs. SCE; (b) CA: $E = 0.2 \text{ V}$ vs. SCE.

Results and Analyses

The AFM topography of commercial ITO and ZnO thin films are shown in **Figure 1A and B**, respectively. The ZnO and ITO thin films were homogeneous. Small cracks were occasionally present on the surface of the ZnO films, with typical depth of 10 nm. Such cracks appeared in samples that were annealed at 100°C. Both the ITO and ZnO surfaces appear free from large aggregates, particulates and pinholes, however, their rms roughness differ markedly, being 12 nm and 2.2 nm, respectively. **Figure 1C** shows the sub monolayered biofilm of *S. loihica* PV-4 on ITO whereas **Figure 1D** shows a more uniform multilayered biofilm of *S. loihica* PV-4 on ZnO.

The sheet resistance of ITO films was on average $60 \Omega/\text{sq}$, while that of ZnO films was approximately 1000 times larger, as expected for nominally undoped ZnO material.¹¹ A weak dependence of the films resistivity on the ambient pressure during growth was also observed. The ZnO films with the lowest resistivity were chosen for EAB growth and characterization.

The XRD data for the ITO and ZnO films are shown in **Figure 2A and B** for ZnO with thickness of 136 nm, 1180 nm and 1720 nm. Only a strong PET peak at $2\theta \approx 26.0^\circ$ is seen in **Figure 2A** indicating that the ITO has an amorphous structure.¹¹ From **Figure 2B**, we see that the 136 nm thick ZnO film is also amorphous, whereas the presence of a single peak at $2\theta \approx 34.7^\circ$ indicates good crystallinity of the 1180 nm and 1720 nm thick ZnO films, with the wurtzite structure and c-axis orientation, i.e., the (0001) wurtzite planes lie parallel to the perspex surface.

The water contact angle was measured at $74 \pm 1.2^\circ$ for the ITO film, indicating that its surface is hydrophilic. For the 136 nm thick ZnO film, this angle was measured at $92 \pm 2.0^\circ$, which means that the surface is slightly hydrophobic.

A typical CA for *S. loihica* PV-4 on the ITO electrode is shown in **Figure 3A**. Following inoculation of the suspended cells, the anodic (oxidation) current decreased. The spent growth medium was changed daily with fresh medium, in order to promote biofilm growth. Following the second medium change (MC), the anodic current started increasing as a result of the catalytic oxidation of lactate in the EAB, as previously described.⁶ The current density reached a maximum value of $3.5 \mu\text{A}/\text{cm}^2$ ($n = 3$) after 90 h. The sudden drops in the current density observed after 62 h and 85 h, respectively, are likely due to the exhaustion of the lactate in the medium and/or to a pH change due to accumulation of metabolic end products.¹² The CV of *S. loihica* PV-4 biofilm on ITO electrode showed a typical sigmoidal curve (**Fig. 3B**) with very low hysteresis, as reported earlier for other EABs grown on ITO.⁸ This result corresponds to the catalytic (turn-over) EET at the EAB/electrode interface.

The current density obtained for *S. loihica* biofilms grown on ZnO did not reach a plateau as observed on the ITO electrode (**Fig. 3C**) and had a maximum value of $0.25 \mu\text{A}/\text{cm}^2$ ($n = 2$) after approximately 50 h. The CV curve showed small sigmoidal behavior and overlapped with the redox peaks of the microbially produced flavins, centered at -0.35 V vs. SCE (**Fig. 3D**). Medium change had no effect on CA and CV behaviors, indicating that the current decrease was not caused by the exhaustion of the lactate. AFM images of *S. loihica* PV-4 biofilms grown on ZnO show a uniform multi-layered structure, whereas biofilms on ITO appear sub-monolayered (**Fig. 1C and D**).

These results indicate that facultative EAB-forming microorganisms such as *S. loihica* PV-4 can grow on ZnO thin film electrodes and produce a small but significant oxidation current. However, the EET rate on ZnO is much lower than that measured on an ITO electrode. This can be attributed in part to the higher hydrophobicity of the ZnO films. It is also possible that the lower surface roughness of ZnO films prevent adsorption of *S. loihica* PV-4 membrane cytochromes on the electrode,¹³ thus decreasing the overall oxidation current. Although ZnO has known antimicrobial properties due to formation of reactive oxygen radicals at the interface,¹⁴ the growth of well structured *Staphylococcus epidermidis* biofilms on ZnO films, identical to those used in this study, has been recently documented.¹⁵ In the present case, it is possible that free-oxygen

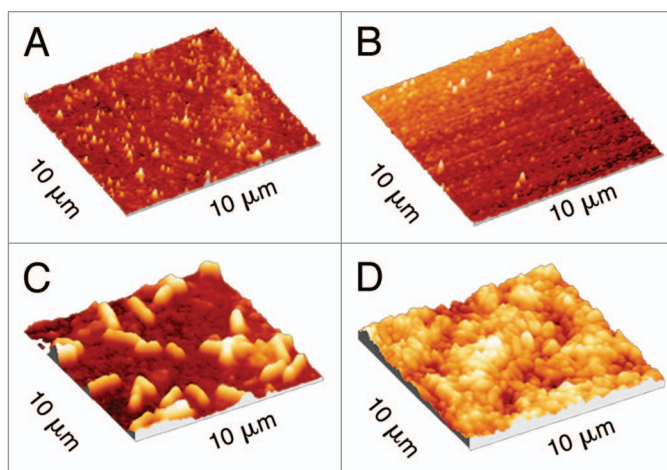


Figure 1. AFM images showing the topography of (A) bare ITO, (B) ZnO grown on prespex by PLD. (C) *S. loihica* PV-4 biofilm formed on ITO and (D) ZnO electrode.

