FEMS IMMUNOLOGY & MEDICAL MICROBIOLOGY

MINIREVIEW

The interconnection between biofilm formation and horizontal gene transfer

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Received 28 October 2011; revised 8 March 2012; accepted 8 March 2012.

DOI: 10.1111/j.1574-695X.2012.00960.x

Editor: Thomas Bjarnsholt

Keywords
biofilm; horizontal gene transfer; social evolution; plasmids; sociomicrobiology.

Abstract

Recent research has revealed that horizontal gene transfer and biofilm formation are connected processes. Although published research investigating this interconnectedness is still limited, we will review this subject in order to highlight the potential of these observations because of their believed importance in the understanding of the adaptation and subsequent evolution of social traits in bacteria. Here, we discuss current evidence for such interconnectedness centred on plasmids. Horizontal transfer rates are typically higher in biofilm communities compared with those in planktonic states. Biofilms, furthermore, promote plasmid stability and may enhance the host range of mobile genetic elements that are transferred horizontally. Plasmids, on the other hand, are very well suited to promote the evolution of social traits such as biofilm formation. This, essentially, transpires because plasmids are independent replicons that enhance their own success by promoting inter-bacterial interactions. They typically also carry genes that heighten their hosts' direct fitness. Furthermore, current research shows that the so-called mafia traits encoded on mobile genetic elements can enforce bacteria to maintain stable social interactions. It also indicates that horizontal gene transfer ultimately enhances the relatedness of bacteria carrying the mobile genetic elements of the same origin. The perspective of this review extends to an overall interconnectedness between horizontal gene transfer, mobile genetic elements and social evolution of bacteria.

Introduction

The evolution, adaptation and ecology of bacteria are intertwined mechanisms. Unveiling how such mechanics work and interrelate is of major importance when trying to understand the biology of bacteria. Grasping the links between adaptation and ecology has the potential to further our understanding of how, why, where and when bacteria evolve into, for example, pathogens or commensals of humans. Contributing substantially to bacterial evolution are genes that are transferred horizontally between bacteria. Whereas gene transfer within a single species results in the propagation of specific traits, interspecific gene transfer may lead to entirely new genetic combinations, which occasionally impose serious consequences to human health. Biofilm formation is, in essence, a product of inter-bacterial relations. Biofilms can be both mono- or multispecies, but the formation of a stable mature biofilm is always the product of copious social interactions that have evolved through adaptations.

For several decades, both horizontal gene transfer (HGT; Box 1) and biofilms (Box 2) have been central areas of microbiological research in environmental as well as medical microbiology, leading to the recognition of their high relevance for bacterial adaptation and evolution. Interestingly, a growing number of observations indicate that plasmid biology and biofilm community structure and functions are intertwined through numerous complex interactions, ranging from the genetic level to the community level. This points towards a principal role of the concerted action of these activities in sociomicrobiology (Box 3) and bacterial evolution. It is therefore now timely and highly relevant to review and discuss evidence of the interconnection between biofilm formation and HGT.

Figure 1 illustrates the interconnectedness between biofilm and plasmid biology and serves as the roadmap of this review. In the first section, we will highlight evidence and

arguments for biofilms as community structures that can promote plasmid transfer and stability. Then, we will flip the coin and focus on evidence for plasmids that in turn promote biofilm formation. Finally, we will discuss evolutionary forces at play in plasmid-driven sociomicrobiology.

Box 1. Biofilm

A biofilm is a gathering of bacterial cells enclosed in a self-produced polymeric matrix composed of extracellular polymeric substances, mainly exopolysaccharides, proteins and nucleic acids. Bacterial biofilms may adhere to an inert or living surface or exist as free-floating communities. Biofilm cells often exhibit an altered phenotype with respect to growth rate and gene transcription, and they display enhanced tolerance towards antibiotics and immune responses. Biofilms provide excellent conditions for bacterial interactions because of (i) the high-density and well-organized diverse microbial community allowing physical cell–cell contact and (ii) the matrix that concentrates various chemical compounds (e.g. communication signals and extra-cellular DNA). Additionally, environmental biofilms are typically multispecies communities. A characteristic feature of biofilms is their organization of cells into matrix-enclosed structures, varying in size from smaller microcolonies to large and sometimes 'mushroom-shaped' structures, which allow nutrient supply and waste product removal for cells placed in the deeper biofilm layers. It is generally accepted that the biofilm mode of growth is predominant in natural bacterial habitats.

The role of biofilm for plasmid transfer and stability

Enhanced HGT in biofilms

Conjugation

Looking at one of the original arguments for interconnectedness between HGT and biofilms, we find that conjugation occurs at higher frequencies between members of biofilm communities than when in a planktonic state (Hausner & Wuertz, 1999; Sørensen *et al.*, 2005). The fact that more transconjugants can be found after mating on a filter compared with mating in liquid culture illustrates this very well. The typical explanation for this observation is that biofilms are dense communities that expedite the spread of mobile genetic elements (MGEs). This is achieved through a spatial and structural advantage whilst keeping the conjugative pili intact.

It has been shown that high horizontal transfer frequencies of mobile plasmids can enable them to persist as molecular parasites (Bahl *et al.*, 2007), whilst other MGEs are merely transmitted vertically. It is likely that a trade-off exists between horizontal and vertical transmission of MGEs – a trade-off that may be facilitated by the costs that the MGE inflict on the host (Andersson & Levin, 1999; Bergstrom *et al.*, 2000). Plasmids that are only maintained through high transfer frequencies may thus only be able to persist in biofilms (Lili *et al.*, 2007). It is noteworthy in this connection that only 28% of all plasmid sequences in GenBank originating from proteobacteria can be grouped as conjugative plasmids (Smillie *et al.*, 2010). Whilst it is evident that the plasmid sequences of GenBank are not generally representative, it helps to underline the important fact that different plasmids have evolved different life strategies (see Box 3). In general, a sufficiently high horizontal transfer rate is critical to the success of MGEs, such as conjugative plasmids. Horizontal transfer can, however, still be an advantage to any MGE even if it is not their principal life strategy.

