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MicroCommentary

Should they stay or should they go? Nitric oxide and the clash of regulators governing *Vibrio fischeri* biofilm formation

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Summary

A key regulatory decision for many bacteria is the switch between biofilm formation and motile dispersal, and this dynamic is well illustrated in the light-organ symbiosis between the bioluminescent bacterium *Vibrio fischeri* and the Hawaiian bobtail squid. Biofilm formation mediated by the *syp* gene cluster helps *V. fischeri* transition from a dispersed planktonic lifestyle to a robust aggregate on the surface of the nascent symbiotic organ. However, the bacteria must then swim to pores and down into the deeper crypt tissues that they ultimately colonize. A number of positive and negative regulators control *syp* expression and biofilm formation, but until recently the environmental inputs controlling this clash between opposing regulatory mechanisms have been unclear. Thompson *et al*. have now shown that Syp-mediated biofilms can be repressed by a well-known host-derived molecule: nitric oxide. This regulation is accomplished by the NO sensor HnoX exerting control over the biofilm regulator HahK. The discoveries reported here by Thompson *et al*. cast new light on a critical early stage of symbiotic initiation in the *V. fischeri*-squid model symbiosis, and more broadly it adds to a growing understanding of the role(s) that NO and HnoX play in biofilm regulation by many bacteria.

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Biofilm formation is a key process for many bacteria, but it can represent a double-edged sword, with the benefits of adherence and aggregation weighed against the costs of limited mobility. Accordingly, bacteria have evolved regulatory mechanisms to make context-dependent decisions whether to stay or go. This dynamic is well illustrated during the initial stages of infection in the symbiosis between the bacterium *Vibrio fischeri* and the Hawaiian bobtail squid, *Euprymna scolopes* (Nyholm and McFall-Ngai, 2004; Norsworthy and Visick, 2013; Stabb and Visick, 2013). As adults, these squid have bioluminescent *V. fischeri* packed into a light-emitting organ, which they are thought to use in a camouflaging effect called "counter illumination" (Jones and Nishiguchi, 2004; Stabb and Millikan, 2009). However, when the squid first hatch, they lack symbionts and must acquire them from the environment in a process that is promoted by ciliated mucus-shedding epithelial cells on the surface of the organ (McFall-Ngai and Ruby, 1991; Nyholm *et al*., 2000; Nyholm and McFall-Ngai, 2003). In this first stage of infection, biofilm formation driven by the eighteen-gene *syp* locus is critically important (Yip *et al*., 2005; Yip *et al*., 2006; Shibata *et al*., 2012; Morris and Visick, 2013). Syp-dependent biofilm formation enables *V. fischeri* cells to form aggregates on the light-organ surface in the transition from a dilute planktonic existence to a symbiotic one. However, in the next stage of their symbiotic journey, swimming motility is absolutely required for *V. fischeri* cells to reach their ultimate home in crypts within the light organ (Graf *et al*., 1994). Thus, in the first few hours of initiating infection, *V. fischeri* cells must first form multi-celled biofilm aggregates and then swim as individuals (Fig. 1). Wild-type *V. fischeri* strains that are most competitive at initiating infection form robust Syp-dependent aggregates but also begin their march toward the light-organ interior quickly (Koehler *et al*., 2018).

Given its importance in the nascent symbiosis, the regulation of biofilm formation has been well studied in *V. fischeri*, and interest in this process is heightened by similarities to biofilm formation and its regulation in

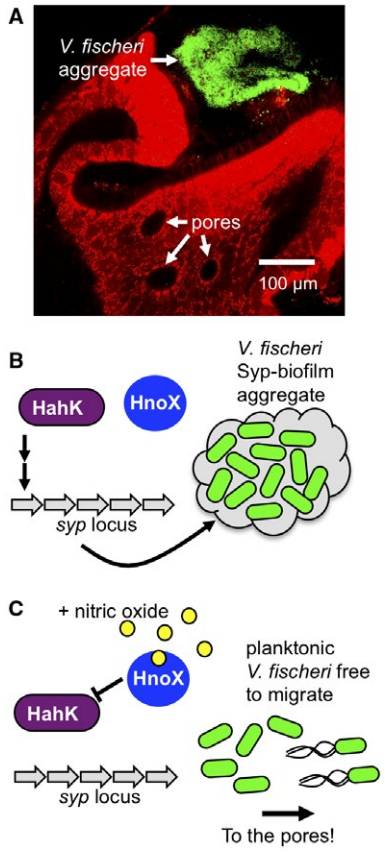
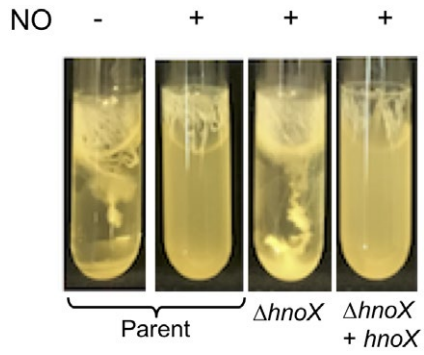


