DOI: 10.1002/cbic.200700781

Rapid Hydrolysis of Quorum-Sensing Molecules in the Gut of Lepidopteran Larvae

Matthias Funke,[a] Rita Büchler,[a] Vertica Mahobia,[a] Alexander Schneeberg,[a] Michael Ramm,[b] and Wilhelm Boland*[a]

Microorganisms compete for nutrients and living space in the gut of plant-feeding insect larvae, such as Spodoptera *spp. Their physiological activities and their organization are generally controlled or synchronised by "autoinducers", such as* N*-acylhomoserinelactones (AHLs). Due to the strongly alkaline milieu in the insect gut, the lactone ring of AHLs is rapidly and spontaneously opened. Further degradation to the inactive components homoserine and the acyl moiety is then achieved by a microbial* N*-acylamino acid hydrolase (AAH) and related enzymatic activities in the insect gut. Initialised by the alkaline milieu, such activities might account for the complete absence of AHLs in the intestinal fluid of the studied* Spodoptera *spp. The AHL-recognition system of* E. coli *RV308pSB40, but not that of* Agrobacterium tumefaciens *NT1/pZLR4 and* Chromobacterium violaceum *CV026, was found to be inhibited by the structurally related* N*-acylglutamines, which are abundantly present in the gut of many lepidopteran larvae. Our observations suggest an active role of the insect in interfering with the quorum sensing of their gut microbiota by several independent strategies.*

Introduction

All insect species are known to harbour a rich and complex community of microorganisms in their guts and other body regions. The microorganisms participate in many types of interactions ranging from pathogenesis to obligate mutualism. One reason for the microbial diversity is that different groups of insects have different feeding habits; this results in different gut structures and functions and promotes the establishment of different phylotypes.[1] Whereas most of the gut microbes are commensals or parasites, some of them play substantial biological roles for their hosts. The gut microbial community of termites is needed for cellulose digestion,[2] and a gut symbiotic fungus is involved in the sterol biosynthesis of anobiid beetles.[3] In stinkbugs a symbiotic bacterium even controls their nymphal development.[4] In other cases metabolites of bacterial origin are adopted by the host insect and required for growth or the generation of functional enzymes (cofactors). Bacterial precursors can even serve the insect as sophisticated signals that control behaviour; for instance, guaiacol is used by locusts as an aggregation pheromone.[5] The population of the gut bacteria is, to a certain extent, influenced by the composition and the nutritional value of the ingested food. For example, locusts that feed on a protein-rich diet display a microbiota that is different from the microbial population found in locusts that feed on a low-protein but fiber-rich diet.[6] These examples clearly demonstrate that the microbial colonisation of the insect gut is a highly dynamic process that is determined by a multitude of factors that are not well understood. Besides the external factors imposed by the fresh and (partly) degraded food, together with intestinal secretions of the insect, microbial exudates can principally contribute to the community structure. Many bacteria produce autoinducers for quorum sensing (QS) that control and coordinate their metabolic activity in communities (Figure 1). The recognition of these signal molecules by specific receptors leads to gene-activation, controlled aggregation (biofilms),[7] and metabolic activities that produce pathogenicity factors,[8,9] toxins,[10] dyes,[11] or that regulate the phenomenon of bioluminescence.[12] Gram-negative bacteria mainly communicate through *N*-acylhomoserine lactones (AHLs; **1a**–**d**),[13] A-factors **2**,[14] quinolones of the type

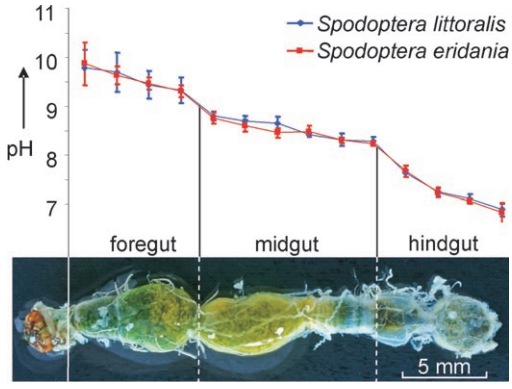
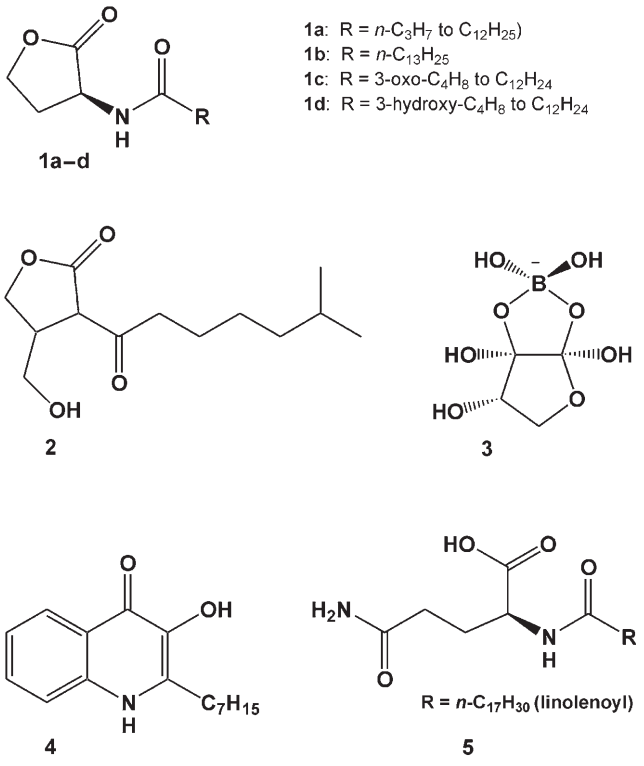


Figure 1. The pH profile in the gut of *Spodoptera littoralis* und *Spodoptera eridania*; the picture shows the gut of *Spodoptera littoralis*.

