Cross-talk in bacterial extracellular signalling systems

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ABSTRACT

Many Gram negative bacteria produce acylated homoserine lactones (AHLs) as extracellular signalling molecules. These signals mediate a wide range of traits including those associated with the colonisation of surfaces. Recently, fatty acid derivatives and cyclic dipeptides have also been identified as extracellular signalling molecules produced by bacteria, and have been demonstrated to mediate starvation and stationary-phase responses. These compounds also cross-talk with AHL signals and constitute families of signals which can induce or repress AHL regulated phenotypes, suggesting a much more complex form of "communication" between bacteria than previously thought. This hypothesis is further supported by the specific interference of each of the three classes of signalling compounds by furanones. These eukaryotic AHL antagonists competitively inhibit AHLs and other signalling molecules which mediate AHL regulated responses, and control colonisation by bacteria.

Introduction

Extracellular signals are important in a broad range of bacterial species and have been proposed to control the expression of phenotypes that are central to many adaptive responses [8, 18]. This allows bacteria to respond to essential environmental parameters such as population densities and levels of nutrients. Here we present how extracellular signals mediate such responses and report on the increasing diversity of extracellular signalling systems. We also discuss how eukaryotic signal antagonists, as well as bacterial signals of different chemical classes, mediate cross-talk between the different signalling systems.

The major means by which we explore how signals and antagonists of different classes communicate is through their interaction with bacterial acylated homoserine lactone (AHL) systems. This offers the use of well established and experimentally advanced molecular and analytical systems. Also, the AHL signals control the expression of phenotypes that are essential for high density and stationary-phase biology.

Three avenues of research are used to illustrate the main concepts of this contribution:

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1. The control of bacterial biofilm formation on the red alga *Delisea pulchra* by halogenated furanones as antagonists of AHL systems;
2. Crosstalk and interference in AHL systems by cyclic dipeptides, a novel class of signals which are produced by several bacterial species; and
3. Crosstalk and interference in AHL systems by uncharacterized extracellular signals that regulate bacterial starvation and stress responses.

Furanones and AHLs

Biofilms form on both inanimate and animate surfaces, the consequences of which are often detrimental. On living surfaces in a marine environment biofilms harbour pathogens which can cause mortality in hosts or degrade host tissues. Biofilms also cue the settlement of other fouling organisms such as epiphytic plants and invertebrates which are also detrimental to their hosts [16]. One mechanism which has been proposed as a means of control of bacterial colonisation on marine organisms is the production of deterrent secondary metabolites [4, 17]. We have investigated this mechanism in the red alga *Delisea pulchra*, a common sub-tidal plant in Southern Australia. *D. pulchra* produces a series of secondary metabolites, furanones, which are unique to this plant. The concentration of these compounds in the plant [3] is inversely proportional to the number of bacteria that colonise the plant surface suggesting a role for furanones as inhibitors of bacterial colonisation [10, 13]. By establishing the identity and concentrations of furanones on the plant surface we are able to conduct biological and molecular experiments that are meaningful in an ecological context [2, 6].

The structural similarity between furanones and AHLs suggests that furanones are mimics of AHLs. This prompted us to study in detail their specificity in well characterized AHL systems.

The hypothesis for the mechanism of action of furanones is that they competitively bind at the AHL receptor site of the AHL transcriptional activator, the LuxR homologue. One line of evidence for this mode of action is that furanones, in a structure-function dependent fashion, displace labelled *N*-(3-oxohexanoyl)-L-homoserine lactone (OHHL) from a LuxR overproducing system in *E. coli* [12]. To verify that furanones have no non-AHL related effects, we employed high resolution proteome analysis and demonstrated that changes in protein expression are limited to very few proteins, the majority of which reflect the changes in the proteins encoded by the structural genes for bioluminescence. We were able to confirm that furanones inhibited AHL signalling using an *E. coli* strain harbouring a bioluminescence reporter plasmid lacking the AHL synthase gene, *luxI*. In this strain the Lux proteins for bioluminescence are produced in response to the addition of the native signal, and their expression is inhibited by the addition of the furanones that were found to displace labelled OHHL from LuxR [12]. Moreover, we demonstrated that swarming, which is a very efficient means of colonisation, is specifically inhibited by furanones [9]. Using a *Serratia liquefaciens* strain which carries a mutation in its AHL synthase gene, *swrI* , we showed that the expression of the AHL controlled gene *swrA* was downregulated. The *swrA* gene product is a lipopeptide biosurfactant that is essential for swarming [11].

Based on the natural concentrations and types of furanones on the surface of *Delisea pulchra*, we tested the hypothesis that furanones specifically interfere with bacterial colonisation traits at the plant surface by coating inanimate surfaces with furanones and performing adhesion and colonisation experiments. This was done directly in the field as well as by using marine bacterial isolates in laboratory experiments. Indeed, field

experiments showed strong inhibition of bacterial adhesion, and marine isolates from different sources tested *in vitro* were specifically inhibited in adhesion as well as swarming at ecologically realistic concentrations [10, 13].

