Mechanisms of biofilm resistance to antimicrobial agents

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Biofilms are communities of microorganisms attached to a surface. It has become clear that biofilm-grown cells express properties distinct from planktonic cells, one of which is an increased resistance to antimicrobial agents. Recent work has indicated that slow growth and/or induction of an *rpoS*-mediated stress response could contribute to biocide resistance. The physical and/or chemical structure of exopolysaccharides or other aspects of biofilm architecture could also confer resistance by exclusion of biocides from the bacterial community. Finally, biofilm-grown bacteria might develop a biofilm-specific biocide-resistant phenotype. Owing to the heterogeneous nature of the biofilm, it is likely that there are multiple resistance mechanisms at work within a single community. Recent research has begun to shed light on how and why surface-attached microbial communities develop resistance to antimicrobial agents.

Bacterial biofilms are formed when unicellular organisms come together to form a community that is attached to a solid surface and encased in an exopolysaccharide matrix. Biofilms can be made up of single or multiple bacterial species. For example, it has been estimated that dental biofilms contain >500 different bacterial taxa[1]; conversely, in the latter stages of the disease, the primary bacterium in the lungs of cystic fibrosis (CF) patients is *Pseudomonas aeruginosa*. It has been observed that the resistance of biofilms to antibiotics is increased compared with what is normally seen with planktonic cells. In fact, when cells exist in a biofilm, they can become 10–1000 times more resistant to the effects of antimicrobial agents[2–5].

It is becoming increasingly clear that biofilms have an enormous impact on medicine. Biofilms can form on many medical implants such as catheters, artificial hips and contact lenses and, owing to their increased resistance to antimicrobial agents, these infections can often only be treated by removal of the implant, thus increasing the trauma to the patient and the cost of treatment. It has been estimated that biofilms are associated with 65% of nosocomial infections[6] and that treatment of these biofilm-based infections costs >$1 billion annually[7–9].

The development of biocide resistance is not understood, but recent studies have used a variety of model systems to determine how and why biofilms are so resistant to antimicrobial agents. As the importance of biofilms in nosocomial infections has increased, much energy has been directed towards the study of the effects of antimicrobial agents on these surface-attached communities. The key question we ask in this review is: what are the mechanisms of biofilm resistance to antimicrobial compounds? What we will emphasize is that there are multiple mechanisms, which vary with the bacteria present in the biofilm and the drug or biocide being applied. These mechanisms include physical or chemical diffusion barriers to antimicrobial penetration into the biofilm, slow growth of the biofilm owing to nutrient limitation, activation of the general stress response and the emergence of a biofilm-specific phenotype. In this review, we will focus our attention on *in vitro*-derived single-species biofilms, although some dual-species biofilm work will be highlighted.

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Failure of the antimicrobial to penetrate the biofilm

The production of an exopolysaccharide matrix, or glycocalyx, is one of the distinguishing characteristics of biofilms. It has been suggested that this matrix, among other functions, prevents the access of antibiotics to the bacterial cells embedded in the community. We will highlight a few of the more recent studies on the subject of antibiotic diffusion through a biofilm. For a more comprehensive review of this subject, the reader is directed to a review by Stewart[10].

Either reaction of the compound with, or sorption to, the components of the biofilm can limit the transport of antimicrobial agents to the cells within the biofilm. Although mathematical models suggest that, for many antibiotics, there should be no barrier to their diffusion into a biofilm, some studies have shown an apparent failure of certain antimicrobial agents to penetrate the biofilm. Chlorine, a commonly used disinfectant, did not reach >20% of the bulk media's concentration within a mixed *Klebsiella pneumoniae* and *P. aeruginosa* biofilm, as measured by a chlorine-detecting microelectrode[11]. In fact, the penetration profile was suggestive of a substrate being consumed within the matrix. Suci *et al.* used infrared spectroscopy to show that the rate of transport of the antibiotic ciprofloxacin to the surface of a colonized surface was reduced compared with transport to a sterile surface[12]. These authors suggested that the ciprofloxacin was binding to the biofilm components.

Other groups have taken different approaches to address the question of whether the biofilm acts as a barrier to antimicrobial agents. On the one hand, *P. aeruginosa* biofilms were formed on one side of a dialysis membrane and the amount of piperacillin that penetrated the biofilm was measured. Consistent with the results discussed above, the *P. aeruginosa* biofilm prevented diffusion of this antibiotic[13]. On the other hand, *Staphylococcus epidermidis* biofilms formed in a similar manner allowed for the diffusion of rifampicin

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and vancomycin across the membrane[14], implying that these antibiotics could efficiently penetrate this biofilm. These results suggest that inhibition of diffusion cannot always explain resistance to antimicrobial compounds.

