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INHIBITION OF BACTERIAL QUORUM SENSING BY THE RUMINAL FLUID OF CATTLE

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ABSTRACT: Rumen fluid contains factors that inactivate autoinducers, monitor microflora development and prevent the mobilization of virulence properties, and thus, the formation of resistant bacteria. The aim of this study was to assess the ability of rumen fluid to suppress the intercellular communication of bacteria by inactivating the N-acyl-homoserine lactones. We used strains of luminescent *Escherichia coli* JLD271 which were transformed with plasmids containing quorum sensing genes and luminescence genes. Bacteria were cultivated in LB agar medium containing 10 ug/ml doxycycline then in LB medium until the early exponential growth phase. The autoinducers used were N-butyryl-L-homoserine lactone (C4-HSL), N-(3-oxo)-hexanoyl-L-homoserine lactone (oxo-C6-HSL) and N-dodecanoyl-L-homoserine lactone (C12-HSL). The ruminal fluid of young calves was collected via a fistula. Equal volumes of ruminal fluid and autoinducers were incubated then added to the cultures of luminescent bacterial strains. Dilution of the ruminal fluid up to 12.5% did not affect the luminescence of the bacterial biosensors. Incubation of ruminal fluid with N-acyl-homoserine lactones decreased their activity, marked by unexpressed specificity. The most effective inhibition of the N-(3-oxo)-hexanoyl-L-homoserine lactone was observed at 10^{-7} M, and above this concentration the efficiency of quorum sensing inhibition decreased. Ruminal fluid was able to inactivate N-acyl-homoserine lactones, and the efficiency of inhibition was not related to the length of the alkyl tail in the signal molecule. This suggests the ability of the ruminant digestive system to control bacteria by the inactivation of N-acyl-homoserine lactones, which regulate the quorum sensing system.

Keywords: Ruminal fluid, Homoserine lactone, Bioluminescence, Quorum sensing inhibitor

1. INTRODUCTION

Cells often interact in bacterial communities via autoregulators, which are various substances that are formed and perceived by the bacterial population [1]. Gram-negative bacteria most commonly communicate via the synthesis of N-acyl-homoserine lactones (AHL), which occurs only when the population reaches a critical density. This phenomenon is called quorum sensing (QS) [2]. This interaction activates target genes, the expression of which is only effective with a sufficient number of cells, which generally alter the functional state of the bacteria following their activation by homoserine lactones (HSL), belonging to the first type of autoinducers (AI-1).

Investigation of the intercellular communication regulation mechanisms is critically important, making it possible to control the expression of genes that are important in the manifestation of certain properties of the bacterium, such as luminescence, antibiotic resistance and virulence [3]. Some bacterial QS systems are responsible for the development of pathogenesis, which increases the risk and spread of infection. However, macroorganisms (animals and plants) tend to have mechanisms to suppress bacterial communication channels [4], allowing them to regulate bacterial activity, including virulence [5]. The most common methods of QS inhibition are the binding of AHL molecules (often non-specific), enzymatic cleavage of the lactone ring by lactonase, and the production of autoinducer analogues which block the active center of the regulatory protein [6].

Certain microbiocenoses encompass a huge variety of different microorganisms, particularly the bacteria in the rumen of ruminants. Some members of the community can not only produce autoinducers of the second type (AI-2) [7], but also AHL [8]. However, this is controversial as several studies have shown the opposite [9]. Bacteria not belonging to the normal rumen flora could potentially receive these regulatory molecules, thereby leading to infection. We assumed that the ruminal fluid contains factors that inactivate autoinducers, especially AHL. This would facilitate control over the development of microflora, prevent the mobilization of virulence properties and the formation of resistant bacteria or their aggregates. In this study, we investigated the ability of molecular factors in the ruminal fluid to suppress intercellular bacterial communication by inactivating AHL.

2. MATERIALS AND METHODS

Bacterial strains of *Escherichia coli* JLD271 that had been transformed with a plasmid

containing both QS genes (*rhlR* and *lasR* from *Pseudomonas aeruginosa* and *luxR* from *Vibrio fischeri*) and luminescence genes (*luxCDABE* from *Photorhabdus luminescens*) [10] were used (Table 1).

The plasmids contained a selective gene (doxycycline resistance), *luxR* homolog gene (only in pAL101, pAL103 and pAL 105) and LuxI homolog promoter. Lux gene expression and luminescence increased in the presence of AHL.

Table 1. The *Escherichia coli* strains used and their characteristics.

