ISSN 0003-6838, Applied Biochemistry and Microbiology, 2014, Vol. 50, No. 4, pp. 353–358. © Pleiades Publishing, Inc., 2014.
Original Russian Text © D.G. Deryabin, A.A. Kamayeva, A.A. Tolmacheva, G.I. El-Registan, 2014, published in Prikladnaya Biokhimiya i Mikrobiologiya, 2014, Vol. 50, No. 4, pp. 391–397.

The Effects of Alkylhydroxybenzenes on Homoserine Lactone-Induced Manifestations of Quorum Sensing in Bacteria

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Received September 16, 2013

Abstract—The effects of four alkylhydroxybenzene (AHB) homologs with different hydrocarbon chain lengths on the synthesis of violacein pigment induced by C_6-homoserine lactone (HSL) and biofilm formation by *Chromobacterium violaceum* NCTC 13274 and on *Escherichia coli* pAL103 bioluminescence in the presence of C_6-oxo-HSL were studied. Alkylhydroxybenzenes inhibit the growth of *C. violaceum* increased in the C_5-AHB → C_{12}-AHB series in the absence of this activity in C_1-AHB. Subinhibitory AHB concentrations reduced violacein production and suppressed biofilm formation. These effects were presented as individual and group regression dependencies between the analysed parameters. Using the bioluminescent model, the regulatory effects of AHBs were not associated with their direct competition with HSL and that they develop as a result of changes in the sensitivity of bacterial cells to the respective quorum sensing inducer.

DOI: 10.1134/S0003683814040036

Small molecules play an important role in intra- and interspecies communication in microorganisms [1]. One striking example is the collective population density-dependent behaviour of bacteria, which is termed ~~as~~ "quorum sensing" (QS) and realized through the biosynthesis and perception of various homoserine lactones (HSL) [2]. The LuxR/I-type QS system was the first to be described and is the most widespread in the world of prokaryotes where the *luxI* gene encodes for HSL synthase and the *luxR* gene encodes for the HSL-receptor protein [3]. At the same time, the general sequence of events includes the accumulation of an autoinducer in the cultivation medium, its interaction with the receptor protein, and the binding of the resultant LuxR-HSL complex with target gene promoters [4]. Bioluminescence enzymes [5], the synthesis of pigments [6] and antibiotics [7], as well as the formation of a wide range of virulence factors [8] are under such control in more than 450 bacterial species; hence, the quorum sensing system can be considered as target for novel agents development for the suppression of bacterial infections [9].

The HSL formation and recognition can estimate the minimum cell population density sufficient for the expression of stationary-phase genes, while other small molecules control the maximum possible cell number in culture. In particular, such activity is inherent in a peculiar group of phenol lipids, the alkylhydroxybenzenes (AHBs) [10], designated in the literature as resorcinol lipids or alkylresorcinols [11]. In contrast to HSL, AHBs are characterised by multitarget interaction with membrane structures [12], proteins [13] and nucleic acids [14], which results in the multiplicity of their regulatory effects. At the same time, the most integral manifestation of AHB biological activity is growth cessation upon reaching the maximum number of cells in culture and the formation of hypometabolic and dormant forms upon a further increase of concentration [15]. The consequence of this mechanism is the ability of AHBs to inhibit ~~the~~ growth of a wide range of pathogenic and opportunistic bacteria [16], suggesting the possibility of using this group of small molecules for the suppression of bacterial infections.

Investigation of the effect of AHBs on HSL-induced collective behaviour of microorganisms is of great significance, as it creates prospects for using the natural mechanisms of cell-to-cell communication for controlling the quorum sensing system of bacterial pathogens.

The aim of this work was to study the effects of four AHB homologs with different hydrocarbon chain lengths on the C_6-HSL-induced synthesis of violacein pigment and biofilm formation by *Chromobacterium violaceum* NCTC 13274, as well as bioluminescence of *Escherichia coli* pAL103 in the presence of C_6-oxo-HSL.

