

Mini-Review

Communications blackout? Do *N*-acylhomoserine-lactone-degrading enzymes have any role in quorum sensing?Declan M. Roche,¹ Joseph T. Byers,¹ Debra S. Smith,¹ Freija G. Glansdorp,² David R. Spring² and Martin Welch¹Correspondence
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A number of bacteria, including some significant pathogens, utilize *N*-acylhomoserine lactones (AHLs) as quorum sensing signals. There is considerable interest in the therapeutic potential of disrupting quorum sensing. Recently, a number of bacteria have been identified which are capable of enzymic inactivation of AHLs. These enzymes show considerable promise as 'quencher' of quorum sensing. However, the assumption that the natural function of these enzymes is to disrupt or modulate quorum sensing has yet to be established. This review surveys the progress made to date in this field and examines what implications these findings have for our understanding of the role played by these enzymes *in vivo*.

Identification of AHL-degrading enzymes

N-Acylhomoserine-lactone-dependent quorum sensing is now recognized as playing a major role in the virulence of a number of pathogenic bacteria and as such is increasingly seen as a useful target for antimicrobial therapy (Hentzer & Givskov, 2003). For example, experiments have shown that the inactivation of either or both of the *rhl* and *las* quorum sensing systems of *Pseudomonas aeruginosa* significantly attenuates virulence (Pearson *et al.*, 2000) and there is evidence that some plants have evolved the ability to interfere with quorum sensing through the release of compounds which mimic the activity of AHL signals (Bauer & Robinson, 2002). It has been suggested that targeting the quorum sensing system and as a result shutting down the expression of virulence traits has the advantage that it does not affect the viability of the individual cells, but rather the virulence of the population as a whole, meaning there is less powerful selection for the evolution of resistance than is the case in, for example, antibiotic therapy (Hentzer & Givskov, 2003). Such targeting of quorum sensing systems is referred to as 'quorum quenching'.

Quorum sensing can be interfered with in a number of ways; one of the more obvious is to target the signal itself for destruction, preventing it from accumulating. The possibility that such an activity may already exist in nature has been investigated by carrying out screens of soil microorganisms in order to identify any that are capable of inactivating AHLs. Such screens have led to the isolation of a number of bacterial strains exhibiting the ability to either inactivate and/or metabolize AHLs (Table 1).

The first such isolate was a *Bacillus* species, 240B1, which was found to produce an enzyme, AiiA, which inactivated AHLs by hydrolysing the ester bond of the lactone ring to give acylhomoserine (Fig. 1A) (Dong *et al.*, 2000, 2001). This enzyme was found to possess two small conserved regions, including a zinc-binding motif, which were shared with the zinc metalloenzymes, a group which includes the glyoxylase II and arylsulfatase enzyme families, and β -lactamases (Daiyasu *et al.*, 2001). Many members of this family target substrates possessing an ester linkage (Aravind, 1998). AiiA has been dubbed an *N*-acylhomoserine lactonase.

Shortly after the discovery of AiiA, a strain of *Variovorax paradoxus* was isolated which was found to be capable of utilizing AHL as a sole energy source (Leadbetter & Greenberg, 2000). This is in contrast to 240B1, which did not further degrade AHL. *Variovorax* was found to degrade AHL through an acylase activity in which the amide bond connecting the HSL ring to the acyl chain is cleaved, releasing homoserine lactone and fatty acid, which is further metabolized (Fig. 1B). This activity was subsequently also found in a strain of *Ralstonia*; in this case the gene encoding the acylase was cloned and found to show homology to a number of other acylases and N-terminal hydrolases of the Ntn-hydrolase superfamily (Lin *et al.*, 2003).

Subsequently a number of other *Bacillus* species with AHL-inactivating properties were isolated, some of which were further investigated and found to possess homologues of *aiiA* (Dong *et al.*, 2002; Lee *et al.*, 2002). An AHL-lactonase,

Table 1. Organisms identified to date exhibiting AHL-degrading activity

The notes give a brief summary of the range of AHLs used in the characterization of the activities in each study and, where appropriate, any findings in relation to the specificity of the activities for particular AHLs.