Strain	Description	AHL detection	AHL used
E. coli pAL101	*rhlR*$^+$*rhlI*::*luxCDABE*	Yes	N-butyryl-L-homoserine lactone
E. coli pAL102	*rhlI*::*luxCDABE*	No	N-butyryl-L-homoserine lactone
E. coli pAL103	*luxR*$^+$*luxI*::*luxCDABE*	Yes	N-(3-oxo)-hexanoyl-L-homoserine lactone
E. coli pAL104	*luxI*::*luxCDABE*	No	N-(3-oxo)-hexanoyl-L-homoserine lactone
E. coli pAL105	*lasR*$^+$*lasI*::*luxCDABE*	Yes	N-dodecanoyl-L-homoserine lactone
E. coli pAL106	*lasI*::*luxCDABE*	No	N-dodecanoyl-L-homoserine lactone

Cultivation was performed in LB agar medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10 ug/ml doxycycline for 18 h at 37°C. The samples were then transferred to LB broth (Sigma-Aldrich) and incubated for 90 min at 37°C to achieve early exponential phase growth.

N-butyryl-L-homoserine lactone (C4-HSL), N-(3-oxo)-hexanoyl-L-homoserine lactone (oxo-C6-HSL) and N-dodecanoyl-L-homoserine lactone (C12-HSL) were used as autoinducers, added at concentrations ranging from 10^{-8} to 10^{-4} M.

Ruminal fluid of young calves was collected through a fistula and centrifuged at 5000 rpm for 10 min. The ability to inhibit the autoinducer activity was assessed by incubating 10 µl of ruminal fluid with an equal volume of ASL for 15 min at 37°C. Then 80 µl of the respective biosensor was added. The measurement was conducted for 120 min using a luminometer (LM-01T; Immunotech, Prague, Czech Republic) in the kinetic mode, and the luminescence level was expressed as relative luminescence units (RLU). The bioluminescence index (BLI) was calculated as the ratio of the RLU at 120 min to that at time zero. The AHL inhibition was calculated as the ratio between the RLU in the experimental probe (with rumen fluid) to RLU in the control probe.

Experimental studies were carried out in five-fold repetitions. Calculations were made using the methods of variation statistics.

3. RESULTS AND DISCUSSION

Ruminal fluid is a multicomponent solution that is acidic, which suppresses bacterial luminescence biosensors. This has been previously demonstrated in lyophilized cultures of recombinant strains of *E. coli* K12 TG1 in the commercial Ecolum system [11]. Among the *E. coli* strains used, only pAL103 and pAL104 had detectable levels of background luminescence of 252 ± 4 and 12440 ± 218 RLU, respectively (Fig. 1).

Despite having sufficient initial luminescence, the pAL105 and pAL106 strains lost their luminescence over time. As a result, the overall quantum yield was much lower than the recombinant *E. coli* strains pAL103 and pAL104.

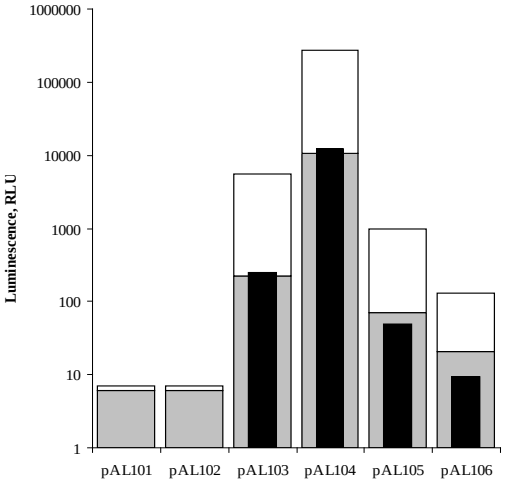

Fig. 1 Initial luminescence (grey columns), average luminescence (black columns) and accumulated luminescence after 120 minutes of exposure (white columns) of the *Escherichia coli* strains.

The cultured strains are characterized by different levels of perception of external factors [12]. The native ruminal fluid markedly inhibited the luminescence of *E. coli* strains pAL103 and pAL104 by up to 49% and 43%, respectively, relative to baseline levels. However, subsequent incubation did not lead to any qualitative changes in bioluminescence parameters. At 60 minutes, the renewal was up to 59% and 53%, respectively (Figs. 2).

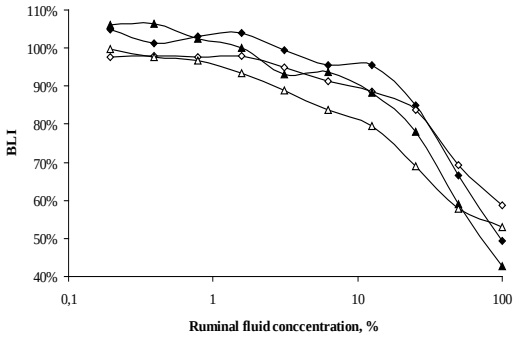

Fig. 2 Dependence of bioluminescent index (BLI) of *Escherichia coli* pAL103 (rhombus) and pAL104 (triangle) strains on the ruminal fluid concentration at 0 (closed points) and 60 minutes (open points).

