Trends in Parasitology

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Opinion

# Gels and cells: the *Leishmania* biofilm as a space and place for parasite transmission

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***Leishmania*** **make an abundant glycoprotein and proteophosphoglycan-rich gel, called the promastigote secretory gel, in the anterior midgut of their sand fly vector. This gel is a multi-faceted virulence factor which promotes the survival and transmission of the parasites between hosts. Here, we present the case that *Leishmania* parasites embedded in the promastigote secretory gel should be redefined as a biofilm as it shares striking similarities in biogenesis, form, and function with biofilms of other unicellular organisms. We believe that this reinterpretation will stimulate new hypotheses and avenues of research to improve our understanding of the developmental programme of *Leishmania* and the interaction these parasites and other kinetoplastids have with their insect hosts.**

## Highlights

*Leishmania* parasites embedded in the promastigote secretory gel in the midgut of its sand fly vector is analogous to a classical attached microbial biofilm.

*Leishmania* biofilm is important for positioning parasites for transmission but also maintenance of sand fly infection.

*Leishmania* proteophosphoglycans within the biofilm are important for the establishment of infection in mice by hijacking the wound-healing response in skin and the activation of macrophages.

Biofilms have been observed during the insect stage of other trypanosomatid parasites, including the honeybee parasite, *Lotmaria passim*.

## Microbial biofilms

The *Leishmania* parasite occupies particular niches in its sand fly vector which enable life cycle progression and onward transmission. This opinion article draws attention to the fact that a key factor in these processes is the formation of a biofilm, consisting of *Leishmania* parasites embedded in the promastigote secretory gel. Here, we highlight the biology of this biofilm, its likely functions in parasite transmission, and make comparisons with generic concepts described in other microbial biofilms. We acknowledge that a *Leishmania* infection of a sand fly does not take place in isolation, and the midgut contains a diverse and varied bacterial microbiome [1,2] – which, in combination with the sand fly immune system, will affect *Leishmania* development [3]. However, here we focus on *Leishmania*.

Microbial cells, both prokaryotes and eukaryotes, exist either as independent planktonic cells or as communities in the form of surface-attached biofilms or as non-attached aggregates. These latter two consist of a heterogeneous aggregations of cells that are embedded in a matrix of **extracellular polymeric substances** (see Glossary) [4,5]. Although the composition may vary, extracellular polymeric substances are organic biopolymers often composed of polysaccharides but can also include proteins, lipids, extracellular nucleic acids, and other molecules [6]. For simplicity, given the commonalities between attached and non-attached microbial community types [4], we refer to both of these types as biofilms. Biofilms are found across a range of different environmental settings. In the clinical arena, microbial biofilms are associated with chronic infections that are difficult to treat or found attached to medical devices, such as stents [7,8]. In industrial settings, biofilms have been implicated in blocked pipes and in the wider natural environment, they can provide protection from a range of pressures, including desiccation [9–11].

The high cell density and competition for nutrients within the biofilm results in the differentiation of cells within the biofilm into subpopulations, with an increase in expression of genes associated with lack of nutrients and oxygen, and those related to slow growth and the stationary phase

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[12–15]. While the majority of biofilm research focuses on single-species biofilms, mixed-species biofilms that contain multiple different bacterial species, and also unicellular eukaryotes such as yeast, are common [16,17]. These mixed-biofilm communities often have metabolic advantages, with waste products of one member utilised by the others [18]. Biofilms also have an important role in protecting the embedded cells from the action of antibiotics and other deleterious stresses, including pH, temperature, UV, and chemicals [19–23]. The aggregation of cells into a biofilm therefore supports a functional differentiation from individual, freely moving cells, enabling persistence in a particular environmental niche. Interestingly, recent work has shown that the kinetoplastid parasite *Lotmaria passim* forms a biofilm in its honeybee host [24] and that led us to consider the role for a biofilm during *Leishmania* development in the sand fly.