Species	Gene name	Activity	Notes	Reference
<i>Bacillus</i> sp. 240B1	<i>aiiA</i>	AHL lactonase	Degrades OHHL, ODHL, OOHL	Dong <i>et al.</i> (2000)
<i>Bacillus</i> strain COT1, strains of <i>Bacillus thuringiensis</i> , <i>B. cereus</i> , <i>B. mycoides</i>	<i>aiiA</i> homologues	AHL lactonase	Degrade OHHL with differing efficiencies	Dong <i>et al.</i> (2002)
<i>Bacillus thuringiensis</i> (numerous subspecies)	<i>aiiA</i> homologues	AHL lactonase	Degrade OHHL with differing efficiencies	Lee <i>et al.</i> (2002)
<i>Arthrobacter</i> sp. IBN110	<i>ahlD</i>	AHL lactonase	Degrades OHHL, OHL, OdDHL, BHL, HHL, DHL	Park <i>et al.</i> (2003)
<i>Agrobacterium tumefaciens</i>	<i>attM</i> , <i>aiiB</i>	AHL lactonase	Degrade OOHL, HHL; <i>aiiB</i> less active	Zhang <i>et al.</i> (2002); Carlier <i>et al.</i> (2003)
<i>Variovorax paradoxus</i> strain VAI-C	ND	AHL acylase	Grew on a wide range of AHLs, with differing efficiencies	Leadbetter & Greenberg (2000)
<i>Ralstonia</i> strain XJ12B	<i>aiiD</i>	AHL acylase	Degrades OdDHL, ODHL, OHHL, BHL	Lin <i>et al.</i> (2003)
<i>Pseudomonas</i> strain PAI-A, <i>Pseudomonas aeruginosa</i> PAO1	<i>pvdQ</i> , unidentified acylase*	AHL acylase	Both strains can degrade long-chain (>6C), but not short-chain AHLs	Huang <i>et al.</i> (2003)
<i>Pseudomonas</i> sp., <i>Variovorax</i> sp., <i>Variovorax paradoxus</i> , <i>Comamonas</i> sp., <i>Comamonas testosteroni</i> , <i>Rhodococcus erythropolis</i>	ND	ND	Degrade a range of AHLs, exhibiting differences in efficiency between oxo- and non-oxo-derivatives	Uroz <i>et al.</i> (2003)

ND, Not determined.

*Although PvdQ exhibited AHL acylase activity, it was shown not to be involved in the AHL-degrading activity identified in this study.

AhlD, has also been identified in an *Arthrobacter* species, in this case conferring upon the cells the ability to grow on AHL as a sole carbon source (Park *et al.*, 2003). Another recent screen has identified additional bacterial isolates with AHL-degrading properties, including *Pseudomonas*, *Variovorax*, *Comamonas* and *Rhodococcus* spp. (Uroz *et al.*, 2003), although in this study the specific enzymic activities were not characterized.

Database searches for homologues of the characterized AHL lactonases and acylase in complete bacterial genomes have shown that relatives of these enzymes exist in a wide range of species, indicating that these activities may be widespread. To date all the enzymes characterized have been shown to be either lactonases or acylases. It would also be theoretically possible to enzymically alter AHLs by optical inversion from the L to the D form (Fig. 1C), which has been shown to be less active in *Erwinia carotovora* (Bainton *et al.*, 1992). This activity has not been found in the screens carried out so far.

Having identified AHL-degrading enzymes, the principal focus of investigations to date has been in exploring their ability to interfere with quorum sensing. Typically researchers have investigated their effectiveness in disrupting the virulence of *Pseudomonas* and *Erwinia* spp. In most cases, the enzymes have proved effective in achieving this. Simple

co-culturing of AHL-inactivating bacteria with AHL-producing bacteria has been shown to inhibit quorum sensing-related activities, both in liquid culture and on plant tissue (Dong *et al.*, 2000; Park *et al.*, 2003; Uroz *et al.*, 2003). In addition, cloning of the AHL lactonase and acylase genes into quorum sensing bacteria has been shown to abolish or dramatically reduce AHL accumulation (Dong *et al.*, 2000; Reimann *et al.*, 2002; Lin *et al.*, 2003). Most significantly, the *aiiA* gene has been cloned into tobacco and potato and found to confer resistance to infection by *E. carotovora* subsp. *carotovora*, inhibiting the production of virulence determinants and allowing the host defences to effectively combat the infection (Dong *et al.*, 2001). Clearly the rationale behind the identification of these enzymes, that of developing new means of interfering with quorum sensing, has been vindicated.

AHL turnover or quorum sensing modulation?