Although higher gene transfer in biofilms is the general observation, there are also examples of spatial constraints within biofilms that may hinder the dispersal of plasmids in an already-established biofilm (Merkey *et al.*, 2011). Król *et al.* (2011) illustrated how the transfer of an incP-1 plasmid has spatial and nutritional constraints and occurred mostly in the oxic zone in an *Escherichia coli* biofilm. We speculate that a prerequisite for successful introduction of certain plasmids in a biofilm community is that the plasmid is present in the initial phases of biofilm formation. This can be accomplished if the biofilm priming probabilities are encoded by the plasmid (to be discussed later).

Transformation

Besides conjugation, transformation is also known to occur at higher rates in biofilms. Higher transformation rates not only involve small DNA fragments, but also big elements such as plasmids, including those that do not encode genes for mobilization (Hendrickx *et al.*, 2003; Maeda *et al.*, 2006; Etchuuya *et al.*, 2011). This research illustrates how plasmids that are currently characterized as nonmobilizable may potentially be transferred horizontally in biofilms. When bacteria enter a competent stage, they often activate a DNA release programme. An essential part of the biofilm matrix is extracellular DNA (eDNA), and this has been shown to have a central role in stabilizing the biofilm matrix (Whitchurch *et al.*, 2002; Box 2). The development of natural competence is coupled to the presence of DNA. It is, therefore, believed that natural competence is triggered in biofilms by eDNA (Molin & Tolker-Nielsen, 2003), thereby potentially increasing the host range of MGEs such as plasmids. Furthermore, eDNA provides cell-surface and cell–cell adhesion properties – both key mechanisms of biofilm development (Molin & Tolker-Nielsen, 2003; Vilain *et al.*, 2009). Hence, there is an increasing amount of research

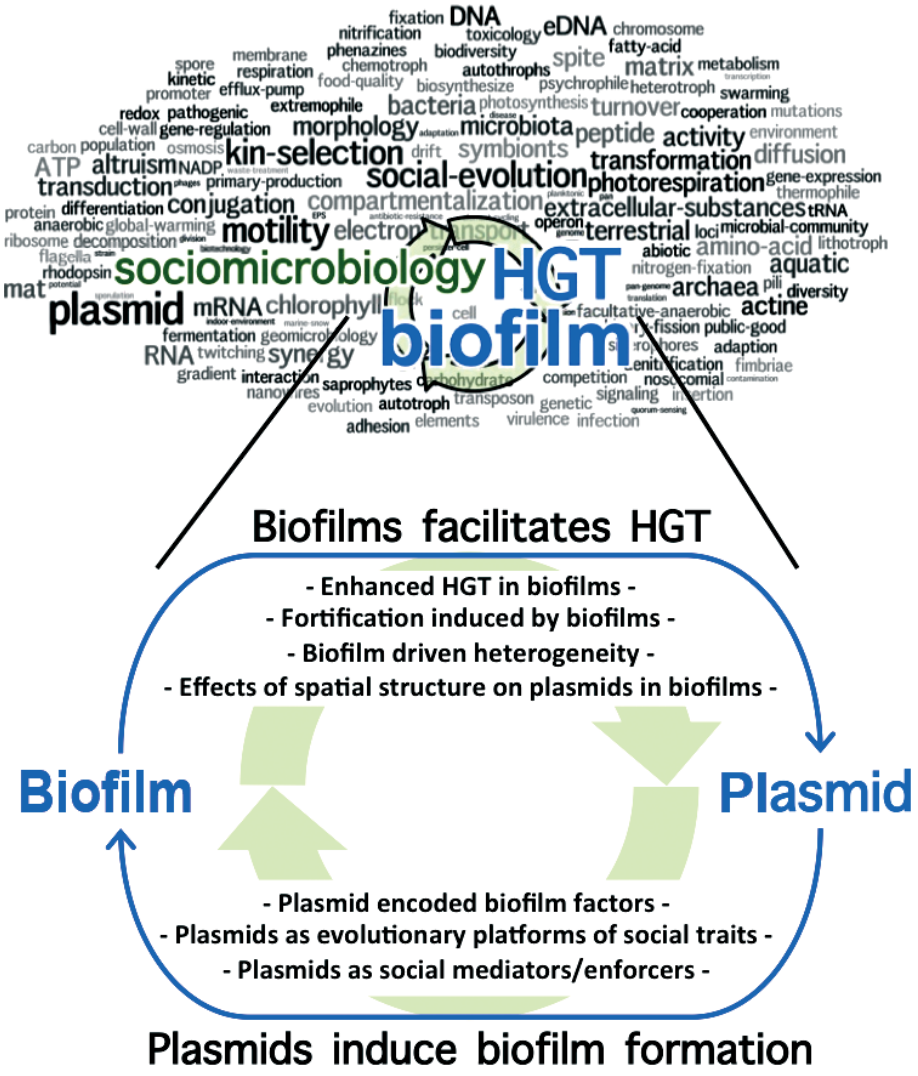

Fig. 1. Top: word cloud representing bacterial and archeal biology. Biofilm and HGT have been highlighted to emphasize their interconnection associated with sociomicrobiology. Bottom: outline of this article and overview of main arguments for the positive loop that biofilms and plasmids impose on each other.

supporting evidence that transformation triggers and stabilizes biofilm development and biofilms may in turn initiate transformation.