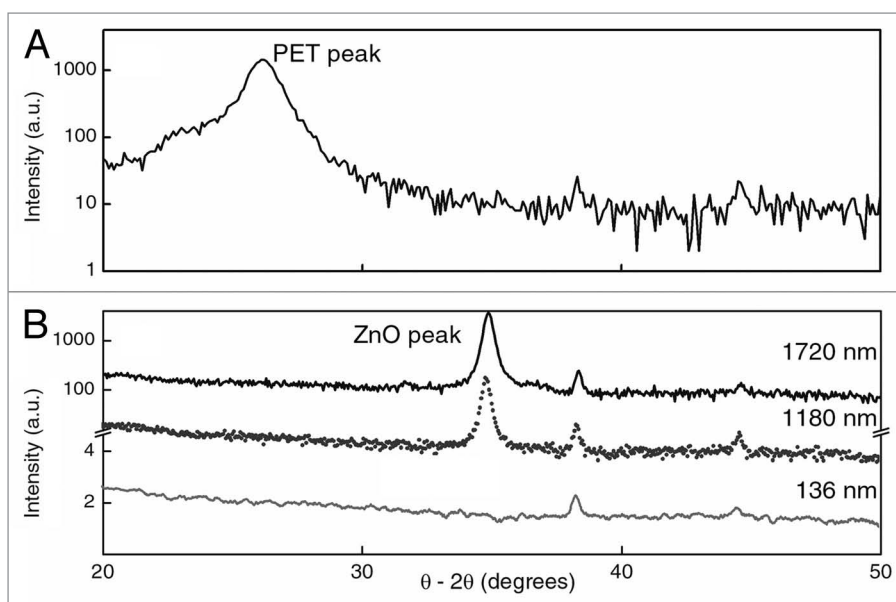


Figure 2. XRD patterns at $(\theta-2\theta)$ scans with the $\text{Cu K}\alpha$ wavelength of 0.154 nm of (A) ITO and (B) PLD deposited ZnO thin films of various thicknesses.

radicals prevent the growth of the facultative anaerobe *S. loihica* PV-4 due to the partial disruption of the cell membrane thereby decreasing the oxidation current.

Finally, the relatively high value of the electrical resistivity of the ZnO material used in the present work may have a limiting influence on the current production at the EAB/electrode interface. This drawback could be easily circumvented by suitable metal doping to produce ZnO material with lower resistivity.

Conclusions

This work showed that ZnO deposited on plastic substrates using pulsed laser deposition are

suitable for electrochemical characterization of facultative EAB forming strains. ZnO thin films with increased hydrophobicity and lower resistivity may result in higher EET at the interface biofilm/electrode.

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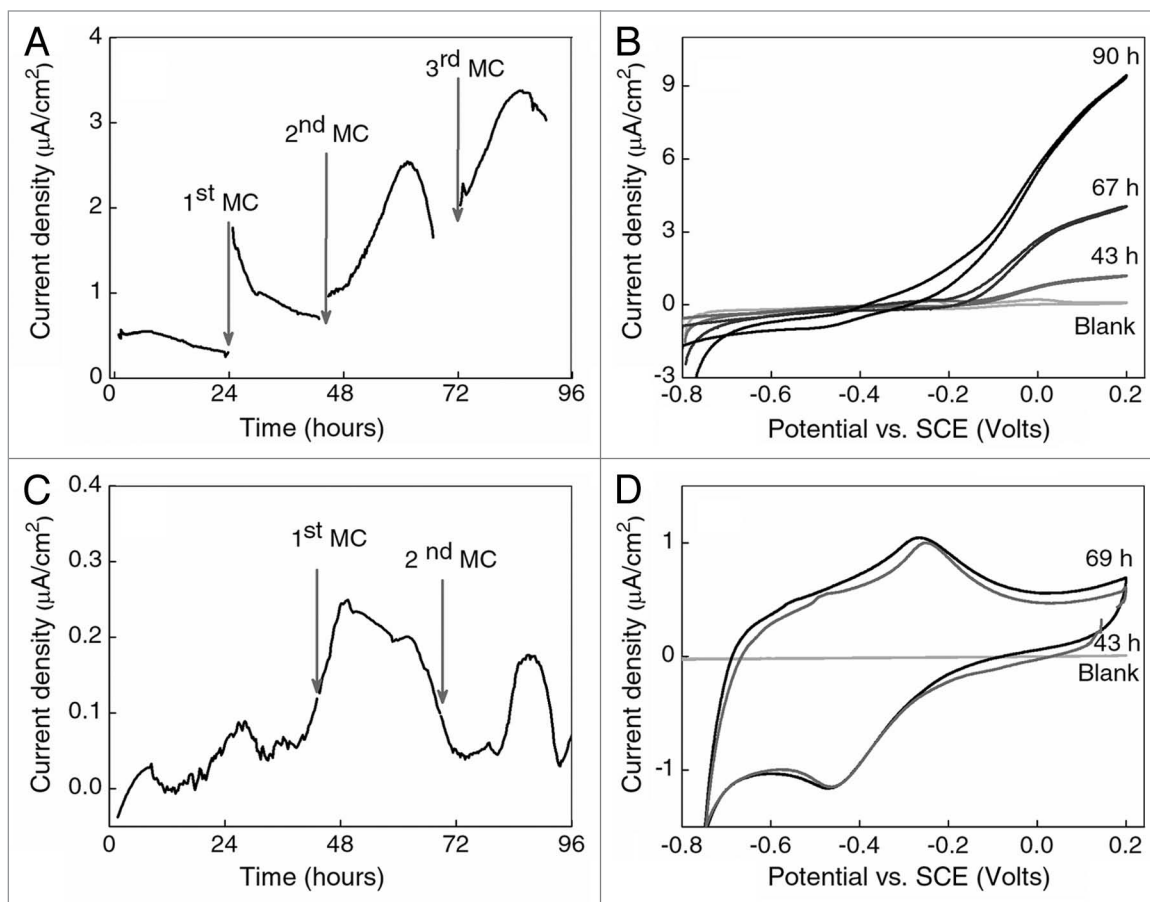