As yet, the role that AHL-degrading enzymes play in their natural ecological niche remains far from clear, and it is by no means certain that AHL degradation is their 'normal' function. In fact, what has been established to date about the activities of these enzymes raises many questions, particularly in relation to their suggested role in modulating the activities of quorum sensing bacteria. That there is

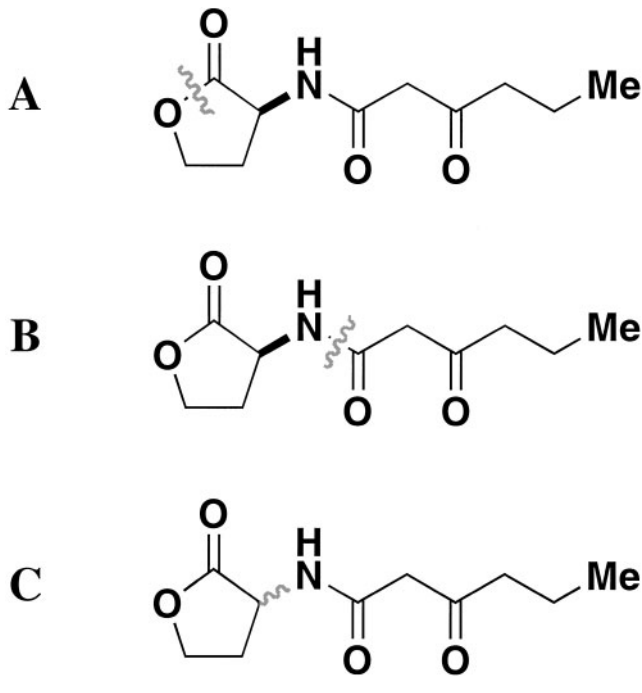


Fig. 1. Inactivation of AHL. In this case the *N*-3-oxohexanoyl-L-homoserine lactone produced by *Erwinia carotovora* is shown. There are three possible routes to inactivation. A, hydrolysis of the lactone ring. B, hydrolysis of the amide bond. Enzymes have been identified capable of catalysing A and B. C, racemization to give *N*-3-oxohexanoyl-D-homoserine lactone. To date, enzymes capable of catalysing this reaction have not been identified.

turnover of AHL in the natural environment is not in question; otherwise the accumulation of AHL would render quorum sensing redundant since the system is predicated on the fact that the concentration of AHL is an accurate reflection of the density of the population, and for this to be the case, only *de novo* synthesis of AHL should be of significance. Indeed, one would expect that the utilization of AHLs, and other molecules, as quorum sensing signals would only have evolved in the context of turnover preventing their buildup in the environment.

AHL has been shown to undergo non-enzymic turnover during laboratory culturing as a result of lactonolysis at high pH, in response to increasing temperature and as a function of acyl chain length (Byers *et al.*, 2002; Yates *et al.*, 2002). AHLs and their breakdown products are likely to be commonly encountered by micro-organisms in many environments and it would be surprising if they were not exploited as a carbon source. The fact that they have not been reported to accumulate under conditions such as low soil pH, which might be expected to protect them from spontaneous breakdown, is evidence that this is likely to be the case. That at least some of the AHL-degrading strains are capable of using these substrates as energy sources is consistent with this. It should also be noted that the fact

that some strains degrade but do not metabolize AHL when grown as pure cultures does not rule out the possibility that they form part of a community of organisms which catalyse different stages of AHL breakdown; it has been shown that growing *Variovorax* and *Arthrobacter* together on AHL gives more efficient growth than that of either strain alone, presumably because their differing enzymic activities complement one another (Flagan *et al.*, 2003). Another point worth noting is that some of the breakdown products of AHL, such as homoserine lactone and homoserine, can be toxic (Flagan *et al.*, 2003) and detoxification could be an explanation for some of the AHL-degrading activities identified.

The possibility exists, of course, that these enzymes have a more complex role than simple degradation. From the first reports of their discovery, there has been speculation that AHL-degrading enzymes may play a role of much greater significance: that of actively interfering with quorum sensing in other bacteria. This speculation naturally emerged from the observations of the effects that these enzymes were seen to have on AHL-utilizing quorum sensing activities in the laboratory. It is easy to see ways in which this could benefit the competitors of quorum sensing organisms. For example, some strains of *E. carotovora* produce carbapenem antibiotics in a quorum sensing-dependent manner, presumably to inhibit the growth of competitors at the site of infection (Axelrood *et al.*, 1988). Any bacteria capable of interfering with quorum sensing in *Erwinia* could inhibit the production of these antibiotics and gain a competitive advantage. It is also possible that these enzymes may play a role in modulating interactions within as yet poorly understood microbial communities, such as multispecies biofilms. However, if bacteria are actively interfering with quorum sensing, this raises the question as to whether quorum sensing bacteria would have evolved counter-strategies, for example producing forms of AHL which are resistant to enzymic degradation, perhaps similar to the synthetic non-hydrolysable AHLs which have been shown to retain the ability to activate quorum sensing systems (Smith *et al.*, 2003). Alternatively, they could utilize completely different signals, such as those found in AHL-independent quorum sensing systems. Clearly, the presence of such enzymic activities in the environment has not prevented the success of AHL-dependent quorum sensing. Nevertheless, the possibility of complex interactions between quorum sensing bacteria and AHL-degrading bacteria cannot be discounted.

