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CONFERENCE PROCEEDINGS

Non-Species-Specific Effects of Unacylated Homoserine Lactone and Hexylresorcinol, Low Molecular Weight Autoregulators, on the Growth and Development of Bacteria

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Abstract—We conducted a comparative study of the effects of α-amino-γ-butyrolactone, the common structural element of extracellular microbial regulators of the homoserine lactone (HSL) group, and of 4-*n*-hexylresorcinol, an autoregulator of the alkylhydroxybenzene (AHB) group, on the growth and development of gram-positive and gram-negative bacteria. We revealed non-species-specific effects of HSL and AHB and characterized their concentration dependencies. The addition of 10^{-5}–10^{-3} M HSL or 10^{-5}–10^{-4} M AHB during the exponential growth phase of the cultures grown on balanced media resulted in cell division arrest and accelerated the transition to the stationary phase that culminated in endospore formation in *Bacillus cereus, Alicyclobacillus tolerans*, and *Sulfobacillus thermosulfidooxidans*. When bacilli grew under the cultivation conditions that resulted in a low–zero spore percentage, 10^{-4}–10^{-3} M HSL cancelled the inhibition of spore formation. In the gram-negative bacteria *Pseudomonas aurantiaca* and *Azotobacter vinelandii*, AHB at concentrations of 10^{-4} to $(1.5–2.5)\times10^{-4}$ M induced the formation of dormant cells. Studies with the actinobacterium *Streptomyces avermitilis* revealed that the HSL effect varied depending on the age of the test cultures. The addition of 10^{-4} M HSL during the lag phase of a submerged streptomycete culture accelerated its transition to the stationary phase and induced the formation of endospores, the dormant cells that are regarded as alternatives to exospores (conidia). If HSL (3.64 and 4.55 mg per 1 cm^2 disc) was locally added to a surface *S. avermitilis* culture, the growing mycelium formed rings that differed in their density, in the extent of the development of aerial mycelium, and in the presence/absence of exospores. Ring-shaped growth of streptomycete mycelia was also induced by 0.075–0.75 mg of AHB; however, unlike HSL, AHB repressed exospore formation. The data on non-species-specific effects of HSL and AHB suggest that they may perform regulatory functions at the microbial community level.

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The operation of bacterial intercellular communication facilities and of the systems controlling behavior on the population level in accordance with changes in environmental conditions is presently receiving increasing attention of researchers in various subfields of microbiology. Density-dependent quorum sensing (QS) autoregulatory systems have been particularly well-characterized. In a number of gram-negative bacteria, the diffusible extracellular communication agents used by QS systems are acylated homoserine lactones (aHSL) [1–3]. In contrast, QS processes in some gram-positive bacteria involve oligopeptides [4], discussed in reviews [2, 3]. The gram-negative bacterium *Vibrio harveyi* was shown to possess two bioluminescence-inducing QS systems based on aHSL and a chemically different autoinducer AI-2 [5] that was identified as furanosyl borate diester [6]. Streptomycetes employ a well-characterized system of cell differentiation control and antibiotic formation that involves the extracellular A factor and A factor-similar autoregulators (butyrolactones) [7]. The addition of the A factor to lag phase cultures of non-spore-forming mutants or butyrolactone-synthesizing streptomycete dissociants unable to form aerial mycelium and antibiotics, results in the generation of streptomycete variants whose properties revert to normal during cultivation on solid media [7]. Presumably, low molecular weight communication factors are involved in controlling gene expression via forming complexes with DNA-binding regulatory proteins [1, 2]. Virtually all studies on extracellular autoregulators

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used microorganisms that synthesize them. However, the interactions of low molecular weight regulators with their molecular targets are based on physical and chemical processes; this fact does not provide for their specificity to receptor molecules and, therefore, to cells that contain them.

Of particular interest to us was the hypothesis [8] that unacylated HSL is involved in the expression of factor σ^S (encoded by the *rpoS* gene), an RNA polymerase subunit responsible for the transcription of stationary phase genes in *Escherichia coli* cells. This hypothesis concerning the positive effector role of unacylated HSL was discussed by a number of scientists who presented evidence both for [9] and against [10] it.

Another well-known control system involved in the transition of a microbial culture to the stationary phase uses extracellular d (differentiation) factors that represent a different type of autoregulators [11–14]. The mode of action of d_1 factors, which were identified as alkylhydroxybenzenes (AHB) in some bacteria [14], is based on physical and chemical interactions (hydrogen bonds, hydrophobic and electrostatic interactions) and complexation with cell biopolymers (enzyme proteins and nucleic acids) [15–17] and with membrane phospholipids [18]. Upon reaching the threshold concentration that varies depending on the cell number in a developing culture, AHB cause cell division arrest and the culture's transition to the stationary growth phase. A further increase in AHB concentrations results in the formation of cystlike dormant cells (CDC) that are characterized by an anabiotic state [12–14]. Importantly, the aHSL threshold concentration related to cell quorum phenomena is the *minimum* level required for autoinducing aHSL synthesis and increasing the HSL intracellular level to enable target gene expression. In contrast, the AHB threshold concentration is the *maximum* level; exceeding it results in cell division arrest and transition to the stationary phase. Non-species-specific effects of AHB were revealed in a number of bacterial species [11–14]. Of particular interest, therefore, was a comparison of non-species-specific effects of HSL and AHB on various test organisms, which was the goal of this work.

