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EXPERIMENTAL ARTICLES

The Effect of Anabiosis Autoinducers on the Bacterial Genome

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Abstract—The mutagenic activity of chemical analogues of microbial anabiosis autoinducers (the autoregulatory d_1 factors of cell differentiation), which act to inhibit cell proliferation, to enhance cell tolerance, and to induce the transition of cells to an anabiotic state, was studied using the Ames test. In the range of concentrations studied (0.1 to 100 μg/ml), alkyl-substituted hydroxybenzenes (AHBs) differing in hydrophobicity, i.e., methylresorcinol (C_1-AHB) and hexylresorcinol (C_6-AHB), as well as unsubstituted resorcinol, showed different growth-inhibiting and mutagenic effects. C_6-AHB was found to inhibit the growth of *Salmonella typhimurium* TA100 and to induce its mutagenesis at a rate of 1.8 revertants/nmol. C_1-AHB taken at low concentrations not only failed to inhibit bacterial growth but even stimulated it and exerted an antimutagenic effect. Unsubstituted resorcinol virtually did not influence bacterial growth and showed weak mutagenic activity. The growth-inhibiting effect of elevated C_6-AHB concentrations correlated with the degree of the transition of the original phenotype producing S-type colonies to a phenotype producing R-type colonies. The role of AHB homologues, as microbial autoregulators with mutagenic activity, in the regulation and correlation of two processes: the phenotypic dissociation of microbial populations and the formation of resting microbial forms, is discussed. The accumulation of AHBs in senescent microbial cultures may induce adaptive mutations, change the expression of genes, and promote the development of minor cell subpopulations (phenotypes), thus providing for the adaptation of these cultures to varying environmental conditions.

Key words: alkyl hydroxybenzenes, growth-inhibiting activity, mutagen, genome, Ames test, phenotypic dissociation, *Salmonella.*

The dissociation of microbial populations into phenotypically different variants (clones, subpopulations) is a well-known mechanism which promotes microbial adaptation to varying environmental conditions and enhances species stabilization and conservation [1, 2]. The dissociation is due to different types of spontaneous intragenomic recombinant rearrangements known as adaptive mutations [3]. Molecular mechanisms responsible for phenotypic stability (or, conversely, variability) of microorganisms have yet to be appropriately studied. Our interest in this problem comes from our observation of a correlation between the induction of the development of minor subpopulations (or colonial morphotypes) in *Bacillus cereus* and the level of extracellular anabiosis autoinducers (or d_1 factors), which control the formation of resting microbial forms [4].

Our earlier studies posed the question of whether the frequency of adaptive mutations in bacteria increases when cells occur in the state of anabiosis or adaptive mutations are generated upon the germination of resting forms during the first stage of DNA replication. There is increasing evidence that mutations may take place in nondividing, proliferatively resting, and probably anabiotic cells of bacteria and animals. Mutagenesis may be due to different mechanisms. The mutagenesis caused by endogenous mutagens may give rise to mutant RNA transcripts. If such transcripts initiate the replication of DNA, DNA lesions will transform into adaptive mutations. Another mechanism lies in errors of polymerases during cryptic DNA synthesis, which, in nondividing cells, may lead to a more considerable renewal of DNA than its mere duplication [5]. With this mechanism, the cryptic synthesis of DNA is considered to be a source of variable genetic material.

Plant di- and trihydroxyalkylbenzenes were found to cause Cu(II)-dependent DNA cleavage, which intensifies with the length of the alkyl group of alkyl hydroxybenzenes (AHBs). The cleavage of DNA by 5-alkylresorcinols (a type of AHBs) begins with the oxidation of the benzene ring of AHBs, which requires alkaline conditions and the presence of Cu^{2+} and oxygen. The modified AHBs cleave DNA in a reaction involving the reduction of Cu^{2+} ions by the hydroxybenzene moieties of AHBs with the formation of active oxygen species. Catalase acts to prevent DNA breaking [6].

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Experiments with liposomes showed that alkylresorcinols possess antioxidant and, therefore, antimutagenic activities, whose values directly depend on the length of the alkyl group [7]. The hydroxyl group of the benzene ring serves as a primary donor of hydrogen after the formation of a free radical. The carbon-containing side chain of the benzene ring may also contribute to the antioxidant activity of alkylresorcinols. The formation of a radical at the hydroxyl oxygen induces changes in the electron density on the entire molecule. This destabilizes the entire system and results in the oxidation of the benzene ring [7].

Therefore, the ability of alkylresorcinols to provide hydroxyl hydrogen for interaction with free radicals may either slow down free radical reactions due to a redistribution of electron density over the entire molecule or activate these reactions due to copper binding to the oxygen atom of the hydroxyl group of alkylresorcinols.

