

**Supporting information for “Possible odour-mediated attraction of flies to  
*Bacillus subtilis* NRS-762 stationary phase culture”**

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## Future work

The preliminary observations reported leaves open several questions concerning the identity of the volatile compound(s) secreted by *B. subtilis* NRS-762, and the molecular mechanisms by which the compound(s) attracted the flies and elicited possible behavioural change (e.g., induction of foraging activity). More broadly, the preliminary observations may potentiate new avenues of study concerning the ecological and physiological roles of interkingdom signalling between *B. subtilis* and flies via volatile odourous compounds secreted by the bacterium. Nevertheless, replication of the preliminary observations should be conducted first - since a variety of factors (some of which lab-specific) could potentially confound the phenomenon.

After the reported observations have passed the replication test, future research should characterize, via gas chromatography mass spectrometry (GC-MS), the compendium of volatile compounds secreted by *B. subtilis* during each growth phase in LB medium. Information of the volatile compounds' identities would allow comparison (via structural homology) with those known to mediate interkingdom signalling between bacteria and airborne vectors. Additionally, the dataset also forms the basis for high throughput screening experiments designed for determining the specific volatile compounds – either alone or in combination – that help attract flies. The species specificity of the profiled compounds should also be evaluated using the same approach, although the high anticipated cost meant that the effort should be conducted by a consortium of investigators. But beyond determining the compound(s) mediating bacteria-fly interactions, a more important but difficult question involves confirming the causal relationship between the profiled compound(s) and hypothesized signaling (communication) function since the putative compounds might elicit a variety of physiological responses.

Given that the set of volatile compounds reported by Ma et al., also activate swarming motility in *P. mirabilis*, screens could also be conducted to determine whether the odourous compound(s) also induces expression of bacterial genes related to motility, stress response, energy conservation, or cell differentiation. Information gleaned would illuminate possible mechanisms by which the compound(s) help prime the population for coping with environmental stressors via the dual strategies of riding out the impending adverse conditions *in situ*, or seeking proactive long-range dispersal and possible preservation of remnants of the population.

Investigating if the postulated interkingdom signalling phenomenon is present during growth of *B. subtilis* NRS-762 in other media would also be an interesting lead to follow. Such a study would illuminate whether a conserved set of metabolites/nutrients trigger specific cell survival response pathways, in particular, volatile compound dependent

interkingdom signaling described herein. Along the same line, efforts can also be directed towards examining if similar sets of volatile compound(s) are secreted by *B. subtilis* NRS-762 grown on solid medium – and their effects on cell metabolism, physiology and motility mode (particularly, swarming motility). Naturally, the first question to be asked is whether flies would be attracted to *B. subtilis* grown on agar plates. Comparative analysis of the effect of liquid and solid culture on the relative amount and types of volatile compound(s) secreted, and their efficacy in attracting flies and modulating the physiology of the bacterium would yield important insights into possible roles played by the type of “lifestyles” (i.e., planktonic or surface-associated) on the bacterium’s ecophysiology. Additionally, since a large fraction of extant bacteria co-aggregate in biofilms on surfaces [1], investigating possible roles by which volatile compound(s) secretion help select appropriate survival strategies for the population would expand our understanding of the methods used by microbes for surviving – and even thriving – in some of the more hostile environments on the planet.

Defining associative relationships (i.e., who is related or associated with whom) and characterizing the biochemical basis of the association is the central tenet of ecology. Specifically, delineation of the ecological interaction is the first step towards identifying the underlying mechanisms and quantifying the rates of nutrient exchange between species [2]. Signalling is the key mechanism connecting stationary phase *B. subtilis* cultures to the observed behavior of flies in the conceptual model presented, while quorum-sensing was hypothesized to underpin the postulated signaling event given the cell density-dependent nature of volatile compound emanation from *B. subtilis* cultures. One possible approach for verifying the postulated interkingdom signaling between *B. subtilis* and flies could be the direct measurement of activity of neurons in fly olfactory bulb or antennal lobe (via electroantennogram) after exposure to volatile compounds secreted by *B. subtilis*, which when combined with molecular identities afforded by GC-MS, would associate specific compound(s) to particular neurological response [3].