MATERIALS AND METHODS

This work used the following chemical analogs of bacterial autoregulatory factors: homoserine lactone (HSL), α-amino-γ-butyrolactone hydrobromide (molecular weight 119, Sigma), and 4-*n*-hexylresorcinol (C_6-AHB) (molecular weight 194, Sigma). Ethanol solutions of HSL or C_6-AHB were added to exponential phase cultures to achieve final concentrations of 10^{-5}–10^{-3} M; the ethanol concentration in the suspension was 0.5% (vol/vol). The same ethanol amount was added to control systems.

The tested species were: (i) the gram-positive spore-forming heterotrophic bacterium *Bacillus cereus* VKM B-504^T; (ii) the moderately thermophilic, obligatorily mixotrophic acidophilic bacterium *Sulfobacillus thermosulfidooxidans* VKM B-1269^T; (iii) the chemolitho-heterotrophic bacterium *Alicyclobacillus tolerans* VKM B-2304^T (formerly *"S. thermosulfidooxidans* subsp. *thermotolerans,"* strain K1) that displays pronounced organotrophic growth but is also capable of oxidizing low amounts of mineral compounds under mixotrophic conditions; (iv) the actinobacterium *Streptomyces avermitilis* JCM 5070^T; (v) the gram-negative cyst-forming bacterium *Azotobacter vinelandii* VKM B-1617^T; and (vi) the gram-negative non-spore-forming bacterium *Pseudomonas aurantiaca* VKM B-1558.

B. cereus cells were grown on a synthetic medium to induce spore formation [13]. However, if the glucose content in this medium was increased to 40 or 60 g/l, spore formation was repressed [13]. The bacteria *S. thermosulfidooxidans* and *A. tolerans* were cultivated under mixotrophic conditions on the liquid Manning (M) medium that repressed endospore formation or on the A medium that provided for spore formation [19]. A submerged culture of the actinobacterium *S. avermitilis* was grown on CP-1 synthetic medium with glucose [20]. The bacterium *A. vinelandii* was cultivated on a nitrogen-free medium containing (g/l): mannitol, 5; K_2HPO_4 – 0.2; $CaCl_2$ – 2; $MgSO_4 \cdot 7H_2O$, 0.2; NaCl, 0.2; K_2SO_4 – 0.1; $Na_2MoO_4 \cdot 2H_2O$, 0.1 (pH 7.5). *P. aurantiaca* was grown on an M-9 synthetic medium with 0.2% glucose. The bacteria were cultivated on shakers (140–160 rpm) in 250 ml flasks containing 50 ml of medium at 28°C (*B. cereus*, *A.vinelandii*, *P. aurantiaca*, and *S. avermitilis)*, 37°C (*A. tolerans*), and 48°C (*S. thermosulfidooxidans*).

The surface culture of *S. avermitilis* was grown on agar-containing oat medium with 0.25% yeast extract [20]. The HSL effect was determined in this system with paper discs (containing 1.82, 2.73, 3.64, and 4.55 mg of HSL per one 1 cm^2 disc) placed on the agar surface either immediately, or 3, 6, 8, and 16 h after inoculating the streptomycete. Additionally, the effects of C_6-AHB (from 0.075 μg to 0.75 mg) or HSL at the concentrations cited above were assessed by the cylinder method (d = 6 mm, V = 38 μl). The plates were incubated at 28°C for 7 days.

The growth of submerged bacterial cultures was monitored by their optical density (λ = 650 nm; l = 10 mm) using a Specord UV VIS spectrophotometer, Carl Zeiss, Jena, Germany or by the cell number per 1 ml (counted in a Goryaev chamber). The number of viable cells was determined from the number of colony-forming units (CFU) after inoculating diluted cell suspensions on agar-containing media. Microscopic studies were conducted with a Zetopan microscope (Reichert, Austria) equipped with a phase contrast device, and vegetative and dormant cells were counted separately after scanning 30–40 frames. The micromorphology of streptomycete mycelium was investigated

Table 1. Biological effects of HSL on growth and development of spore-forming and non-spore-forming bacteria