Figure 3. (A) CA of *S. loihica* PV-4 biofilm on ITO-coated PET electrode; (B) CV of the plain ITO electrode (blank) and of the biofilm at 43, 67 and 90 h; (C) CA of *S. loihica* PV-4 biofilm on ZnO-coated perspex electrode; (D) CV of the plain ZnO electrode, and of the biofilm at 43 and 69 h. In all experiments, MC was performed to replace the spent growth medium with fresh medium.

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Abstracts from the COST workshop

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Control of oral biofilms: Retention of beneficial properties

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A diverse range of microorganisms, of which bacteria are the most predominant, colonize and grow on the surfaces of the oral cavity. The largest accumulations of bacteria are found as

biofilms on the tooth surface (dental plaque) while on mucosal surfaces epithelial turnover controls the microbial load. Dental plaque was probably the first biofilm to have been studied

with respect to its microbial composition and sensitivity to antimicrobial agents. It is now one of the best-studied biofilms displaying all of the typical characteristic features. The

presence of dental plaque is regarded as natural and essential to the normal physiological development of the oral cavity. However, microbial homeostasis in dental plaque can be disrupted and disease occurs including dental caries and gingivitis. As part of the resident oral microflora, dental plaque acts as an important component of the host defenses by excluding exogenous populations through colonization resistance and contributes to the regulation of the inflammatory host response to commensal bacteria. Recent studies have

also shown the nitrate reducing activity of the oral microflora to be a key factor in the production of beneficial antimicrobial and vasoactive molecules. Oral healthcare products should therefore attempt to control the levels of plaque rather than attempting to eliminate it, so as to retain the beneficial properties. Control of dental plaque-related diseases has traditionally relied on non-specific removal of plaque by mechanical means. However, as our knowledge of oral disease mechanisms increases, future treatment is likely to be

more targeted, for example at small groups of organisms, single species or at key virulence factors they produce. The potential of nanoparticles to control the formation of biofilms within the oral cavity, as a function of their biocidal, anti-adhesive and carriage capabilities is also coming under scrutiny. Thus, more effective products that are able to improve clinical efficacy while preserving the beneficial aspects of the normal oral microflora are likely to be the development goals in the future.

Cell-to-cell communication (quorum sensing)

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Yersinia pseudotuberculosis biofilm development on *Caenorhabditis elegans* is regulated by quorum sensing-dependent repression of the Type III secretion system. Many Gram-negative bacteria employ a cell population density dependent regulatory system known as quorum sensing (QS) that couples gene expression with growth phase to control a variety of virulence-associated phenotypes. These systems are dependent on the synthesis and transduction of N-acylhomoserine lactone (AHL) signal molecules and in the human pathogenic species of *Yersinia* AHL synthase orthologs of LuxI (AHL synthase) and LuxR (response regulator) have been identified in *Yersinia pseudotuberculosis* (*ypsrI*, *ytbR/I*), *Yersinia enterocolitica* (*yenR/I*) and *Yersinia pestis* (*yprR/I*, *yepR/I*). We have shown that

Y. pseudotuberculosis and *Y. enterocolitica* synthesize long and short chain AHLs and that QS is important for activating flagella-mediated motility which, in *Y. pseudotuberculosis* acts by regulating the expression of two key regulators, *flhDC* and *fliA*. *Y. pseudotuberculosis* and *Y. pestis* form biofilms on the surface of the nematode worm *Caenorhabditis elegans* and this tractable host-pathogen model is therefore an important tool for examining biofilm development on biotic, motile surfaces. We show that *Y. pseudotuberculosis* biofilm development on *C. elegans* is governed by AHL-mediated QS since biofilms are attenuated when nematodes are infected with *Y. pseudotuberculosis* expressing an AHL-degrading enzyme or when the AHL synthase or response regulator genes respectively

are mutated. Similarly, when motility genes such as *flhDC*, *fliA* and *flhA* are mutated biofilms are also attenuated. While examining whether the flagella export apparatus was involved in the biofilm phenotype SDS-PAGE revealed that several proteins associated with the type three secretion (TTS) system were upregulated in motility mutants in a temperature and calcium independent manner. Similar observations were made for the *Y. pseudotuberculosis* QS mutants indicating that the TTS virulon is repressed by QS. By curing the virulence plasmid or by introducing a *yscJ* mutation we then showed that biofilms can be restored in *flhDC* and *ypsl/ytbI* mutants. Taken together these data show that biofilm formation is dependent on QS-dependent repression of TTS.