Fortification induced by biofilms

One of the early realizations within biofilm research was that the bacterial residents of mature biofilms are better protected against various biotic and abiotic exposures than their free-living counterparts. Plasmids that enhance the survivability of their hosts under a given selective pressure will consequently enhance their own persistence. Priming and stabilizing biofilm formation is one such example. Biofilm induced protection is of major concern when trying to eradicate bacterial infections of humans. The initial problem with biofilm-associated infections is that biofilm embedded bacteria are protected against key elements of the immune system such as macrophages. Higher tolerance against antibiotics can also be observed, further complicating the eradication of these infections. By now, it is well accepted that most chronic infections are biofilm related (Costerton *et al.*, 2003).

Improved protection of bacteria, induced by biofilms, has been shown to vary depending on the bacteria and the nature of the antimicrobial exposure (Høiby *et al.*, 2010). Examples also include better protection against oxidative stress (Burmølle *et al.*, 2006; Geier *et al.*, 2008),

various cleaning detergents (Simöes *et al.*, 2010), grazing by protozoans (Matz & Kjelleberg, 2005) and phage invasion (May *et al.*, 2011). Biofilm plasmids also gain from priming the biofilm, as plasmid invasion of an already-established biofilm may be hard. The biofilm, thus, shields the embedded microbial community from both competing plasmids and destructive phage invasion. Obtaining the ability to form biofilms may, in this regard, provide an advantage for both the host bacteria and its associated MGEs.

Biofilm-driven heterogeneity

Various barriers against the spread of plasmids have been identified by prior research. Two good examples include the well-known restriction–modification systems as well as the recently discovered CRISPR/cas system (clustered regularly interspaced short palindromic repeats coupled with *cas*-genes) that in some sense resembles an adaptive immune system. The latter is also an example of a system where a single cell may obtain immunity to a specific foreign nucleic acid sequence and eliminate MGE's carrying this sequence upon entry of the cell. The immunity becomes encoded and thus stored in the chromosome of the cell, further ensuring immunity in the coming cell linage (Horvath & Barrangou, 2010). This type of inherited plasmid immunity will consequently indirectly promote inter-clonal plasmid transfer. The bacterial communities within biofilms are heterogeneous, often multi-species, and variation is high even in specific bacterial linages (Stewart & Franklin, 2008). Therefore, biofilms may be communities where plasmids are more likely to encounter an accessible recipient than in a more homogeneous planktonic culture, subsequently being established in the community. Therefore, plasmids within biofilms are likely to experience a larger host range.

In a stressful environment, biofilm formation is a response that can help protect bacteria, but a 'waiting out the storm' strategy does not guarantee the bacterium's success if no adaptation takes place, and the environment continues to change. Bacteria are likely to encounter such situations frequently in natural environments. The process of biofilm formation most likely has an important role as a provider of structures where communal genes can be shared. The ability to use this opportunity for accelerated adaptation based on gene shuffling may play an important role in the initiation of biofilm development for both bacteria and MGEs (Jeffterson, 2004). HGT is an important mechanism for maintaining genetic heterogeneity, but it has also been shown that living as part of a biofilm promotes and maintains bacterial heterogeneity through mutation (Boles *et al.*, 2004; Heuer *et al.*, 2008; Conibear *et al.*, 2009). Selection and mutational evolution works on the entire genome both chromosome and extra chromosomal elements (Rankin *et al.*, 2011b). The stimulation of biofilm formation by MGEs will therefore ensure heterogeneity through both modular and mutational adaptation of the genome.

Effects of spatial constrains on plasmids in biofilms

The biofilm can provide the bacterial population with a spatially structured community (Burmølle *et al.*, 2010). But, like in the case with plasmid transfer frequencies in biofilms, spatial effects have a significant influence on overall functions within biofilms. Employing a computational approach, Mc Ginty *et al.* (2010) initially found that plasmids encoding cooperative traits could not withstand invasion by a social cheater. Interestingly though, they found that the cooperative plasmid was maintained regardless of the presence of a defector (cheater) plasmid when a spatially structured environment was applied to the model. They also observed that strains carrying the cooperative plasmid dominated in the metapopulation under these settings, because they provide conditions for their own persistence (e.g. higher productivity and horizontal transmission). Thus, biofilms provide a structured environment that confines defectors by buffering against the advancement of social cheater genes on plasmids. The role of plasmids as mediators of social traits in biofilms will be discussed further below.

Another well-known spatial effect on bacteria in biofilms is that of bacterial activity. Gradients and spots of nutrients and substrates typically transpire in biofilms and influence the bacterial composition and structure as well as the bacterial activity in regions of a biofilm. Biofilms that are grown in flow chambers are more active in their outer layers than in their inner and more subdued layers. This is a consequence of the formation of substrates and nutrient gradients that are established because of the spatial structure (Stewart & Franklin, 2008).

Plasmids are maintained in bacterial linages via plasmid-encoded mechanisms that ensure its vertical transmission, which is critical for the success of any plasmid. However, when bacterial activity is high and cells divide frequently, the rate of plasmid loss is potentially heightened, and plasmid-free cells can arise. Bacteria in biofilms are in general less active than their planktonic counterparts. Lower bacterial activity leads to fewer cell divisions, which in turn can result in a high degree of plasmid maintenance within a biofilm, because plasmid loss is less likely to occur. Also, if cell division is more infrequent, then less energy is being spent on plasmid replication, thus reducing the metabolic burden of plasmid maintenance on the bacterium (O'Connell *et al.*, 2006). Biofilms

can, therefore, enhance the maintenance of plasmids within bacterial populations.

Box 2. Horizontal gene transfer

HGT in bacteria can be conducted through three different mechanisms: direct cell–cell contact (conjugation), bacteriophage-mediated DNA transfer (transduction) and uptake of naked DNA by competent cells (transformation). By conjugation, plasmids and conjugative transposons may be spread from a bacterial cell to members of its own and other species, between Gram-negative and Gram-positive bacteria, and even from bacteria to yeast, plants and mammalian cells. In Gram-negative bacteria, the conjugative transfer depends on specific pili and is sometimes quorum-sensing-regulated. In contrast, Gram-positive conjugation is pili-independent and often relies on production and detection of specific pheromones. The conjugative plasmids encode genes that mediate their own transfer and ensure segregation to both daughter cells during cell division.