Culture (variant)	HSL	
	Concentration, M	Effect
B. cereus (on the spore formation-promoting medium)	10^{-5}	No effect
	10^{-3}–10^{-4}	Cell division arrest (1–3 h after the addition of HSL), accelerated transition to the stationary phase and, accordingly, accelerated spore formation
B. cereus (on the medium that partially represses spore formation)	10^{-5}	Cell division arrest (1–3 h after the addition of HSL), accelerated transition to the stationary phase
	10^{-3}–10^{-4}	Cell division arrest (1–3 h after the addition of HSL), accelerated transition to the stationary phase, removal of the inhibition of spore formation
B. cereus (on the medium that completely represses spore formation)	10^{-5}–10^{-3}	Cell division arrest (1–3 h after the addition of HSL), accelerated transition to the stationary phase culminating in the formation of dormant forms (DF)
S. thermosulfidooxidans (on the M medium that completely represses spore formation)	10^{-5} 10^{-4}	Cell growth arrest (after 3 h) Removal of the inhibition of spore formation
A. tolerans (on the spore formation-promoting A medium)	10^{-5}–10^{-4}	Cell division arrest (5 h after the addition of HSL), accelerated spore formation
	10^{-3}	Cytotoxic effect
A. tolerans (on the M medium that partially represses spore formation)	10^{-5}–10^{-4}	Cell division arrest (after 5 h), removal of the inhibition of spore formation
	10^{-3}	Cytotoxic effect
S. avermitilis (submerged culture)	10^{-5}	No effect
	10^{-4}	Accelerated transition to the stationary phase, resulting in the formation of endospores, the DF that are an alternative to exospores
	10^{-3}	Growth inhibition. Hypha thinning and partial lysis of mycelium
S. avermitilis (surface culture)	3.64 and 4.55 mg per disc	Induction of a ring-shaped pattern of mycelium growth; the rings differ in density, the degree of development of aerial mycelium, and the presence/absence of exospores
A. vinelandii	10^{-5}	Growth and cell division arrest (after 1 h), stimulation of extracellular pigment formation
	10^{-4}–10^{-3}	Cell division arrest (after 1 h), accelerated transition to the stationary phase ending in DF formation
P. aurantiaca	10^{-5}	No effect
	10^{-4}–10^{-3}	Cell division arrest (after 1–3 h), accelerated transition to the stationary phase ending in DF formation

using a Lumipan microscope (Carl Zeiss, Jena, Germany) equipped with a phase contrast device.

Our studies included three series of experiments; three repeats of each experiment were done. The results were considered reliable if the standard deviation values of cell groups did not exceed $p = 0.05$.

RESULTS

The addition of the autoregulators HSL and C_6-AHB at concentrations of 10^{-5}–10^{-3} M to exponential phase cultures resulted in cell division arrest in all tested bacteria (Table 1). The inhibitory effect of HSL was non-species-specific. It varied depending on the HSL concentration and manifested itself 1–3 h after the addition of HSL to bacterial cultures, the time comparable to the generation time values of the cultures involved. Autolytic processes only occurred after the addition of 10^{-3} M HSL to a submerged *S. avermitilis* culture (Fig. 1d) and to an *A. tolerans* culture on a spore formation-repressing medium. The HSL-induced inhibition of cell proliferation is not accompanied by any toxic effect of HSL and may be due to the onset of the

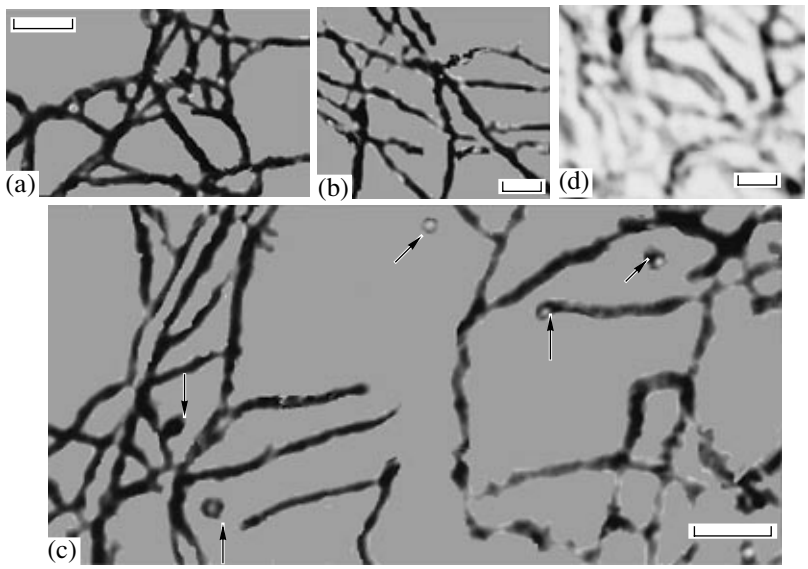

Fig. 1. Micrograph of submerged *S. avermitilis* mycelium (phase microscopy) developing in the presence of HSL: (a) control (without HSL); (b) 10^{-5} M; (c) 10^{-4} M; (d) 10^{-3} M; bar, 10 μm; arrows point to spores.

stationary phase. In line with this suggestion, we observed stationary phase-related events such as the formation of dormant cells. The HSL effects were investigated in *Bacillus* cultures grown on both media promoting and repressing spore formation, because the developmental program of microbial cultures, including spore formation, varies to a considerable extent, depending on medium composition.