[a] *M. Funke, Dr. R. Büchler, Dr. V. Mahobia, A. Schneeberg, Prof. Dr. W. Boland*
Max Planck Institute for Chemical Ecology
Hans-Knöll-Strasse 8, 07745 Jena (Germany)
Fax: (+49) 3641-571202
E-mail: boland@ice.mpg.de

[b] *Dr. M. Ramm*
Leibniz Institute for Natural Product Research and Infection Biology
Hans Knöll Institute, Beutenbergstrasse 11a, 07745 Jena (Germany)

4[15] and autoinducers-2 that comprise the often boron-containing dihydroxypentanediones of the type **3** from *Vibrio* spp.[16] The acyl side-chains of the AHLs range in length from 4 to 18 carbons and vary in the substitution at the β position of the acyl moiety. The differences might be considered to be a means of diversification of the signal molecules. The communication between Gram-positive bacteria mostly relies on oligopeptides rather than AHLs.[13] Several bacterial strains from the gut of *S. littoralis* have been isolated that are principally able to produce and respond to AHLs, for example, *Acinetobacter* spp., *E. coli*, *Pseudomonas* spp., *Enterobacter* spp., *Ochrobactrum* spp. and *Erwinia* spp.[14,17] However, a very important factor that might have an important impact on the QS of the bacterial communities in the insect gut is the local pH along the intestinal tract. Many lepidopteran larvae maintain a strongly alkaline pH in the fore- and midgut[18] (Scheme 1).



Scheme 1. Selected quorum-sensing factors of microorganisms. *N*-Acylhomoserine lactones (AHL) with different side-chains and functionalities: *N*-acyl- (**1 a**), *N*-(myristoleyl)- (**1 b**), *N*-(3-oxoyacyl)- (**1 c**), *N*-(3-hydroxyacyl)homoserine lactone (**1 d**), the A-factor 2-isocaproyl-3-hydroxy-methyl-γ-butyrolactone (**2**), 2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran-borate from *Vibrio harveyi* (**3**), *Pseudomonas* quinolone signal 2-heptyl-3-hydroxy-4(1*H*)-quinolone (**4**), *N*-linolenoylglutamime (**5**) present in the insect gut.

Because many of the QS molecules, especially the AHLs and A-factors, possess a base-sensitive γ-lactone moiety,[19] the half-life of such molecules might be rather limited in the different segments of the insect gut. In addition, the gut of lepidopteran larvae contains high concentrations of *N*-acylglutamines **5** that could compete with the binding sites of the QS molecules because of their close structural similarity[20,21] and high local concentration in the fore- and midgut. Here we demonstrate that the various series of *N*-acylhomoserine lactones indeed suffer rapid ring opening under the alkaline conditions of the insect gut. Moreover, due to the presence of a bacterial *N*-acylamino acid hydrolase (AAH), the ring-opened products are rapidly further degraded into the amino acid and fatty acid building blocks.

Results

pH profiles in the gut of *Spodoptera* larvae

Gut pH is important in the regulation of enzymatic reactions in digestion, the dissociation or coagulation of ingested proteins, the control of solubility of food components and the determination of the gut microbial flora. In particular, alkaline gut regions are known from many insect orders, and a connection to dietary preferences has been claimed.[22,23] In lepidoptera high trypsin and chymotrypsin activities correlate with pH values in the range from 7.0 to 11.0.[24] To analyse the impact of the gut pH on the half-life of the base-sensitive γ-lactone moieties of the AHLs (Figure 1) from Gram-negative bacteria, the pH profile of the gut from larvae of *Spodoptera littoralis* and *Spodoptera eridania* was determined by microsensors. The larvae of *S. littoralis* represent a generalist herbivore, whereas larvae of *S. eridania* mark a more specialised herbivore that feeds on cotton, soybean or lima bean, the latter two of which contain considerable amounts of cyanogenic glycosides.[25] The caterpillar gut (3rd to 4th instar) is roughly 26 mm long and can be easily separated into four regions on the basis of key nodal points. On an average, the foregut is about 8 mm, midgut is about 14 mm and colon and rectum are about 4 mm each.

For both species the pH in the foregut was found to be in the alkaline range (about pH 10±0.5). Along the gut a nearly constant decrease in the pH up to the hindgut could be observed (Figure 2). The midgut showed a moderately reduced pH with a mean range from pH 8.75 to 8.25. In the posterior gut sections the pH decreased strongly to values near to neutrality. In the colon and rectum, the pH range was 7.55 to 6.58, descending towards the end part of the gut.

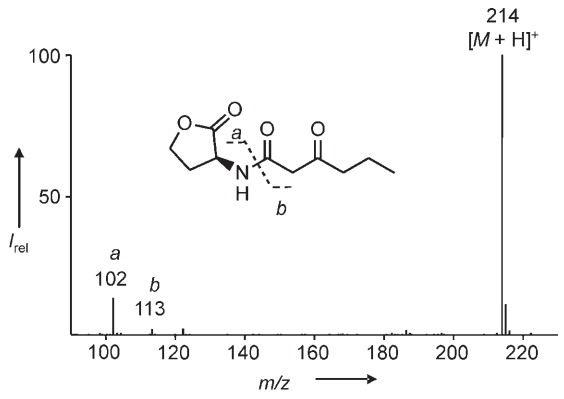


Figure 2. APCI mass spectrum of *N*-(3-oxo-hexanoyl)homoserine lactone.

Screening for AHL in the gut of *Spodoptera* larvae

Due to the presence of a multitude of bacteria in the gut of *Spodoptera* larvae,[26] many of which are principally able to produce and respond to AHLs, we analysed the gut segments of *S. littoralis, S. eridania* and *Helicoverpa armigera* for these compounds by HPLC–MS and GC–MS after derivatisation with MSTFA.