Transduction describes HGT mediated by bacteriophages. When new phage particles are produced, DNA originating from the phage-infected bacterial cell may accidentally be packed into the phage particles and transferred to new bacterial hosts. Here, the DNA must be integrated into the chromosome or plasmids to become stabilized and expressed. Transformation is uptake of free DNA from the surrounding environment. Most often, cells reach an inducible, competent state that enables the DNA uptake. Following this, the DNA must be circularized into a plasmid or recombined into the chromosome for the DNA to be maintained within the new genome.

The role of plasmids in biofilm formation and stabilization

Plasmid-encoded biofilm factors

The typical plasmid genome can be divided into backbone and accessory regions. The traits encoded in the backbone include replication, partition, stability and mobilization functions and can be thought of as the essentials of a functionally stable minimal plasmid entity. Genes that encode functions, which enhance the fitness of the plasmid's host under a given selective pressure, are typically described as accessory genes (Smillie *et al.*, 2010). Examples of functions of accessory genes include resistance towards antibiotics, metals, bacteriocines, metabolic functions and attachment to specific surfaces, to name a few (Norman *et al.*, 2009).

Biofilm-associated factors (BAFs) can be encoded both by genes in the backbone and the accessory regions of plasmids. This indicates that biofilm formation may be of importance for some plasmids. This is especially true for the BAFs encoded by the backbone genes (e.g. conjugal pili), as such genes are well-integrated parts of the plasmids biology.

Although only a few BAFs of plasmids have been studied in details, we will give a few examples to illustrate the variety of factors that can be involved. As the understanding of the interconnectedness between biofilms and plasmids is further explored, many more examples will, no doubt, be uncovered.

The plasmid backbone and biofilm formation – the conjugative pili

Conjugation is, in itself, aggregative in nature, promoting cell–cell contact between donors and recipients, thus demonstrating that the backbone of conjugative plasmids by default promotes interactions, which in time may lead to biofilm formation. Surprisingly, Ghigo (2001) found that all investigated conjugative plasmids could also prime surface-associated biofilms by providing cell-surface adhesive properties. These results were supported by Reisner *et al.* (2006) who found that biofilm formation was most common for natural *E. coli* isolates that harboured conjugative plasmids. It was also shown that biofilm formation was most pronounced during derepression of plasmids. This phenomenon has mostly been studied using incF-type plasmids. Naturally repressed incF plasmids also promote biofilm formation, but to a lesser degree. Research on biofilm formation primed by conjugative incF plasmids indicates that the expression of the conjugative pili is implicated in biofilm priming, but it seems that the pili is not the main factor directly facilitating the adherence. Cell-surface adherence may, however, be initiated mainly by activating the host biofilm system. This was shown to be the case by May & Okabe (2008), who discovered that expression of colonic acid and curli in *E. coli* was induced by a natural incF plasmid. They therefore proposed that the conjugative pili promotes cell–cell contact whilst the induction of colonic acid and curli production enables cell-surface adherence in addition to overall stability and structure of the biofilm.

The genetics and mechanics behind biofilm priming by conjugative incF plasmids are not well understood as the interactions between host chromosome and plasmid have proven to be very complex (González Barrios *et al.*, 2005; Yang *et al.*, 2008; May *et al.*, 2010; Nuk *et al.*, 2011). The connection is, nevertheless, well documented, and unveiling such interactions is important to understand the interconnectedness between biofilm formation and plasmid biology. The example of conjugative incF biofilm priming shows us that what can be considered the plasmid backbone may have evolved in such a manner that biofilm priming is intrinsic to many plasmids.

Biofilm factors encoded in accessory regions of plasmids

In the accessory regions of plasmids, different examples of biofilm priming factors have been found. The best understood examples of these are fimbriae and nonconjugative pili that are known to mediate cell–cell (bacteria and/or eukaryotic) contact but also cell-surface adherence. Fimbriae and some types of pili are structures that are dedicated to mediating adherence and thus biofilm priming. Looking at members of *Enterobacteriaceae*, three assembly pathways of surface-associated fimbriae have been identified: the type IV pili pathway, the nucleation pathway (curli assembly) and the chaperone/usher pathway (Clegg *et al.*, 2011). These three pathways have given rise to a wealth of fimbriae structures that show enormous diversity in genetic structure and regulation, but also in specificity and function. It is typical to identify multiple different types of chaperone/usher fimbriae amongst different genera and also in a single bacterial genome (Clegg *et al.*, 2011). Within *E. coli* genomes, there are fimbriae-encoding genes both on mobile elements such as plasmids and amongst core genes of the chromosome. Plasmid-encoded fimbriae are identified both on conjugative and nonmobilizable plasmids. We have, in our group, characterized different biofilm-enabling plasmids that encode type 3 fimbriae. Some of these plasmids were isolated from environmental samples based on a PCR screening for IncX plasmid replicons, whereas others were isolated because of the enhanced ability of their host to attach to abiotic surfaces (Norman *et al.*, 2008). Based on complete nucleotide sequencing of a number of these plasmids, the origin of the type 3 fimbriae encoding *mrkABCDF* cassette was identified. Investigations of the composite transposons, which mobilize the *mrkABCDF* cassette, revealed the likely modular mobilization of this gene cassette from a *Klebsiella pneumoniae* chromosome to incX1 type plasmids (Norman *et al.*, 2008; unpublished data). Besides enhancing attachment and biofilm formation, the *mrkABCDF* cassette of the incX1 plasmid, pOLA52, also resulted in elevated transfer frequencies (when compared to a *mrk*-knock-out mutant of pOLA52; Burmølle *et al.*, 2008). Finding biofilm primers such as fimbriae in the accessory regions of plasmids from many independent origins indicates that the adhesive properties must be advantageous to the host bacterium.

