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Karrikins from plant smoke modulate bacterial quorum sensing†

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The discovery that plant smoke contains germination stimuli has led to the identification of a new class of signaling molecules named karrikins. Here we report a potential second role for these molecules: in various bacterial species – *A. tumefaciens*, *P. aeruginosa* and *V. harveyi* – they modulate bacterial quorum-sensing (QS), with very different outcomes.

Nature has evolved many different mechanisms to modulate cross-talk between different organisms (*e.g.* animals, plants, bacteria, fungi) and most of these are based on secretion and recognition of small signaling molecules.[1–3] It was discovered recently that naturally occurring butenolides, derived from the smoke of burnt plant material, stimulate seed germination in a wide range of plant species. One specific family of compounds, the karrikins, was identified and characterized as active compounds that promote this intriguing phenomenon.[4–7] While current studies focus on the mechanism and mode of action of karrikins in plants, we decided to explore whether these compounds are able to affect bacterial group behavior especially in QS systems. QS describes the mechanism used by a population of microorganisms to act as a single multicellular organism in a cell-density dependent manner through secretion and sensing of small diffusible molecules, enabling intercellular communication leading to synchronized gene expression.[8] Given the known ubiquitous interactions between plants and bacteria and the structural similarities between karrikins and certain QS molecules (*e.g.* short chain AHLs and AI-2, Fig. 1) as well as QS inhibitors (*e.g.* patulin, Fig. 1),[9–15] we chose to examine potential interactions between karrikins and three different bacterial species. Interestingly, the lactone moiety of karrikins resembles one class of signaling molecules, of Gram-negative bacteria, autoinducers-1 (AI-1) and the pyran moiety resembles a second class of QS molecules, autoinducers-2 (AI-2) (Fig. 1).

We hypothesize that in a post-fire environment, if a plant can sense these molecules to their advantage (more space, less competition) to promote germination it is reasonable to assume that bacteria have developed means to sense this opportunity to proliferate.

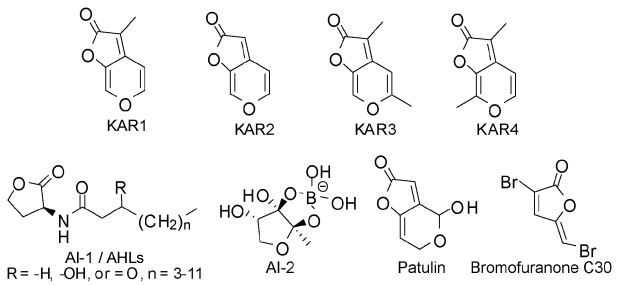

Fig. 1 Karrikins and structural similarities with known QS molecules and inhibitors. *P. aeruginosa* AIs: *N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL), *N*-butyryl-L-homoserine lactone (C4-HSL). *A. tumefaciens* AI: *N*-(3-oxooctanoyl)-L-homoserine lactone (3-oxo-C8-HSL). *P. syringae* AI: *N*-(3-oxohexanoyl)-L-homoserine lactone (3-oxo-C6-HSL). *V. harveyi* AIs: AI-1, *N*-(3-hydroxybutanoyl)-L-homoserine lactone (3-OH-C4-HSL); AI-2, (*S*)-4,5-dihydroxy-2,3-pentanedione (DPD).

Another hypothesis – not mutually exclusive with the first – is that karrikins are used by the plants to manipulate certain bacteria to their advantage in a post-fire environment, to provide them nutrition, protection from other pathogens, while repressing virulence factor production and pathogenicity. It is known that plants recognize certain QS molecules, such as the primary *P. aeruginosa* AI, 3-oxo-C_{12}-HSL, which influence gene expression in plants and trees.[13,16]

By studying the effects of these molecules on bacteria we hope to gain a better understanding of the role of karrikins in nature and more general principles of chemical guidance of coexistence and warfare.