The APCI mass spectra of the AHLs displayed the ion $[M+H]^+$ as the base peak and displayed low-abundance fragments that corresponded to the protonated lactone moiety (*m/z* 102) and the acyl fragments (*m/z* 113) after cleavage across the amide bond.[13] For common AHLs, such as *N*-hexanoyl-, *N*-decanoyl-, *N*-dodecanoyl-,*N*-(3-oxo-hexanoyl)- and *N*-(3-hydroxy-hexanoyl)homoserine lactone the detection limit was found to be about 5.0 pmol.

To avoid ex post formation of AHL by bacterial growth, samples from the gut were immediately sterilised by filtration through a membrane (0.22 μm). The pH was adjusted to pH 7 to prevent additional ring opening after withdrawal of the sample from the gut. However, careful investigation of the different gut sectors by LC–MS and GC–MS gave no evidence for the presence of AHL and/or their ring-opened derivatives.

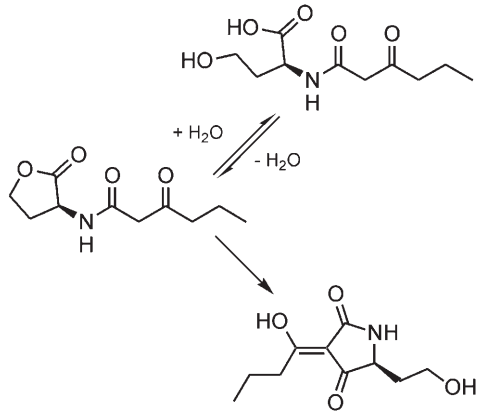
Besides mass spectroscopy, AHL-sensitive reporter organisms, such as *Agrobacterium tumefaciens* NT1/pZLR4,[27] *Chromobacterium violaceum* CV026[11] and *E. coli* RV308pSB40,[27] were used to evaluate the presence of AHLs in the insect gut. The detection limit was found around 5 pmol.[13] In line with the analytical findings, this approach also gave no evidence for the presence of AHLs in the insect gut when applied to the sterilised and pH-adjusted (pH 7) gut lumen of *Spodoptera* larvae. Surprisingly, the gut lumen from insect larvae that were fed on an artificial diet containing defined amounts of AHLs, also gave no evidence for either intact or ring-opened AHLs. Accordingly, additional and unknown factors must contribute to the rapid and complete degradation of internally produced or externally added AHL in the gut.

Stability of AHLs under alkaline conditions

AHLs of Gram-negative bacteria (Scheme 1) possess a lactone moiety that is rather stable at neutral and slightly acidic pH, but unstable under basic conditions.[19,28] The ring-opened product, namely the corresponding *N*-acylhomoserine is believed to lack biological activity.[29] To judge the dynamics of the AHL cleavage along the larval gut, the stability and the half lives of various classes of AHL were determined by alkaline hydrolysis at different pH values. The progress of the hydrolysis was monitored and quantified by HPLC–MS. During the reaction, the intensity of the quasimolecular ion $[M+H]^+$ of the AHLs continuously decreased, but the intensity of the quasimolecular ions of the corresponding ring-opened products $[M+H_2O]^+$ increased. As expected, the AHLs with saturated acyl moieties proved to be more stable with a half-life time of 17.5 min at pH 10 and of 5 min at pH 11, which was determined for the very common *N*-hexanoylhomoserine lactone. AHLs that carry an oxygen function in the β position of the fatty acid chain proved to be much more sensitive (Table 1). The *N*-(3-oxo-acyl)homoserine lactones were not only rapidly hydrolysed, but, in addition, suffered a rearrangement to tetramic acids at a ratio of 2:1, in advantage of tetramic acids. For example, *N*-(3-oxo-hexanoyl)homoserine lactone was predominantly converted into [4-(1-hydroxybutylidene)-5-oxo-pyrrolidin-2-yl]-acetic acid, which can be considered to be a product of an intramolecular Claisen-type condensation (Scheme 2).[30]

Table 1. Half-life times of C_6-homoserine lactones under alkaline conditions.

AHL	$t_{1/2}$ [min]		
	pH 9	pH 10	pH 11
N-hexanoylhomoserine lactone	–	17.5	5
N-(3-hydroxyhexanoyl)homoserine lactone	23	11	4.5
N-(3-oxo-hexanoyl)homoserine lactone	17	4.5	3



Scheme 2. Ring opening of 3-oxo-hexanoyl-HL and formation of tetramic acids.

The ring-opened aliphatic *N*-acylhomoserines proved to be rather stable in the pH range 8–11 and did not suffer further cleavage into the building blocks homoserine and hexanoic acid to a significant extent. Accordingly, other and additional factors that must be present in the insect gut are responsible for the complete degradation of AHL described above.

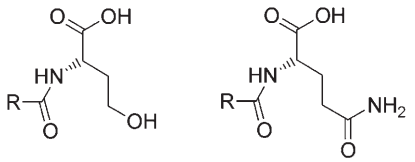
Enzymatic degradation of ring-opened *N*-acylhomoserines

An important factor that determines AHL stability in a microhabitat, such as the insect gut, is the biologically-driven signal degradation. Such phenomena are particularly important for multispecies environments. AHL degradation is mainly achieved via two different pathways. Lactonases[29] open the lactone ring, similar to the spontaneous reaction in the alkaline medium, and acylases/hydrolases[31] cleave the amide bond between the ring and the side-chain.

Recently we characterised an *N*-acylamino acid hydrolase (AAH) from *Microbacterium arborescens* that was previously isolated from the gut *S. exigua* larvae.[32] This type of hydrolytic

activity appears to be rather widespread in insect gut bacteria and might account for the rather rapid degradation of the *N*-acylglutamines within the posterior segments of the digestive system.[33]

AAH catalyses the cleavage of *N*-linolenoylglutamine and several other *N*-acylglutamines that are abundantly present in the gut of lepidopteran larvae.[32] Although the enzyme rapidly cleaves long and medium-chain *N*-acylglutamines, it does not attack the intact, cyclic *N*-acylhomoserine lactones (Table 2).