Cyclic-di-GMP signaling and biofilm formation of *Escherichia coli*

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The ubiquitous bacterial signaling molecule cyclic-di-GMP, which is produced and degraded by diguanylate cyclases (carrying GGDEF domains) and specific phosphodiesterases (EAL domains), respectively, regulates transitions between the motile-planktonic and sedentary biofilm "life-styles."¹ c-di-GMP controls a variety of targets, including transcription and the activities of enzymes and complex cellular structures. Many bacterial species possess many GGDEF/EAL proteins (29 in *E. coli*), which has led to the concept of temporal and functional sequestration of c-di-GMP control modules.¹ Some GGDEF/EAL domain proteins

(four in *E. coli*) have degenerate GGDEF/EAL motifs, are enzymatically inactive and can act by direct macromolecular interactions. In *E. coli*, c-di-GMP signaling is tightly integrated with the general stress response, as many GGDEF/EAL genes are regulated by RpoS.³ Moreover, c-di-GMP-dependent down-regulation of motility and induction of biofilm-associated functions such as the production of (auto)adhesive curli fimbriae occur during entry into stationary phase and require RpoS.²

The talk will present an overview of the molecular mechanism of switching from motility to adhesion, which is based on a mutual

inhibition of the FlhDC/motility and RpoS/CsgD/curli control cascades involving c-di-GMP signaling, and the molecular function of a degenerate EAL domain protein, which acts as a direct anti-repressor in a pathway that modulates biofilm architecture.⁴ Taken together, these studies have also generated a novel general concept of the evolution of complex bacterial second messenger signaling.¹

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Biofilms and their significance in clinical microbiology

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In spite of the large amount of experimental data currently available, a number of open issues is still to be addressed on the significance of the microbial biofilms in clinical microbiology and in the pathogenesis of biofilm-based infections, including:

(1) The disclosure of interaction mechanisms in multispecies biofilms;

(2) The validation of a model to explain the higher antimicrobial resistance of biofilm-growing microorganisms;

(3) The detection of specific agents able to disrupt mature biofilms or to interfere with quorum sensing phenomena;

(4) The finding of advanced anti-biofilm materials to prevent biofilm-based device-related infections.

Even if Gibbons and Nygaard described since 1970 the co-aggregation as a specific phenomenon of recognition and mutualistic or competitive interaction among different species in the oral cavity, most of the investigations have concerned single-species biofilms, so disregarding the pivotal role of multispecies biofilms in causing acute and chronic infections in the respiratory, gastrointestinal and genitourinary apparatus.

Anyway, single- and multispecies biofilms are difficult to eradicate with conventional antibiotics. In fact, an antimicrobial resistance 10–1,000 times higher has been observed in sessile growing microorganisms in comparison with the planktonic forms. However, we haven't so far a general model able to properly take into account our present knowledge on the higher antimicrobial resistance of biofilm-growing microorganisms, even if the occurrence of the following phenomena has been reported: (1) the reduced and slow antibiotic penetration due to the physical barrier represented by microbial exopolysaccharides; (2) the high conjugation frequency that promotes the exchange of plasmids, including genes coding for multidrug-resistance; (3) the decreased metabolic activity due to the top-bottom gradients of oxygen and nutrients and the related prolonged doubling time of microbial cells; (4) the aspecific binding of antimicrobial molecules with microbial catabolites; (5) the emergence of dormant and persister cells. In this regard, it should be remembered that most of the studies have been carried out in vitro on single-species biofilms.

To overcome the difficulties encountered in the antimicrobial treatment of

biofilm-associated infections, alternative approaches based on specific agents able to disrupt mature biofilms or to interfere with quorum sensing phenomena have been proposed. Among those experimented in the recent years, the most promising agents seem to be the matrix-disrupting enzymes Dispersin B and alginate lyase while different molecules extracted from natural sources are under investigation as possible quorum sensing inhibitors.

Finally, a serious health issue is represented by the increasing occurrence of infections related to the implant of medical devices, including dental prostheses, catheters, stents and orthopedic prostheses.

Our team is currently involved in research projects addressing some aspects of the above mentioned open issues: (1) the ability of anaerobic bacterial species isolated from clogged biliary stents to form dual-species biofilms; (2) the in vitro study on the efficacy of antibiotics reported to be more active as "biofilm killers" and the possible synergistic role of matrix-disrupting enzymes; (3) the development of novel anti-biofilm coatings able to counteract microbial adhesion and biofilm formation on medical device surfaces.

Study of biofilm formation by foodborne pathogenic and technological bacteria on model stainless steel surfaces, under monospecies and dual-species conditions, and evaluation of resistance of these sessile communities to chemical disinfectants

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Biofilm formation is a natural phenomenon that happens almost wherever microorganisms and surfaces exist in close proximity. Attachment of pathogenic bacteria onto food-contact surfaces and subsequent biofilm formation is undesirable, since detachment of cells from biofilm structure can lead to cross-contamination of food products and cause foodborne diseases. The risk becomes even more serious since bacteria enclosed in biofilms have been shown to express increased resistance to many antimicrobial treatments compared with their planktonic counterparts. In the majority of natural environments, monospecies biofilms are relative rare. Conversely, microorganisms are associated with surfaces in complex multispecies communities. In the

food industry of fermented products (dairy, fermented sausages, vegetables etc), mixed bacterial biofilms may contain strains beneficial to the safety and quality of the final product (e.g. lactobacilli and staphylococci). In the present study, the ability of nine bacterial strains to form biofilms onto model stainless steel surfaces was studied. These strains were selected from totally 30 strains that had been initially screened for biofilm formation on different abiotic substratums under various environmental conditions. They belonged to the well-known foodborne pathogens *Listeria monocytogenes* and *Salmonella enterica*, as well as to the useful species *Lactobacillus sakei*. Strains of *L. sakei* are nowadays used for the production of fermented meat products in