METHODS

HSL and AHB chemical analogs (≥99% purity) were used as small regulatory molecules for cell-to-cell communication in bacteria (Fig. 1). N-hexanoyl-

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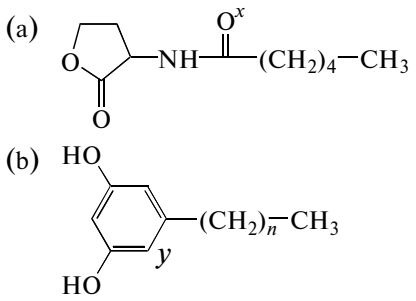

Fig. 1. The structural formulas of HSL (a) and AHBs (b), where $n = 0, 4, 5,$ or 11; C_6-oxo-HSL ~~comprises~~ an additional oxo-group (x); the hydrocarbon radical in the C_6-AHB molecule is in position 4 (y).

L-homoserinelactone (C_6-HSL) (Cayman Chemical, USA) and N-(oxo)-hexanoyl-L-homoserinelactone (C_6-oxo-HSL) (Sigma, USA) were used as quorum sensing inducers. The AHB homologs 1,3-dioxy-5-methylbenzene (C_1-AHB), 1,3-dihydroxy-5-pentylbenzene (C_5-AHB) and 1,3-dioxy-4-hexylbenzene (C_6-AHB) were obtained from Sigma (USA); and 1,3-dioxy-5-dodecylbenzene (C_{12}-AHB) was obtained from Enamine (Ukraine).

The effects of HSL and AHBs were studied using *C. violaceum* NCTC 13274 (Health Protection Agency, Great Britain) containing an insertion of the mini-Tn5 transposon into the *cviI* gene (the *luxI* analog), responsible for the synthesis of the intrinsic C_6-HSL autoinducer [17] (Table 1). As a result, this microorganism lacks the ability for the independent development of QS-dependent manifestations, but recovered ~~it after~~, the exogenous introduction of HSL with ~~a~~ hydrocarbon chain length of C_4 to C_8 [18].

The second target was *E. coli* pAL103; *E. coli* pAL103; *luxR* + *luxI_luxCDABE*; Tet^r p15A [19]. The characteristic feature of this strain was the presence of the pAL103 plasmid which contained the *luxCDABE* genes responsible for bioluminescence, cloned under the control of the *luxR* gene exogenously-added C_6-oxo-HSL. The sensitivity of this reporter system was enhanced by transferring it into *E. coli* JLD271 (host strain) with a mutation in the *sdiA* gene [20], where its product recognizes HSL and could compete for binding with the LuxR protein (Table 1).

C. violaceum NCTC 13274 was grown in LB broth (AppliChem GmbH, Germany) containing glucose (10 mg/mL) at 27 ± 1°C. The resultant culture (20 μL) was added to 1 mL LB broth containing AHB homologs at 4.5–1000 μM, as well as 0.005 μM C_6-HSL necessary for QS induction. Samples without HSL and AHBs were used as a negative control and samples with HSL (but without AHBs) were used as a positive control.

After 18–24 h of cultivation, growth intensity was assessed by measuring OD_{450} with a Stat Fax 303 6VIS photometer (Awareness Technology Inc., ~~United States~~). The development of QS-dependent reactions was estimated by the formation of violacein pigment in biomass precipitated at 12000 *g* for 5 min, extracted with 96% ethanol and quantified at 575 nm (OD_{575}) after repeated centrifugation [21]. Depigmented and air-dried residual was used in the crystal violet (CV) binding assay for quantitative characterization of the total biofilm biomass [22] in which 200 μL 0.1% aqueous CV was added to the precipitate, incubated for 45 min, washed twice with distilled water, bound CV extracted with 200 μL of 96% ethanol and measured at 540 nm (OD_{540}).