Another adhesion structure protruding from bacterial surfaces are the type 4 pili. Type 4 pili are interesting biofilm primers because these pili are found widely distributed amongst *Bacteria*. Research even suggests that type 4 pili-like organelles are found in *Archaeae* (Pohischroder *et al.*, 2011). Type 4 pili are remarkable in that they enable not only attachment but also a variety of other functions including gliding motility, twitching motility, DNA uptake and signal transduction (Craig & Li, 2008). In *E. coli*, we see examples of type 4 pili encoded in the chromosome but also on plasmids and other MGEs (Pelicic, 2008). Intriguingly, the type 4 pili often found on conjugative plasmids of *E. coli* (the incI-cluster), a type associated with a so-called shufflon (Gyohda *et al.*, 2004), seem to have been partly incorporated into the conjugative apparatus and are required for conjugation between cells in liquid culture (Sampi *et al.*, 2010). Regulation of the type 4 pili of the incI1 plasmid R64 shares regulatory genes with the conjugative pili (Kim *et al.*, 1993). The related IncI2 plasmid R721 has fragmented type 4 pili where two of its genes are located away from the type 4 pili operon and situated amongst genes of the conjugative pili (Kim & Komano, 1992; accession number: AP002527). These type 4 pili thus represent a biofilm priming module that is somewhere between an accessory and a backbone-related element. This implies that biofilm priming associated with the accessory region might be incorporated to become part of the backbone. It is most likely that accessory genes are gained and lost at relatively higher rates than backbone genes, and for genes to be incorporated into the backbone of a plasmid implies that the function of the genes is of general importance for the success of the plasmid.

It is noteworthy that plasmids that have been associated with biofilm formation belong to the incF (incFI – incFV), incX and the incI cluster and have also been shown to be amongst the most relatively abundant plasmids harboured by *E. coli* (Reisner *et al.*, 2006, unpublished data), a bacterium that, in the absence of these plasmids, shows weak biofilm forming capabilities on abiotic surfaces.

Many of the aforementioned examples of biofilm-enabling plasmids originate from *E. coli*, but biofilm priming encoded on plasmids has been identified in both Gram-negative and Gram-positive bacteria such as the *Pseudomonas putida* TOL plasmid (D'Alvise *et al.*, 2010), *Lactococcus lactis* pAMβ1 (Lou *et al.*, 2005), *Azospirillum brasilense* plasmids (Pentrova *et al.*, 2010) and the *Enterococcus faecialis* pBEE99. Similar to *E. coli*, *E. faecialis* commonly occupy a commensal niche in the gastrointestinal tract but also appear as opportunistic pathogens. Random mutagenesis revealed that knocking out the *bee* cassette of conjugative plasmid pBEE99 lowered biofilm formation of the parent strain by 70%. The *bee* cassette seems to be located on an element resembling a transposon. It is likely that *bee* encodes a pilus-like structure that showed distant relatedness to genes found in the chromosome of *Leuconostoc mesenteroides* (Tendolkar *et al.*, 2006; Coburn *et al.*, 2010).

Above we discuss the role of pili and fimbriae in biofilm priming. These are the examples that are best studied in connection with MGEs. Reports of plasmids that enable biofilm formation through increased EPS production were recently made; pO157 is such an example and enables a hyper-adherent *E. coli* variant (Lim *et al.*, 2010). If biofilm priming/formation is a lucrative strategy for a plasmid and its host, then we can expect alternative BAFs to exist and function in various ways as a result of convergent adaptation. Hence, new plasmid-encoded BAFs will be identified as more focus is directed towards the interconnectedness between plasmid and biofilm biology.

Plasmids as social evolutionary platforms

Plasmid mechanics – invention and reinvention of biofilm factors

Plasmids are unique genetic elements that are found ubiquitously in bacteria. The role of plasmids as both independent units, but also as potential synergistic symbionts, has influenced the evolution of bacteria immensely. Over the last two decades, a great deal of research has provided us with a better understanding of how MGEs have transferred, invented and reinvented genes and genetic networks horizontally, generating new traits moulded by intrinsic selective pressures in various environments (Ochman *et al.*, 2000; Martínez, 2008). Here, we wish to give examples of genes that are believed to have, through the course of evolution and by means of HGT, gained new functions. These examples should underline the role of MGEs and HGT as key players in the creation of certain original functions. Plasmids are evolutionary platforms for the invention of new traits and genetic networks. This, coupled with the fact that plasmids are dependent on the fitness of their host, in addition to the potential for vertical and horizontal transmission, makes the mechanics of plasmids an important facilitator in the rise of novel biofilm promoting traits. The evolution of plasmid-encoded BAFs will, as a consequence, vary depending on the lifestyle strategy of the plasmid. However, the interconnectedness between plasmids and biofilm formation indicates that plasmids have an important role in microbial evolution and more specifically in shaping of social interactions amongst bacteria.

There are several factors making plasmids function as social evolutionary platforms. (1) Importantly, the success of a plasmid is reliant on its host fitness and its own maintenance in a population/community. (2) Genes encoded on plasmids are generally present in many copies compared with those encoded on the chromosome because numerous copies of a plasmid exist inside the cell. Consequently, plasmid-encoded genes are typically expressed to a higher extent. (3) When genes are moved from the bacterial chromosome to a plasmid, new stronger promoters may be associated with the genes that are moved. This normally happens when genes or gene cassettes are mobilized by insertion sequences, transposons or integrons. (4) Gene-regulation is typically lost or altered in the event of interspecies HGT, and genes may, therefore, be expressed constitutively or at changed rates. (5) The turnover of plasmid-encoded genes is high. Also see Fig. 2.