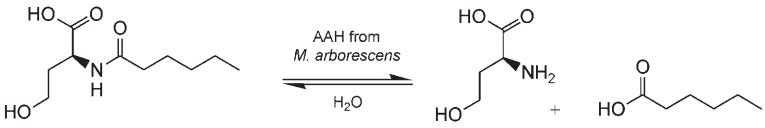


Scheme 4. Structural similarity of acyclic *N*-acylhomoserines and *N*-acylglutamines that are present in the insect gut.

Table 2. The K_m values for enzymatic degradation of *N*-acylhomoserines by the *N*-acylamino acid hydrolase (AAH) from *Microbacterium arborescens*.

N-Acylamino acid	K_m [μmol L^{-1}]
N-linoylglutamine	36
N-hexanoylhomoserine	43.4
N-hexanoylhomoserine lactone	no reaction
N-decanoylhomoserine	44.7
N-dodecanoylhomoserine	125.3

On the other hand, we observed that AAH efficiently cleaved the amide bond of the ring-opened *N*-acylhomoserines lactones to yield homoserine and the corresponding short-chain fatty acid (Scheme 3). To estimate whether or not the catalytic



Scheme 3. Hydrolysis of ring-opened *N*-hexanoylhomoserine by the *N*-acylamino acid hydrolase (AAH) from *Microbacterium arborescens*.

efficiency of AAH would be sufficient to cleave the ring-opened homoserine lactones in the alkaline fore- and midgut of the insects, we determined the Michaelis–Menten constants (K_m) of AAH for the cleavage of different *N*-acylhomoserines.

According to Table 2 the K_m values for the hydrolysis of ring-opened homoserine lactones is of the same order as the K_m value for the cleavage of the long-chain *N*-linolenoylglutamine by AAH.[32] The high K_m for the C_{12}-homoserines (C_{12}-HL) is due to the rather low solubility of the compound.

Interference of *N*-acylglutamines from the insect gut with quorum sensing in *E. coli*

The close structural similarity of the *N*-acylglutamines (Scheme 4) and the ring-opened *N*-acylhomoserines prompted us to look for cross-reactions between the AHL recognition system and the *N*-acylglutamines, which are highly abundant in the front parts of the insect gut.[20,21] To test for potential cross-reactions, *E. coli* RV308pSB40 was exposed to liquids from the gut lumen (regurgitate) or to *N*-linolenoylglutamine in concentration that resemble those in the insect gut. Thus, addition of buffered *N*-C_6-HL (100 μg mL^{-1}) to the bacterial culture led to bioluminescence within a few minutes. However, repetition of the experiment in the presence of regurgitate (50 μL) from *S. littoralis* resulted in a significant reduction of the effect; this indicates that compounds are present in the insect gut that interfere with the *E. coli* AHL-recognition system. Owing to the structural similarity of *N*-linolenoylglutamine with ring-opened *N*-C_6-HL (Scheme 4) we tested next *N*-linolenoylglutamine at a concentration of 100 μg mL^{-1}, which corresponds to the natural concentration in the gut of *Spodoptera* larvae.[21]

As shown in Figure 3, the addition of the *N*-linolenoylglutamine reduced the bioluminescence to a similar extent as the regurgitate. After treatment of the gut fluid by an ion exchange resin (Dowex 50WX8-200), which removes the *N*-acylglutamines and other basic compounds,[34] the bioluminescence of the *E. coli* system was no longer affected; this suggests that the *N*-acylglutamines might be responsible for this effect. No interference of the *N*-acylglutamines was observed with the quorum-sensing systems of the reporter strains *Agrobacterium tumefaciens* NT1/pZLR4 or *Chromobacterium violaceum* CV026.

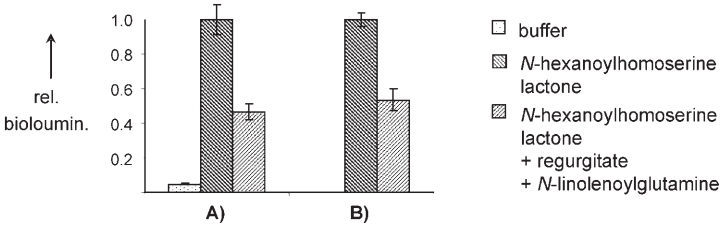


Figure 3. The effect of *N*-acylglutamines and insect regurgitate on the bioluminescence of *E. coli* RV308pSB40 was stimulated by *N*-hexanoylhomoserine lactone. The effect of A) insect regurgitate and B) *N*-linolenoylglutamime on the bioluminescence of the *E. coli* reporter system. Data were normalised with respect to the effect of *N*-hexanoylhomoserine lactone; relative bioluminescence (rel. biolumin.): 1.

Discussion

Amongst the physicochemical parameters in the caterpillars' gut, the pH has long been an object of attention and analysis. It is well known that the hydrogen-ion concentration is a regulating factor for enzymatic reactions, solubility and dissociation