A difference between thick and thin biofilms and their resistance to antibiotics has been observed. Penetration of a thin biofilm-covered bead [average cell density ~3.5 log colony-forming units (cfu) cm^{-2}] by hydrogen peroxide was observed directly, even though the cells within the biofilm were more resistant to the compound compared with planktonic cells[15]. By contrast, thicker biofilms, grown on glass slides (average cell density ~7.6 log cfu cm^{-2}), presented a barrier to the penetration of hydrogen peroxide. Interestingly, hydrogen peroxide was able to penetrate a thick biofilm formed by a mutant strain of *P. aeruginosa* that lacked one of the major catalase genes, *katA* (Ref. 16). As catalases are enzymes that neutralize hydrogen peroxide, this result suggested that, in thick biofilms, cells were protected from hydrogen peroxide penetration by the catalase-mediated destruction of this compound.

Anderl *et al.* formed *K. pneumoniae* colony biofilms on agar plates with or without antibiotic[17]. By placing a filter at the top of the colony, essentially sandwiching the colony, they were able to assay directly for antibiotic diffusion from the agar plate through the colony by performing a standard zone of inhibition assay with the filter. This breakthrough study showed that ampicillin was unable to penetrate the biofilm and that the production of the ampicillin-degrading enzyme β-lactamase was responsible for this phenomenon, as the ampicillin was able to penetrate a biofilm formed by a β-lactamase mutant. Surprisingly, the β-lactamase mutants grown in a biofilm were still resistant to ampicillin, suggesting that other mechanisms contribute to the resistance of these cells. Furthermore, ciprofloxacin was able to penetrate the biofilm, yet, as was the case with ampicillin, it was unable to kill the biofilm bacteria[17]. This simple method allowed for the differentiation between transport effects and other mechanisms and thus provides a powerful tool for the further analysis of the molecular mechanism of biofilm resistance to antimicrobial agents.

From these studies, and others, it is clear that the exopolysaccharide matrix (or other components of biofilms) does not form an impenetrable barrier to the diffusion of antimicrobial agents, and other mechanisms must be in place to promote biofilm cell survival. However, for certain compounds, the exopolysaccharide matrix does represent an initial barrier that can delay penetration of the antimicrobial agent. The experiments described above strongly suggest that multiple mechanisms are required for overall antimicrobial resistance.

Slow growth and the stress response

When a bacterial cell culture becomes starved for a particular nutrient, it slows its growth. Transition from exponential to slow or no growth is generally accompanied by an increase in resistance to antibiotics[18,19]. Slow growth of the bacteria has been observed in mature biofilms[20,21]. Because cells growing in biofilms are expected to experience some form of nutrient limitation, it has been suggested that this physiological change can account for the resistance of biofilms to antimicrobial agents.

By paying close attention to the growth phase of planktonic cells and biofilm cells, recent studies have been able to examine specifically the contribution of a slow growth rate to biofilm cell survival against antibiotics. Gilbert and colleagues examined growth-rate-related effects under controlled growth conditions for planktonic cultures and biofilms of *P. aeruginosa*, *Escherichia coli* and *S. epidermidis*[22–24]. They made the general observation that the sensitivities of both the planktonic and biofilm cells to either tobramycin or ciprofloxacin increased with increasing growth rate, thus supporting the suggestion that the slow growth rate of biofilm cells protects the cells from antimicrobial action. For *P. aeruginosa* at slow growth rates, both the planktonic and intact biofilm cells were equally resistant to ciprofloxacin. However, as the growth rate was increased, the planktonic cells became more susceptible to ciprofloxacin than the biofilm cells. This result supports the idea that some other property of the biofilm, and not just growing slowly, was important for the observed recalcitrance of biofilms to antimicrobial treatment[22]. The same group reached a similar conclusion while working with *Burkholderia cepacia*. Desai *et al.* compared the resistance of planktonic and biofilm cells at different stages during exponential growth up to the entry into stationary phase[25]. They found that resistance increased as the planktonic cultures and the biofilm cells approached stationary phase. The maximal resistance of both cultures occurred in stationary phase where the biofilm cells were 15-times more resistant than the planktonic cells. These results suggested that some determinant other than growth rate is responsible for a certain level of resistance, and slow growth adds additional protection. This determinant could be related to the fact that cell density increases during this late stage of exponential growth (see below). Other studies have suggested that mechanisms differ for different antibiotics. For example, although the slow growth rate in a *P. aeruginosa* biofilm seemed to account for biofilm resistance to tetracycline, it did not seem to affect resistance to tobramycin[26].