## Defining the *Leishmania* biofilm

*Leishmania* parasites have a complex life cycle, with multiple developmental forms, during which they alternate between their mammalian hosts and their insect vector, the sand fly (Figure 1A; [25]). In the mammalian host, the *Leishmania* **amastigote** resides in the parasitophorous vacuole of a macrophage. When a sand fly takes an infected blood meal the blood cells – including the macrophages – are lysed, releasing the amastigotes, which transform into the **procyclic** promastigote form, followed by the **nectomonad** form [26]. The nectomonad form escapes from the blood meal remnants encased in the **peritrophic matrix** and interdigitates its flagellum between the midgut microvilli to ensure that the parasite persists in the sand fly as the blood meal remnants are defecated. The nectomonads then migrate to the cardia and stomodeal valve region of the anterior midgut and transform to the **leptomonad** form [26,27]. In this region of a *Leishmania*-infected sand fly midgut two additional forms are subsequently observed: the metacyclic form, which is important for onward transmission to the mammalian host, and the **haptomonad** form, which is attached to the stomodeal valve [28]. The leptomonad, metacyclic, and haptomonad forms are all embedded in the promastigote secretory gel, and together the gel and the parasites constitute a biofilm (Figure 1B) [27,29]. From now on, we will refer to the embedded *Leishmania* parasites in the promastigote secretory gel as a biofilm.

The developmental relationship between the parasite forms – leptomonad, metacyclic, and haptomonad – in the thoracic midgut has not been fully defined but it is likely that the leptomonad form is the progenitor of both the metacyclic and haptomonad, with a recent preprint describing single-cell RNAseq analysis of *Leishmania*-infected sand flies supporting this conclusion [29,30]. There are currently limited molecular markers to distinguish these forms, with metacyclics characterised by expression of SHERP [31–33] and a recently identified set of adhesion proteins associated with the haptomonads [34]. However, transcriptomic analysis of leptomonads and metacyclics isolated from sand flies showed that these stages expressed genes associated with nutrient stress and slow growth [35], as predicted for stratified microbial biofilms [13].

The promastigote secretory gel element of the biofilm has been most extensively studied in *Leishmania mexicana* [36–38]. Biochemically, the promastigote secretory gel is comprised of a number of proteophosphoglycan moieties and glycoproteins, the most abundant of which are the filamentous proteophosphoglycans and secreted acid phosphatase [39]. However, secreted acid phosphatase activity has not been identified in *Leishmania major* [40], likely indicative of a different promastigote secretory gel composition. In addition, a membrane-bound proteophosphoglycan was identified in *L. major* [41], which may act to create different layers of extracellular polymeric substances around the parasites, as has been observed for microbial communities in sludge [42]. Ultrastructurally, the biofilm contains parasites embedded in a dense matrix of glycoproteins and filamentous proteophosphoglycans [36,39].

## Glossary

**Amastigote:** a *Leishmania* developmental stage, found within the macrophage parasitophorous vacuole, which has a small cell body, with a short flagellum that barely protrudes from the cell body.
**Extracellular polymeric substances:** organic polymers of microbial origin composed of combinations of polysaccharides, proteins, nucleic acids, and lipids which are used for embedding cells as part of a biofilm.
**Haptomonad:** a *Leishmania* developmental stage found stably attached to the sand fly stomodeal valve through a heavily modified flagellum. This stage has a shorter and wider cell body, with a much-reduced flagellum.
**Leptomonad:** a *Leishmania* developmental stage found in the thoracic midgut and cardia of the sand fly. This form is the transformation product of the nectomonad and has an elongated cell body and long flagellum; it is the likely precursor of the haptomonad and metacyclic form.
**Lipophosphoglycans:** an important class of molecules found on the surface of the *Leishmania* parasite. It consists of a glycolipid core that is attached to the cell membrane through a glycosylphosphatidylinositol (GPI) anchor, with a series of phosphosaccharide repeats forming the phosphoglyan chain that extends from the cell surface.
**Nectomonad:** a *Leishmania* developmental stage associated with parasite escape from the digested blood meal. The cells are large and highly motile in comparison to the procyclic form, with its long flagellum able to interdigitate with the midgut microvilli. This form marks the transition of the *Leishmania* parasite from the abdominal midgut to the thoracic midgut.
**Peritrophic matrix:** a structure, formed from glycoproteins and chitin microfibrils, which encases the blood meal separating the midgut epithelium from the blood, as it is digested.
**Procyclic:** a *Leishmania* developmental stage into which the amastigote form differentiates. This stage has an elongated cell body with a short flagellum, offering the cell limited motility. The term 'procyclic' is also used by many authors to collectively refer to all promastigote stages preceding metacyclics in the *Leishmania* life cycle.