Fig. 1. Image and model of *V. fischeri* initiating symbiotic infection. A. A confocal micrograph shows *V. fischeri* (green) forming an aggregate of cells in a mucus matrix associated with ciliated epithelial cells (red) on the surface of the host-squid light organ. In this initial interaction with the host, Syp-mediated biofilms promote aggregate formation. However, swimming motility is then required as the cells migrate to pores and eventually traverse ducts and gain access to deeper crypt spaces. The host delivers nitric oxide to the surface mucus.
(B & C): A new model for biofilm regulation. HahK promotes activation of the *syp* locus and Syp-mediated aggregate formation (panel B), but accumulation of nitric oxide (yellow circles) causes HnoX to repress HahK activity, reducing Syp-biofilm formation and freeing more cells to migrate toward the pores (panel C). Panel A is taken from Yip et al. (2006) with permission of the authors.

pathogenic *Vibrio* species (Visick, 2009; Yildiz and Visick, 2009). In the current model of *syp*-locus regulation, SypG is a key response regulator that when phosphorylated promotes *syp* transcription and biofilm formation. SypG is phosphorylated and activated by the sensor kinase SypF, which itself can be modulated by another sensor kinase RscS (Darnell *et al*., 2008; Hussa *et al*., 2008; Norsworthy and Visick, 2015). Conversely, SypF can reduce SypG activity, although the conditions determining whether SypF has a net positive or negative effect on SypG activation are not yet understood (Thompson *et al*., 2018b). The versatile SypF also modulates the activity of SypE, which represses Syp-biofilm formation post-transcriptionally (Morris *et al*., 2011; Morris and Visick, 2013). Two additional regulators, BinK and HahK, have negative and positive effects on Syp-biofilm formation respectively (Brooks and Mandel, 2016; Thompson *et al*., 2018; Tischler *et al*., 2018). Interestingly, *V. fischeri* isolates from non-squid sources often are poorly adapted for infecting *E. scolopes*, but they can become more proficient through mutations in *binK* (Pankey *et al*., 2017) or the acquisition of *rscS* (Mandel *et al*., 2009), which result in more robust Syp-biofilms. These experimental evolution studies have underscored the importance of *syp* regulation in establishing this symbiosis.

Despite our detailed understanding of this clash between positive and negative regulators governing the *syp* locus, the underlying environmental cue(s) determining aggregate formation and dispersal have remained elusive, particularly with respect to the conditions relevant in the symbiotic environment. The fact that Syp biofilms are essentially not made by wild-type grown in standard culture media has proven an experimental obstacle in this and other regards. Recently, manipulation of salt and nutrient conditions has led to increased *sypA* transcription in culture (Marsden *et al*., 2017), and calcium in particular is *syp*-inducing (Tischler *et al*., 2018). However, neither of these studies has yet pointed to a clearly documented symbiotic cue, and two critical open questions remain. What elements of the symbiotic environment stimulate *V. fischeri* to generate a Syp-mediated aggregate? And what other cue(s) stimulate the dissolution of the biofilm to favor migration toward the site of infection?

In this issue of *Molecular Microbiology*, Thompson, Tischler *et al*. (2018) now connect a negative regulator of Syp biofilm formation to a regulatory cue with its own rich history of study in this and other symbioses: nitric oxide (NO). As noted above, HahK is an indirect activator of *syp*. Because *hahK* appears to be in a two-gene operon with *hnoX*, which encodes an NO-sensor, Thompson and her colleagues tested whether there is a regulatory connection between NO, HahK and biofilm formation. They now report that NO inhibits Syp-biofilm formation by *V. fischeri* and that this inhibition requires HnoX and HahK (Fig. 2), suggesting a new model for symbiotic biofilm regulation (Fig. 1). The study provides further evidence supporting a model for HahK entering the previously known regulatory cascade and exerting effects directly on SypF, and thereby indirectly affecting SypG. Although NO can be antimicrobial, the results show biofilm inhibition is not simply the result of growth inhibition.Fig. 2. Nitric oxide (NO) inhibition of biofilm formation in cultured *V. fischeri* cells. NO treatment reduces biofilm formation (visualized as aggregates) and concomitantly increases the number of planktonic cells (illustrated by culture turbidity). NO does not have this effect on an *hnoX* mutant (Δ*hnoX*) but is restored to the mutant by genetic complementation



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(Δ*hnoX*+hnoX). Images are taken from Thompson, Tischler *et al*. (2018) courtesy of K. Visick

The connection between Syp-mediated biofilm regulation and NO is particularly exciting given that NO and its effects have been documented in the *V. fischeri-E. scolopes* symbiosis. Davidson *et al*. (2004) showed that the squid produce a nitric-oxide synthase (NOS) and NO in symbiotic tissues. Moreover, vesicles appear to deliver NO to the mucus on the ciliated surface of the light organ where the Syp-mediated aggregates first form and then disperse during initiation of the symbiosis (Davidson *et al*., 2004). Once the host is infected, microbe-associated molecular patterns (MAMPs), specifically lipopolysaccharide and peptidoglycan, from *V. fischeri* trigger down-regulation of NO production (Altura *et al*., 2011).