Heterogeneity

The experimental conditions resulting in the tight control of growth described in the studies summarized above allowed investigators to focus on the effect of a specific growth rate on bacterial susceptibility to antimicrobial agents. However, when thinking about biofilms, a logical assumption is that

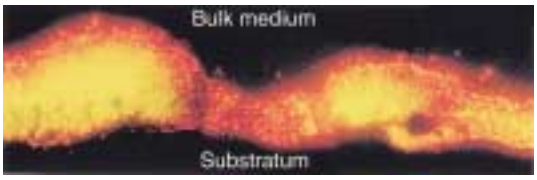

Fig. 1. Physiological heterogeneity in biofilms. The spatial pattern of growth rate within a *Klebsiella pneumoniae* biofilm, as judged by acridine orange staining. In this figure, areas of red–orange staining correspond to a high relative RNA content and thus rapid growth. Cells staining yellow/green have low relative RNA content and a slower growth rate. There are clearly distinct regions of faster and slower growth throughout the biofilm. The bottom of the image is the portion of the biofilm attached to the substratum and the top of the image is the portion of the biofilm exposed to the bulk medium. Reproduced, with permission, from Ref. 21.

any given cell within the biofilm will experience a slightly different environment compared with other cells within the same biofilm, and thus be growing at a different rate. Gradients of nutrients, waste products and signaling factors form to allow for this heterogeneity within the biofilm.

Recent advances in technology have resulted in the ability to visualize the heterogeneity within a biofilm. A staining method utilizing acridine orange was employed to identify regions of biofilms that contain rapidly or slowly growing cells based on their relative RNA–DNA content[21]. The method was first used on bacterial colonies and the regions of the colonies that turned orange (high relative RNA content) were correlated to fast growth rates, whereas regions that were stained yellow/green (low relative RNA content) represented slowly growing cells. When this method was used on seven-day-old biofilms, orange marked the biofilm–bulk-liquid interface and yellow/green marked the center of the biofilm (Fig. 1). This heterogeneity within biofilms has also been shown for protein synthesis and respiratory activity, whereas DNA content remained relatively constant throughout the biofilm[27,28].

There is also evidence for gradients of physiological activity in response to antimicrobial treatment. For example, the pattern of respiratory activity of a *K. pneumoniae* biofilm in response to monochloramine (an oxidatively active biocide) treatment showed that cells closest to the biofilm–bulk-liquid interface lost activity first[29] (Fig. 2). Similarly, when biofilm cells were treated with the antibiotic fleroxocin, cell elongation was observed and was most extreme in cells located close to the exposed side of the biofilm[30]. These studies reveal that the response to antimicrobial agents can greatly vary, depending on the location of a particular cell within a biofilm community.

General stress response

Recently, it has been suggested that the slow growth rate of some cells within the biofilm is not owing to nutrient limitation *per se*, but to a general stress response initiated by growth within a biofilm[31]. This

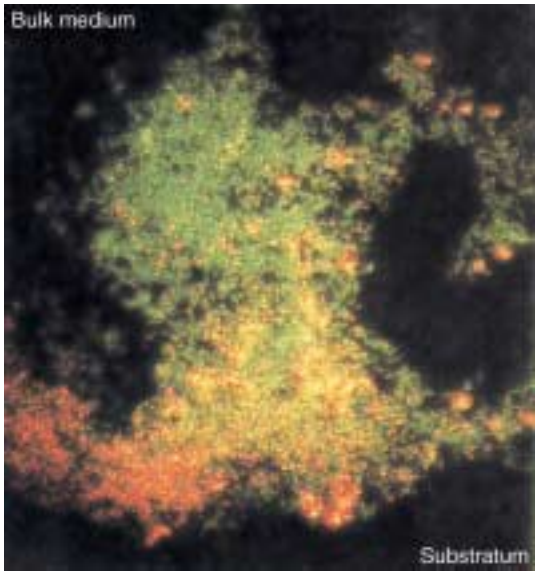

Fig. 2. Susceptibility to biocide treatment. A two-species biofilm treated with the oxidatively active biocide monochloramine. This figure illustrates that there is heterogeneity within the biofilm in terms of the response of individual cells to biocide treatment. Areas of red–orange staining correspond to respiratory activity. Green cells have no respiratory activity. Yellow regions represent a mixture of respiring and non-respiring bacteria. The bottom of the image is the portion of the biofilm attached to the substratum and the top of the image is the portion of the biofilm exposed to the bulk medium. Reproduced, with permission, from Ref. 29.