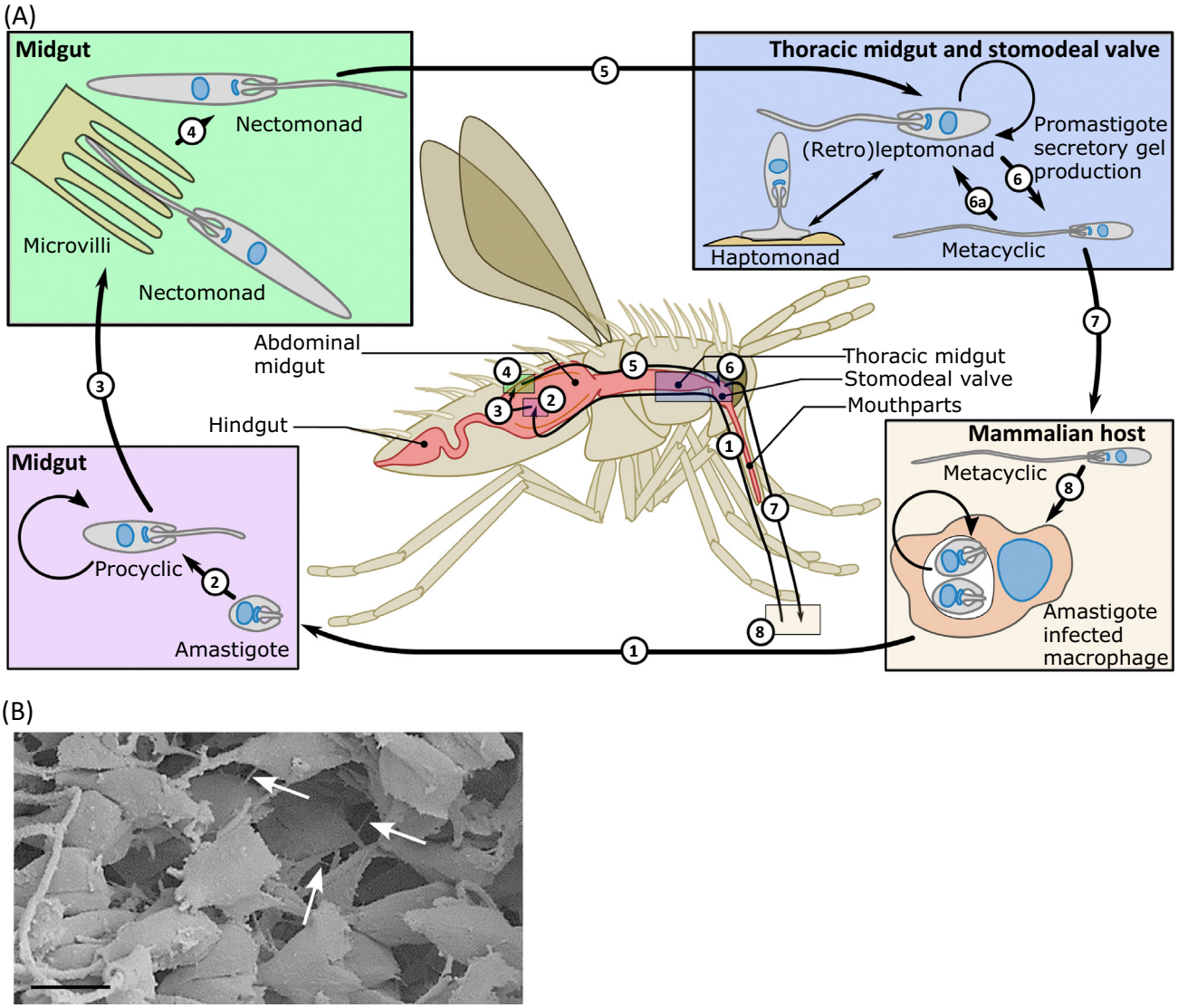


Figure 1. The *Leishmania* life cycle. (A) Cartoon highlighting the location of critical development steps in the *Leishmania* life cycle following an infected bloodmeal (adapted from [25]). (1) Blood is taken into the midgut and encased in a peritrophic matrix. (2) Amastigotes transform into weakly motile procyclic promastigotes and replicate in the nutrient-rich environment of the bloodmeal. (3) Procyclics transform into nectomonad forms which escape the peritrophic matrix and attach to the midgut microvilli to resist expulsion during defecation. (4) Nectomonads, strong swimmers, detach from the microvilli and migrate anteriorly towards the stomodeal valve. (5) Nectomonads transform into leptomonads in the anterior midgut, then replicate. It is likely that leptomonads that encounter the chitin on the stomodeal valve transform into the haptomonad form and attach by modifying their flagellum. (6) In the gel, leptomonads differentiate into infectious metacyclic promastigotes and accumulate next to the stomodeal valve. (6a) Another blood meal stimulates the expansion of leptomonad-like forms, termed retroleptomonads, possibly from the dedifferentiation of metacyclics. (7) Metacyclic, non-metacyclic forms, and a proportion of the promastigote secretory gel are regurgitated together into the skin of the host when the infected sand fly feeds. (8) Metacyclics invade host macrophages, then transform into and replicate as amastigotes, to complete the life cycle. (B) Scanning electron micrograph of *Leishmania* biofilm. *Leishmania* promastigotes embedded in the promastigote secretory gel. White arrows point to strands of promastigote secretory gel connecting different cells. Scale bar: 2 μm. Image adapted from [50].