E. coli pAL103 was cultivated for 24 h at 37 ± 1°C on LB agar which contained 12 μg/mL of doxycyclin, and the effect of AHBs on the development of QS-dependent bioluminescence was analyzed in 2 experiments. In the first, the culture was grown in the LB broth for 3 h to $OD_{450} = 0.5$ U and 100 μL transferred into the appropriate wells of 96-well microplates (Microlite 2+ opaque plastic, Thermo, USA) containing AHB homologs (100 μL) at 31.25–500 μM and C_6-oxo-HSL at 0.0125–0.2 μM. In the second, *E. coli* pAL103 was cultivated for 24 h in the presence of various AHB homologs, with consideration of the final OD_{450} value in each sample.

E. coli pAL103 suspension (100 μL) was transferred into 96-well microplates which contained an inducing dose of C_6-oxo-HSL and the luminescence of experimental and control samples recorded dynamically for 60 min (LM 01T bioluminometer, Immunotech, Czech Republic) using the KILIA software for data evaluation and backup. The degree of bioluminescence induction (I) was defined as the ratio between sample luminescence intensity at 60 min and time 0, and relative luminosity values were related to biomass (OD_{450} values).

~~All experiments were repeated no less than 3 times. The results were processed by the methods of analysis of variance using Excel and Origin software and the numerical and graphical methods of descriptive statistics.~~

RESULTS AND DISCUSSION

The AHB effects on HSL-induced QS manifestations in *C. violaceium* NCTC 13274 were analysed. Optical flow (450, 540, and 575 nm) in combination with sequential extraction of stained products provided information about direct antibacterial activity of the compounds, as well as their influence on QS-dependent synthesis of violacein pigment and biofilm formation.

Most of the compounds showed antibacterial activity consisting of the inhibition of *C. violaceum* NCTC 13274 growth that depended on the structural peculiarities of AHBs, primarily the length of the hydrocarbon radical (Table 2). The shortest-chain homolog C_1-AHB did not completely inhibit bacterial growth,

Table 1. Bacterial strains used for the estimation of AHB effects on QS-dependent reactions of bacteria

Strain	Peculiarities of genetic structure	QS inducer	Reporter indicator	Reaction rate	Source
C. violaceum NCTC 13274	Insertion of mini-Tn5 transposon into the *cviI* gene	C_6-HSL	Production of the blue-violet pigment violacein	18–24 h	[6, 18]
E. coli pAL 103	Availability of the *luxR* + *luxI_luxCDABE* gene cassette	C_6-oxo-HSL	Development of bioluminescence	15–60 min	[21]

but decreased OD_{450} to 51.7% compared to the control at the highest concentration tested (1000 μM). C_5-AHB, C_6-AHB and C_{12}-AHB completely inhibited growth of *C. violaceum* NCTC 13274 at ~~the~~ minimum inhibitory concentrations of 500, 250 and 125 μM. Subinhibitory concentrations of long-chain AHB homologs maintained moderate growth-inhibiting activity up to 31.25 μM, where OD_{450} values decreased ~~by~~ 37.9–77.1% compared to control. These data were in good agreement with Nikolayev et al. [16], who demonstrated similar activities for a wide range of natural and chemically-synthesized phenol lipids, which increased at a higher degree of their hydrophobicity and depended on the size and position of hydrocarbon substituents in AHB molecules.

The effects of subinhibitory AHB concentrations on QS-dependent violacein production and biofilm formation were estimated by calculating EC_{50} values (50% reduction compared to the positive control) (Table 2). Violacein synthesis is strictly controlled by the QS system [17] and only induced in the presence of C_6-HSL [18], ~~proved to be~~ most sensitive to AHBs. In the presence of low C_1-AHB activity, the ability to inhibit this pigment substantially increased in the C_5-AHB → C_{12}-AHB series, which was characterized by a decrease in EC_{50} concentrations from 39.05 to 19.52 μM.

Biofilm exopolysaccharide synthesis indirectly controlled by the QS system [22, 23] responds to AHBs in different ways. Biofilm formation was suppressed by C_1-AHB with an EC_{50} = 502 μM. The increased hydrocarbon radical length in AHB molecules resulted in enhancement of biological activity, with EC_{50} values of 46.875, 39.05 and 23.42 μM for C_5-, C_6- and C_{12}-AHB, respectively (Table 2).