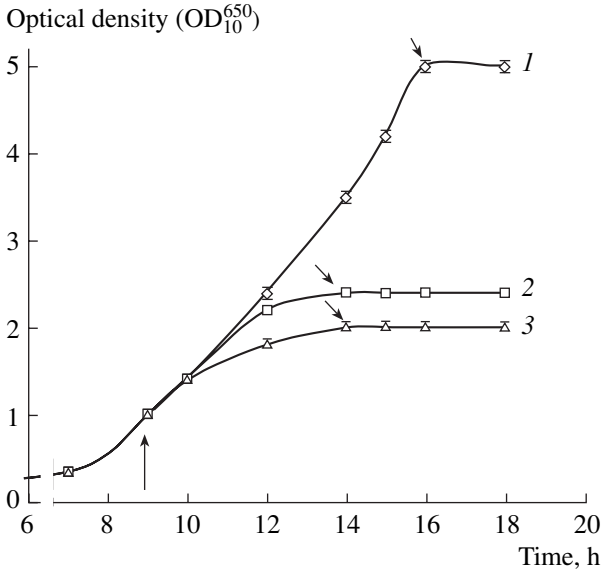

Fig. 2. Growth and development of a *B. cereus* culture on the spore-formation-promoting medium. (*1*), control system; (*2*) and (*3*), with 10^{-4} and 10^{-3} M HSL, respectively. Vertical arrow, time of the addition of HSL; slanting arrow, onset of the stationary phase.

A *B. cereus* culture grown on a spore-formation-promoting medium made the transition to the stationary phase in the presence of HSL (10^{-4} and 10^{-3} M) 4–5 h earlier than a control culture (Fig. 2); therefore the onset of spore formation occurred earlier. The share of spore-forming cells after adding 10^{-4} M HSL was 22 and 71% after 6 and 16 h, respectively. It was significantly higher than in the control system (0 and 16%, respectively, Table 2). However, the total percentage of forespores and spores was virtually equal in the control and the experimental system after 40 h of growth (Table 2). Hence, the transcription events associated with the HSL-induced stationary phase followed the normal pattern, and the developmental cycle of the *Bacillus* culture culminated in endospore formation. If *B. cereus* grew on a medium with 4% glucose, which yields a low endospore percentage (6%), the addition of HSL (10^{-4} M) reversed the spore formation process. The total forespore and single spore content was 88% in this system vs. 6% in the control system (Table 3), i.e. virtually the same as with bacilli on the spore formation-promoting medium (Table 2). However, if spore formation was completely inhibited by cultivating *B. cereus* on a medium with 6% glucose, the addition of HSL failed to remove the inhibition (Table 1) irrespective of its concentration (within the 10^{-5}–10^{-3} M range). Based on the results of our microscopic studies, the developmental cycle of these cultures ended in the formation of refractive cells that represent dormant forms (DF), an alternative to spores. However, the properties of dormant cells formed on HSL addition were not investigated within the frame of this study. Thus, additional research is to be carried out in the future.

An analogous effect of HSL was revealed in studies with the bacilli *A. tolerans* and *S. thermosulfidooxi-*

dans, whose cultivation conditions (nutrient medium, temperature, and pH) differed from those of the heterotrophic bacterium *B. cereus*. The addition of 10^{-4} HSL to an *A. tolerans* culture grown on the A medium to promote spore formation resulted in the transition to the stationary phase and, therefore, in an earlier onset of spore formation than in the control system, but had no effect on the number of spore-forming cells (Table 2). The total forespore and endospore yield did not exceed 15% if *A. tolerans* was grown on the M medium that partially represses spore formation. Adding HSL (10^{-5}–10^{-4} M) to this system increased the yield to 45% (Table 3). As for *S. thermosulfidooxidans* grown on the same medium, the inhibition of spore formation was removed, and the total percentage of spore-forming cells and spores was 20% in cultures treated with 10^{-4} M HSL (Table 3). Importantly, no spore formation occurred in control systems.

Subsequent studies were conducted with the gram-negative bacterium *A. vinelandii* that can form cysts, specialized dormant cells, under appropriate conditions (not in our experimental system). The addition of 10^{-4}–10^{-3} M HSL to an *Azotobacter* culture growing on a nitrogen-free medium with mannitol caused its transition to the stationary phase, resulting in the formation of refractive DF that were distinct from cysts (Table 1) and retained their viability. After storing the cultures for three months, their CFU number was 2.7×10^7 ml^{-1}; this corresponded to 60% of the CFU number in the early stationary phase. Therefore, the addition of HSL to *A. vinelandii* failed to increase the cyst yield (less than 1% both in the experimental and control systems) under these cultivation conditions, but caused the formation of dormant cells that were characterized by a comparatively simple morphology. We also noted that *A. vinelandii* cells formed a fulvous pigment in the presence of 10^{-5} M HSL earlier than in the control system (Table 1). The synthesis of this pigment, a secondary metabolite, is linked to the stationary phase.