The accumulation of anabiosis autoinducers (in some bacteria and yeasts, these are AHBs, particularly alkylresorcinols [8, 9]) in resting microbial forms [10, 11], whose germination leads to the phenotypic dissociation of microbial populations, suggests that anabiosis autoinducers may be mutagenic. To study the effect of the hydrophobicity of AHB homologues on the mutagenesis of DNA, we used chemical analogues of microbial growth autoregulators (AHBs with different alkyl groups and unsubstituted resorcinol), since natural autoinducers of anabiosis represent undefined mixtures of AHB isomers and homologues [8, 11].

The aim of the present work was to investigate possible alterations in the bacterial genome induced by elevated concentrations of chemical analogues of microbial anabiosis autoinducers during the transition of bacteria to a proliferatively resting state.

MATERIALS AND METHODS

Two chemical analogues of the d_1 autoregulatory factors of bacteria, hexylresorcinol or C_6-alkyl hydroxybenzene (C_6-AHB, M_r = 196) and methylresorcinol or C_1-alkyl hydroxybenzene (C_1-AHB, M_r = 124), as well as unsubstituted resorcinol (M_r = 112), were used in the form of solutions in dimethylsulfoxide (DMSO). These substances were added in amounts such that the concentration of DMSO in the medium did not exceed 3%. The control reaction mixtures contained equivalent amounts of DMSO. C_1-AHB and C_6-AHB had a purity of 99.9%. Commercial high-purity resorcinol was purchased from Khimreaktiv (Russia).

The growth-inhibiting activity of resorcinols was evaluated using a test strain grown either in liquid (nutrient broth) or solid (nutrient agar) medium. The test strain *Salmonella typhimurium* TA100 (*hisG46, rfa*, Δ*uvrB, pKM101*) was obtained from the Research Institute for the Biological Testing of Chemicals, Kupavna, Russia. The strain allowed frameshift and base pair substitution mutations to be detected. In experimental variant I, 0.1-ml aliquots of solutions containing 0.03, 0.3, and 3 mg/ml resorcinols were mixed with 2.9 ml of 0.8% soft agar containing an exponential-phase *S. typhimurium* culture, and the mixtures were poured onto layers of hardened 2% nutrient agar in petri dishes. Experimental variant II was similar to experimental variant I, except that liquid test cultures grown in nutrient broth in the presence of the aforementioned concentrations of resorcinols were plated onto 2% nutrient agar. Bacterial growth was expressed in the number of colony-forming units (CFU) per ml of cell suspension.

Table 1. Effect of alkyl resorcinols on the growth of *S. typhimurium* TA 100 on nutrient agar

Compound, μg/ml	Number of viable cells, CFU × 10^6/ml
Control	409 ± 17 (100)
Hexylresorcinol	
100	0*
10	12 ± 3 (2.9)*
1	184 ± 13 (45.0)*
Methylresorcinol	
100	330 ± 5 (80.7)*
10	435 ± 15 (106.3)*
1	392 ± 17 (95.8)*

Note: Parenthesized is the percentage of viable cells with respect to the control.
*The difference between the experiment and control is statistically significant with $P < 0.001$.

The genotoxic activity of AHBs was assayed by the Ames mutagenicity test without metabolic activation [12]. A substance was considered to be mutagenic if the frequency of revertants induced by this substance exceeded at least twofold the frequency of spontaneous revertants. The mutagenicity of the substance was calculated as the ratio of the number of revertants to the concentration of this substance within a straight-line portion of the respective experimental curve. In these experiments, the widely used mutagen *N*-nitroso-*N'*-methylurea (Sigma) at a concentration of 10 μg/plate served as the positive mutagenicity control.

The results were statistically processed in terms of the Student's *t*-test using the Microsoft Excel 2000 program. The *t*-statistic was calculated for significance level $P < 0.05$.

RESULTS

The d_1 factors of microorganisms (in some bacteria and yeasts, these are AHBs) perform the functions of endogenous growth inhibitors (or population density factors). These factors limit the growth of microbial populations at threshold concentrations and induce the transition of microbial cells to the anabiotic state at ele-