Here, we report the effects of two members of the karrikin family, KAR1 and KAR2, on QS systems of different types of bacteria: (i) a plant pathogen, *Agrobacterium tumefaciens*;[2,3,14,16] (ii) an opportunistic pathogen, *Pseudomonas aeruginosa*, that is able to infect both humans and plants;[12,13] (iii) *V. harveyi* a luminescent marine bacterium and opportunistic pathogen of marine animals.

The synthesis of KAR1 and KAR2 was performed following procedures described by Goddard-Borger *et al.* with minor modifications (Scheme S1, ESI†).[17] We first examined the effects of the karrikins on *P. aeruginosa* wild-type strain PAO1-lux, containing the

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luminescent reporter gene luxCDABE cloned downstream of *lasI*.[18] We also examined a potential agonistic effect, using the *P. aeruginosa* PAO-JP2 (PAO1-lux *lasI*$^-$, *rhlI*$^-$) strain,[18] which is unable to produce *N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL) and *N*-butanoyl-L-homoserine lactone (C4-HSL). No effects were observed for both compounds in the two reporter assays, and we conclude that KAR1/KAR2 does not interact with the *las* system in *P. aeruginosa*, which is the higher in hierarchy between the two primary QS systems in *P. aeruginosa.* We then investigated the *rhl* system of *P. aeruginosa*, using the luminescent reporter strain PAO-JP2 (pKD-*rhlA*) (PAO-JP2 with the *rhlA* promoter fused upstream of the *luxCDABE* operon).[18] Interestingly, KAR1/KAR2 showed concentration dependent inhibition of the *rhl* system in the presence of 10 μM C4-HSL (Fig. 2a). In order to examine the physiological relevance of *rhl* inhibition in wild-type *P. aeruginosa*, pyocyanin assays were conducted, as the production of this important virulence factor is controlled in part by RhlR. Addition of KAR1/KAR2 to *P. aeruginosa* strain PAO1 (wild-type) reduced production of pyocyanin (Fig. S1, ESI†). Interestingly, when we tested the effects of the karrikins on the quorum sensing system of the related plant pathogen *P. syringae* (Fig. S2, ESI†),[19] which employs 3-oxo-C_6-HSL as its autoinducer, we observed neither agonist nor antagonist effects – suggesting that the response to KAR1/2 is highly specific, and the QS inhibitory effects in *P. aeruginosa* appear to be based on direct competition with C4-HSL for binding to RhlR (Fig. 2a).

We then examined whether the plant pathogen *A. tumefaciens* would be affected by karikkins. We used a luminescent reporter strain, *A. tumefaciens* A136 pCF218 pMV26 (lacking Ti plasmid, TraR response regulator, *traI*$^-$ and TraI promoter fused to *luxCDABE*).[20] Addition of KAR1/2 at different concentrations in the absence of *N*-(3-oxooctanoyl)-L-homoserine lactone (3-oxo-C_8-HSL) resulted in activation of the QS response cascade. Both karikkins served as agonists in a concentration dependent manner (Fig. 3). These results suggest that KAR1/2 may be sensed by *A. tumefaciens* as early alerts

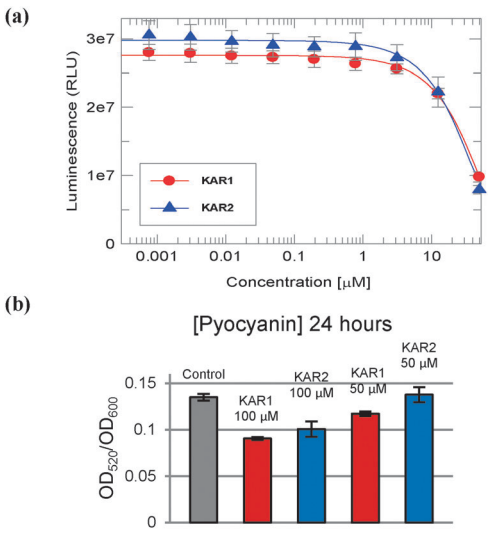

Fig. 2 (a) Inhibition of QS by KAR1/KAR2, in *P. aeruginosa* PAO-JP2 (pKD-*rhlA*) in the presence of 10 μM C4-HSL, after 6 hours. At 200 μM and beyond KAR1 caused some growth inhibition. (b) Inhibition of pyocyanin production in *P. aeruginosa* strain PAO1 by KAR1 and KAR2.