many European countries. Three strains were employed for each species and cells were left to form biofilms, at 15°C and 30°C, under two different mixed-culture conditions; that is either three strains of same species together, or six strains of two different species together. It is believed that microbial interactions influence the biofilm forming capacity of individual strains, as well as their resistance to antimicrobial treatments. However, present results did not reveal any significant differences at the levels of biofilm populations for each bacterial species separately, among the two different culture conditions (monospecies, dual-species). The minor differences which were observed were found to be dependent on both the species employed and the

incubation temperature (15, 30°C). Afterwards, the disinfection ability of three common chemical antimicrobial compounds (benzalkonium chloride 50 ppm, chlorine 10 ppm and peracetic acid 10 ppm) against biofilm cells was studied. Under current conditions, *L. monocytogenes* presented higher resistance

to the three disinfectants compared to the other two bacterial species. Not important differences, with regard to antimicrobial resistance of the three bacterial species separately, were revealed among the two different culture conditions (monospecies, dual-species). Present study offers important knowledge on

the abilities to form and eliminate bacterial biofilms, under conditions simulating real food processing environments. In the future, we plan to evaluate the individual contribution of each strain both in the formation of the complex biofilm community, as well as in the resistance of it to antimicrobials.

Electro-active biofilms: When microbiology and electrochemistry meet

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Microbial fuel cells (MFC) can transform the chemical energy from cheap organic compounds directly into electrical energy. The concept was known since the 70s, but with little prospect of industrial development in the short or medium term. A fundamental breakthrough occurred in 2002: the unexpected discovery of microorganisms capable of catalyzing the oxidation process at the surface of graphite electrodes. These microorganisms can attach spontaneously to the surface of materials and form a three-dimensional structure more or less organized called electro-active biofilm (EAB). The EABs can be formed artificially in laboratory from pure culture of microorganisms (*Geobacter sulfurreducens*, *Rhodospirillum rubrum*, *Shewanella oneidensis*, etc.) or can develop spontaneously on electrodes immersed in natural environments rich in microorganisms such as sediments or sludge from wastewater treatment plants. With this new type of electro-catalysis assisted by microorganisms, the MFC technology produce electrical energy by oxidizing a large diversity of organic compounds (acetate, volatile

fatty acids, saccharides...) contained in natural environments. Currently, EAB catalyzing reactions of microbial electro-oxidation are the most commonly studied by the international scientific community working on MFC. At the "Laboratoire de Génie Chimique" (CNRS – University of Toulouse), a new field of research was opened on microbial catalysis of oxygen electro-reduction following the progress achieved in the field of biocorrosion. Indeed, marine EABs able to catalyze the electrochemical reduction of oxygen have been detected from seawater subsurface in 2005.¹ The reconstruction of this type of marine aerobic EAB was then perfectly controlled in laboratory five years later, in 2010.² In the same time, culture-dependant isolations led to the identification of three pure electro-active strains catalyzing the oxygen electro-reduction on stainless steel electrodes with performances similar to the oxygen electro-reduction on a pure platinum electrode.²

Now the project is to develop the research on these aerobic EAB beyond the current knowledge in two complementary ways:

- Building the necessary basic knowledge on "Stainless Steel/EAB" interfaces. The tools of microscopy and molecular biology will then be combined to understand how EAB is structuring on the surface of stainless steels. Advanced techniques in electrochemistry at the local level (as SVET for example) will be used to pierce the mysteries of electron transfer between microorganisms and the surface of stainless steel.

- Using these fundamental advances in designing optimal microbial cathodes that will lead the MFC technology to a pre-industrial development. The combination of different scales of electrodes structuring (nano- and/or micro-structuring) to promote bacterial adhesion and the electron transfer is an original approach to maximize the catalytic performance of the interfaces. Also, a strategy combining the stabilization of the reaction and the improvement of mass transport inside the biofilm structure should consistently improve the stability of microbial cathodes.

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Is *Candida* spp. biofilm eradication possible in vitro?

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Candida species represent the fourth most common cause of nosocomial bloodstream infections. The management of candidiasis associated with catheters remains difficult since these medical devices can act as substrates for biofilm growth. Fungal biofilm is a complex phenomenon that may occur within three days of catheterization. *Candida* species, especially *C. albicans*, are the most common fungal species associated with biofilms. Different approaches which could contribute to the prevention or inhibition of *Candida*

biofilms associated with invasive indwelling medical devices or superficial devices have been developed and evaluated. This talk deals with the strategies which could contribute to fight *Candida* biofilms in vitro. We especially investigated *C. albicans*, *C. glabrata* and *C. parapsilosis* species because of their major implication in device-related candidiasis. Our studies tried to mimic three inhibition strategies able to prevent *Candida* spp. adherence and/or subsequent biofilm formation. The first one corresponded to long-term systemic

therapy (at least 24 h) using antifungal agents; some of them could significantly inhibit biofilms with different maturation ages. This strategy mimicked a curative way. The anti-biofilm interest of natural compounds, especially terpenic derivatives, was also investigated in this way. The second one was to prevent or delay fungal colonization in vitro, by coating or grafting active compounds on the catheter surface. Antiseptic as well as antifungal agents and natural compounds have been tested. This second strategy corresponded to

a prophylactic way. Finally, the third one corresponded to the use of antifungal lock solutions. The antifungal-lock technique involves in vitro the static instillation of a concentrated solution of antifungal agent (corresponding to 100–500 MIC) into the catheter, and allowing it to remain for an extended period, usually no more than 12 h. It could represent a logical step

to prevent colonization of long term devices and thereby reduce the rate of catheter related bloodstream infections. This one could also be used in a curative approach, if associated with systemic therapy. In conclusion, *Candida* spp. biofilm inhibition involves in vitro a lot of various approaches and we can hope that some of these will really contribute to reduce fungal

in vivo colonization and prevent candidiasis associated with medical devices. However, our in vitro results suggested that once the *Candida* spp. biofilms were developed on the device surface, it was quite impossible to obtain their overall eradication, whatever the used approach, suggesting the possible interest to use combined strategies.