This reaction is related to both the staining peculiarities of the ruminal fluid, conditioning the absorption of light quanta and is a consequence the reduction of total luminescence intensity and acute toxicity expressed by considerable ionic strength of the test samples. This leads to destabilization of the metabolic processes of the bacterial cell and loss of contact within the first few seconds. Dilution of the ruminal fluid expectedly led to a reduction in the above-described phenomenon. At concentrations of 25% and below, the luminescence-quenching activity was reversed.

Within the first minute, the luminescence was higher than in the second minute of contact. The threshold concentration of the biological fluid was 12.5%. At this concentration there was no significant effect on luminescence of the bacterial biosensors, as determined by EC_{20} (80% luminescence compared to the control indicators). Thus, this concentration of ruminal fluid was used to inhibit the activity of acyl homoserine lactone derivatives in order to avoid suppression of luminescence.

In the second stage, we studied the ability of the ruminal fluid to inactivate AHL molecules. The luminescent response was decreased when compared to samples that only contained the autoinducer at the relevant concentration. It was found that the effectiveness of the impact on HSL differed depending on the acyl tail. Thus, this was not due to the specific impact, but more likely due to the biosensor reaction.

We formed three groups of biosensors to evaluate the activity of various AHL. Each group consisted of a strain containing the gene that encodes the receptor protein and a corresponding strain without the specified gene. The *E. coli* pAL101 strain reacted with C4-HSL at concentrations of 10^{-6} M and above. Peak luminescence was registered at an autoinducer concentration of 10^{-4} M, for which the luminescence level was 7030 ± 255 RLU (Fig. 3).

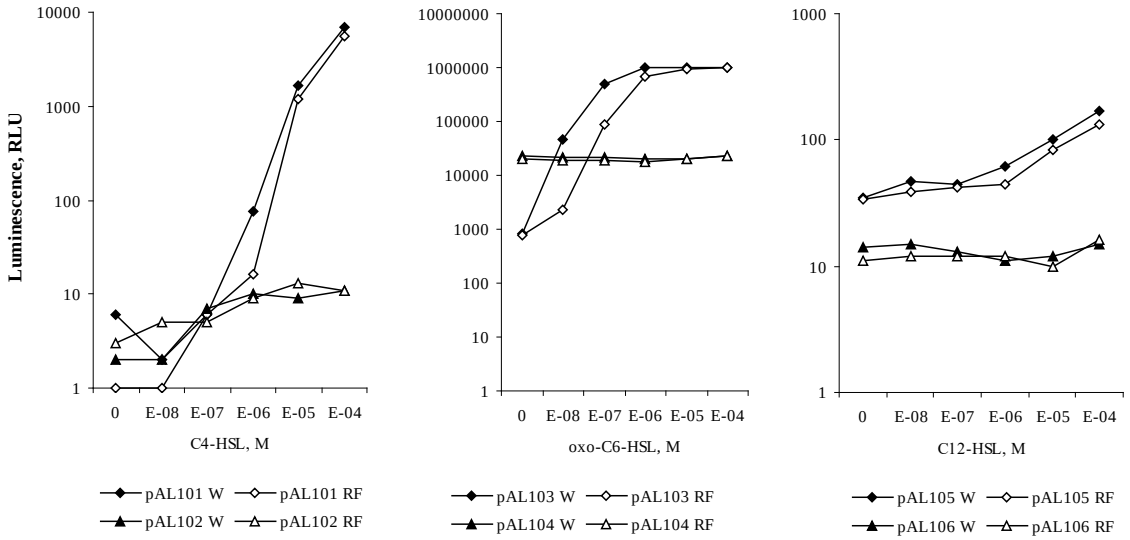

Fig. 3 Dependence of *Escherichia coli* strain luminescence on N-acyl-homoserine lactone (AHL) concentration in samples containing water (W) or ruminal fluid (RF).

At the same time, the biosensor as a whole showed a lower reaction in response to the sample with the specified AHL and ruminal fluid. The highest inhibitory effect (79%) was observed at the lowest detectable concentration (10^{-6} M). The inactivation efficiency decreased up to 19.5% at 10^{-4} M with the simultaneous increase in autoinducer content, for which the biosensor luminescence was 5656 ± 193 RLU (Table 2).

Table 2. Percentage of luminescence inhibition of recombinant *Escherichia coli* strains upon contact with ruminal fluid (RF) and N-acyl-homoserine lactone (AHL) in comparison to samples that only contained AHL.