The major structural components of the promastigote secretory gel are the filamentous proteophosphoglycans, a set of proteins that have a highly repetitive central domain which is heavily glycosylated – a clear extracellular polymeric substance. Three different variably substituted

phosphoglycans are attached to the serine-rich repeat domain of the proteophosphoglycans, with the core unit composed of Gal-Man-$PO_4$ phosphodisaccharides [36]. A central repetitive core of serine/threonine-rich sequences is also seen in the *Saccharomyces cerevisiae* flocculins, which are important for yeast cell adhesion and biofilm formation [43], suggesting that this is a common solution to enable microbial aggregation.

There are five filamentous proteophosphoglycan genes encoded in the *L. major* genome, with an array of four genes adjacent to each other on chromosome 35 – PPG1, PPG3, PPG4, PPG5 – while PPG2 is present as a single copy on chromosome 33 [36,39,44,45]. The filamentous proteophosphoglycans are predominantly expressed in the promastigote forms, except for PPG2 which is also expressed in the amastigote, with a life cycle-dependent pattern of glycosylation [46]. The specific PPG gene expressed by the different *Leishmania* stages – and which form is the dominant player in biofilm formation – are unknown. Moreover, the modification pattern of the filamentous proteophosphoglycan may vary between stages, as is found for the surface embedded **lipophosphoglycan** [47]. A potential added complexity is that other organisms present in the midgut may alter the chemical composition of these modifications through the action of transferases and/or hydrolases to epimerise sugar units or to transfer or remove sugar moieties, as seen in other biological systems [48].