A role for these enzymes in quorum sensing systems?

Of greater significance than the above speculation in terms of our understanding of the natural role played by these enzymes is the discovery of homologues of both kinds of AHL-degrading enzyme in bacteria that actually produce AHLs. The first such enzyme was found in *Agrobacterium tumefaciens*. AttM was found to show homology to AHL lactonases and indeed characterization of the gene revealed

it to be a potent blocker of AHL accumulation; constitutive expression of *attM* abolished the production of native AHL (Zhang *et al.*, 2002). *attM* had previously been identified as belonging to a cluster of genes involved in attachment to host cells (Matthysse *et al.*, 2000). It was noted that AHL levels in *A. tumefaciens* cultures decline rapidly when the cells enter stationary phase (Zhang *et al.*, 2002), which correlated with the fact that a quorum sensing-mediated activity, Ti plasmid transfer, is initiated at mid-exponential phase and declines thereafter. This decline in AHL levels was attributed to AHL lactonase activity; however, the authors did not report the level of expression of *attM* at this point in the growth cycle, and it is important to note that the decline of AHL levels during stationary phase in other bacteria has been attributed to non-enzymic turnover. Recently, two other AHL lactonase homologues, AiiB and AiiC, were identified in *A. tumefaciens*; AiiC did not exhibit lactonase activity, while AiiB was expressed in *E. carotovora* subsp. *atroseptica* and found to reduce endogenous AHL levels and virulence (Carlier *et al.*, 2003). The activity of these enzymes in *A. tumefaciens* was not examined. Since Ti plasmid transfer takes place at a specific point in the growth cycle, it has been suggested that AHL degradation may be important in ensuring the correct timing of this activity.

Recently, a homologue of an AHL acylase, *pvdQ*, has been identified in another quorum sensing bacterium, *Pseudomonas aeruginosa* (Huang *et al.*, 2003). The enzyme was found to efficiently degrade long-chain-acyl AHLs, though not short-chain-acyl AHLs, and constitutive expression prevented accumulation of the native 3OC12HSL quorum sensing signal, but not the short-chain-acyl C4HSL signal. However, the ability of the bacteria to grow on AHL as a sole carbon source was not affected when *pvdQ* was inactivated, which may suggest that other enzymes are involved in this activity. Furthermore, growth on AHL was characterized by very long lag phases and doubling times, suggesting that the bacteria are not adapted for AHL metabolism in these growth conditions. It has been suggested that the function of long-chain-acyl AHL turnover in *P. aeruginosa* may be related to a need for the bacteria to manipulate the ratio of their two AHL signals, allowing fine-tuning of the level of expression of the genes under their respective control.

Of particular interest in both of these species is evidence that the enzymes identified as AHL degraders may be involved in other functions in the cell, suggesting that assigning them a role in quorum sensing modulation may be premature. Knockout mutants of *attM* have been shown to have a non-attaching and avirulent phenotype, exactly the opposite of what would be expected in cells which were free to accumulate AHL (Matthysse *et al.*, 2000). Against this, it should be added that loss of the pAt plasmid which carries *attM* results in accumulation of AHL to higher levels than the wild-type (Vaudequin-Dransart *et al.*, 1998), suggesting that some AHL turnover at least can be ascribed to its

activity. In addition, *attM* has been shown to be part of an operon containing two other genes: *attK*, identified as a homologue of succinate semialdehyde dehydrogenase, and *attL*, a homologue of alcohol dehydrogenase (Matthysse *et al.*, 2000; Zhang *et al.*, 2002). The operon is under the control of a repressor protein, AttJ. This suggests that the three enzymes are likely to be involved in an as-yet-unidentified metabolic pathway. Whether the other two enzymes can be assigned roles in AHL degradation remains to be seen.

Similarly, the *pvdQ* gene of *P. aeruginosa* has recently been identified as an iron-regulated component in the pathway for the biosynthesis of pyoverdine, a fluorescent siderophore involved in iron acquisition (Lamont & Martin, 2003; Ochsner *et al.*, 2002). This suggests that the acylase activity it possesses is directed towards another function than that of AHL degradation, a fact consistent with the observation that inactivation of *pvdQ* has no effect on the ability of the cells to utilize AHL (Huang *et al.*, 2003), raising the likelihood that this is not in fact its natural role. This is particularly significant as it represents the first definite role established for an AHL-degrading enzyme, and given the high efficiency with which *pvdQ* has been shown to degrade AHL, it raises a question mark over the function ascribed to AHL-degrading molecules in general.