The concept of NO acting as an inter-kingdom signal in this and other symbioses has been well considered (e.g. (Nyholm and McFall-Ngai, 2004; Wang and Ruby, 2011)), but to a large degree *V. fischeri*'s response to NO has been viewed through the lens of *V. fischeri* resisting NO's antibacterial effects. For example, it has been proposed that *V. fischeri*'s multiple mechanisms for resisting NO may contribute to the specificity of this symbiosis. In *V. fischeri*, the NO-responsive regulator NsrR governs NO-resistance mechanisms that include the NO-detoxifying flavohaemoglobin protein Hmp (Wang *et al*., 2010b) and the heme-independent NO-resistant alternative oxidase Aox (Dunn *et al*., 2010). Bioinformatic analyses further predict that the *nnrS* gene, which encodes another putative heme-containing NO-resistance determinant, is likewise regulated by NsrR (Rodionov *et al*., 2005). As noted above, *hnoX* encodes another NO-sensor in *V. fischeri*, and HnoX responds to NO by repressing genes involved in iron uptake (Wang *et al*., 2010a). Keeping cytoplasmic iron levels low, along with the ability of Aox oxidize NADH even when other heme-dependent oxidases are blocked by NO, could prevent buildup of ferrous iron and subsequent oxidative stress due to Fenton chemistry (Dunn, 2018). All of these responses reflect the fact that NO can be lethal, and indeed cells can succumb to NO even with all of these defense mechanisms. Interestingly, when hatchling squid were treated with NOS inhibitors, bacterial aggregates on the light-organ surface were larger than usual (Davidson *et al*., 2004), an effect that could easily be viewed as being caused by the loss of antimicrobial NO activity, although this possibility remains speculative.

The results now presented by Thompson *et al*. (2018) encourage viewing such previous data from the perspective of NO as a cue. Perhaps suppressing NO production by the host yields larger bacterial aggregates not because fewer bacterial cells are killed by NO but because NO encourages cells to leave the aggregate. An alternative but similar possibility is that the delivery of NO to the light-organ surface is spatially heterogeneous, limiting aggregate formation to low-NO areas. Although NOS inhibitors also caused larger aggregates by the non-symbiont *Vibrio parahaemolyticus* (Davidson *et al*., 2004), this bacterium along with the pathogen *Vibrio vulnificus* resemble *V. fischeri* in having both a *syp*-gene cluster and a *hahK-hnoX* operon, so they may likewise share NO-mediated Syp-biofilm regulation. On the other hand, while it is tempting to speculate that NO triggers aggregate dispersal on the light organ, this possibility also remains to be tested directly. Although NO represses biofilm formation, its effect on established *V. fischeri* aggregates warrants deeper inspection. Whatever the role of NO, it should be noted that *hnoX* is not required for migration from the aggregate to the crypts (Wang *et al*., 2010a), and it seems likely that HnoX and NO are only a part of the regulatory decisions made by would-be *V. fischeri* symbionts during the transition from seawater to aggregate to migrating cells.

Although there is a good deal of evidence supporting the idea that *V. fischeri* responds to NO produced by the host, Thompson, Tischler *et al.* (2018) rightly point out that endogenous NO production by *V. fischeri* cannot be ruled out as a contributing signal. In *Azospirillum brasilense*, endogenous NO production as a byproduct of denitrification can stimulate biofilm formation, and this stimulation can be blocked by a disruption of the Nap periplasmic nitrate reductase system (Arruebarrena Di Palma *et al*., 2013). Although *V. fischeri* lacks a canonical denitrification system, it does appear to contain an assimilatory pathway for converting nitrate to ammonia, encoding both Nap and the Nrf nitrite reductase, and NO might arise as a result of their activity. Consistent with this possibility, a bioinformatic analysis suggested that in *V. fischeri* the *nap*-activating regulator NarP may also activate expression of the NO-detoxifying Hmp and/or the NO-responsive

regulator NsrR, thus implicating a coordination of nitrate reduction and NO-resistance mechanisms (Rodionov *et al*., 2005). Further investigation of possible endogenous NO production by *V. fischeri* should help clarify this issue.

The current work by Thompson, Tischler *et al*. (2018) not only generates new questions in the *V. fischeri-E. scolopes* symbiosis, it also contributes to a larger interest in NO and biofilm regulation. Several other bacteria regulate biofilm formation in response to NO, in many cases NO leads to biofilm dispersal (Barraud *et al*., 2015), and HnoX is often (although not always) a key player in such regulation (Bacon *et al*., 2017). Thus, the observations here fit into a larger picture of NO-mediated regulation that is coming into focus, and they suggest avenues for further study. For example, based on the homology and synteny of the *syp* and *hnoX* genes noted above, it will be interesting to learn whether the regulation discovered in *V. fischeri* is also at work in the pathogens *V. parahaemolyticus* and *V. vulnificus.* Likewise, further elucidating the interplay between HnoX and SypF could provide mechanistic insight into NO-mediated biofilm regulation in other bacteria. In another broader sense the current study fits in with a growing appreciation that host-associated bacteria can use host-derived NO as part of their decision to adhere in a biofilm structure or to disseminate and spread.

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