Probe type	AHL concentration (M)				
	10^{-8}	10^{-7}	10^{-6}	10^{-5}	10^{-4}
E. coli pAL101 + C4-HSL + RF	-	-	79%	27%	20%
E. coli pAL103 + oxo-C6-HSL + RF	95%	82%	32%	5%	1%
E. coli pAL105 + C12-HSL + RF	-	-	27%	16%	21%

The *E. coli* pAL102 strain (without *rhlR*) did not respond to the C4-HSL, and the luminescence level did not change significantly in the sample containing the autoinducer and ruminal fluid.

A somewhat different pattern was observed with respect to oxo-C6-HSL. The *E. coli* pAL103 strain was selected as a specific biosensor. These bacterial cells perceived oxo-C6-HSL at a minimum concentration of 10^{-8} M, and were characterized by a saturation threshold of 10^{-6} M. The luminescence did not change above this level, at which it was maintained at 1008720 ± 3806 RLU. Thus, there was an excess of autoinducer which activated luminescence in all cultured bacteria.

The ruminal fluid showed pronounced inactivation of oxo-C6-HSL. The maximum value of 95% inactivation was recorded at a concentration of 10^{-8} M. At 10^{-7} M and 10^{-6} M, AHL inhibition was 82% and 32%, respectively. Application of the sample with a high concentration of oxo-C6-HSL (from 10^{-5} to 10^{-4} M) and ruminal fluid to the *E. coli* pAL103 strain led to a luminescent response that was comparable to the luminescence of the samples ($P > 0.05$) containing only the autoinducer (986400 ± 17706 RLU). This confirms that the system contained a sufficient quantity of oxo-C6-HSL to induce a luminescent recombinant strain, even after contact with the ruminal fluid.

The *E. coli* pAL104 strain could not synthesize the LuxR protein, and there was no change in luminescence in the presence of the same autoinducer at any concentration. The average luminescence level was 21648 ± 376 RLU. Samples containing different concentrations of oxo-C6-HSL as well as ruminal fluid were characterized by stable luminescence which was indistinguishable from the control level (19961 ± 722 RLU). This confirms a lack of influence of either the autoinducers or ruminal fluid on the luminescence genes (*luxCDABE*). This suggests that the inhibition of *E. coli* pAL103 luminescence depends on QS.

Finally, we also tested AHL with a high alkyl chain length (twelve carbon atoms). This was detected using the *E. coli* pAL105 strain. This biosensor could detect C12-HSL from 10^{-6} M, with a subsequent increase in dose-dependent induction. However, the luminescence response of the biosensor was low (at 10^{-4} M of C12-HSL the luminescence was 170 RLU). This led to a less significant change in luminescence. Thus, samples containing ruminal fluid and 10^{-6} M C12-HSL showed 27% lower luminescence than samples with the same concentration of autoinducer without ruminal fluid.

As expected, increasing concentrations of AHL reduced the activity of the ruminal fluid, with reductions of 16% and 21% at 10^{-5} and 10^{-4} M C12-HSL, respectively. Similarly, the *E. coli* pAL106 strain without the genes to synthesize the receptor protein (in this case, *LasR*) showed no change in luminescence with any of the analytes. The luminescence level in the samples was indistinguishable from that of the controls.

In this study, we demonstrated the inhibition of bacterial intercellular communication by ruminal fluid for the first time. Despite conflicting reports in the literature about the presence of AHL in mammalian species [13], we used ruminal fluid bioassays as a quorum quenching factor and recombinant able to detect AHL biosensors of *E. coli* [14].

The microbial community of ruminal fluids can reach a high density [15], and most of it is associated with feed particles [16]. Our data suggest that regulation of the microbial population may occur via QS signaling systems. Our results complement the results of studies previously conducted on QS second type autoinducer (AI-2), containing the *luxS* gene. Their participation in the formation of biofilm in rumen was assumed [17], and the results are consistent with those obtained by several authors [8] [18] [19]. In addition, it may represent one of regulatory pathways to prevent the growth and infection of some pathogenic

bacteria such as enterohemorrhagic *E. coli*, which can adapt via QS and develop in the rumen, including through the perception of AHLs [20].

4. CONCLUSION

We observed the ability of ruminal fluid to inactivate AHLs, which resulted in reduced reactivity of the bacterial biosensors. In our opinion, the nature of inhibition factors is more likely to be non-specific, and can degrade or absorb different molecules. Moreover, the inhibition does not appear to be related to the length of the alkyl tail in the HSL molecule, and the resulting differences are caused by variations in the sensitivity of biosensors. However, development of this approach will help to control the activity of undesirable microflora by selective or total suppression of their communication signals, and will allow us to further investigate the relationship between micro- and macroorganisms.

5. ACKNOWLEDGEMENT

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