idea is an attractive possibility because the stress response results in physiological changes that act to protect the cell from various environmental stresses. Thus, the cells are protected from the detrimental effects of heat shock, cold shock, changes in pH and many chemical agents[32]. The central regulator of this response is the alternate σ factor, RpoS, originally thought to be expressed only in stationary phase[32]. However, recent studies suggest that RpoS is induced by high cell density and that cells growing at these high densities seem to have undergone the general stress response, as judged by the production of trehalose (an osmoprotectant) and catalase[33]. As cells in a biofilm experience high cell density, it is logical to propose that these cells would express RpoS. Accordingly, it has been shown by RT-PCR that *rpoS* mRNA is present in sputum from CF patients with chronic *P. aeruginosa* biofilm infections[34].

Another link between RpoS and biofilms was recently identified: *E. coli* cells that lack *rpoS* are unable to form normal biofilms whereas planktonic cells are apparently unaffected by the absence of this σ factor[35]. In *P. aeruginosa*, it has been suggested that an additional σ factor, AlgT, acts in concert with RpoS to control the stress response[31,34]. Cochran *et al.* found that thin biofilms formed by null mutants of *rpoS* and *algT* on alginate gel beads were susceptible to hydrogen peroxide but not to monochloramine[36]. However, when these mutants formed thick biofilms on glass slides, they were as resistant to both oxidative biocides as the wild-type cells. Thus, although there is some evidence to suggest that *rpoS* and *algT* have a

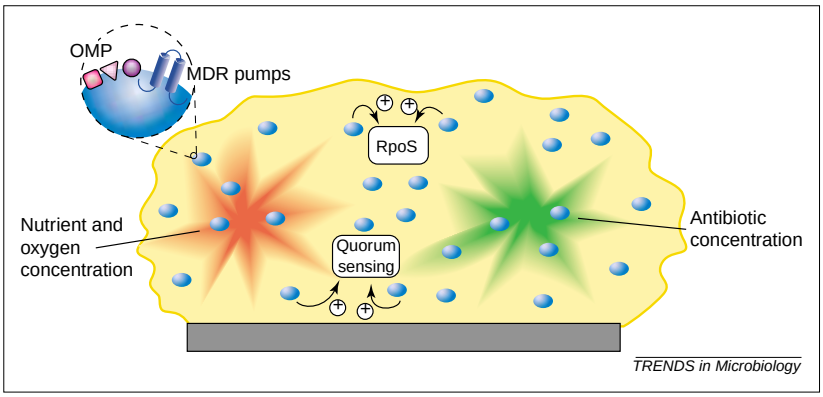

Fig. 3. Drug resistance in biofilms. A schematic of mechanisms that can contribute to the resistance of biofilm-grown bacteria to antimicrobial agents. The extracellular polysaccharide is represented in yellow and the bacteria as blue ovals. Biofilms are marked by their heterogeneity and this heterogeneity can include gradients of nutrients, waste products and oxygen (illustrated by colored starbursts). Mechanisms of resistance in the biofilm include increased cell density and physical exclusion of the antibiotic. The individual bacteria in a biofilm can also undergo physiological changes that improve resistance to biocides. Various authors have speculated that the following changes can occur in biofilm-grown bacteria: (1) induction of the general stress response (an *rpoS*-dependent process in Gram-negative bacteria); (2) increasing expression of multiple drug resistance (MDR) pumps; (3) activating quorum-sensing systems; and (4) changing profiles of outer membrane proteins (OMP).

role in biofilm resistance to oxidative biocides, it is clear that other factors must contribute to this resistance.