Box 3. Social bacterial behaviours

Social behaviours are classified into four categories according to their effect on the direct fitness (fitness gained through reproduction) of the actor and the recipient. If both the actor and the recipient increase their direct fitness as a consequence of the social behaviour, this is defined as mutualism. If the behaviour increases the direct fitness of the actor but decreases that of the recipient, this would be selfishness. Spite occurs when the behaviour of an actor reduces both its own and the recipient's direct fitness. The last category, altruism, is seen when the actor's direct fitness decreases, whilst the recipient's increases (Hamilton, 1964).

The social behaviours classification scheme is applicable in social interactions between bacteria, but can also be applied when considering the relationship between plasmid and bacterium. The lifestyle strategy outcome of the specific plasmid can be defined according to the classification of social behaviours where the plasmid serves as the actor and the host as the recipient.

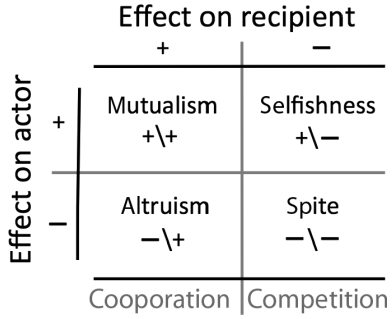

Illustration of the four categories of social behaviours

From chromosome to plasmid and back – regulation of gene expression

One of the better-studied examples of proteins that are believed to have gained new functions through HGT by MGEs is that of some antibiotic resistance functions. We refer to Martínez (2008) for a fuller perspective on this subject. The basic idea is that proteins that confer antibiotic resistance have developed from household genes of various functions, mainly though HGT and selection.

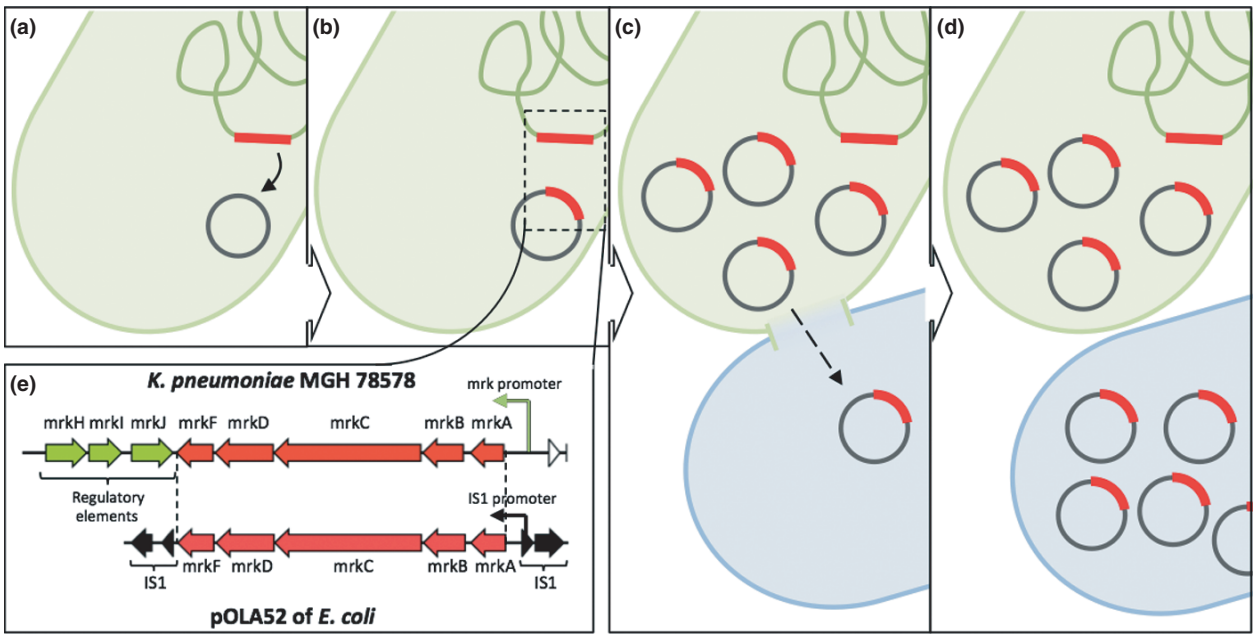

Fig. 2. Horizontal transfer of a BAF. (a–b) A BAF (red) is moved from the chromosome (green) to a MGE (black). (c) A copy of the BAF-encoding MGE is transferred horizontally to a new host. (d) The MGE replicates to multiple copies in its new host. (e) One example of comparable events is the mobilization of the *mrkABCDF* cassette (coding for type 3 fimbriae) from the chromosome of *K. pneumonia* to an incX1-type plasmid via a composite transposon: flanking insertion elements (IS1). Regulatory elements (*mrkHIJ*) and possibly also the original promoter of the *mrkABCDF* cassette were lost when it was mobilized to a plasmid (see main text for further discussion).

One such example is that of the OqxAB pump. The OqxAB pump is encoded in the chromosome of *K. pneumonia* where its function is unknown but it does not confer resistance to antibiotics (Hansen *et al.*, 2007). The OqxAB pump does, however, provide multidrug resistance when overexpressed on plasmids in a variety of enterobacteria (Hansen *et al.*, 2007). Such natural plasmids have been isolated from pigs that were treated with olaquindox, which is one of the drugs to which the OqxAB renders its host resistant (Hansen *et al.*, 2007). Plasmids encoding OqxAB have, in addition to farm animals, also been isolated from humans (Kim *et al.*, 2009; Zhao *et al.*, 2010). Although this example is not biofilm related *per se*, it helps to illustrate an event where few proteins can change the host cell phenotype if they are taken out of their normal chromosomal context and expressed on a plasmid.