Quorum-sensing is commonly depicted as a cell density-dependent signalling modality, but recent studies have highlighted more subtle aspects of the cell-cell communication system; specifically, the signaling molecules are construed as cues or coercive elements in chemical manipulation of other species [4]. For example, quorum-sensing molecules could be used as “cues” to elicit specific actions from the target organism or serve as elements of coercion to force a response from others [4]. Thus, in the absence of more information concerning the nature of the interactions between *B. subtilis* and flies, the proposed quorum-sensing signalling mechanism could be interpreted as a cue or a form of chemical coercion where *B. subtilis* is attempting to obtain a response from flies. In the latter case, production of the signaling molecule by *B. subtilis* would need to force a costly response from the flies. Put in another way, production of the substance has evolved to benefit the sender rather than the receiver [5].

While the current research note postulated that the volatile compound(s) secreted helped attract flies for long-range dispersal of *B. subtilis* during nutritional stress; however, no evidence was collected on demonstrating the feeding of the bacterium by flies, or its deposition at new localities. Thus, controlled lab or field experiments could be conducted to demonstrate the dispersal of *B. subtilis* by flies (e.g., with *D. melanogaster* as model organism), and the establishment of a new clonal population in an alternative locality with more favourable environmental conditions. Olfactory responses of *D. melanogaster* to a variety of synthetic and natural volatile compounds have been investigated from multiple perspectives ranging from understanding fly-plant interactions to the development of electronic nose [3, 6]. More important, the presence of a fair number of olfactory bulbs (i.e., 40) housing odour sensing neurons in the organism would also provide experimental utility; in particular, the detection of good signal response to volatile compounds at low concentrations. Another factor in favour of *D. melanogaster* serving as model organism is the recently observed capability of the fly in processing volatile compounds (i.e., synthetic chemicals) it is not evolved to process [6]; hence, implying that the repertoire of compounds that the fly could detect and respond to is potentially large. Ability of responding to diverse volatile compounds would facilitate the dissection of the physiological response of flies to the compendium of volatile compounds in *B. subtilis*'s secretome.

Complementary to the broad-based survey approach outlined above, the reductionist methodology of linking specific molecules to particular phenotypes in flies would provide the basis upon which further molecular level studies could determine the mechanistic factors underlying the observed phenomenon. Specifically, given that odours are complex mixtures of myriad molecules, the model fruit fly's ability at discriminating between different compounds within the mixture could be examined, which would help identify compounds of physiological importance. While using a model organism such as *D. melanogaster* may yield insights generalizable to other fly species, another possible experimental approach could investigate the species of flies attracted to the "cocktail" of volatile compounds secreted by *B. subtilis*. Doing so would provide empirical information concerning the species range targeted by the bacterium and, by inference, provide a lens into the selective forces shaping evolutionary relationships between *B. subtilis* and different fly species. Finally, studies could also be conducted to examine if the observed phenomenon extends to other species of *B. subtilis* or flies.

More intriguingly, would other vectors such as mosquito respond to the volatile compounds secreted by *B. subtilis* NRS-762? Specifically, given that mosquitoes are attracted to humans and animals via a combination of odour, heat and carbon dioxide [7], could the same apply in the attraction of flies to *B. subtilis* cultures? Are we missing other elements such as heat and carbon dioxide in the equation? In particular, would heat and elevated carbon dioxide concentration serve to synergistically increase the number of flies attracted to *B. subtilis* NRS-762 cultures? At a more fundamental level, efforts could be directed to

understand the interface between extracellular signalling and secondary messenger signal transduction, which would help delineate the entire set of molecular actors transmitting and translating extracellular information into intracellular actions that potentiate a phenotypic response.

Other related studies – of a more microbiome theme - could also investigate the roles of *B. subtilis* in conferring additional metabolic capabilities to flies, or helping maintain the robustness to perturbation of the flies' GI tract microbiota's community structure. Findings elucidated would help define, in more concrete terms, the nature of mutualistic interactions between flies and *B. subtilis* NRS-762. Compared to intracellular microbial symbionts with reduced opportunities for disengaging from the symbiotic relationship given the vertical transmission of symbionts to the host's offspring, extracellular symbionts do have greater latitude in leaving the relationship through horizontal exchange or *de novo* host acquisition of new symbionts from the environment, which displaces existing species [8]. Thus, an interesting follow-up question would be examining the stability of the above postulated mutualistic relationship between bacteria and flies, and possible factors that may potentiate an unravelling of the relationship either initiated by the bacterium or fly.

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