Microbial Interactions in Biofilms: Role of Siderophores and Iron-Dependent Mechanisms as Biocontrol Strategies

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Biofilms are ubiquitous in nature and can cause significant problems in public health, medicine and industry. Antimicrobial approaches to treat bacterial proliferation and biofilm formation constitute a focal point of modern research. We are entering a post-chemical antimicrobial era, not only due to the need to delivering of environmentally-friendly products, but also due to the increasing resistance of some pathogens against the most common antimicrobials, and the recalcitrance of biofilms even when treated with high concentrations of chemicals. An innovative approach to control biofilms can be the based on application of the concepts of microbial ecology, already under study in the development of biocontrol on seeds, roots and plants. The modes of action of those biocontrol systems can include, among others, the inhibition of microorganisms by antimicrobial compounds and competition for iron through the production of siderophores. To sequester and solubilise ferric iron, many microorganisms utilise an efficient system consisting of low-molecular mass compounds with high iron affinity termed siderophores (iron-scavenging agents). Siderophore production is a virulence factor of many microorganisms, as they can act as a biocontrol factor. In this review we will present some knowledge on the potential of siderophores and iron-dependent mechanisms, mainly related with Pseudomonas *spp., as a new line of biofilm control strategies.*

Microbial Biofilm Control Using Microbial Products

As has been well-documented, biofilm growth and biofouling are global problems causing tremendous economic problems in medicine and industry (Costerton *et al.* 1995). Bacteria in biofilms have additional resistance mechanisms that protect them against stress conditions, namely the exposure to conventional antimicrobial agents (Costerton *et al.* 1995; Simões 2005). Accordingly, there has been a great deal of research to better understand biofilm development and to identify improved strategies for biofilm control (Simões

2005). It is clear that new strategies for biofilm control are urgently needed, due to the failure of current procedures (Simões *et al.* 2003; 2005). For many years, researchers thought of bacteria as individual cells designed to proliferate under various conditions but unable to interact with each other and to collectively respond to environmental stimuli, as it is typical for multicellular aggregates. This view began to change few years ago with the discovery that microorganisms coordinate their behaviour *via* the secretion of specific signalling molecules in a population density-dependent manner (Davies *et al.* 1998). Biofilm formation and recalcitrance to conventional control procedures are related to cell-to-cell interactions, commonly designated quorum-sensing (QS), and with the involvement of secondary metabolites in biofilm development, mainly the mechanisms involved in iron sequestering (Banin *et al.* 2005). Quorum signalling is now recognized as a global regulatory mechanism for biofilm formation. Bacterial cells can produce and sense signal molecules (autoinducers), allowing the whole population to initiate a concerted action once a critical concentration (corresponding to a particular population density) of the signal has been reached, a QS phenomenon (Surette *et al.* 1999). It is conceivable that QS inhibition may represent a natural, wide spread, antimicrobial strategy with significant impact on biofilm formation (Bauer and Robinson 2002; Dong *et al.* 2002; McLean *et al.* 2005). QS inhibition is also associated with food spoilage events, mainly due to the production of extracellular enzymes (Dunstall *et al.* 2005).

Interspecies interactions constitute an improved and alternative biocontrol strategy. Studies revealed that co-cultures of microorganisms can improve the production of antimicrobial metabolites, as found out by Røssland *et al.* (2005) by the co-culture of *Lactobacillus* spp. and *Lactococcus* spp. with *Bacillus cereus*, microorganisms commonly found in dairy industry. Furthermore, although most laboratory biofilm studies involve only a single species, it is important to note that biofilms formed in nature often include multiple bacterial species. Interactions between microorganisms are well-known phenomena, and in many environments, substrate and space competition and antagonism is a powerful selective force which has led to the evolution of a variety of effective strategies for colonising and growing on surfaces (Fux *et al.* 2005; Gram *et al.* 1999). Studies of such interactions in certain niches, e.g., in the rhizosphere, have been very detailed, but other niches, such as food industry, have received less attention (Hass and Défago 2005). It seems important to learn more about these interspecies interactions as they may play a crucial role in both competition and cooperation between different species in natural environments. These studies can provide information about the bioregulation effects within a multidrug resistant biofilm. As an example, a single species, by microbial inclusion or cross-species induction, can produce an extracellular metabolite that can act as a biofilm control agent in a complex multi-species system (Dong et al. 2002; Orsi 2004, Yan *et al.* 2003).