Another example is the transcriptional factor SoxR. Recently, Dietrich & Kiley (2011) argued that SoxR has different functions in enteric and nonentric bacteria, although the genes are clearly related. In enteric bacteria (*E. coli*), SoxR regulates only one gene, *soxS*, that subsequently regulates many (> 100) targets. In nonenteric bacteria, SoxR, which has been identified in *Alpha-*, *Beta-*, *Delta-* and *Gammaproteobacteria* and *Actinobacteria* (Dietrich *et al.*, 2008), regulates multiple targets directly, including aspects of biofilm development (*Pseudomonas aeruginosa* and *Streptomyces coelicolor*). The *soxR* gene is believed to have been transferred horizontally from a nonenteric to an enteric bacterium. This represents an example of a single gene that has been transferred horizontally and, because of this and recombination events, subsequently functions differently in the new host bacterium (Dietrich *et al.*, 2008).

Looking at the biofilm priming *mrkABCDF* system previously mentioned has proven to be very convenient because the nucleotide sequence of chromosomally encoded *mrkABCDF* in *K. pneumoniae* and plasmid-encoded *mrkABCDF* are almost identical, indicating a relatively recent mobilization of the *mrkABCDF* cassette to the incX1 plasmids. Lately, important regulatory elements of type 3 fimbriae encoded in the chromosome of *K. pneumoniae* have been revealed (Johnson & Clegg, 2010; Johnson *et al.*, 2011; Wilksch *et al.*, 2011). These regulatory key elements are not present on the *mrkABCDF* encoding plasmids (Norman *et al.*, 2008; Ong *et al.*, 2009, unpublished data), indicating that either new regulatory functions have progressed or constitutive expression occurs. This example illustrates how plasmids can function as evolutionary and adaptive templates for biofilm-related mechanisms. The

regulatory networks directly connected to the BAFs found on plasmids are generally less complex than the ones found in the chromosome.

More complex examples of BAFs encoded on plasmids include type 4 pili as mentioned earlier. Tracking the evolutionary events of type 4 pili is complicated because these pili are very diverse and widespread. The ubiquity and the diversity do, however, indicate that the horizontal spread and evolution (modular included) of such BAFs have shaped the type 4 pili to have many properties related to biofilm formation and microbial socialness (Craig & Li, 2008; Pelicic, 2008).

The presence of biofilm-enabling incF plasmids in *E. coli* has been shown to affect the expression of numerous genes of the chromosome (González Barrios *et al.*, 2005; Yang *et al.*, 2008; May *et al.*, 2010; Nuk *et al.*, 2011). Further expanding the knowledge about how the innate biofilm system works in bacteria will help in the assessment of the importance and implications that genes encoded on MGEs have in cross-regulating and interacting with biofilm-related genes of the rest of the genome.

The pAA plasmids enable aggregative adherence amongst enteroaggregative *E. coli* (EAEC). The aggregative pAA behaviour is controlled through an AggR regulon. Intriguingly, there are indications that the plasmid-encoded AggR regulon can regulate a chromosomal operon of a pathogenic island of its host (Harrington *et al.*, 2005). In turn, this indicates interactions across the genome. When BAFs of the communal gene pool are integrated into the chromosome, such as in the case of genomic islands, these MGEs are expected to become less independent and more integrated with the innate biofilm system of the bacterium.

Plasmids as social mediators

Based on computational modelling and sequence analysis of genomic and metagenomic data, Nogueria *et al.* (2009) found that HGT promotes cooperation as MGEs heighten genetic relatedness, enforce cooperation and, consequently, drive inter-bacterial cooperation. In this study, the secretome was used as the factor imposing social interactions leading to cooperation. For a complete discussion on this subject, we refer to Nogueria *et al.* (2009), Rankin *et al.* (2011a,b), and Giraud & Shykoff (2011).

As many, though not all, BAFs can be considered part of the secretome, we here briefly consider key arguments and relate these to the interconnectedness of biofilm and plasmid biology. Nogueria *et al.* (2009) inferred the localization of putative proteins predicted from annotated genes in 21 genomes – 20 *E. coli* and one *Escherichia fergusonii*. By doing this, they showed a pattern as to the localization of core, ancestral and recent genes (gene classes). Proteins that were predicted to be secreted, as well as proteins localized at the outer membrane, were in the majority of cases encoded by recently acquired genes and very few ancestral core genes. This indicated that social traits are typically located in the mobile part of the genome. When analysing human gut metagenomes of unweaned babies, infants and adults, significantly more nonancestral *E. coli* genes were predicted to belong to the secreted and outer-membrane protein groups than those localized elsewhere. Furthermore, they illustrated how secreted and outer-membrane proteins were encoded more often on plasmids than in mobilizable hotspots and again in these hotspots more often than nonmobilizable parts of the genomes.

Interestingly, the biosynthetic energetic cost per residue of secreted and outer-membrane proteins were found to be lower than that of the proteins in the periplasm, cytoplasm and inner membrane, demonstrating a clear association between protein cost and localization (Nogueria *et al.*, 2009). For MGEs to carry social traits, they impose a lower cost on the host bacterium, compared with genes coding for proteins, otherwise localized to the other compartments. Many of the secreted proteins found in the study were annotated as virulence factors (Nogueria *et al.*, 2009), illustrating how virulence factors can be a product of bacterial social interactions.

The production of outer-envelope molecules that benefit the neighbouring bacteria can potentially end up as a fitness loss because of a cost that ultimately benefits other bacteria that have not invested in the production of the molecules. Compared with molecules that are secreted into the environment and diffuse away, the surface-attached outer-membrane molecules, such as fimbriae, can be recycled or re-scavenged, thus lowering the cost of this type of public good (Nogueria *et al.*, 2009). Additionally, this may explain why structures such as fimbriae and pili are the typical BAFs that are identified on plasmids.