Cyclic dipeptides and AHLs

In our studies of diverse signalling systems in bacteria, we hypothesised that signals other than AHLs are produced by bacteria and may play a key role in modulating the same regulatory pathway induced by AHLs. In collaboration with the University of Nottingham, we identified that cyclic dipeptides (CDPs) interfere with AHL mediated phenotypes. Cyclic dipeptides produced by *Pseudomonas* spp., and by *E. coli* effect the expression of AHL regulated phenotypes, as determined by AHL reporter bioassays, for bioluminescence, pigment production in *Chromobacterium violaceum* and *Agrobacterium tumefaciens*, and swarming in *Serratia liquefaciens*. This interference appears to be due to the competition between CDPs and the native AHL signals. Competition experiments using a *S. liquefaciens* swarming bioassay and the *Vibrio fischeri* bioluminescence based *E. coli* reporter system, suggest that CDPs specifically compete for binding with the native AHL signals.

Cyclic dipeptides are stationary-phase produced extracellular signals in *E. coli* and they inhibit the expression of two stationary-phase genes, *fic* and *bolA*. We are further investigating the signalling role of this widespread class of compound [1, 5]. While no specific function has yet been assigned to these compounds in bacteria, CDPs are evolutionarily conserved and have been ascribed signalling functions in higher organisms [14].

Novel stationary-phase induced extracellular signals and AHLs

Extracellular signals have long been postulated to play a role in the starvation-stress response in non-differentiating bacteria. We have demonstrated that extracellular signals regulate a substantial part of the starvation or stationary-phase response in the marine bacterium *Vibrio angustum* S14 [15]. A starvation supernatant extract was found to specifically upregulate carbon starvation specific gene products, as mapped by proteome analysis, as well as specifically downregulate carbon starvation repressed proteins. The supernatant extract also induces several AHL bioassays which led us to employ furanones as antagonists of these novel signals.

Furanones displayed a high degree of specificity in these experiments. Of the carbon starvation proteins that are upregulated by the extracellular signals and downregulated by the furanone antagonist, 92% are specifically induced by carbon starvation [15]. Only 3 of 637 logarithmic phase expressed proteins were effected by furanones, suggestive of a specific mode of action. The hallmark of the carbon starvation response is that it accommodates successful transition to small starved ultramicrocells that are starvation and stress resistant. Furanones at non-growth inhibitory concentrations resulted in a loss in culturability during starvation. Also, the stress resistance that develops during carbon starvation is lost by addition of furanones. By simultaneously adding furanones and starvation supernatant extract we obtained protection against loss in culturability and stress resistance during starvation of *V. angustum* S14, providing strong support for the notion that furanones and the extracellular signal(s) compete.

Using the latter experiment as a bioassay, the simultaneous addition of furanones and a series of liquid chromatography generated fractions of the starvation supernatant revealed the presence of several signals. Moreover, one of the fractions contained an inhibitor, i.e.

the cells lost culturability during starvation upon addition of this fraction to the starvation bioassay. We propose that extracellular signals, in conjunction with the intracellular fine tuning accommodated by inducers and repressors, would allow the cell to optimise its response to changes in environmental conditions by the induction or inhibition of the expression of carbon starvation proteins.

Further to these studies, signals similar to those produced by *V. angustum* S14 operate in other bacteria. We have recent evidence that the regulation of the low temperature induced viable but non-culturable (VBNC) response in *Vibrio vulnificus* is in part mediated by extracellular signals which strengthens the hypothesis that the VBNC state is a genetically programmed response. This response is inversely related to starvation, i.e. prestarvation prevents or delays VBNC formation. As with *V. angustum* S14, several extracellular signals appear to be produced during stationary-phase by *V. vulnificus* cells. At least one of these signals mediates the carbon starvation response and prevents the entry into VBNC.

Some of the extracellular signals in *V. angustum* S14 have been tentatively identified as fatty acid derivatives and are currently being further characterized. These compounds have strong structural similarities with another recently identified signal, the long chain fatty acid derivative in *Ralstonia solanacearum*, 3-hydroxypalmitic acid methyl ester [7]. Furthermore, long chain fatty acids also serve as signals in prokaryotic differentiation systems suggestive that signals of this class of molecules could be wide spread in bacteria.

Conclusion

Bacteria survey their environment and respond accordingly by either signalling members of their own populations to coordinate activities, or by interfering with the communications network of other competing bacteria. It is becoming evident that bacteria use a wide range of compounds for these purposes. This paper has presented information on such novel signalling compounds in different bacteria, and highlighted the fact that signalling languages are used in communication and crosstalk even though members of a bacterial community speak different dialects. Furthermore, we have demonstrated that eukaryotes are also able to interfere with bacterial communication through their own chemical signals. Table 1 summarizes how the bacterial signals discussed in this paper cross-talk with AHL signalling systems, and how furanones which are produced by a eukaryotic host interfere with the AHL systems as well as function as antagonists of the different bacterial signals.

Table 1. Cross talk between different signalling systems.

Organism	Signal type	Cross talk in AHL systems	Signals antagonized by furanones
Pseudomonas spp.	AHL, cyclic dipeptide (CDP)	yes yes	yes yes
Escherichia coli	CDP	yes	yes
Serratia liquefaciens	AHL	yes	yes
Vibrio angustum S14	unknown	yes	yes
Vibrio vulnificus	unknown	yes	yes

It is anticipated that further exploration of the chemical language of both microorganisms and higher organisms will reveal additional signals and regulatory pathways which are designed to respond to the complexity of the ever changing external environment.

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