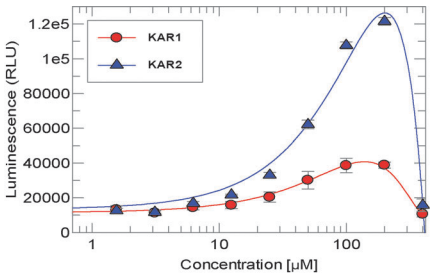

Fig. 3 Activation of QS by different concentrations of KAR1/2, in the absence of 3-oxo-C8-AHL in *A. tumefaciens* A136 pCF218 pMV26 after 15 hours.

for an opportunity to colonize new plants, though an argument against this hypothesis would be that the time scale of new plant growth is not compatible with such a signaling scenario – although one may argue that forest fire smoke can linger for days. Still, the maximal QS activation in this strain was roughly 50-fold less for the karrikins compared to the natural autoinducer 3-oxo-C8-HSL, prompting us to examine whether karrikins might also serve as QS inhibitors through partial agonism. Employing the same experimental conditions used for the agonist assay, but with added synthetic 3-oxo-C_8-HSL (400 pM), we observed no modulatory effects for the karrikins. These results suggest that the affinity of 3-oxo-C_8-HSL to the primary QS receptor TraR is much higher compared with KAR1/KAR2. Still, KAR1/KAR2 may very well interact with an unknown protein that can regulate the activation of QS in *A. tumefaciens*.

Next, we examined the effects of KAR1/2 on the AI-2 signaling pathway, as given the putative importance of AI-2 based interspecies signaling.[3,21–24] We used modified strains of *V. harveyi*, BB170 (*luxN*$^-$, AI-1 receptor),[23] and MM32 (*luxN*$^-$, *luxS*$^-$),[21,24] which respond only to the presence of AI-2. While potential effects of plant smoke on marine bacteria would be far fetched at best, and structural similarities between karrikins and AI-2 are not strong, we did observe a clear synergistic effect in a concentration dependent manner (Fig. 4a) for KAR1/2 on BB170. In addition, we added KAR1/2 to *V. harveyi* strain MM32 in the absence of exogenous AI-2. No response was observed, suggesting that KAR1/2 does not act as agonist in these bacteria. However, upon addition of synthetic AI-2 (133 nM) the same synergistic effect was observed as in BB170 (Fig. 4b), suggesting that KAR1/KAR2 is affecting the AI-2 signaling pathway in some manner, for instance through interaction with the AI-2 receptor, LuxP. Alternatively, KAR1/KAR2 may be recognized by an unknown receptor, which can affect AI-2 induced gene expression, the biosynthesis of AI-2 or both.

Finally, we examined the effects of karrikins *in vivo* and studied their physiological relevance with regard to bacterial pathogenesis in plants. Blackwell and coworkers showed that QS inhibitors effectively inhibited virulence of *Pectobacterium carotovera* in bean and potato rot models.[25] We conducted two experiments, based on *P. aeruginosa* plant infection models described by Rahme and coworkers.[26] We focused on the activity of KAR1 in *P. aeruginosa*, as the QS inhibitory or agonist effects in the other bacteria were less pronounced, and we tested the effect of KAR1 on the infection of *Arabidopsis thaliana* plants and lettuce midriffs. While KAR1 did not prevent infection of living plants, we did observe a significant reduction in loss of plant leaves from 24 h to 48 h post-infection (Fig. 5), in the presence of 100 μM KAR1