Secreted proteophosphoglycans are detectable by antibodies from the earliest stages of sand fly infection, when the parasites are still within the blood meal and appear to be synthesised throughout the lifetime of the infection [27,49]. They condense into a gel that can be observed when the parasites have migrated to the anterior midgut. Typically, the biofilm extends posteriorly from the stomodeal valve as the parasite numbers increase, allowing it to extend into the posterior portions of the abdominal midgut [29]. The presence of the biofilm puts pressure on the midgut walls and can triple the volume of the anterior midgut, forcing open the stomodeal valve which separates the midgut from the foregut and proboscis [50]. In sand flies fed a second blood meal the biofilm expands even further and manages to push its way into the pharynx and proboscis [50,51].

Overall, there are clear commonalities in organisation and gene expression between a classic attached microbial biofilm and the *Leishmania* biofilm:

(i) A mixture of developmental stages, including the haptomonad attached to the stomodeal valve that anchors the biofilm.
(ii) Cells are embedded in an extracellular polymeric substance.
(iii) Gene expression profiles are associated with nutrient stress and slow growth.

## Functions of the *Leishmania* biofilm

The *Leishmania* biofilm has four key functions that facilitate transmission: (i) changing sand fly feeding patterns; (ii) spatial positioning and maintenance of infective parasites in the sand fly; (iii) protection from the immune system; and (iv) physiological effects on the mammalian host.

### Sand fly feeding patterns

The *Leishmania* biofilm plays an important role in parasite transmission by partially blocking the sand fly gut, reducing the efficiency of feeding, resulting in the sand fly making more feeding attempts – each associated with regurgitation of the embedded parasites [49,52]. The bacterium *Yersinia pestis* – which causes plague, and is transmitted by fleas – uses an analogous mechanism to enhance transmission. In the flea, *Y. pestis* forms a biofilm in the gut and proventriculus that results in increased feeding attempts and hence bacterial transmission [53,54]. Ultimately, the *Y. pestis* biofilm is lethal to the flea as it leads to its starvation and dehydration within days.

Although not as extreme, the *Leishmania* biofilm may also reduce the longevity of its vector, while also promoting the biting frequency and persistence of sand flies [33,52].

### Parasite positioning and maintenance

The *Leishmania* biofilm is likely to be important for positioning the parasites in the thoracic midgut for transmission, with the spatial distribution of metacyclics and leptomonads changing as the biofilm develops, resulting in accumulation of metacyclics near the stomodeal valve [29]. In combination with the haptomonad form, the biofilm will stop all parasites being transmitted during sand fly feeding, ensuring maintenance of infection. Reinforcing this, intake of a second blood meal can substantially increase the size of the biofilm [50] and the number of haptomonads attached to the stomodeal valve [51].

### Protection from the immune system

The *Leishmania* biofilm has a protective effect mirroring that seen for other microbial biofilms, as proteophosphoglycan (i) is a potent activator of the complement system, depleting it before complement can damage the *Leishmania* parasites regurgitated by the feeding sand fly [55,56], and (ii) can protect transforming amastigotes from the lethal effect of sand fly midgut hydrolases *in vitro* [57]. By extension, proteophosphoglycans secreted by the parasite could therefore offer protection from digestive enzyme attack in sand flies during the earliest stages of amastigote-to-procyclic differentiation. Additionally, proteophosphoglycans from the mature biofilm may protect transforming amastigotes, if they were introduced into the *Leishmania*-infected blood meal, as the blood meal was ingested by a sand fly, with an existing *Leishmania* infection.

### Effects on the mammalian host

The *Leishmania* biofilm is also important for establishing the mammalian infection. Proteophosphoglycans from the biofilm transmitted with the parasites influence cutaneous and visceral leishmaniasis in mice, and macrophages *in vitro*, through insulin-like growth factor-1 signalling and arginase 1 expression [33,58–60]. This and the biofilm's ability to potently attract macrophages and neutrophils to the bite site manipulates the host by exaggerating the wound response to the sand fly bite, resulting in the influx of more parasite host cells. The net result is exacerbated infections which are more transmissible to other sand flies [58,59]. Similarly, the regurgitated *Y. pestis* biofilm offers protection from the host's innate immunity, potentially giving them an advantage in establishing infection [61,62]. It is important to note that while these four functions of the *Leishmania* biofilm outlined above are supported by experimental evidence, the effect on these functions of the specific deletion of the proteophosphoglycan genes, which would be expected to block biofilm formation, has yet to be examined.