Bacterial-fungal interactions and new model hosts for the study of biofilms

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Candida albicans is an important cause of morbidity in hospitalized and immunosuppressed patients. Virulence factors of *C. albicans* include: filamentation, proteinases, adherence proteins and biofilm formation. Prokaryote-eukaryote interactions are ubiquitous and have important medical and environmental significance. Although bacterial-fungal interactions shape microbial virulence during polymicrobial infections in biofilms, a limited number of studies have evaluated this interaction on a genetic level. We have developed a novel alternative to study microbial pathogenesis and host responses: the use of invertebrate model hosts and especially the nematode *Caenorhabditis elegans*. *Candida albicans* and the Gram-negative bacterial pathogen *Salmonella enterica* serovar *typhimurium* are pathogenic to the nematode *Caenorhabditis elegans*. We observed that when *C. elegans* is infected with *C. albicans* and *S. typhimurium*, *C. albicans* filamentation is inhibited. Importantly, the antagonistic interaction was also observed in a *C. albicans* biofilm environment.¹ The interaction is mediated by *sopB*, an effector of a Type III secretion system (TTSS)

of *S. typhimurium*. Using quantitative real time-PCR assays, we found that the *Candida* supernatant upregulated the *S. typhimurium* genes associated with *C. albicans* killing (*sopB* and *sipB*). Interestingly, the *sopB* effector negatively regulated the transcription of *CDC42*, which is involved in fungal viability.² Similarly, in vitro coinfection assays in planktonic and biofilm environments supported the inhibitory effects of *Acinetobacter baumannii* toward *C. albicans*.³

Moreover, there is an urgent need for the development of new agents with activity against biofilms. However, antimicrobial drug discovery has been limited by numerous obstacles including the failure to identify new cultivable microorganisms, a high background of toxic compounds or compounds with poor pharmacokinetic properties in synthetic compound libraries and the inability of most synthetic leads to penetrate across the multidrug resistance (MDR) pump barrier of Gram-negative bacteria. We have bypassed some of these obstacles by developing whole-animal high-throughput screens that utilize *C. elegans* as a model host to

simultaneously identify new classes of antimicrobials with antivirulence or immunomodulatory efficacy and evaluate toxicity/efficacy. We devised a *Candida*-mediated *C. elegans* assay that allows high-throughput in vivo screening of chemical libraries for antifungal activities, while synchronously screening against toxic compounds. Compounds identified in the screen that affect the virulence of *Candida* in vivo can potentially be used as “probe compounds” and may have antifungal activity against other fungi. Importantly, *C. albicans* hyphae and biofilm formation were also disrupted in the presence of natural products identified through these screens.⁴ In conclusion, our investigations have identified novel virulence factors, cross kingdom pathogen-pathogen interactions and evolutionarily conserved traits that are involved in biofilm formation.

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Efficacy of a *Staphylococcus aureus* biofilm-embedded bacterin against coagulase-negative staphylococci intramammary infections in dairy cows

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Mastitis is the most important disease in dairy cattle, including both clinical and subclinical infections, that affects quality and quantity of milk and is accompanied by increased health care costs, higher culling rates and sometimes even death.

Intramammary infections are usually initiated either by colonization of the teat canal with bacteria derived from infected epidermis or by an influx of contaminated milk entering the gland in milking machines.

Many chronic infections are associated with a bacterial growth in the form of adherent colonies surrounded by an extracellular matrix, constituting a biofilm. Because of their aggregate size, biofilms are not susceptible to macrophage or neutrophil phagocytosis and become resistant to some antibiotics. On the other hand, detachment of biofilm bacteria cells is a cause of new colonizations, while the production of endotoxins and exotoxins (within the biofilm and by detached

planktonic cells) produce inflammation and tissue damage.

Various studies have demonstrated the in vitro biofilm formation ability in the main pathogens isolated from bovine mastitis: *Staphylococcus aureus*,¹⁻³ *Escherichia coli*⁴ and *Streptococcus uberis*.⁵ With regard to coagulase-negative staphylococci (CNS) isolated from bovine mastitis (the most frequently isolated bacteria in cows' milk samples), the in vitro biofilm formation have been determined

in *S. epidermidis*² and other coagulase-negative species.⁶

The aim of this study was to characterize the CNS species isolated from cows included in a field trial to check the efficacy of Startvac[®] vaccine in a commercial dairy herd with sub-clinical CNS mastitis problems. The Startvac[®] vaccine contains a bacterin from a strong biofilm producing *S. aureus* strain expressing SAAC (Slime Associated Antigenic Complex).⁷ A total of 331 healthy lactating cows and heifers with somatic cell counts lower than 150,000 cells/ml were randomized to receive either vaccine or placebo. The animals were

milk sampled on a monthly basis for five months, for enumeration of somatic cell count (SCC). In those animals showing SCC > 150,000 cells/ml, California Mastitis Test (CMT) was performed by quarter. CMT-positive quarters were sampled for microbiological analysis, and followed up until they had two consecutive negative isolations. A total of 93 CNS were isolated: 64 in the placebo group and 29 in the vaccinated group. All the 93 CNS isolates were examined for the in vitro slime production ability by the Congo red agar (CRA) plate test: slime production was detected in the 37% of the control group isolates and in the

31% of the vaccinated isolates. CNS were also identified to the species level by means of tDNA-PCR.