A number of transition metals, such as iron, are needed by bacteria as vital constituents, but their availability in the environment may not be sufficient to support microbial growth, even in biofilms where bacteria show a decreased metabolic activity. To sequester and solubilize ferric iron, many microorganisms synthesise siderophores, low-molecular mass (<1000 Da) compounds with high iron affinity. According to the generally accepted definition, siderophores are ferric-specific microbial iron-chelator compounds whose biosynthesis is regulated by the availability of iron in the surrounding medium (Orsi 2004). Under conditions of high iron concentration the production of these compounds is repressed. Iron Fe^{3+} ions have a very low solubility at neutral pH and therefore cannot be utilized by microorganisms. Siderophores dissolve these ions, essential for microbial survival, interactions and biofilm formation (Banin *et al.* 2005; Gram *et al.* 1999). As soluble Fe^{3+} complexes they can be taken up by active transport mechanisms. A universal method to detect and determine siderophores was developed using their high affinity for Fe^{3+} with the complex chrome azurol S (CAS)/Fe^{3+}/hexadecyltrimethylammonium bromide serving as indicator (Schwyn and Neilands 1987). Other methodologies were developed using the principles of Schwyn and Neilands (1987), as can be found in Table 1.

Table 1 Methodologies currently used to assess microbial siderophore production

Reference	Technique	Purpose
Schwyn and Neilands (1987)	Original methods for detection and determination of siderophores	Liquid assay for siderophores in solution, agar testing for solid inocula
Milagres *et al.* (1999)	Schwyn and Neilands (1987) solid assay method modification for application to a wider range of microrganisms	Solid assay: 2-types agar for better growth of microorganisms
Gram *et al.* (1999)	Spectrophotometric detection of siderophores using liquid CAS	Liquid assay for siderophore presence/determination
Vellore (2001)	Schwyn and Neilands (1987) solid and liquid modified assay for siderophore detection using	
Machuca and Milagres (2002)	Correlation between siderophore production in solid and liquid media (for fungi)	Siderophore production profiling
Sritharan and Asuthkar (2004)	Schwyn and Neilands (1987) solid assay method modification	2-layer agar assay for maximizing bacterial growth
Carrilo – Castañeda *et al.* (2005)	Spectrophotometric method to determine siderophore production	Spectral assessment of siderophore presence

Siderophore production can be thought of as a virulence factor in many microorganisms, acting as a biocontrol factor (Gram *et al.* 1999). In particular, such biological mechanisms, alone or as part of combination procedures could provide a new line of efficient biofilm control compounds. A pioneer study indicated that siderophore-containing *Pseudomonas* spp. culture supernatants inhibited growth of *Shewanella putrefacians*, as did the addition of iron-chelators (Gram 1993).

Siderophores – Biofilm Architects

The role of iron, and thus of siderophores, in the bacteria world goes beyond that of basic survival. Iron has an important structural function, determining biofilm and aggregate formation. When deprived of this key element, bacteria and their non-adherent aggregates, tend to abandon the planktonic state. Nevertheless, before adhering to a surface bacteria already establish a thin floating biofilm. As soon as it becomes well-attached to a surface, a biofilm thickness-augmentation starts taking place (Berlutti *et al.* 2004).