Enforced bacterial interactions, reprogramming bacterial cheaters and enhancing relatedness

Any public good is prone to exploitation by cheaters – an evolutionary dilemma that most public good factors are supposedly confronted with (Nadell *et al.*, 2009). The ultimate consequence is that the cheater will outcompete the noncheater resulting in what is known as 'the tragedy of the commons'. This could suggest that exploitable social traits such as public goods are not expected or, at best, are unlikely to evolve. Social

traits have nonetheless evolved and are very widespread in bacteria. A few examples include swarming, quorum sensing, siderophore production and biofilm formation.

For explanations as to how these public good traits have evolved, modern social evolutionary theory and experimental research point towards mechanisms that negate the advancement of cheaters and stabilize the evolution of social traits in bacteria (Hamilton 1964; West *et al.*, 2006; Xavier *et al.*, 2011). Mechanisms opposing cheaters may function directly or indirectly. Such examples include spatial and nutritional factors, which have been suggested to be important during bacterial biofilm formation (Xavier & Foster, 2007). The spatial factor of a biofilm may also be of importance if we look at the specific example of biofilm priming as a social trait. In this case, the cheater is a MGE that does not produce the BAF but still gains from being embedded in a biofilm – a scenario likely to be found in nature. Computational modelling by Mc Ginty *et al.* (2010) indicates that plasmid-encoded public good cheaters were not able to outcompete noncheaters in a meta-population when structure was embedded in the model. When no structure was appointed, the cheaters could outcompete the noncheater. When socialness, primed via fimbriae, only benefits bacteria in a close proximity to the donator, odds are that the neighbouring bacteria are more closely related than those at greater distances. This is the case simply because bacteria grow by binary fission, providing a spatial advantage against cheaters in a confined matrix as biofilms.

Another and more direct example of a way expected to negate cheaters and stabilize the evolution of social traits is what could be described as mafia methods (toxin/antitoxin, colicin production and restriction–modification systems – see Box 4) encoded on plasmids leading to social enforcement.

It has been shown that plasmids can be in conflict with their host, and the evolutionary outcome has in some cases been explained as the employment of mafia strategies by plasmids to ensure their stability within a population (Zielenkiewicz & Ceglowski, 2001). Plasmids that enable social interactions such as biofilm formation push the donor bacterium to interact with a recipient. This interaction may heighten the net success of the MGE but is also likely to open the door to multiple social dilemmas. The employment of mafia methods could be a mechanism that ensures that cheaters are less likely to evolve. Biofilm priming encoded on plasmids can be viewed as a cooperative trait that has a built-in enforcement strategy because of the typical addiction and stability factors encoded on plasmids.

Box 4. Mafia methods ensuring plasmid stability

Central to plasmid maintenance in populations are plasmid addiction systems that give the bacterial hosts an *offer they cannot refuse*. In one system, plasmids carry restriction–modification modules where plasmid-encoded methyltransferases modify host DNA and thus prevent its digestion by a restriction endonuclease also encoded by the plasmid (e.g. EcoRI from pMB1). If the plasmid is lost, a slow degradation of the methylase causes unmethylated sites in the host chromosome. The remaining endonuclease then causes double-strand breaks in the host chromosome.

Similarly, postsegregation killing (PSK) systems such as the CcdA/CcdB *or* HOK/SOK (host killing/suppressor of killing) systems (*parB* locus), encoded by *E. coli* plasmids F or R1, respectively, are efficient systems ensuring the killing of host cells that lose the plasmids. PSK systems are composed of an operon of at least two essential genes encoding a stable toxin and a corresponding unstable antitoxin.

In contrast to these systems are the more violent bacteriocins, which are toxins mostly encoded by plasmids. A well-studied example of this is the ColE1 plasmid, which encodes colicin that kills neighbouring *E. coli* cells if they do not contain the ColE1 plasmid. The plasmid contains three genes essential to the colicin system that code for colicin production (*cea*), immunity to colicin (*imm*) and the *kil* gene causing cell lysis phenotypes. In the event of DNA damage (e.g. dying cells), large quantities of colicin and lysis protein is produced. The lysis of the host cell causes colicins to be released into the extracellular medium whereby ColE1-free cells (*imm-)* are killed.

The study by Nogueria *et al.* (2009) showed in congruence that social traits (the secretome) were co-located with integrases, restriction–modification and toxin/antitoxin regions. This was interpreted as an adaptation to further enhance the enforcement of cooperation through the mafia method. It was also argued that cooperation was enforced and maintained through gene transmission by reprogramming of defectors. Rankin *et al.* (2011a,b) argued that HGT increases relatedness whilst a reduction occurs when such genes are lost. Plasmids are thus linked to cooperation because increased local relatedness favours cooperation. Such heightened relatedness is not only restricted to already related species, because the mobilization of plasmid-encoding public goods can cross species barriers. Thereby, cooperative traits can establish new relatedness between previously unrelated bacteria.

Concluding remarks

Our belief is that to understand how virulence mechanisms such as biofilm formation are established, we need to understand social dilemmas raised by microbial inter-

actions. To achieve the above-mentioned goal, it is essential that we gain a better understanding of the molecular mechanisms involved. HGT and MGEs, such as plasmids, are at the very heart of this. In this review, we have argued that the interconnectedness between biofilm formation and plasmid biology may act as a positive loop that promotes both. The perspective extends to an overall interconnectedness between HGT, MGE and social evolution of bacteria.

Acknowledgements

This study was partly funded by grants from the Danish Innovation Consortium, SiB, ref no: 11804520 (Jonas Stenløkke Madsen) and The Danish Science Research Council, Technology and Production, ref no: 09-090701 (Mette Burmølle).

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