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Towards understanding gene expression in multispecies biofilms containing *Salmonella typhimurium*

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Salmonella is still one of the most important food-borne pathogens causing millions of infections each year. An important problem of Salmonella infections is the fact that the pathogens are frequently incorporated in surface-bound multicellular structures, i.e. biofilms, which are difficult to combat. Currently used anti-biofilm strategies are often not effective against these biofilms due to differences in global gene expression and regulation patterns as compared to planktonic cells. Moreover, in situ biofilms exist of more than one microbial species. These multispecies characteristics make metabolic regulations and gene expression profiles even more complex. In order to develop novel anti-Salmonella therapeutics, also effective against biofilms, profound insight in these processes under these alternative physiological states (i.e. monospecies as well as multispecies) is needed. As such, our research focuses on the expression patterns of *Salmonella typhimurium*

genes in monospecies and multispecies biofilms. Previous studies in monospecies *S. typhimurium* biofilms using the Differential Fluorescence Induction (DFI) technique identified 26 promoter fusions showing biofilm-specific upregulation.¹ The major advantage of performing this type of a genome-wide screening is the generation of data on single cell level instead of a mean value for the whole population. This way, the heterogeneity, which is inherent to biofilms, can be taken into account. This heterogeneity is even more important in complex in situ biofilms containing multiple species. Therefore, we are using a library of defined promoter constructs (from previous in house research as described in ref. 1 and literature) in a model multispecies biofilm containing two *S. typhimurium* serotypes and *Escherichia coli*. Moreover a genome-wide study, identical to the DFI monospecies research, is being optimized in this specific multispecies context. Due to the effects of the

complex heterogeneous environment in such multispecies biofilms, a change in gene expression patterns and regulation is to be expected, as our preliminary results indicate. This way, it becomes possible to discriminate between genes that are important (i.e. upregulated or downregulated) during biofilm formation in general and genes that are specifically involved in biofilms with a certain microbial composition. Hereto, we will not only use the above mentioned model multispecies biofilm, but we will also try to reconstitute (qualitatively and quantitatively) in situ biofilms, especially from the food processing industry. Identified upregulated genes (and their products) in biofilms can lead to new targets to combat biofilms. Therefore, knowledge generated during this research can help the identification and rational design of potential biofilm-inhibiting molecules, that specifically target biofilm formation.

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Developed microbial biofilms for sustainable agriculture

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Conventional agricultural systems have been threatened by indiscriminate use of chemical inputs in the long run. Consequently, they have depleted the beneficial microbial communities resulting in declined soil fertility and low crop productivity. This study examined the effect of developed microbial biofilms with N₂ fixers on restoration of soils deteriorated by conventional agricultural practices in tea cultivation.

The lower chemical fertilizer applications (i.e. reducing recommended chemical fertilizer use by 50%) coupled with the biofilm-based biofertilizers known as biofilmed biofertilizers (BFBFs) increased biological nitrogen fixation significantly. Simultaneously it increased soil microbial biomass and decreased soil NO₃⁻ and pest infestation. Further, the combined application significantly increased soil organic C by 20%,

and reduced leaf transpiration by 40%. It also supported plant growth, rhizoremediation and soil moisture conservation in comparison to the 100% chemical fertilization. It is apparent from this study that replenishing the depleted soil microbial communities by applying such biofertilizers is likely to be beneficial in agroecosystems with chemical N fertilizer use, if they are to be sustained for crop production.

Developed microbial biofilms: A novel rhizoremediation tool to heal soil sickness

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In Sri Lanka, tea [*Camellia sinensis* (L.) O. Kuntze] is the main agricultural foreign exchange earner. However, dieback of tea bushes and yield decline have been reported from some tea estates in many regions of the country. It is hypothesized that this is due to collapse of beneficial soil microbial communities under conventional agriculture and gradual accumulation of phytotoxic allelochemicals in the tea soil. This study was done to investigate the effect of application of developed microbial communities like Biofilmed Biofertilizers (BFBFs) on soils with phytotoxic allelochemicals.

To evaluate the phytotoxic effect, lettuce seed germination test was employed with BFBFs treated and untreated soil samples which were collected from affected tea estates. Then organic solvent extracts of above soils were subjected to Fourier transform infrared (FTIR) analysis. Finally, accumulations of allelopathic phytotoxins in drought conditions were simulated in the laboratory using PEG 6000, and plant responses were measured. The lettuce seed germination test in original soil samples showed zero or low germination (0-17%), while BFBFs treated soil samples showed

significantly high germination percentage (73-84%). In the FTIR spectra, the untreated soil gave a peak at 1744 cm⁻¹ that is responsible for a quinone that acts as an allelopathic phytotoxin, but BFBFs had evidently depleted the quinone, as reflected from the peak degradation of the spectra. Thus, it is clear that the well developed microbial biofilms like BFBFs can revive the microbial diversity and action in tea soils, which help biodegrade the accumulated allelopathic phytotoxins, and reduce the severity level of drought stress, favoring plant growth.