Quorum sensing

The role of quorum sensing in biocide resistance is not yet clear. Previous work by Davies and colleagues showed that a mutant in the *lasR–lasI* quorum-sensing system in *P. aeruginosa* was unable to form a biofilm with normal architecture[37]. Moreover, these authors presented data showing that *lasI* mutant biofilms were abnormally sensitive to treatment with SDS, although the question of whether these mutant biofilms had altered antibiotic resistance was not addressed[37]. However, a recent study by Brooun and co-workers showed that mutants defective in quorum sensing were unaffected in their resistance to detergents and antibiotics[26]. Further complicating the interpretation of these studies is a report suggesting a role for RpoS in regulation of quorum sensing[38]. Additional experimentation is required to elucidate the role (direct or indirect) of quorum sensing in biocide resistance.

Induction of a biofilm phenotype

Thus far, the mechanisms discussed have been based on general strategies to slow the effect of antimicrobial agents on cells in the biofilm. An emerging idea in the field is that a biofilm-specific phenotype is induced in a subpopulation of the community that results in the expression of active mechanisms to combat the detrimental effects of antimicrobial agents[15,39–41].

When cells attach to a surface, they will express a general biofilm phenotype and work has begun to try to identify genes that are activated or repressed in biofilms compared with planktonic cells[42]. Furthermore, it is possible that all or just a subset of these biofilm cells could express increased resistance to antimicrobial agents. This resistant phenotype might be induced by nutrient limitation, certain types of stress, high cell density or a combination of these phenomena. As summarized below, recent work has focused on the identification of genes that could contribute to this increased-resistance phenotype.

Multidrug efflux pumps can extrude chemically unrelated antimicrobial agents from the cell. In *E. coli*, upregulation of the *mar* operon results in a multidrug-resistant phenotype. The efflux pump thought to be responsible for this resistance is AcrAB. To address the question of whether this known multidrug-resistance system is involved in biofilm resistance to antimicrobial agents, expression of *mar* was monitored in batch, chemostat and biofilm cultures by *lacZ* fusion[41]. Overall, the results did not support the idea that the *mar* operon is upregulated in biofilms, as the level of *mar* was lower in biofilms compared with the level seen in equivalent stationary-phase culture grown in batch. Furthermore, this same group made use of *mar*- and *acrAB*-deleted strains to determine if the resistance of *E. coli* to ciprofloxacin was affected by loss of these loci[40]. Loss of *mar* and *acrAB* did not adversely affect the *E. coli* biofilms, but constitutive expression of *acrAB* did provide a certain level of protection against ciprofloxacin. Although these results suggest that upregulation of the *mar* operon specifically does not account for *E. coli* biofilm resistance to antimicrobial agents, they do not discount the possibility of other multidrug-resistance pumps being induced in response to life in a biofilm.

There are three known multidrug-efflux pumps in *P. aeruginosa* and there are several other putative pumps that have been identified by the *P. aeruginosa* genome project. One study has suggested the importance of one of these pumps in the resistance to the antibiotic ofloxacin[26]. Using strains of *P. aeruginosa* that either lacked or overexpressed the MexAB–OprM pump, it was shown that, at low concentrations of ofloxacin, biofilms lacking the pump were more susceptible to this drug than biofilms that overexpressed the pump. However, for a different quinolone, ciprofloxacin, there was no difference. Therefore, as was the case with the *E. coli* studies, the question of whether induction of pumps is one of the key alterations conferring resistance to biofilm cells awaits further experimentation.

Another resistance mechanism that can be induced in biofilm cells is the alteration of the membrane-protein composition in response to antimicrobial agents. This change could result in decreased permeability of the cell to these compounds. Mutations in *ompB* (a regulator of the

Questions for future research

- What other factors are important for antimicrobial resistance in multi-species biofilms?
- What genes are induced in biofilm cells that allow for increased resistance to antimicrobial agents?
- What signals are involved in *rpoS* regulation in biofilms?
- Are multidrug efflux pumps important for biofilm resistance to antimicrobial agents?
- What is the role of quorum sensing in the biocide resistance developed by biofilms?

genes encoding the outer membrane porin proteins OmpF and OmpC) and in *ompF* increased the resistance of *E. coli* to a β-lactam antibiotic[43]. Mutants that lack OmpF have been shown to be more resistant to chloramphenicol and tetracycline[44]. Furthermore, in starving cells, the relative proportions of the major *E. coli* porins OmpC and OmpF were altered, favoring the expression of the smaller porin, OmpC (Ref. 45). The above results support the suggestion that altering porin expression affects the intrinsic resistance of bacteria to antimicrobial agents. Recently, it was shown that the expression of *ompC* and three other osmotically regulated genes was increased in biofilm bacteria compared with planktonic cells[46]. These data suggested that bacteria in a biofilm are indeed living in an environment of increased osmotic stress. Thus, the environmental conditions within the biofilm can lead to alterations within the cell envelope that protect the bacteria from the detrimental affects of antimicrobial agents.