The effect of iron on biofilms is not, however, linear. The virtual absence of iron leads to the same effect as its excess: biofilm formation inhibition. For each species there will be an optimal iron concentration interval, maximizing biofilm establishment and development. When outside those limits, the bacteria tend to exist solely in the planktonic state (Musk *et al.* 2005). Furthermore, an iron excess will not only impair biofilm formation, but also increase disruption, culminating in destruction, of pre-established biofilms. This fact might be linked with the oxidative damage, originated by an iron overload. However, in particular cases, it has been noticed that iron excess leads to biofilm formation. Such reaction might be in response to unfavorable, or less favorable, conditions (Visca *et al.* 2006). This iron unavailability limits bacterial growth itself, *in vivo*, being able to act, *per se*, as a therapy to control the infection. Nevertheless, experiments designed to mimic conditions found by bacteria in the human lung, have revealed a direct and positive link between iron deficiency and *P. aeruginosa* growth (Musk *et al.* 2005).

From the suspicion that iron plays a main role in the *P. aeruginosa* biofilm formation scheme, studies were carried out seeking to assess how exactly iron interferes with these communities. It has been found that the lack of a functional iron uptake system will still lead to biofilm formation, but with the cells presenting a different phenotype. Normal strains, producing at least one of these species siderophores (pyochelin and pyoverdine), form mushroom-like structures. Mutants that do not produce any, originate flat biofilms even if inorganic iron is present in the medium. This can be reverted by addition of other iron sources, such as ferric dicitrate or desferrioxamine (Banin *et al.*

2005). Furthermore, the iron sequestration capacity of certain molecules, such as lactoferrin, is reflected on a reduced bacterial growth and, consequently, biofilm establishment. This results from the effect that low-iron concentrations has on bacteria. The lack of this iron instigates them to twitch and wander, instead of clustering and/or settling on a surface. Although limiting biofilm formation, lactoferrin does not affect biofilms already formed, contrary to an iron excess.

The influence that some molecules have on biofilm establishment and development might, on the other hand, be an indicator that conditions are not favorable to their formation. By acting as a signal, it can prevent an energy spoil, which would result from the attempt to form biofilms, in contexts where they would most likely fail (Singh *et al.* 2002).

Mashburn *et al.* (2005) found that co-culturing *P. aeruginosa* together with *Staphylococcus aureus* leads to a change in its physiology and pathogenesis, being such a change closely related with iron availability. Whether by inducing *S. aureus* autolysis, or by directly provoking its lyses, *P. aeruginosa* detects higher iron levels when grown together with this species, than when grown alone. The possible use of iron derived from *S. aureus*, probably from its proteins, is likely to be less-expensive and a more profitable alternative to siderophore biosynthesis, release and uptake, as a source of iron.

In human oral cavities, although not being the main factor determining *Streptococcus mutans* biofilm formation, iron is responsible for its noticeable augmentation. Such effect was registered both in biofilms attached to surfaces, and in those floating in a fluid phase. The iron deficiency promptly impels bacteria to aggregate among themselves, originating thin sheets of floating aggregates that, with the perpetuation of iron deficiency, tend to adhere to an available surface. In the same way, once iron becomes available, this tendency is inverted (Berlutti *et al.* 2004). Whether in biofilm communities, or as planktonic individuals, iron can play an additional role of trade coin in the bacterial society. Similarly to some aspects of human societies, bacteria can share their siderophores with non-producer strains that identically have the need of iron for their proper development. This molecule sharing, also known as cooperation, does carry additional costs for the producers. It would then be expect that, with selection, the solitary living would be favored so as to avoid exploitation. Despite this, producer individuals seem to still bear such burden. Such an option might be taken due to a possible higher positive counterpart, resulting from the social type of living. Hence, siderophore sharing could indeed be worth it (Velicer 2003).

This particular type of cooperation agreement is, furthermore, associated with relatedness and scale of competition. A broader scale of competition, affecting

wider levels of assemblage, usually leads to a higher cooperation. On the other hand, as the competition level becomes narrower, and thus more local, relatedness seems to be less of a reason for altruistic behaviors. Ultimately, in a one–on–one competition, individuals seek self protection. Relatedness then becomes a less important cooperation motif, in inter bacterial relations, as competition extends itself to closely related individuals (Griffin *et al.* 2004). Siderophores can, additionally, act as signaling molecules, and therefore regulate proteins production, even their own.

Siderophores – Friends and Foes?