or coagulation of ingested proteins, and the determination of gut flora.[22] For example, at pH values greater than pH 9 some enzyme–tannin complexes might not form; this would prevent disruption in insect digestion and immobilise gut enzymes by unspecific blockage of active sites.[35] The insect gut is hub to a consortium of bacterial species[26,36–38] and in larvae of *Spodoptera littoralis* and *Spodoptera eridania* that are fed on an artificial diet,[39] these numbers exceed the range of 10^7 mL^{-1} (V.M., unpublished data). The AHLs that are generally used by various members of the gut community are likely to be affected by the insect gut environment. Studies on *N*-(3-oxo-hexanoyl)homoserine lactone produced by *Erwinia* species showed that under stationary growth-phase conditions, the compound's concentration starts to diminish when the alkalinity of the culture increases to pH 7.0 or higher due to bacterial growth.[19] In another biochemical investigation by using ^{13}C NMR spectroscopy, it was found that ring opening of homoserine lactone (HL), C_3-HL and C_4-HL increased as the pH increased. By acidifying the growth media to pH 2.0, lactonolysis could be reversed.[28] Screening of the regurgitate by using LC–MS as well as by using the reporter strains *A. tumefaciens* NT1/pZLR4, *C. violaceum* CV026 and *E. coli* RV308pSB40 provided no evidence for these compounds. Even after ingestion of the *N*-C_6-HL along with the food, the compound could not be found in the intestinal fluid of the insect; this suggests a rapid and irreversible degradation of the compound in the insect gut. Accordingly, the strongly alkaline pH, especially in the foregut of *Spodoptera* larvae, could represent a key factor that controls the stability of the AHL, their formation and also their degradation by subsequent enzymatic reactions. Rapid degradation of the *N*-acylglutamines in the gut fluid has been also described for the tobacco hornworm, but the origin of this activity has remained unsolved.[40] In this context, the recently isolated and characterised *N*-acylamino acid hydrolase (AAH) from *M. arborescens*, which was isolated from the gut of *S. exigua*[32] might represent a key factor. Because the ability to cleave *N*-acylglutamines appears to be very widespread among gut bacteria,[33,41] the activity of the corresponding AAH's from the microbial population of the insect gut might be sufficient to explain the complete hydrolysis of homoserine lactones. Because AAH does not cleave intact AHLs, a two-step sequence has to be postulated. First, the strongly alkaline conditions of the fore- and midgut open the lactone moiety within minutes, and, second, hydrolytic enzymes of the type of AAH cleave the AHLs into the free amino- and fatty acid building blocks. The situation is different in the less alkaline area (pH about 7.0–8.0) of the hindgut, but even there no AHLs were found by analytical methods. In addition to the described scenario other microbial enzymes, such as the AHL lactonases,[29] might be operative. These enzymes catalyse the ring opening of the lactones, and, therefore, have the same effect as the alkaline pH. Lactonases and other quorum-quenching enzymes benefit the producing microorganism in their fight for nutrients and living space, especially in a highly competitive environment such as the insect intestine.[1]

In questioning the biological significance of these observations, one may speculate that the insect requires the quorum-quenching cascade to control the metabolic activities of its gut microbiota. Because AHLs often promote the formation of biofilms,[42] the secretion of siderophores,[43] antiobiotics[44] or proteolytic enzymes,[45] the suppression of this microbial communication system might benefit the insect by avoiding or reducing detrimental metabolic activities. The inhibitory effect of small amounts of *N*-linolenoylglutamine on the quorum-sensing system of *E. coli* RV308pSB40 might support this view. Due to their structural similarity to AHL and due to their high concentration in the insect foregut (200 μg mL^{-1}) these compounds might interfere with AHL reception. This effect is, however, not general, because *Agrobacterium tumefaciens* NT1/pZLR4 and *Chromobacterium violaceum* CV026 proved to be insensitive to the regurgitate or *N*-linolenoylglutamine. Because the latter organisms are known to respond to shorter AHLs, for example to C_4-, C_6- or to C_8-HL's, it remains to be clarified whether *N*-acyl glutamines of shorter chain length can interfere with their quorum-sensing system.

Our results suggest that a reaction cascade resulting in quorum quenching might exist in the lepidopteran intestine. This phenomenon might have developed during evolution to allow an insect to adapt to the inhabiting microflora and to control its metabolic activities. However, owing to their short generation cycle and the ease of adaptation, bacteria might have a hitherto underestimated impact on the coevolution of gut microbiota–insect and, indirectly, plant–insect interactions, which requires further study.

Experimental Section

Chemicals: Tetracycline, kanamycine, gentamycine, and *N*-(3-oxo-hexanoyl)homoserine lactone were purchased from Sigma–Aldrich. X-Gal was obtained from AppliChem (Darmstadt, Germany). AHLs acylated with *n*-alkyl fatty acids were synthesised according to Chhabra et al.[46] *N*-(3-Hydroxy-hexanoyl)homoserine lactone was obtained by reduction of *N*-(3-oxo-hexanoyl)homoserine lactone with $NaBH_4$.

Bacterial reporter strains; culture conditions and bioassays: For cultures of *Chromobacterium violaceum* CV026 the LB medium was additionally treated with kanamycin (30 μg mL^{-1}), and for cultures of *E. coli* RV308pSB40 tetracycline (1 mg mL^{-1}) was added. *Agrobacterium tumefaciens* NT1/pZLR4 was cultured in a medium that contained peptone (5 g), meat extract (3 g), mannitol (10 g), KH_2PO_4 (0.5 g), yeast extract (0.4 g), $MgSO_4{\cdot}7H_2O$ (0.2 g) and NaCl (0.1 g) in distilled H_2O (1 L).

Agrobacterium tumefaciens NT1/pZLR4[27] and *Chromobacterium violaceum* CV026[11] were grown on LB agar plates at 28 °C. Liquid cultures were incubated overnight. Assay plates were produced by dilution of liquid cultures with tenfold amount of liquid medium that contained agar (1.5 %). In case of *Chromobacterium violaceum* CV026 kanamycine (30 μg mL$^{-1)}$ was added. After drying, the plates were covered with sterile paper (0.5 cm^2) that contained the test solution (20–50 μL). After incubation for 10 to 24 h, the plates were inspected for zones of pigment production around the discs. Colour development later than 24 h was not considered to be relevant.