Quorum quenchers or something else?

Since these enzymes have been identified as a result of their AHL-degrading capabilities, it is not surprising that the roles proposed for them have been informed by this fact. However, it does not follow that because they exhibit this activity, they should automatically be classed as AHL lactonases or acylases. That caution should be exercised in relation to their function is further indicated by findings in relation to the range and specificities of their enzymic activities. AiiC of *A. tumefaciens* did not degrade lactone despite its homology to other lactonases (Carlier *et al.*, 2003). A number of the enzymes identified exhibit activity only against certain types of AHL. The specificity of the lactonase activity of the characterized pseudomonads for long-chain-acyl AHLs has already been described. In other species the enzymic activity differs markedly between oxo-substituted and non-substituted forms of AHL, and the efficiency of degradation also varies considerably (Uroz *et al.*, 2003). It is likely that one reason for the differing specificities observed has been variation in the method used to isolate the bacteria; growth on a specific type of AHL may select for activities optimized for that substrate, and it may be that other specific activities against other AHL types would be found if they were used in the screening process. Of course this specificity could also be explained in terms of the AHL-degrading system having been fine-tuned to target specific AHLs in the context of specific ecological interactions or, as has been suggested for *P. aeruginosa*, specific regulatory functions.

These findings also need to be considered in the context of the nature of the enzyme families to which the

AHL-degrading enzymes belong. The fact that AHL lactonase can hydrolyse the ester bond and AHL acylase can cleave the amide bond of AHLs may not imply that these activities are targeted toward these molecules at all, notwithstanding the fact that in some cases at least, they do so at high efficiencies. It has been suggested that acylases tend to be very specific in their targets, showing little activity towards close homologues of their substrates (Lin *et al.*, 2003). However, a porcine acylase has been shown to exhibit AHL-degrading abilities when used at a sufficiently high concentration (Xu *et al.*, 2003), and it must be noted that the expression levels of these enzymes when introduced into other bacteria or into plants may not reflect their activities at their normal expression levels. Similarly, zinc metallohydrolases exhibit diverse enzymic activities (Daiyasu *et al.*, 2001) and care needs to be taken in assigning specific function to an enzyme in the light of its ability to degrade one particular substrate. Interestingly, however, Wang *et al.* (2004) have recently reported that the AiiA enzyme exhibits high specificity for *N*-acylhomoserine lactones and that, unlike other metallohydrolases, AiiA does not require zinc or any other metal ions for activity, suggesting it possesses a novel catalytic site.

Conclusions

To date, characterization of the specific role played by AHL-degrading enzymes has taken a back seat to investigations into their potential as quenchers of quorum sensing – understandably so, given the potential utility of such activities. However, this approach means that what insights we have gained into their natural function have been arrived at as a by-product of these investigations, and as yet there has not been a detailed functional characterization of any of these genes. It will be necessary to carry out such characterization before we can be confident that the enzymes identified do indeed function as quorum quenchers. This scenario seems elegant in principle, but we have already seen how previously existing information about the activities of AttM and PvdQ complicates attempts to attribute to them a definite role in quorum sensing modulation, and it would not be surprising to see similar complications emerge in relation to other genes which have not yet been fully characterized. It will be particularly interesting to see to what extent they turn out to have properties consistent with their proposed functions. For example, in the case of putative AHL-degrading bacteria, is the expression of the enzymes activated by the presence of AHL, or are they expressed constitutively? Similarly, in the case of the AHL-utilizing quorum sensing bacteria which have these enzymic activities, how consistent is the timing of expression of these genes with the observed levels and/or ratios of AHLs during the growth cycle and in different environmental conditions? It will be necessary to examine what factors control the expression of the genes. In addition, there should be careful screening for potential roles other than AHL degradation by observing what other activities, if any, are affected by knocking out the genes. To date, only

pvdQ has been assigned a specific function in another context, in the course of an unrelated study. Of course, it is possible that the enzymes may be bifunctional, having a role in quorum quenching in addition to any other activity identified. Careful study of the phenotypes of knockout mutants, e.g. through ‘global’ methods of analysis such as proteomics or transcriptomics, will be necessary to establish definite functions for these genes.

That these enzymes can degrade lactones, often with great efficiency, is clearly not in dispute; neither is their potential utility; however, careful investigation is needed before we can be confident of their function.

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