## *Leishmania* biofilm development

To interpret biofilm function in detail, it is critical to understand the processes driving its assembly, maturation, and dispersal. Currently, we lack the resolution on the composition and workings of *Leishmania* biofilm development to give a detailed description and can only provide an overview (Figure 2). During a sand fly infection, leptomonad forms colonise the thoracic midgut and cardia where they duplicate and also differentiate to metacyclic and haptomonad forms, while the biofilm is being established [26,29]. After this point the number of metacyclics continues to increase, as does the volume of the biofilm, while the number of leptomonads falls [29]. A mature sand fly infection, before a second blood meal, is characterised by a high cell density of metacyclics embedded in a biofilm, with many haptomonads attached to the stomodeal valve and with fewer leptomonads present.

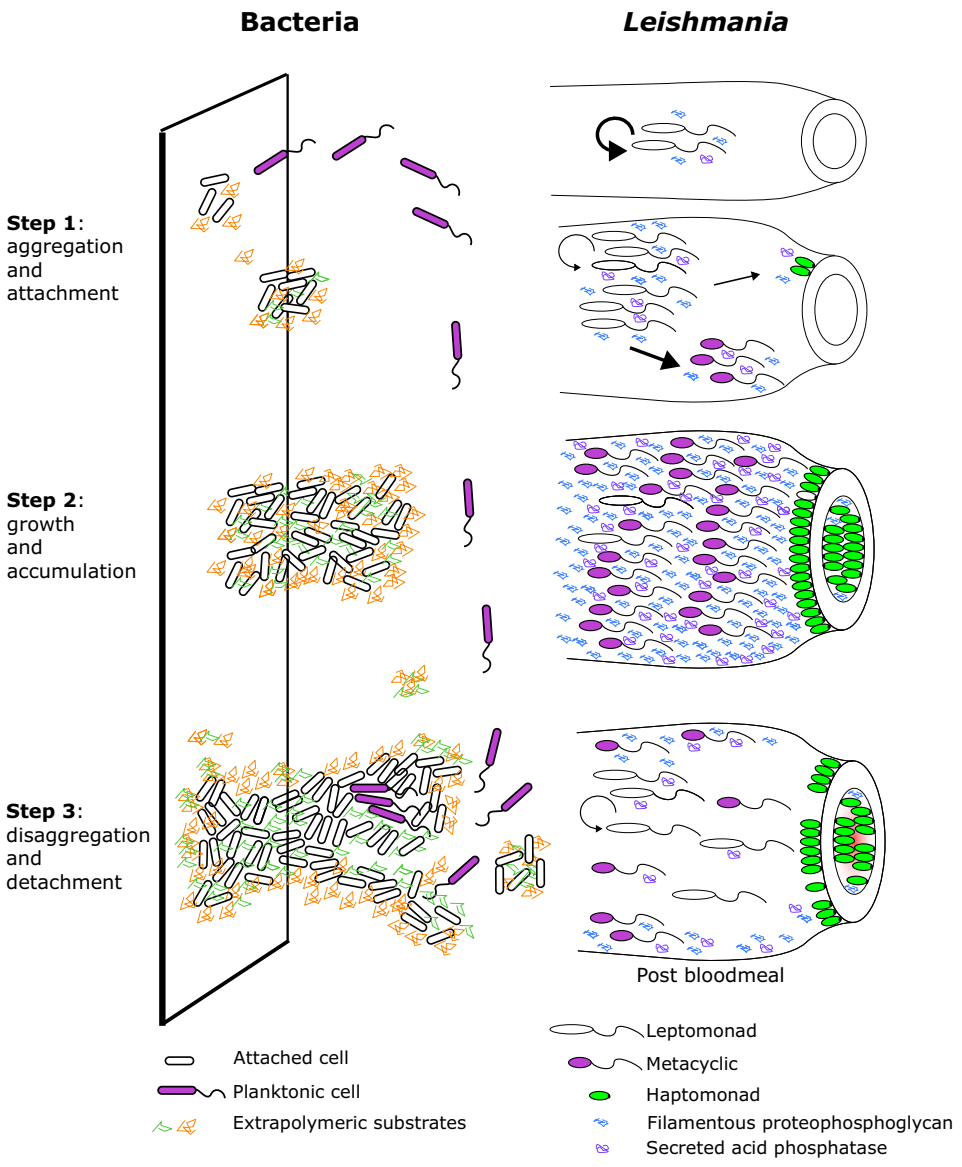


Figure 2. Comparison of bacterial and *Leishmania* biofilm development. Three-step model of biofilm dynamics from initial aggregation to release of cells back into the environment. For the development of the *Leishmania* biofilm, the circular arrows indicate parasite replication, with the linear arrows representing a differentiation step, and the thickness gives an indication of the relative amount of replication and/or differentiation occurring at that point.

Given our aim of understanding the *Leishmania* biofilm in the context of other microbial biofilms, we asked how similar this process is to models of biofilm development. The original five-step model of surface-attached biofilm development was outlined in 2002 and used *Pseudomonas aeruginosa* to show that specific stages of biofilm formation were associated with defined morphological and metabolic changes [63]. Step 1: reversible attachment, defined by cells making unstable contact with a surface. Step 2: irreversible attachment, defined by a cellular commitment to attach to a surface that triggers a genetic reprogramming, resulting in the production of extracellular polymeric substances and loss of flagella-based motility. Step 3: maturation I, defined by

clusters of cells that are embedded in a matrix of extracellular polymeric substances. Step 4: maturation II, defined by the growth and development of the cell clusters to form mature microcolonies. Step 5: dispersion, defined by the breakdown of biofilm and release of motile cells back into the environment. However, this original five-step model was highly specific for certain bacterial species grown in a closed, defined culture system and attaching to a surface [63].