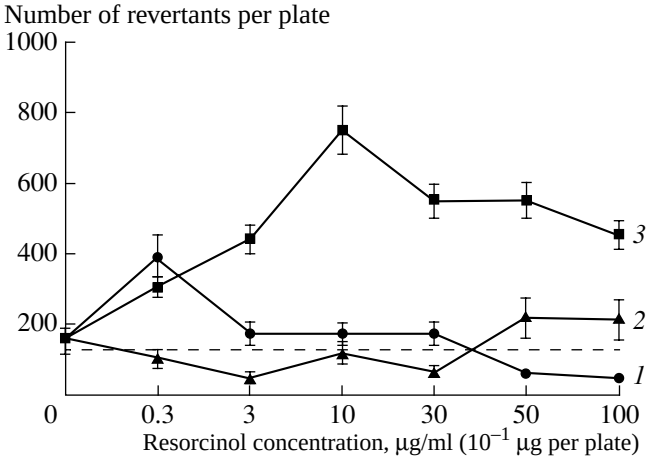
Table 2. Effect of unsubstituted and alkyl-substituted resorcinols on the proliferative activity of *S. typhimurium* TA100 grown in nutrient broth

Compound, μg/ml	Number of viable cells, CFU × 10^6/ml	Number of R-type colonies, × 10^6/ml
Control	1196 ± 43 (100)	32 ± 4 (2.7)
Hexylresorcinol		
50	35 ± 5 (2.6)*	27 ± 4 (85.7)
5	33 ± 3 (2.7)*	6 ± 1 (18.5)
Methylresorcinol		
50	1120 ± 19 (99.1)	47 ± 6 (4.2)
5	1268 ± 28 (106.0)	26 ± 5 (2.1)
Resorcinol		
50	920 ± 22 (76.9)	2 ± 1 (0.2)
5	992 ± 37 (82.9)	5 ± 1 (0.5)

Note: Parenthesized is the percentage of viable cells with respect to the control (column 2) or the percentage of R-type colonies with respect to the total number of colonies in a given experiment (column 3).

*The difference between the experiment and control is statistically significant with $P < 0.01$.

vated concentrations [8–11]. With the mutagenicity test of Maron and Ames, the mutagenic effect of tested substances on a microbial culture cannot be correctly evaluated if they inhibit the colony-forming ability of the culture by more than 3% [12]. In the light of this, to correctly evaluate the possible mutagenic effect of the d_1 factor analogues on the genome of *S. typhimurium* TA 100 by the Maron–Ames procedure, it was necessary to determine the threshold concentrations of C_1-AHB and C_6-AHB, above which these substances would inhibit the proliferative activity of the test culture. Analysis of the colony-forming ability of *S. typhimurium* TA 100 grown on nutrient agar showed that C_6-AHB exerted a considerably higher inhibitory effect than C_1-AHB (Table 1). Actually, the mutagenic effect of C_1-AHB could be investigated at any concentration of this substance, whereas that of C_6-AHB could be studied only at concentrations lower than 10 μg/ml (ca. ~5×10^{-6} M).



Induction of revertants by (*1*) hexylresorcinol, (*2*) methylresorcinol, and (*3*) unsubstituted resorcinol in the Ames mutagenicity test. The dashed line shows the frequency of spontaneous revertants.

In another set of experiments, the growth-inhibiting activity of C_1-AHB, C_2-AHB, and unsubstituted resorcinol was studied by growing the test culture in nutrient broth in the presence of different concentrations of the resorcinols, which were added at the time of inoculation. After 5 h of growth, the number of proliferatively active cells was determined by plating culture aliquots onto nutrient agar. As can be seen from the results presented in Table 2, only about 3% of *S. typhimurium* TA 100 cells grown in the presence of 5 and 50 μg/ml C_6-AHB remained proliferatively active as compared with the control. At the same time, C_1-AHB and unsubstituted resorcinol added at the concentrations mentioned failed to inhibit the growth of the test culture in nutrient broth.

Noteworthy is the fact that elevated concentrations of AHBs, especially C_6-AHB, exhibited enhanced growth-inhibiting effects and induced the transition of the original cell phenotype producing smooth, slimy, translucent, round colonies of the S type to a phenotype giving rise to rough, dull, dry colonies of the R type (Table 2). Almost all colonies produced by bacterial cells grown at a high C_6-AHB concentration (50 μg/ml) were of the R type. The effect of C_6-AHB on the population variability of *S. typhimurium* is of particular interest, since the toxicogenicity of this bacterium is associated with its variability. The R-type salmonella phenotype is avirulent and, as a rule, cannot be isolated from clinical sources [13].