The transition of cultures to the stationary phase and DF formation under the influence of 10^{-4}–10^{-3} M HSL was also demonstrated in the gram-negative non-spore-forming bacterium *P. aurantiaca*. After storing its culture for 3 months, the pseudomonads retained a viability level of 3.2×10^8 CFU ml^{-1}, corresponding to 40% of the maximum CFU yield in a stationary-phase culture.

In the actinobacterium *S. avermitilis*, we established that the effect of HSL on the streptomycete growth varied depending, apart from its concentration, on the physiological age of the bacteria involved (i.e., on the time of the addition of HSL). The HSL concentration of 10^{-5} M exerted no influence on the development of the submerged culture and on the cell morphology of streptomycete mycelium (Fig. 1b), regardless of the time of the HSL addition. The HSL concentration of 10^{-4} M induced early endospore formation (on the second day of cultivation, Fig. 1c), in contrast to the control system (Fig. 1a) where endospore formation started on the third or fourth day. Interestingly, this effect occurred if HSL (10^{-4} M) was added during the lag phase (0 or 3 h after inoculation). A comparatively weaker effect was produced by HSL added to the culture 6 or 8 h after inoculation; virtually no effect occurred if HSL was added 16 h after inoculation (during the linear growth phase).

Table 2. Effect of HSL (10^{-4} M) on the development of *B. cereus* and *A. tolerans* cultures growing on balanced spore formation-promoting media (data of microscopic studies)

Time after inoculation, h	Percentage of total cell number*		
	dividing cells	single (stationary-phase) cells	spore-forming cells + spores
B. cereus			
HSL			
1	79	21	0
6	5	73	22
16	0	29	71
40	0	21	27 + 52
Control (without HSL)			
1	92	4	0
6	45	55	0
16	0	84	16
40	0	24	29 + 47
A. tolerans			
HSL			
24	0	77	23
72	0	63	37
168	0	52	38 + 10
Control (without HSL)			
24	0	97	3
72	0	85	15
168	0	57	33 + 10

* The mean values of the data obtained by scanning 30–40 frames are presented; the standard deviation values are not shown.

Of particular interest were our results concerning the effect of HSL on the development of a surface culture of *S. avermitilis* (Fig. 3, Table 1). A continuous growth pattern (a "lawn") formed in systems with 1.82 or 2.73 mg of HSL per disc and in the control system. Increasing the HSL dose to 3.64 and 4.55 mg per disc resulted in a ringlike growth pattern (Fig. 3a), i.e., the formation of alternating zones characterized respectively by repression and stimulation of growth and development of aerial mycelium with spiral sporophores. Hence, similar to submerged cultures, HSL is particularly efficient in a surface streptomycete culture when added during the lag phase. The maximum effect occurred if HSL-containing discs were placed on the

Table 3. Effect of HSL (10^{-4} M) on the development of *B. cereus*, *A. tolerans*, and *S. thermosulfidooxidans* cultures growing on spore formation-repressing media (data of microscopic studies)

Time after inoculation, h	Percentage of total cell number*		
	dividing cells	single (stationary-phase) cells (share of refractive or lysed cells)	spore-forming cells + spores
		B. cereus (medium with 4% glucose)	
		HSL	
1	76	24	0
19	0	43	57
40	0	12	22 + 66
		Control (without HSL)	
1	92	8	0
19	10	~89	<1
40	0	94 (63–refractive)	4 + 2
		A. tolerans (M medium)	
		HSL	
5	0	72	28
22	0	70 (10–refractive)	24 + 6
120	0	55 (20–lysed)	31 + 14
		Control (without HSL)	
5	75	24	<1
22	0	95 (60–lysed)	5
120	0	85 (60–lysed)	9 + 6
		S. thermosulfidooxidans (M medium)	
		HSL	
24	0	99	<1
72	0	99 (10–lysed)	<1
168	0	79 (20–lysed)	14 + 7
		Control (without HSL)	
24	23	77	0
72	0	100 (20–lysed)	0
168	0	100 (30–lysed, 10–refractive)	0

* The mean values of the data obtained by scanning 30–40 frames are presented; the standard deviation values are not shown.

agar surface immediately after spreading the inoculum (conidia) over the plate or after 3 h of cultivation. Since HSL is a lactonized derivative of homoserine, we conducted parallel studies concerning the effect of serine on the development of a surface *S. avermitilis* culture. We established that serine failed to induce a ringlike growth pattern at concentrations five times higher than those of HSL (Fig. 3b).