The bacterial need for iron is not, in some cases, restricted to that of need for growth. It is frequently related with virulence determination. Although sometimes not in full agreement, some authors have found that iron availability might directly influence the colonization capacity of the most problematic bacteria in cystic fibrosis: *P. aeruginosa*. Pyoverdine and pyochelin, its siderophores, have shown to be required for virulence expression in this bacterium. Nevertheless, it was found, at the same time, that any of these two siderophores holds a fundamental role in both infection and virulence (Takase *et al.* 2000).

The iron concentration additionally regulates other virulence factors such as Shiga toxin (from *Shigella dysinteriae*), Shiga-like toxin I (from enterohemorrhagic *Escherichia coli*), and diphtheria toxin (from *Corynebacterium diphtheriae*). The role of iron in gene expression is related with its own highly limited availability. Any alteration in its concentration will, therefore, constitute a signal which will alter bacteria genetic expression (Litwin and Calderwood 1993).

In the particular case of *Listeria monocytogenes*, iron availability affects several bacterial properties. An iron-deficient growth leads to a decrease in this bacterium surface hydrophobicity, together with a deep alteration of surface protein composition (Conte *et al.* 1996). Moreover, the capacity of iron to influence bacterial growth depends not only on its concentration, but also on the bacterial species themselves. Iron-binding proteins, such as lactoferrins (mammalian non-immune natural defences), have been found to hold a bacteriostatic capacity. These proteins are able to hinder the growth of several of the most fastidious bacteria, namely *Campylobacter jejuni, Legionella pneumophila, Salmonella typhimurium* and *Vibrio cholerae*. Such capacity is based in iron sequestration, as a way to make it unavailable for bacteria. Nevertheless, an increase in iron availability will reverse the bacteriostatic activity and consequently allow bacteria to reassume growth.

When in cognate iron-deprived situations, bacteria present a strategy to overcome and detour the lack of iron that impairs their growth. Such strategies involve the use of siderophores as competitors for this sequestration

(Valenti and Antonini 2005). Therefore, one of the major approaches of microbiological control, by bacteria, derives from their capacity of directly altering the iron availability. By depriving other species of it, bacteria inhibit the growth of the less competitive ones in terms of iron chelating capacity. Nevertheless, this siderophore-based bacteriostatic capacity can back fire, and lead to a competitive disadvantage, as some species, although not able to produce iron chelators themselves, can capture them from the medium. Thus, non-producer bacteria can then use these foreign siderophores for their own survival and growth, at practically no cost (Visca *et al.* 2006).

The bacterial siderophore capture can also be utilized as a strategy to lead them to, indirectly, uptake antibiotics. Trying to take advantage of this iron transport system, some antibiotics have been coupled with siderophores moieties, being therefore transported jointly into bacteria, and so, acting inside it. In this case, bacterial resistance to antibiotics can arise through mutations in the transport system. The major difference is that resistance is accompanied by negative outcomes for the bacteria itself. The lack of iron by faulty transport system implies a disturbance, even incapacity, of iron acquirement that, consequently, leads to a bacterial inability to grow and multiply (Martínez and Baquero 2002).

Siderophores can, furthermore, be used has control agents not due to their antimicrobial capacities, but to their crucial role in bacteria development. If bacteria have an absolute requirement of these iron scavengers to proceed with their growth, multiplication, and even virulence, it just might be possible to impede infections. Based on this, Ferreras *et al.* (2005) developed a new antibiotic that targets siderophores biosynthesis pathways. It will, consequently, interfere with the growth of their study-subject bacteria, *Mycobacterium tuberculosis* and *Yersinia pestis*, under iron limiting conditions.
Similarly, new alternative treatments can be developed against infections caused by bacteria already resistant to several therapies, constituting this a whole new approach, and a possible way of detouring the everyday more problematic resistance issue.

Acknowledgments

The authors acknowledge the financial support provided by the Portuguese Foundation for Science and Technology (Project CHEMBIO - POCI/BIO/61872/2004, SFRH/BD/31661/2006 – Lúcia Simões and SFRH/BPD/20582/2004 – Manuel Simões).

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