E. coli RV308pSB40[27] were grown on LB plates at 37 °C. For the preparation of a liquid culture, medium (20 mL) was treated with

E. coli RV308pSB40 and incubated, overnight, on a rotary shaker at 37 °C. This culture (2 mL) was treated with fresh medium (18 mL) and incubated for 1 h. The bioluminescence was analysed by using a luminometer (Lumiskan TL Plus, Labsystems). To demonstrate the presence of AHL, the bacterial culture (900 μL) was treated with test solutions from the insect gut (100 μL). The bioluminescence of the sample was recorded in 1 h intervals, and became constant after about 24 to 30 h. Each sample was shaken for 5 s prior to the measurement. Tris buffer (pH 7) was used for negative controls, and standardised solutions of *N*-hexanoylhomoserine (100 $\mu g\,mL^{-1}$) in the same buffer served as a positive control and for the determination of the detection limit. The values for the positive control in Figure 3 were normalised with respect to the effect of *N*-hexanoylhomoserine lactone.

Rearing of insect larvae: Larvae of *Spodoptera littoralis* (Bayer Cropscience, Monheim, Germany) or *Spodoptera eridania* (BASF Corporation, Florham Park NJ, USA) were hatched from egg clutches and reared on an agar-based artificial diet in plastic cages at 23–25 °C under a light–dark regime with 16 h of illumination. For the artificial diet ground white beans (500 g) were soaked, overnight, in water (100 mL). Ascorbic acid (9.0 g), parabene (9.0 g), aq. formaldehyde (4.0 mL, 36.5 %) and agar (75 g) were added to distilled H_2O (1000 mL) and boiled. After being cooled, the mixture solidified to a white waxy solid.[39] Alternatively, the larvae were reared on Lima beans at 23–25 °C under a light–dark regime with 16 h of illumination.

Determination of the gut pH profile: The pH profile along the larval gut was determined by using miniaturised glass electrodes PH-50 (Unisense A/S, Aarhus, Denmark);[47] outer diameter of the electrode tip: 40–60 μm; length of the pH-sensitive glass segment: 200–300 μm. The working microelectrode was connected to the input terminal of a high-impedance millivoltmeter ($Ri > 10^{14}\ \Omega$) and to the reference electrode (REF-500), which was further connected to the measuring chamber's Ringer solution, which consisted of NaCl (7.5 g), KCl (0.35 g) and $CaCl_2$ (0.21 g) per litre via a KCl-filled agar bridge. The microelectrodes were calibrated against standard pH solutions. Standard solutions with pH 4–9 were from Sigma Aldrich and stock solutions (50 mM) in the range of pH 10–12 were freshly prepared from $NaHCO_3$ (pH 10–11), Na_2HPO_4 (pH 11–12) and KCl (pH 12–12.5), respectively. Solutions were adjusted to the respective pH with NaOH (0.1 N). Solutions above pH 10 were kept in glass-stoppered bottles to prevent absorption of CO_2. Microelectrodes were calibrated before, during and after each set of experiments with three different pH buffers that covered the range of operation. When the pH microelectrode responded linearly between various pH standards with an optimal slope of 50–70 $mV\,pH^{-1}$ unit, the experimental millivolt readings were converted to the corresponding pH values.

Third or fourth instar larvae of *S. littoralis* and *S. eridania* were thoroughly cleaned with distilled H_2O (pH 7.0) and immobilised by freezing at −80 °C for 2–4 min. Larvae were then dissected by a ventral longitudinal incision exposing the gut. The gut was kept intact, still attached to skin from anterior and posterior regions. The dissected larvae were immediately laid open and fully extended onto a solidified layer of 0.8 % agarose in Ringer's solution and quickly embedded in an identical layer of cool molten agarose (56 °C) that solidified instantly.[48] Microelectrodes were positioned with a manual micromanipulator HS6 (Bio-Medical Instruments, Warren, MI, USA). The millivoltmeter measurements were taken at ambient temperatures (22 ± 2 °C). In each set, recordings were taken for five independent replicates.

Hydrolysis of AHLs: AHLs were hydrolysed in the following buffers: pH 7: tris(hydroxylmethyl)aminomethan (Tris) HCl (500 mM); pH 8: Tris–HCl (100 mM); pH 9: Tris–HCl (100 mM); pH 10: Na_2CO_3/$NaHCO_3$ (100 mM each); pH 11: CAPS, NaOH (100 mM). All buffers were sterilised by ultrafiltration prior to use. Hydrolysis was achieved by adding the buffer (2.0 mL) to a well-stirred solution of the AHLs (5 mM *N*-hexanoylhomoserine lactone, 1 mM *N*-(3-oxo-hexanoylhomoserinelactone, and 1 mM *N*-(3-hydroxyhexanoylhomoserine lactone) in H_2O (2.0 mL); this was followed by pH control at room temperature. The solution was stirred further at room temperature and aliquots (0.5 mL) were taken after 15 s, 5 min, 10 min, 20 min and 50 min, respectively, and mixed with Tris buffer (pH 7, 0.5 mL) to stop hydrolysis. For LC–MS analysis, synthetic *N*-(*n*-phenylpentanoyl)-glutamine[49] (4 μL of a stock solution in MeOH ($2.0\ mg\,mL^{-1}$) was added as an internal standard.