Recently, a more general model has been proposed to cover the development of all biofilm types, including non-attached aggregates [4,5]. This focuses on three key steps: (i) aggregation and attachment, (ii) growth and accumulation, and (iii) disaggregation and detachment (Figure 2). *Leishmania* biofilm development, in our opinion, aligns well with this three-step model of biofilm development. The initial colonisation of the stomodeal valve region represents Step 1: aggregation and attachment. The increase in *Leishmania* parasite numbers and the blocking of the midgut with the biofilm represents Step 2: growth and accumulation. While the breakdown of the *Leishmania* biofilm has not been observed, the next sand fly blood meal would cause an element of disaggregation and detachment, which combined with the ability of the metacyclic to move through the promastigote secretory gel [29] would align with Step 3.

We speculate that a quorum sensing (QS) mechanism may have a role in regulating *Leishmania* biofilm development, as bacterial biofilm development is regulated by QS [64] and differentiation to the stumpy form in the mammalian host of the related kinetoplastid parasite, *Trypanosoma brucei*, is also regulated through a QS mechanism [65]. Proteophosphoglycans are secreted by all promastigote forms and are detectable using antibodies from as early as 2–3 days post-infection in sand flies [27,49]; therefore, QS is unlikely to have a role in promastigote secretory gel production. However, QS may influence parasite differentiation, including to the haptomonad form, which will help to anchor the biofilm to the sand fly anterior midgut. The microbiota of the sand fly midgut is known to change considerably with blood feeding and during the course of infection; it is essential to support *Leishmania* growth and metacyclogenesis [1], and part of this effect may be the provision of QS sensing molecules. Of course, *Leishmania* may generate their own QS molecule. For example, if leptomonads secrete such a molecule, the levels of this molecule will increase with increasing leptomonad numbers to an extent that it could trigger haptomonad and/or metacyclic differentiation, and hence the development of a mature *Leishmania* biofilm. Interestingly, while QS has not yet been established for *Leishmania*, the horizontal transfer of drug-resistance genes via extracellular vesicles has [66] – indicating that promastigote-to-promastigote communication in the biofilm is possible.