Hence, unacylated HSL blocks cell division and the transition to the stationary phase in a non-species-specific fashion. It exerts its influence on both spore-forming and non-spore-forming bacteria belonging to diverse taxonomic and physiological groups. In bacilli and streptomycetes, the HSL effect was accompanied by the expression of genetic programs responsible for spore formation. In gram-negative non-spore-forming bacteria, HSL caused the formation of refractive dormant cells.

An analogous system was used to test the regulatory effect of hexylresorcinol, another density-sensing autoregulator belonging to the AHB group. The results of this work confirm the data obtained earlier [12–14] on the mode of action of AHB as density-sensing regulators and testify to their non-species-specific activity and a clear-cut concentration dependence of their effects. At concentrations of about 10^{-4} M, C_6-AHB caused cell division arrest and transition of a *B. cereus* culture to the stationary phase. The addition of 7×10^{-4} M during the growth deceleration stage of a culture on a spore-formation-repressing medium (with 6% glucose)

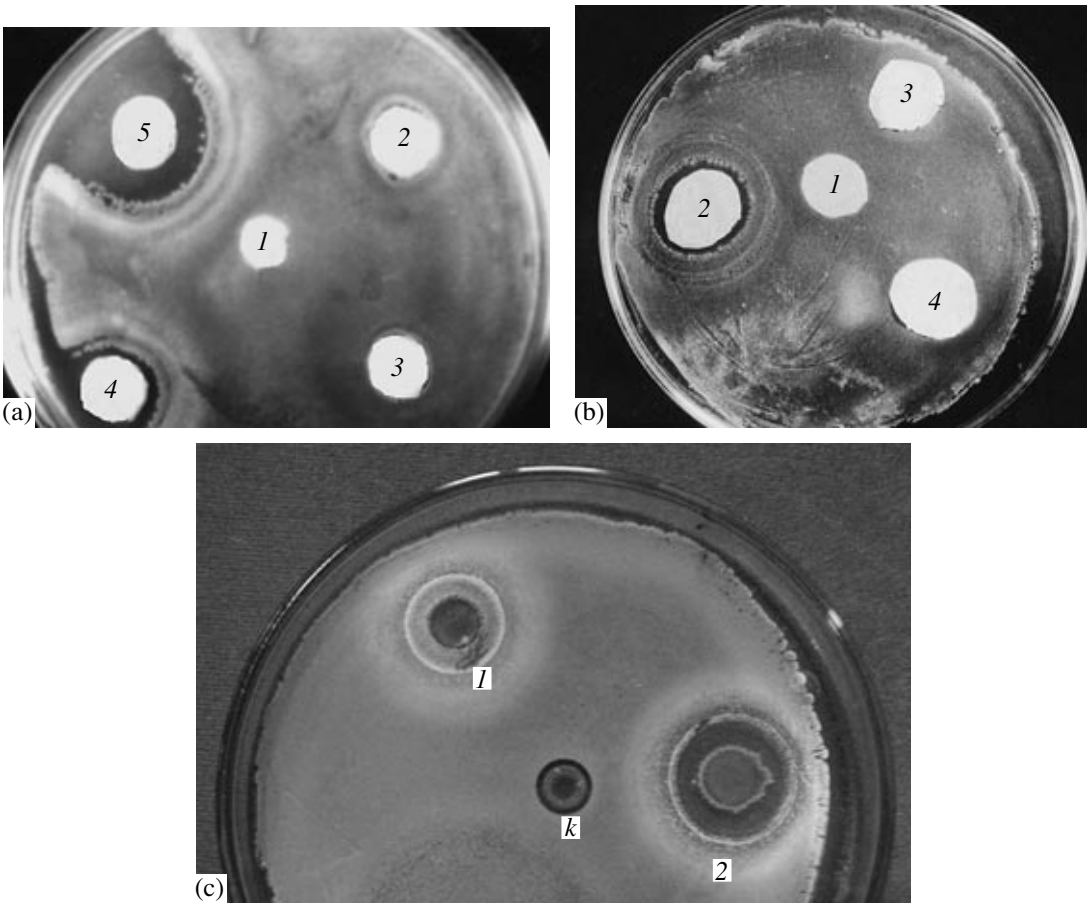

Fig. 3. Formation of alternating concentric zones in a surface culture of *S. avermitilis* with HSL and C_6-AHB. (a) disc 1, control (without HSL); discs 2–5, HSL amounts of 1.82, 2.73, 3.64, and 4.55 mg per disc, respectively; (b) disc 1, control; disc 2, HSL amount of 4.55 mg per disc; discs 3 and 4, serine amounts of 4.2 and 22.75 mg per disc, respectively; (c) k, control; 1 and 2, C_6-AHB amounts of 0.37 and 0.75 mg per cylinder, respectively.

induced the formation of anabiotic cystlike dormant cells that represent an alternative to endospores and were described in detail earlier [12–14]. Growth inhibition and the transition of bacterial cultures to the stationary phase resulting in DF formation also occurred under the influence of C_6-AHB on the other tested bacterial species, *P. aurantiaca* and *A. vinelandii.* A further increase in C_6-AHB concentration to 2×10^{-3} M caused the formation of "micromummies," i.e., cells that remain morphologically intact but lack metabolic activities and the colony-forming capacity. The formation of mummified bacterial and yeast cells in the presence of supercritical AHB concentrations was revealed by us earlier [21]. In contrast to AHB, HSL did not cause cell mummification at supercritical concentrations.