The Ames mutagenicity test showed that C_6-AHB and unsubstituted resorcinol possess mutagenic activity (see figure), which was detected as a twofold or higher excess of the number of induced histidine revertants over the number of spontaneous histidine revertants. At concentrations above 3 μg/ml, the high growth-inhibit-

ing activity of C_6-AHB masked its mutagenic effect, whereas resorcinol, whose inhibitory activity is low, exhibited a distinct mutagenic effect at all the concentrations studied. Earlier, the mutagenic effect of resorcinol was described with respect to the induction of chromosomal aberrations in plant cells and of daughter chromatid exchange in the marrow cells of rats in vivo [14]. C_1-AHB did not influence the growth of the test culture and exhibited an antimutagenic, rather than mutagenic, activity. These data are in agreement with the observations of Pashin *et al.*, who showed that 5-methylresorcinol diminishes the mutagenic effect of benzo[a]pyrene and γ-radiation on the golden hamster V-79 cells and murine marrow reticulocytes [15]. Unlike the mutagenic activity of the known mutagens β-naphthylamine, nitropyrene, and aflatoxin B1, which induce 8.5, 453, and 1057 revertants/nmol, respectively [16], the mutagenic activity of C_6-AHB and resorcinol was low (1.8 and 0.7 revertants/nmol, respectively).

DISCUSSION

Analysis by the Ames test procedure [12] showed that alkyl-substituted hydroxybenzenes, the primary components of the autoregulatory d_1 factors of bacteria, may induce frameshift and base pair substitution mutations in a manner dependent on their concentration and the hydrophobicity of their molecules. Among the three resorcinols studied, C_6-AHB possesses the highest mutagenic activity, although it is considerably lower than the mutagenic activity of the commonly used mutagens. As is evident from the chemical structure of C_6-AHB, it may act as both intercalating and alkylating agent. At concentrations higher than 3 μg/ml, the mutagenic activity of C_6-AHB evaluated by the Ames mutagenicity test was masked by its growth-inhibiting activity. Alternatively, C_6-AHB self-mitigated its own mutagenic effect. Earlier, the protective activity of C_6-AHB was shown for the *ras*-transformed murine fibroblasts exposed to the genotoxic concentrations of RNase or 5% ethanol [17]. These data agree with the observations that some microbial metabolites (their structure was not described) may stabilize and thus metabolically inactivate DNA during cell transition to a resting state [18]. The accumulation of autoregulatory substances in a nutritionally starved culture was found to reduce the frequency of spontaneous revertants as the population density of the histidine auxotroph *S. typhimurium* BA13 increased [19].

C_1-AHB, which is less hydrophobic than C_6-AHB, did not exhibit mutagenic activity within the entire range of concentrations studied. Conversely, unsubstituted resorcinol, which acts only as an intercalating agent, showed weak mutagenic activity. All these data agree with the above physicochemical consideration of the interaction of different AHB homologues with the nucleic acids of cells.

It should be emphasized that the effect of AHBs on the phenotypic stability of salmonella populations is not species-specific. Both natural autoinducers of anabiosis (d_1 factors) and their chemical analogues affect the dissociation of microbial cultures, particularly *Bacillus cereus* [4] and *Staphylococcus aureus* [20]. The effect of AHBs on species metastability is implemented through the segregation of minor cell variants (clones, serotypes, subpopulations), which substitute for the dominant type. Culture dissociation takes place during the germination of resting microbial forms [4], the transition to which is controlled by elevated concentrations of the d_1 factors (AHBs) in developing microbial cultures [10, 11].

The relation between the phenotypic variability of microorganisms and mutations was discussed by many researchers [1–4]. As was emphasized by Prozorov, the population variability of bacteria is related to the recombinant rearrangement of their genome [3]. This rearrangement may be due to specific spontaneous highly reversible mutations occurring at a high rate (10^{-2} to 10^{-4} per one cell generation). The experimental data presented here are indicative of the important role of microbial anabiosis autoinducers (AHBs) in the metastability of species and the conjunction of two processes, the development and maintenance of the resting state and the phenotypic dissociation of microbial populations.

The mutagenic activity of AHBs should be taken into account when discussing the variability of senescent microbial cultures. The gradual increase in the fraction of minor cell variants (phenotypes) in developing microbial cultures with a maximum of proliferatively resting cells in the stationary growth phase was first described by Krasil'nikov for streptomycetes [21] and then by other researchers for some other microbial taxa. The age-related changes in the population variability of *Bacillus cereus* described in our recent work [4] may be due to the accumulation of the d_1 factors (AHBs), which possess both growth-inhibiting and mutagenic activities. In this case, bacterial phenotype may change from the S type to the R type and vice versa. The threshold concentrations of AHBs possessing mutagenic activity may induce adaptive genetic rearrangements (mutations) in senescent microbial cultures and promote the development of a subpopulation of cells that is more adapted to altered environmental conditions. The high frequency of reversion to the original phenotype suggests that adaptive mutations may be hereditarily fixed, which is one of the possible mechanisms responsible for the stabilization and conservation of species within the norm of their responses to varying environmental conditions.

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