Conclusion

There is no one answer to the question of why and how bacteria growing in a biofilm develop increased resistance to antimicrobial agents. We have seen that there are many possible mechanisms that account for bacterial resistance to antimicrobial compounds (summarized in Fig. 3). Depending on the bacterial complement of the biofilm, and the antimicrobial agent used to treat the biofilm, different mechanisms will account for resistance to the antimicrobial compound. Furthermore, the environmental heterogeneity that exists within a biofilm might promote the formation of a heterogeneous population of cells, such that different levels of resistance can be expressed throughout the community. For example, the cells closest to the liquid–biofilm interface might be protected to a small degree by the exopolysaccharide matrix and by enzymes that inactivate certain antimicrobial agents. The cells in an intermediate position might be growing slowly and could also be protected by the outermost layer of cells. Finally, another sub-population of cells might express a biofilm-specific resistance phenotype induced by the particular environmental factors influencing these cells. It is clear that additional studies must be performed to further elucidate how and why bacteria growing in complex surface-attached communities can protect themselves from the insults of antimicrobial agents.

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Viruses of the extremely thermophilic archaeon *Sulfolobus*

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Viruses of *Sulfolobus* are highly unusual in their morphology, and genome structure and sequence. Certain characteristics of the replication strategies of these viruses and the virus–host interactions suggest relationships with eukaryal and bacterial viruses, and the primeval existence of common ancestors. Moreover, studying these viruses led to the discovery of archaeal promoters and has provided tools for the development of the molecular genetics of these organisms. The *Sulfolobus* viruses contain unique regulatory features and structures that undoubtedly hold surprises for researchers in the future.

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Since their recognition as the third domain of life, Archaea, particularly those that thrive in extreme environments, have been the focus of a great deal of recent research, including complete genome sequence determination[1]. However, the viruses of these organisms are poorly understood. A survey of the extra-chromosomal elements of the extremely thermophilic and acidophilic archaeon *Sulfolobus* has revealed the presence of many novel viruses, and both conjugative and cryptic plasmids[2,3]. The viruses of *Sulfolobus* have been assigned to four novel families – Fuselloviridae (viruses SSV1, SSV2 and SSV3)[3–5], Rudiviridae (SIRV1 and SIRV2)[6], Lipothrixviridae (SIFV)[7] and Guttaviridae (SNDV)[8] – on the basis of their unique morphology.

Morphology

The structure of typical virus particles from each of the four families is shown in Fig. 1. The flexible filamentous virions of the lipothrixvirus SIFV (2000 × 24 nm) contain a nucleosome-like core of linear DNA wound as a superhelix around a zipper-like array of 80-kDa protein subunits. The core is covered by a lipid envelope (Fig. 1a). By contrast, the stiff rod-shaped virions of rudiviruses (830–900 × 23 nm) do not possess an envelope. In the Rudiviridae, the tube-like superhelix formed by linear DNA and a single 15.8-kDa DNA-binding protein is closed at its ends by 'plugs' to which tail filaments are attached (Fig. 1b). The spindle-shaped fusellovirus virions (100 × 60 nm) have a core of positively supercoiled circular DNA associated with a DNA-binding protein, packaged in a hydrophobic protein envelope, which has a short tail (Fig. 1c). Finally, virions of the guttavirus SNDV (100–185 × 70–95 nm) take the form of droplets, which, on their pointed end, carry a dense 'beard' of long thin filaments (Fig. 1d).

Natural hosts and geographical distribution

Sulfolobus viruses appear to be ubiquitous in acidic hot-spring environments. Fuselloviruses have been found in *Sulfolobus* strains isolated from solfataric fields in Japan, Iceland and North America. The natural carriers of rudiviruses and the lipothrixvirus SIFV are *Sulfolobus* isolates from diverse locations in Iceland, although viruses of similar morphology have been observed in samples from North America. The guttavirus SNDV has been found in a *Sulfolobus* isolate from a field sample from New Zealand.

Virus–host relationships

None of the *Sulfolobus* viruses is lytic: the fuselloviruses are temperate, and the others are present in their hosts in a more-or-less-stable carrier state. This strategy could help the viruses escape prolonged direct exposure to the low pH (1–3) and

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