## Concluding remarks and future perspectives

Biofilms enable microorganisms to co-ordinate colonisation and exploitation of new environments and niches. Our view outlined here is that *Leishmania* parasites, embedded in the promastigote secretory gel, fulfil all the established criteria that define a microbial biofilm. It is useful and important to understand the implications this has for parasite development and onwards transmission. The defined microenvironment created by the *Leishmania* biofilm – in which there is an interplay between the different cell types, nutrient availability, and signalling molecules – will play a critical role in regulating *Leishmania* life cycle dynamics (see Outstanding questions). For example, this defined environment may influence *Leishmania* hybridisation by positioning hybridisation-competent cells in close proximity to each other, increasing the efficiency of the formation of parasite clumps by natural IgMs ingested in the blood meal of a sand fly with a mature *Leishmania* biofilm [67].

Furthermore, the *Leishmania* biofilm is constructed from a variety of different proteophosphoglycans and glycoproteins, and these may change with time, while also being influenced by both the parasite

### Outstanding questions

Which cells are the dominant source of the filamentous proteophosphoglycan, and what are the dynamics of its formation?

Does the biofilm play a role in cell differentiation and growth?

Is there a specific trigger for metacyclic (and haptomonad) differentiation – for example, nutrient stress, QS – or do leptomonad forms stochastically differentiate?

What are the biophysical properties of the biofilm, and how do the different promastigotes interact with it?

Does disruption of the *Leishmania*–biofilm–vector interactions provide a new opportunity for biocontrol?

producer and the anatomy and physiology of the sand fly midgut. It seems likely that leptomonad, haptomonad, and metacyclic promastigotes are likely to interact differently with the biofilm as their cell size and shape alters, their motility changes, and their surface glycocalyx undergoes modification and rearrangement. Moreover, it is feasible that, with a rapidly changing climate [68–70], we may see changes in biofilm dynamics and/or constitution to ensure continued *Leishmania* transmission. A recent study has shown such an effect in *Y. pestis* with an increase in biofilm production linked to changes in climate [71].

Much of the early work on the promastigote secretory gel was done in the pre-genome era, and proteophosphoglycan mutants were generated by blocking specific glycosylation steps that impacted many different molecules [36,72]. Now, with modern high-quality genomes and more sophisticated tools for genetic modification [73–75], it is possible to generate specific proteophosphoglycan mutants and define the functions of the *Leishmania* biofilm.

Finally, it is important to note that the biofilm concept is not restricted to *Leishmania*; it has been observed in other kinetoplastid parasites. Recent work has shown that *L. passim* forms a biofilm in the hindgut of the honeybee [24], and a fibrillar matrix surrounding the cells attached to the hindgut was described in *Trypanosoma theileri*-infected tabanids and for *Crithidia mellificae* and *Crithidia acanthocephali* in honeybees [76–78]. Moreover, orthologs of proteophosphoglycan genes, including the serine-rich repeat region, are present in the genomes of parasites in related genera, *Endotrypanum*, *Crithidia*, *Leptomonas*, and *Lotmaria* [44]. As attachment to arthropod tissues is common to kinetoplastid parasites, it is therefore likely that there is an underappreciated role for biofilms in the life cycle and transmission potential of important human and livestock trypanosomatid parasites, for example during *Trypanosoma cruzi* attachment to the hindgut of the triatome and *Trypanosoma congolense* attachment to the proboscis of the tsetse fly.

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## Declaration of interests

The authors declare no competing interests.

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