In the studies with a surface *S. avermitilis* culture, we demonstrated that a local addition of C_6-AHB in amounts of 0.075, 0.37, and 0.75 mg per cylinder resulted in the formation of ringlike zones characterized by the development of aerial mycelium that alternated with growth repression zones (Fig. 3c). In contrast to HSL, C_6-AHB did not cause the formation of sporophores and exospores during a long cultivation period (up to 1 month). A lack of spore-forming capacity was also characteristic of the first mycelium subculture, which reverted to spore formation only after a subsequent reinoculation.

Despite the differences in the effects produced by HSL and C_6-AHB on bacterial cultures, the results obtained enable us to conclude that the tested autoregulators in principle perform a similar biological function. They limit the increase in cell number in a population and control the transition of a growing culture to the stationary phase that culminates in the formation of metabolically quiescent viable cells (cystlike cells).

DISCUSSION

With the exception of the *Pseudomonas* species, none of the studied representatives of gram-positive and gram-negative bacteria of various taxonomic and physiological groups was reported to produce aHSL, and the involvement of HSL in regulating their development was not demonstrated in earlier studies [1–3, 22].

The actinobacterium *S. avermitilis* is of special interest because its submerged cultures form endospores of the *Bacillus* type that represent an alternative to the exospores forming in a surface culture [23].

We chose unacylated HSL because its molecule is the common structural component (aHSL) of quorum sensing (QS) factors in a number of gram-negative bacteria. These factors vary in terms of the length and structure of the acyl radical [1–3], which provides for their partial species specificity [2, 3]. A mechanism counteracting the aHSL-mediated quorum sensing system was described. In *Bacillus* sp. 240B1, an AiiA-acyl-homoserine lactonase enzyme was detected that cleaves the lactone ring in the aHSL molecule, yielding acylhomoserine that is not a quorum-sensing regulator [24]. Another enzyme, the AiiD deacylase synthesized by the bacterium *Ralstonia* sp., breaks down aHSL with the formation of unsubstituted HSL and fatty acids. The expression of the AiiD-encoding gene in *Pseudomonas aeruginosa* cells resulted in the inactivation of the quorum-sensing motility induction system and in discontinuation of virulence factor synthesis [25]. Presumably, other microorganisms also contain this deacylase [25]. The assumption that unacylated HSL can be formed from acylated precursors provided the rationale for this work. For comparison, we also used hexylresorcinol, a representative of another group of autoregulators (AHB) synthesized by a wide range of microorganisms [11–14] and plants [26].

The regulatory action of unacylated HSL was assessed using the test systems developed earlier for the investigation of the biological functions of AHB [11–14]. In this work, we established a non-species-specific, concentration-dependent effect of HSL that is involved in down-regulating the increase in cell numbers during the cultivation of bacteria of various taxa. For most cases, we proved that growth inhibition was not the result of HSL toxicity. The toxic effect of HSL was previously demonstrated for a wild-type *E. coli* strain (the minimum toxic concentration was 4.2×10^{-3} M) [27] and for *Arthrobacter* VAI-A (the minimum toxic concentration was over 10^{-3} M) grown on a synthetic medium with HSL as the nitrogen source [28]. Evidence of a regulatory activity of unacylated HSL was provided, apart from the cessation of growth of bacterial cultures, by earlier transcription steps related to the stationary phase, i.e., activation of the spore formation program in bacilli and of pigment synthesis in *Azotobacter.* Importantly, the efficient concentrations (10^{-5}–10^{-3} M) of unacylated HSL used in our studies exceeded those of aHSL operating as species-specific signal factors of QS systems. For instance, the concentrations of the *V. fischeri* bioluminescence autoinducer were 1–10 μg/ml, i. e., 4×10^{-6}– 4×10^{-5} M [2]. Therefore, the intra- and interspecies growth-limiting and stage-transition signals operate in different concentration ranges. Another important conclusion to draw is that the HSL effect varies depending on the recipient's status, as is indicated by the reliable data obtained with *S. avermitilis.* In this system, the regulatory effect was produced by HSL added during the lag phase, not at the later developmental stages. Hence we revealed for the first time an analogy between HSL and the A factor (a γ-butyrolactone derivative) that exhibited activity only when added to the streptomycete test culture in combination with the inoculum (spores) [7].