The complex influence of AHBs on ~~the~~ growth and QS-dependent reactions in *C. violaceum* NCTC 13274 were the basis for analysing the interrelationships between the above parameters presented as a series of individual and group regression dependences (Fig. 2). The interrelationships between values (OD_{450}), AHBs and the respective values of violacein production (OD_{575}) or biofilm formation (OD_{540}) could be most correctly described by exponential regressions such as $y = a\exp(kx)$, where $x = OD_{450}$, $y = OD_{575}$ or OD_{540}, a and k are the normalizing constants (Fig. 2a,b).

Statistical comparison of these constants with due consideration to standard deviations (σ) and representation errors (m) was evidence of their substantial similarity in a description of the C_5-, C_6- and C_{12}-AHB effects ($P > 0.05$). The above circumstance made it possible to formalize "OD_{450}–OD_{575}" and "OD_{450}–OD_{540}" using the common coefficients $a_{(C5, C6, C12)}$ and $k_{(C5, C6, C12)}$ (0.0334 and 2.4192, 0.0868 and 0.9097, respectively), which were used to characterize the biological activity of the compounds. In their turn, the analogous values for C_1-AHB were 0.4389 and 0.8190, as well as 0.0082 and 3.2864, with statistically significant differences ($P < 0.05$) from both individual and group coefficients in the exponential regressions describing the effects of longer chain AHB homologs.

Correspondence between OD_{575} and OD_{540} values was the basis for the formation of another group of regression dependencies, in this case formalized by linear equations such as $y = ax + b$ (Fig. 2c), where $x = OD_{575}$, $y = OD_{540}$, a and b are the normalizing constants. Comparison of these constants made it possible to form a single regression for long chain AHB homologs, where $a_{(C5, C6, C12)} = 0.2148$ and $b_{(C5, C6, C12)} =$

Table 2. The effects of AHBs on the growth and QS-dependent reactions in *C. violaceum* NCTC 13274

Homologs tested	Minimum inhibitory concentration, μM	EC_{50} values, μM	
		violacein production	biofilm formation
C_1-AHBs	>1000	>1000	502
C_5-AHBs	500	39.05	46.87
C_6-AHBs	250	23.42	39.05
C_{12}-AHBs	125	19.52	23.42

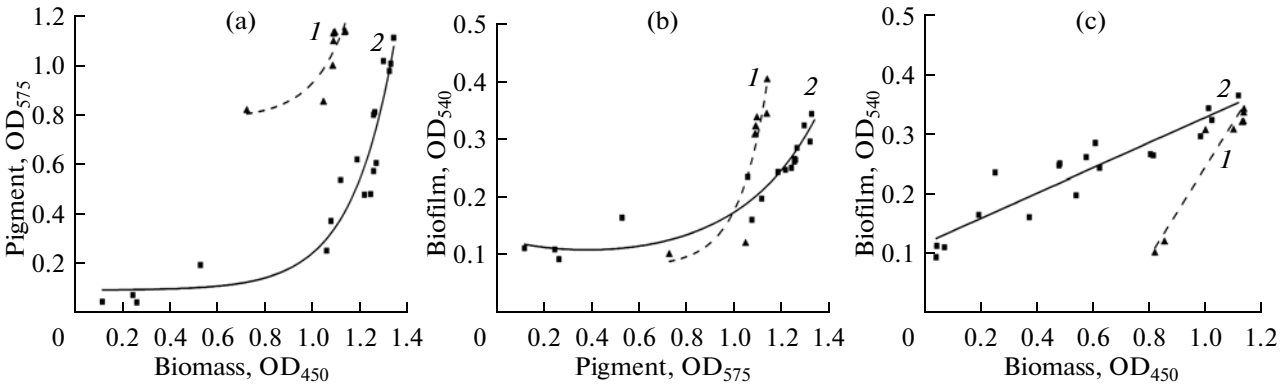

Fig. 2. The interrelationships between AHB effects on the growth of *C. violaceum* NCTC 13274 (OD_{450}), QS-dependent synthesis of violacein pigment (OD_{575}) and biofilm formation (OD_{540}). *1*, individual dependencies for C_1-AHB; *2*, group dependencies for C_5-, C_6 and C_{12}-AHB.