Development of anti-biofilm agents and elucidation of their mode of action

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The objective of our interdisciplinary research consortium is the development of anti-biofilm agents that specifically target bacterial biofilms without affecting the planktonic growth of the bacteria. These specific anti-biofilm agents have the advantage of being less prone to the development of resistance than classical biocides or antibiotics. Moreover elucidation of their biofilm-specific mode of action reveals insights into the molecular events of biofilm formation and is likely to result in the identification of new cellular targets for biofilm inhibitors. The anti-biofilm agents have potential to be used in a preventive fashion (e.g. as coatings on medical implants and devices) or to eradicate biofilms (possibly in combination with antibiotics or disinfectants). *Salmonella Typhimurium*, *Pseudomonas aeruginosa* and *Escherichia coli* are the model organisms, against which the anti-biofilm agents are being developed. Our development strategy consists of (1) in vitro and in silico screenings of compound libraries for anti-biofilm agents, (2) chemical synthesis or purchase of a series of analogues of the identified hits, (3) structure-activity relationship studies (SAR) and structure-toxicity relationship studies, (4) mode of action studies of the lead compounds and (5) further evaluation and development of the lead compounds (e.g. synergy studies,

use of more applied test systems, formulation, ...). To this end a microbiological laboratory (CMPG), a laboratory for biomolecular modeling (BioMOL) and two chemistry groups (COK, LOMAC) of the K.U. Leuven are taking part in the consortium.

Results and perspectives

- Brominated furanones have previously been shown to inhibit quorum sensing and biofilm formation of several bacterial species. We synthesized a library of 25 1'-substituted and -unsubstituted 3-alkyl-5-methylene-2(5H)-furanones and 3-alkyl-maleic anhydrides, by using known and newly developed chemistry, and tested their effect against *Salmonella* biofilms. The most active furanones have IC50 values around 1 μM. No evidence was found that furanones act on the currently known quorum-sensing systems in *Salmonella*. However, the furanones were found to interfere with the synthesis of flagella by *Salmonella*. Interestingly, pretreatment with furanones rendered *Salmonella* biofilms more susceptible to antibiotic treatment.

- An in silico screening of the commercial 'Specs' small molecule library revealed a 2N-substituted 2-aminoimidazoline as inhibitor of biofilm formation. Analogs with either a 2-aminoimidazoline or a 2-aminoimidazole scaffold were synthesized by using newly

developed chemistry. Among the 250 2-aminoimidazole-based compounds synthesized, (4)5-phenyl-2-amino-1H-imidazole was found to have a moderate inhibitory activity against *Salmonella* and *Pseudomonas* biofilm formation. However, substitution of the N1-position, the 2N-position and the 4(5)-phenyl ring enhanced the activity up to 100 times (IC50 ~1 μM). Furthermore, we demonstrated the biofilm inhibitory effect of the (2-hydroxy-2,3-dihydro)-imidazo[1,2-a]pyrimidinium salts, the chemical precursors of the 2-aminoimidazoles (135 compounds). Mode of action studies revealed two possible cellular targets of the imidazoles, which are currently under further investigation. The 2-aminoimidazoles have been introduced in (1) a battery of anti-biofilm challenge tests (with industrial partners) and (2) a project on anti-biofilm coatings for implants.

- A high throughput screening of a library of >20,000 very diverse compounds (CD3) resulted in the identification of 140 anti-biofilm agents. Four compound families have been selected for which extensive SAR studies are currently being performed. Furthermore, screening of a library of 672 novel peptides, isolated from mouse and man, resulted in the identification of five peptides with anti-biofilm activity.

Biofilm growing intestinal anaerobic bacteria

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Sessile growth of anaerobic bacteria from the human intestinal tract has been poorly investigated even if recent literature data on observations by confocal laser scanning microscopy (CLSM) and experiments performed by using fluorescence in situ hybridization (FISH) have reported that mucosal anaerobes, including bacteroides and bifidobacteria, occur in microcolonies and are distributed throughout the mucus layer. Since very few facultative anaerobes can tolerate small levels of oxygen concentration, the investigation on the development of anaerobic bacteria as biofilm growing microbial communities is only obtainable by the use of appropriate gas mixtures in anaerobic cabinets, thus realizing the optimal conditions for their cultivation in

complete absence of oxygen. Our group has recently reported data on the close association existing between the clogging of biliary stents and the development in their lumen of a polymicrobial biofilm constituted by aerobic and anaerobic bacterial species other than by fungal species. Presently, it is believed that microbial colonization of the inner surface of the stent plays a major role in the clogging process. In fact, when a biliary stent is inserted across the sphincter of Oddi, the loss of the antimicrobial barrier represented by the sphincter itself and the low pressure in the common bile duct allow the reflux of duodenal content thus promoting an ascending microbial colonization. By using the clogged biliary stent as a model and exploiting the

explained stents as a generous source of biofilm growing anaerobes, our investigations have been focused on the in vitro ability of the isolated strains, belonging to the species *Bacteroides*, *Clostridium*, *Fusobacterium*, *Peptostreptococcus*, *Prevotella* and *Veillonella*, to form biofilms. Preliminarily, at least one strain belonging to each isolated species has been characterized for its ability to grow as biofilm by the in vitro slime production assay. Then, Field Emission Scanning Electron Microscopy (FESEM) and CLSM have been employed to observe and evaluate the mode of growth of both strong and weak slime-producing strains and to analyze the tridimensional structure of biofilms produced by the isolated anaerobic strains.