Our data on the HSL involvement in initiating the stationary phase of bacterial cultures indirectly support the hypothesis [8] that unacylated homoserine lactone is synthesized by the cells of *E. coli*, an organism lacking an aHSL-based QS system; in this case it functions as an intracellular starvation signal. After reaching the threshold concentration, unacylated HSL activates the expression of a specific sigma factor (σ^S), an RNA polymerase subunit. It is responsible for the transcription of stationary phase genes that are involved in the realization of the stress response and stationary phase (cell differentiation) genetic programs. However, the evidence discussed in detail in [10] contradicts this hypothesis. One piece of evidence is that neither exogenous HSL (up to a concentration of 10^{-3} M) nor its acylated derivatives (the autoinducers of *V. fisheri* and *V. harveyi*) influenced the expression of the *rpoS* gene in *E. coli* cells [29]. Apparently, the transition of microbial cultures to the stationary phase is mediated by a complex mechanism involving multiple intracellular HSL targets and alternative regulators. For example, σ^S expression in *E. coli* is regulated by homocysteine lactone [30], cAMP, UDP-glucose, and guanosine tetraphosphate [8–10]. Unacylated HSL probably interacts non-specifically with proteins, including those involved in transcription regulation. This suggestion is based on the data that HSL can form complexes with enzyme proteins [31]. Nonspecific interactions of AHB with cell biopolymers (enzyme proteins and DNA) are sufficiently well characterized [14–17, 26, 31, 32].

The pleiotropic effects of HSL and AHB manifest themselves in their impact on the ring-shaped growth pattern of a *S. avermitilis* surface culture. The data obtained can be explained as follows. Because the HSL/AHB concentration decreases with increasing distance from the point of the local HSL/AHB introduction, the inhibition zone caused by the effects of toxic HSL/AHB concentrations is changed by a growth zone (Fig. 3). In the growth zone, a sufficient (effective) HSL/AHB concentration causes the transition of the streptomycete to the stationary phase. This results in switching on the genes responsible for the biosynthesis of the factors controlling cell differentiation, including the growth of the aerial mycelium and probably the synthesis of a spore-formation-inducing extracellular regulator [33]. Its diffusion toward the disc and away from it should enhance the growth-inhibiting effects of HSL and AHB in the preceding and succeeding inhibition zones. The alternation of ring-shaped inhibition and growth zones results from the superposition of the effect of the decreasing HSL/AHB concentrations and of the influence of an endogenous regulator. The fact

that no ring-shaped growth pattern occurred with *S. avermitilis* without HSL or AHB raises the possibility that their interaction with the endogenous regulator has a cooperative effect. The inhibition of exospore formation in the presence of AHB, in contrast to HSL, may be due to a change in the cell differentiation program of the streptomycete. The regulatory effect of AHB on intragenomic alterations (demonstrated by us using the Ames test) and on the structural organization of the DNA was revealed by us earlier [17, 32, 34]. It is still an open question why the partial (not complete) repression of spore formation in *B. cereus* when grown on dedicated media is removed by HSL. It seems likely that HSL influences the phenotypic dissociation of *Bacillus* cultures, resulting in segregation of dissociants (similar to the AHB effect [32, 34]) whose cultures possess distinct properties including resistance to glucose repression [35]. Further studies are required to prove that HSL plays a functional role in intraspecies variability at the population level.

The point to re-emphasize is that the HSL effect is non-species-specific; this finding may be of relevance to the issue of regulation of the activities of microbial communities. The extracellular pool of HSL can build up as a result of aHSL destruction by AiiD deacylases [25] produced by a large number of microorganisms. In addition, unacylated HSL that accumulates in starving cells of *E. coli* [8] and other bacteria can be excreted via transmembrane proteins, RhtB [27]. The resulting cumulative signal signifies starvation or an increase in cell density to the threshold level. In response to extracellular HSL accumulation, some components of a microbial community that occupy specific microzones may synchronize their behaviors by stopping cell division and assuming the state of proliferative (in the stationary phase) or metabolic dormancy (individual dormant cells). This suggestion implies an interplay of regulatory networks involving QS mechanisms and "starvation-sensing" and other stress response systems both at the intraspecies [36] and interspecies levels. Importantly, both intra- and interspecies communication in bacteria also implicate the operation of another type of density-sensing autoregulators (AHB) that was detected in various microorganisms [11–14]. These are characterized by nonspecific effects in a wide variety of species. The mechanism of action of AHB is based on changing the structural state and, accordingly, the functional activities of biopolymers (proteins and DNA) and of supramolecular structures (membranes) [15–18]. Possibly, the similarity between the effects of HSL and AHB is due to the presence of a cyclic group in their molecular structure. The data obtained contribute to our knowledge of the role of small-size molecules in initiating stationary phase-related processes not only in the producer cultures investigated, but also in other gram-positive and gram-negative bacteria. The non-species-specific effects of HSL and AHB demonstrated by us raise the possibility that they perform regulatory functions on the microbial community level.

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