0.1157, with its substantial difference from the individual regression describing the result of biological activity of C_1-AHB. Analysis of plotted regressions showed the existence of two complementary dependencies: the high degree of proportionality between OD_{540} and OD_{575} values as a reflection of the coupled suppression of QS-dependent parameters under the influence of AHB described by these values and, on the other hand, the incomplete identity of their reaction consisting in the maintenance of the basic level of biofilm formation ($OD_{540} = 0.1$) under the complete inhibition of violacein biosynthesis ($OD_{575} = 0$).

AHB homologs inhibited the growth of *C. violaceum* NCTC 13274 at high concentrations and suppressed its QS-dependent manifestations at subinhibitory concentrations. The most common dependencies were the enhancement of these activities, along with an increase in the size of nonpolar hydrocarbon radical in AHB molecules, the nonlinear combined variation of bacterial biomass density and QS-dependent parameters, and a differently expressed sensitivity to violacein and biofilm formation under the influence of AHBs. However, the low rate of these reactions in *C. violaceum* NCTC 13274 gave no information about the potential interference mechanisms between AHBs and HSL, which was a basis for further studies with the sensitive luminescent strain *E. coli* pAL 103.

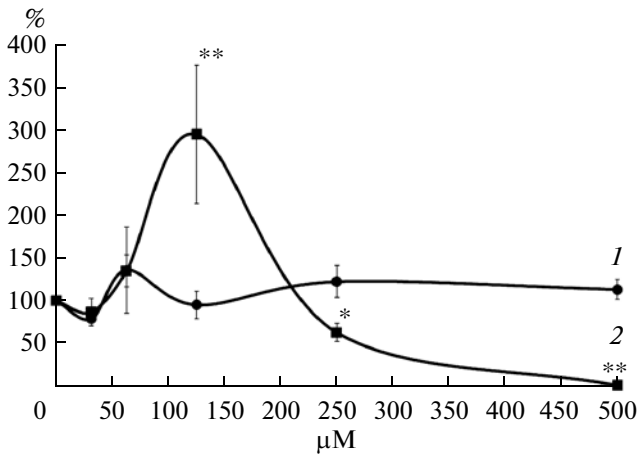

Fig. 3. The effects of different C_6-AHB concentrations (μM) on the intensity of QS-dependent bioluminescence of *E. coli* pAL103; *luxR* + *luxI_luxCDABE* (% of the control) during simultaneous introduction with C_6-oxo-HSL (*1*) or under preliminary influence on bacterial cells followed by C_6-oxo-HSL induction (*2*). * $P < 0.05$; ** $P < 0.01$.

The development of QS-dependent bioluminescence in the given reporter system was assessed in two experiments presupposing the possibility of direct competition between AHBs and HSL for binding with the LuxR protein, as well as the influence of AHBs on the sensitivity of microorganisms to subsequent HSL induction.

In the first experiments, the structurally similar molecules ~~of~~ C_6-AHB and C_6-oxo-HSL were used at a wide range of effective concentrations. Analysis of the bioluminescent response kinetics of *E. coli* pAL 103 in the presence of C_6-oxo-HSL at concentrations of 0.0125 to 0.2 μM confirmed a dose-dependence induction of luminescence, with increases of 17- to 505-fold compared to the respective control. The simultaneous effects of C_6-AHB and C_6-oxo-HSL did not reliably affect of *E. coli* pAL 103 with C_6-AHB substantially changed the character of bioluminescence response during subsequent C_6-oxo-HSL induction (Fig. 3). High C_6-AHB concentrations (500 and 250 μM) significantly reduced QS-dependent bioluminescence intensity to 1% ($P < 0.01$) and 63% ($P < 0.05$) of the respective controls, which corresponded to EC_{50} = 343 μM in the given indicator system. Decreasing C_6-AHB concentration to 125 μM was accompanied by a relative increase in bioluminescence intensity, with its maximum exceeding the control values 2.5- to 4.5-fold. Addition, expression showed a dependence on the inducing C_6-oxo-HSL concentration: the combined effects of luminescence