Enzymatic cleavage of the ring-opened AHLs: *N*-acylhomoserine lactones (2.1 mmol) were dissolved in Tris buffer (50 mM) adjusted to pH 10. After being shaken for 15 h the ring opening was complete without competing cleavage into glutamine and the acyl moiety. Next, the solutions were buffered to pH 8.0 and sterilised by filtration through a membrane (0.22 μm). These solutions were shown to be stable under the experimental conditions. The initial concentration of ring-opened *N*-acylhomoserine was determined by LC–MS. A purified protein fraction of *N*-acylamino acid hydrolase (AAH) from *Microbacterium arborescens*[32] was added to a final concentration of about $0.2\ mg\,mL^{-1}$ enzyme in Tris–HCl (50 mM) at pH 8.0. The protein concentration was chosen to achieve a linear turnover, which was followed by LC–MS. The substrate concentrations were 8×10^{-5} to 3×10^{-4} M for *N*-dodecanoylhomoserine, 1.5 to 7.3×10^{-5} M for *N*-decanoylhomoserine and 9.2×10^{-8} to 4.6×10^{-7} M for *N*-hexanoylhomoserine. The assay mixture was shaken at 37 °C, and aliquots (100 μL) were taken after 30 min (*N*-hexanoylhomoserine and *N*-decanoylhomoserine) or 6 min (*N*-dodecanoylhomoserine), which represented the linear phase of the enzymatic reaction. The reaction was stopped by precipitation of the enzyme by the addition of MeOH (100 μL), and *N*-(*n*-phenyldodecanoyl)-L-glutamine[50] was added as an internal standard in a final concentration of 4.9×10^{-5} M. For statistical treatment, kinetic experiments were repeated four to six times. The K_m values for the hydrolysis of the different AHLs were determined by a double-reciprocal plot according to Lineweaver and Burk.

HPLC and mass spectroscopic analysis of *N*-acylhomoserine lactones: AHLs were analysed by HPLC–MS by using a Thermoquest LCQ (Thermoquest, Egelsbach, Germany) in the APCI mode (vaporizer temperature: 430 °C) that was connected to an Agilent HP1100 HPLC-system. Separation was achieved on a Grom-Sil ODS3CP column (120 mm × 2 mm, 3 μm) by using gradient elution at $0.2\ mL\,min^{-1}$ (solvent A: H_2O, 0.5 % AcOH; solvent B: MeCN, 0.5 % AcOH) starting with 100 % A (3 min), programmed to 100 % B in 27 min. Elution with 100 % B was maintained for 15 min prior to equilibration with the initial solvent mixture. *N*-(*n*-Phenylpentanoyl)glutamine was used as an internal standard (4 μL of a $2\ mg\,mL^{-1}$ solution). Parallel UV detection allowed quantification of the tetramic acids ($\lambda_{max} = 278$ nm) that resulted from ring opening and rearrangement of the 3-oxo-AHL.[30]

Gas chromatography and mass spectroscopy of *N*-acylhomoserine lactones: AHL were additionally analysed by combined gas chromatography and mass spectroscopy by using a Trace GC (Thermo Finnigan, San Jose, CA, USA) that was connected to a Trace MS detector (Thermo Finnigan, San Jose, CA, USA). Compounds were separated by using an EC-5 column (15 m × 0.25 mmx0.25 μm, Alltech, Deerfield, IL, USA). Helium at a flow rate of

1.5 mL min^{-1} served as a carrier gas and a split-mode injection (1:10) was employed. The GC injector, transfer line and ion source were kept at 220 °C, 280 °C and 280 °C, respectively. Spectra were taken in the total-ion-scanning (TIC) mode at 70 eV. Compounds were eluted under programmed conditions starting at 50 °C (3 min isotherm) followed by being heated at 16.4 °C min^{-1} to 300 °C, and were maintained for further 3 min; injected amount: 1 μL.

Acknowledgement

We thank Dr. B. Trautmann, Bayer AG, Monheim, for supplying with egg clutches of Spodoptera littoralis and Peggy Kießling, HKI Jena, for providing AHL-sensitive bacteria strains. We thank Dr. L. Ping for proofreading of the manuscript.

Keywords: lactones · lepidoptera · metabolism · *N*-acylglutamines · *Spodoptera littoralis*

