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Title

United we stand, divided we fall

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Standfirst

Bacteria can compete in the environment using antibacterial Type VI Secretion Systems. A recent study reveals that the simultaneous deployment of an arsenal of different toxins promotes both synergy between those toxins and an optimized answer in the face of inconstant environments.

Main text

Although discovered more recently than other protein secretion systems, the Type VI Secretion System (T6SS) is unexpectedly one of the most widespread among Gram-negative bacteria. Notably, it was the first secretion system discovered that injects antibacterial effectors into target bacteria[1]. The T6SS can thus confer a fitness advantage in environmental niches against rival bacteria (inter- and intraspecies competitiveness have been described) and in the eukaryotic host (i) between commensal bacteria[2] and (ii) between pathogenic and commensal bacteria[3]. In addition, some T6SSs are specialized for disarming the eukaryotic host cell.

The opportunistic pathogen *Pseudomonas aeruginosa* employs three independent T6SSs as antibacterial weapons, two of which also allow interaction with epithelial cells[4]. The H1-T6SS was the first antibacterial T6SS ever described[1] and only an antibacterial role has so far been reported. Seven H1-T6SS effectors have been found, and they disable various cellular components in target bacteria. Immunity proteins, also called antitoxins, bind to the cognate effector proteins to prevent sister cell attack and, in the case of cytoplasmic toxins, prevent self-intoxication. While a single effector protein can kill a competitor, *P. aeruginosa*, like many bacteria, deploys an arsenal of effector proteins. In this report LaCourse and colleagues raised a simple but pertinent question: how do bacteria benefit from the delivery of effectors with diverse activities? The authors proposed two hypotheses that may not be mutually exclusive. Do environmental conditions impact effector activity and are some effectors more adapted to particular environments? Or, do the multiple effectors act synergistically?

LaCourse *et al*. have developed a powerful assay, called PAEE (Parallel Analysis of

Effector Efficacy), with six of the *P. aeruginosa* H1-T6SS effectors as a model. In a classical competition assay, the CFU numbers of target bacteria would be counted for each different condition (i.e. wild-type versus mutant, or according to various growth parameters), using many plates and much time. PAEE is a high-throughput pooled-method based on sequencing unique barcodes introduced into a library of 21 mutants in which one or two of the six effector-immunity gene pairs have been deleted. The library was grown under a variety of environmental conditions in contact with an excess of the unbarcoded parental strain that acts as a donor for the six effectors. The authors tested whether pH, oxygen availability, temperature, or salinity influenced the ability of the parental strain effectors to kill the different effector-immunity deficient mutants. PAEE allowed the authors to observe exciting phenotypes, and showed that the activities of the six effectors are conditioned by the environment. For instance, the killing activity of the effector Tse5 increases between pH 6 and pH 8, with increasing osmolarity, and decreases at higher temperatures. Strikingly, the authors demonstrated effector activity was not linked to gene regulation, and for instance with transcriptional induction in the optimal condition. These toxins tend to be intrinsically more active in a particular environment even if this point has not been firmly established here. The authors tested whether toxins might act in synergy by studying the different effector-immunity double mutants. They observed that Tse1, Tse4 and Tse6 act strongly in synergy with other toxins. The muramidase Tse3 functions in synergy with Tse4 in all conditions, including at high pH where its activity in the absence of Tse4 decreases greatly. Furthermore, some synergistic pairs depend on the environmental conditions: Tse4 and the amidase Tse1 were synergistic at high salinity, and Tse2 and the NAD(P)+ glycohydrolase Tse6 during anaerobiosis. These results demonstrate that effector pair relationships can be conditional. Finally, in one case anti-synergy was observed between Tse4 and Tse5, whose cumulative activities were at or below the sum of their individual activities. Not all possible interactions were studied, and for example the use of triple or higher order mutants might be expected to reveal synergy between groups of toxins that are not synergistic in pairs. The level of synergy between toxins is thus probably underestimated compared to the situation *in vivo*. In conclusion, the arsenal of T6SS effectors acts both synergistically and activity is modulated in response to varying environmental conditions.

Interestingly, PAEE revealed a key role for the poorly characterized effector Tse4[6]. Tse4 was found to act in synergy with Tse1, Tse3 and Tse6, the two firsts being bactericidal toxins by targeting the peptidoglycan, while the latter is bacteriostatic by depleting NAD(P)+ in the cytoplasm. One can wonder at what kind of activity that can allow synergy with effectors that harbor such distinct activities? The authors use a series of experiments to demonstrate the Tse4 is likely to function as a novel ion selective membrane pore. Tse4 is found in the inner membrane of *P. aeruginosa*, and contains glycine zipper motifs that are implicated in transmembrane domain multimerization in pore-forming proteins. The authors demonstrated that these glycine zipper motifs are required for Tse4 toxicity. By constructing a self-intoxication strain by deletion of *tsi4*, the gene coding Tse4 immunity, they showed that Tse4 led to the permeability of the inner membrane to ions, and this was due to an altered $\Delta\Psi$ component of the proton motive force. Membrane disrupting activities have been previously described for T6SS effectors with VasX that binds phosphoinositides[7] or the Tle (Type VI lipase effectors)[8], but never a pore-forming activity as found for Tse4 here. The authors propose that the synergy between Tse4 and cell wall-degrading effectors (Tse1, Tse3) may rely on PMF-sensitive autolysin activation and that Tse4 could exacerbate the consequences of NAD(P)+ depletion by Tse6 through inhibiting $\Delta\Psi$ dependent transporters.

In conclusion, the work of LaCourse and colleagues establishes that the concomitant delivery of multiple toxins allows both synergy between these effectors and an optimized action in a wide range of environmental conditions. Another benefit of a broad repertoire of

effectors, as discussed by the authors, may be to minimize the emergence of resistant among competitors such as the therapeutic use of antibiotic combination to prevent resistance. This work raises important questions as to what extent synergy can occur between effectors from different species, as could occur in complex environments such as the gut or in polymicrobial infections such as those that occur in cystic fibrosis sufferers. And finally how bacteria can defend against these synergies?

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