Table 3. The effects of AHBs on QS-dependent bioluminescence of *E. coli* pAL103; *luxR* + *luxI_luxCDABE*

Homologs tested	EC_{50} values, μM	
	under simultaneous influence of AHBs and C_6-oxo-HSL	under preliminary influence of AHBs followed by C_6-oxo-HSL induction
C_1-AHBs	>500	>500
C_5-AHBs	>500	>500
C_6-AHBs	>500	343
C_{12}-AHBs	>500	225

suppression/stimulation were more marked at ~~the range of~~ low concentrations, 0.0125–0.05 μM, varying slightly when the inducer dose was increased to 0.1–0.2 μM.

The second series of experiments with the optimal inducing C_6-oxo-HSL concentration (0.05 μM) included comparative analysis of the regulatory effects of C_1-, C_5-, C_6- and C_{12}-AHB (Table 3). Simultaneous use of AHBs and HSL did not change development of QS-dependent bioluminescence, while pretreatment of *E. coli* pAL 103 with AHBs substantially influenced the level of bioluminescence during subsequent HSL induction. The intensity of such an effect again showed the dependence on the peculiarities of the influencing AHB molecules' chemical structure, which consisted in the absence of reliable effect on quorum sensing development in C_1-AHB and C_5-AHB, while EC_{50} values characterizing 50% luminance suppression of the control were 343 μM and 225 μM for C_6-AHB and C_{12}-AHB, respectively. This indicated the *E. coli* pAL 103 quorum sensing system was less sensitive to AHB, though it was fundamentally similar to *C. violaceum* NCTC 13274 (see above). This indicator system ~~has~~ demonstrated ~~that~~ the inhibitory effect of AHBs on QS-dependent manifestations was not due to direct competition with HSL for binding with the receptor protein LuxR, but due to variation in bacterial cells sensitivity to the influence of the respective autoinducer as a consequence of multitarget interaction between AHBs and ~~the~~ wide range of molecular targets [12–14].

This study indicated AHBs at subinhibitory concentrations suppressed the quorum sensing system in bacteria through variation in their sensitivity to HSL induction. The findings broaden the spectrum of phenol compounds represented by molecules with one aromatic ring: eugenol (4-allyl-2-methoxyphenol) [23] and vanillin (4-hydroxy-3-methoxybenzaldehyde) [24], as well as the polyphenol isomers vescalagin and castalagin [25], which were previously shown to inhibit QS-dependent reactions. At the same time, AHBs, as well as the compounds above, are typical plant products, so that mono- and polyphenols can be characterized as important components of phytoimmunity, acting not only via direct inhibition of the growth of bacterial pathogens but also via inhibition of the mechanisms of their collective behavior with replacement of the virulent phenotypes by avirulent ones [26].

These effects also demonstrate the possibility of functional interference between different groups of small regulatory molecules of bacterial origin, previously shown for homoserine lactones with different hydrocarbon chain length [6] and for the first time for AHBs and HSL. This also suggests the existence of analogous interactions in natural microbiocoenoses, where the development of HSL-producing cultures can be disturbed by the presence of the dormant part of the bacterial population, where the hypometabolic state was determined by the formation and accumulation of a pool of extracellular AHBs.

This study evaluated the possibility of using AHB homologs as QS-suppressing agents for biotechnology and medical/veterinary purposes. In particular, the results of this study indicated the potential for development of a new generation of antimicrobials with combined abilities to suppressing the growth and quorum sensing system of bacterial pathogens ~~on the~~ basis upon natural and chemically synthesized AHBs.

ACKNOWLEDGMENTS

The studies were supported by the Russian Foundation for Basic Research (projects no. 13-04-97044 and no. 13-04-01145) and the Federal Task of the Ministry of Education and Sciences of the Russian Federation (project no. 148).

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Translated by E. Makeeva

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