[1] R. J. Dillon, V. M. Dillon, *Annu. Rev. Entomol.* **2004**, *49*, 71–92.
[2] G. Tokuda, H. Watanabe, *Biol. Lett.* **2007**, *3*, 336–339.
[3] H. Nasir, H. Noda, *Arch. Zootec. Arch. Insect Biochem. Physiol.* **2003**, *52*, 175–182.
[4] T. Fukatsu, T. Hosokawa, *Appl. Environ. Microbiol.* **2002**, *68*, 389–396.
[5] R. J. Dillon, C. T. Vennard, A. K. Charnley, *Nature* **2000**, *403*, 851.
[6] J. W. Santo Domingo, M. G. Kaufman, M. J. Klug, W. E. Holben, D. Harris, J. M. Tiedje, *Mol. Ecol.* **1998**, *7*, 761–767.
[7] J. W. Costerton, P. S. Stewart, E. P. Greenberg, *Science* **1999**, *284*, 1318–1322.
[8] L. L. Li, J. E. Malone, B. H. Iglewski, *J. Bacteriol.* **2007**, *189*, 12, 4367–4374.
[9] M. R. Parsek, E. P. Greenberg, *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 8789–8793.
[10] P. S. Coburn, M. S. Gilmore, *Cell. Microbiol.* **2003**, *5*, 661–669.
[11] K. H. McClean, M. K. Winson, L. Fish, A. Taylor, S. R. Chhabra, M. Camara, M. Daykin, J. H. Lamb, S. Swift, B. W. Bycroft, G. S. A. B. Stewart, P. Williams, *Microbiology* **1997**, *143*, 3703–3711.
[12] E. G. Ruby, K. H. Nealson, *Biol. Bull.* **1976**, *151*, 574–586.
[13] I. Wagner-Döbler, V. Thiel, L. Eberl, M. Allgaier, A. Bodor, S. Meyer, S. Ebner, R. Pukall, S. Schulz, *ChemBioChem* **2005**, *6*, 2195–2206.
[14] S. Swift, P. Williams, G. S. A. B. Stewart in *Cell–Cell Signalling in Bacteria*, (Eds.: G. M. Dunny, S. C. Winans), American Society for Microbiology, Washington, D.C., **1999**, pp. 291–313.
[15] E. C. Pesci, J. B. Milbank, J. P. Pearson, S. McKnight, A. S. Kende, E. P. Greenberg, B. H. Iglewski, *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 11229–11234.
[16] X. Chen, S. Schauder, N. Portier, A. van Dorsselaer, I. Pelczer, B. L. Bassler, F. M. Hughson, *Nature* **2002**, *415*, 545–549.
[17] M. Pirhonen, D. Flego, R. Heikinheimo, E. T. Palva, *EMBO J.* **1993**, *12*, 2467–2476.
[18] H. M. Appel, L. W. Maines, *J. Insect Physiol.* **1995**, *41*, 241–246.
[19] J. T. Byers, C. Lucas, G. P. C. Salmond, M. Welch, *J. Bacteriol.* **2002**, *184*, 1163–1171.
[20] T. Alborn, T. C. J. Turlings, T. H. Jones, G. Stenhagen, J. H. Loughrin, J. H. Tumlinson, *Science* **1997**, *276*, 945–949.
[21] G. Pohnert, V. Jung, E. Haukioja, K. Lempa, W. Boland, *Tetrahedron* **1999**, *55*, 11275–11280.
[22] T. Lemke, U. Stingl, M. Egert, M. W. Friedrich, A. Brune, *Appl. Environ. Microbiol.* **2003**, *69*, 6650–6658.
[23] J. F. Harrison, *Annu. Rev. Entomol.* **2001**, *46*, 221–250.
[24] M. J. Lee, J. H. Anstee, *Insect Biochem. Mol. Biol.* **1995**, *25*, 49–61.
[25] R. D. Wyndham, T. A. Henry, *Proc. R. Soc. London* **1903**, *72*, 285–294.
[26] D. Spiteller, W. Boland, *Rundgespräche der Kommission für Ökologie,* Bayrische Akademie der Wissenschaften, **2002**, *23*, 111 – 122.
[27] C. Cha, P. Gao, Y. Chen, P. S. Shaw, S. K. Farrand, *Mol. Plant Microbe Interact.* **1998**, *11*, 1119–1129.
[28] E. A. Yates, B. Phillip, C. Buckley, S. Atkinson, S. R. Chahabra, R. E. Sockett, M. Goldner, Y. Dessaux, M. Cámara, *Infect. Immun.* **2002**, *70*, 5635–5646.
[29] Y. Dong, L. Wang, J. Xu, H. Zhang, X. Zhang. , L. Zhang, *Nature* **2001**, *411*, 813–817.
[30] G. F. Kaufmann, R. Sartorio, S. Lee, C. J. Rogers, M. M. Meijler, J. A. Moss, B. Clapham, A. P. Brogan, T. J. Dickerson, K. D. Janda, *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 309–314.
[31] Y. Lin, J. Xu, J. Hu, L. Wang, S. Ong, J. Leadbetter, L. Zhang, *Mol. Microbiol.* **2003**, *47*, 849–860.
[32] L. Ping, R. Büchler, A. Mithöfer, A. Svatos, D. Spiteller, K. Dettner, S. Gmeiner, J. Piel, B. Schlott, W. Boland, *Environ. Microbiol.* **2007**, *9*, 1572–1583.
[33] D. Spiteller, Dissertation, Friedrich-Schiller-Universität, Jena (Germany), **2002**.
[34] R. Halitschke, U. Schittko, G. Pohnert, W. Boland, I. T. Baldwin, *Plant Physiol.* **2001**, *125*, 711–717.
[35] M. Berenbaum, *Am. Nat.* **1980**, *115*, 138–146.
[36] J. A. Breznak, *Am. Zool.* **2000**, *40*, 954–955.
[37] D. L. Cruden, A. J. Markovetz, *Annu. Rev. Microbiol.* **1987**, *41*, 617–643.
[38] C. Toth-Prestia, I. N. Hirshfield, *Appl. Environ. Microbiol.* **1988**, *54*, 1855–1857.
[39] R. Bergomaz, M. Boppré, *J. Lepidopterists' Soc.* **1986**, *40*, 131–137.
[40] H. T. Alborn, M. M. Brennan, J. H. Tumlinson, *J. Chem. Ecol.* **2003**, *29*, 1357–1372.
[41] D. Spiteller, K. Dettner, W. Boland, *Biol. Chem.* **2000**, *381*, 755–762.
[42] B. Huber, K. Riedel, M. Hentzer, A. Heydorn, A. Gotschlich, M. Givskov, S. Molin, L. Eberl, *Microbiology* **2001**, *147*, 2517–2528.
[43] S. Lewenza, B. Conway, E. P. Greenberg, P. A. Sokol, *J. Bacteriol.* **1999**, *181*, 748–756.
[44] N. J. Bainton, B. W. Bycroft, S. R. Chhabra, P. Stead, L. Gledhill, P. J. Hill, C. E. D. Rees, M. K. Winson, G. P. C. Salmond, G. S. A. B. Stewart, P. Williams, *Gene* **1992**, *116*, 87–91.
[45] C. Kooi, B. Subsin, R. Chen, B. Pohorelic, P. A. Sokol, *Infect. Immun.* **2006**, *74*, 4083–4093.
[46] S. R. Chhabra, C. Harty, D. S. W. Hooi, M. Daykin, P. Williams, G. Telford, D. I. Pritchard, B. W. Bycroft, *J. Med. Chem.* **2003**, *46*, 97–104.
[47] N. P. Revsbech, B. B. Jørgensen, *Adv. Microb. Ecol.* **1986**, *9*, 293–352.
[48] A. Brune, D. Emerson, J. Breznak, *Appl. Environ. Microbiol.* **1995**, *61*, 2681–2687.
[49] T. Krumm, W. Boland, *Molecules* **1996**, *1*, 23–26.
[50] G. Görgen, W. Boland, U. Preiss, H. Simon, *Helv. Chim. Acta* **1989**, *72*, 917–928.

Received: December 27, 2007
Published online on July 18, 2008