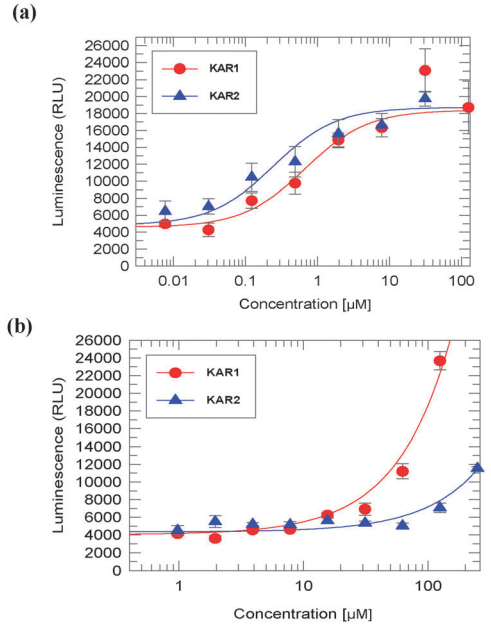

Fig. 4 (a) Activation of QS by synergism by different concentrations of KAR1/2 in *V. harveyi* BB170. (b) Activation of QS by synergism by different concentrations of KAR1/2 in the presence of exogenous synthetic 133 nM AI-2 in *V. harveyi* MM32. Above 200 μM KAR1 was toxic.

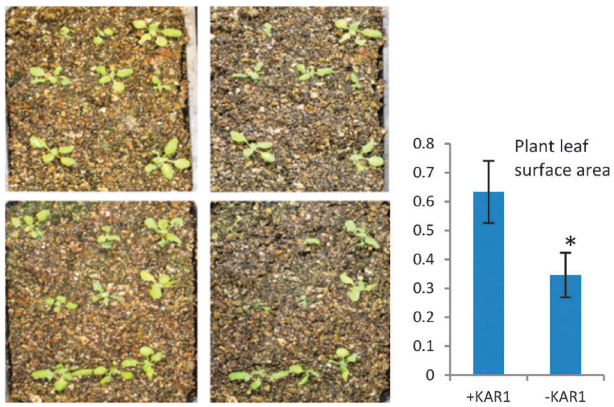

Fig. 5 Effect of KAR1 on infection of *Arabidopsis thaliana* Colombia by *P. aeruginosa* wild type strain PAO1; upper panel, left: with 100 μM KAR1, after 24 h, and right: after 48 h; lower panel, left: without KAR1, after 24 h, and right: after 48 h. The bar graphs reflect the ratio of average leaf area between 24 and 48 h. Plant leaf surface areas were calculated using ImageJ 1.47t.

(36 ± 11% *vs.* 65 ± 8%, $p < 0.03$), indicating that the karrikin mediated reduction in *P. aeruginosa* virulence slows down plant loss.[27] This hypothesis was further strengthened by a slightly reduced induction of soft rot in lettuce midriffs upon infection with *P. aeruginosa* in the presence of increasing amounts of KAR1 (Fig. S2, ESI†).

In this study we focused on the potential role of the karrikin family in the modulation of QS (agonist, antagonist or synergism) in different bacterial species. Whether the presence of karrikins, in the post-forest-fire environment, simultaneously influences plant seed germination and bacterial group behavior (*e.g.* suppression of virulence factors, nutrient production, antibiotic production) for the benefit of habitat rehabilitation is still a major question to answer, especially since the origin of karrikins is still unknown. However, here we provide evidence of the ability of two molecules of the karrikin family, KAR1/2, to affect QS in three different bacteria, two of which (*P. aeruginosa, A. tumefaciens*) are known to interact with plants/trees in nature. While in *P. aeruginosa* a clear QS antagonist effect was observed on the *rhl* system, which controls the expression of pyocyanin, in *A. tumefaciens* we only measured a mild agonist effect. Although the marine bacterium *V. harveyi* is not likely to encounter karrikins in nature, the results were interesting given the proposed interspecies signaling role ascribed to AI-2.

The ability of KAR1/2 to activate or inhibit QS pathways might reveal a new type of interkingdom communication. Further studies on the activity and mechanisms of action of these molecules are needed in order to answer more fundamental questions. Although we determined that KAR1/2 most likely interferes with *P. aeruginosa* QS through inhibition of the *rhl* system, direct identification of proteins that bind KAR1/2 will give us further insight from a mechanistic point of view. The presence, localization and identity of KAR1/2 receptor in bacteria are currently under investigation.

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