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CONFERENCE PROCEEDINGS

A Comparative Study of the Inducing Effect of Homoserine Lactone and Hexylresorcinol on Phenotypic Dissociation in Bacteria

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Abstract—It has been shown that the phenotypic dissociation of *Bacillus subtilis* SK1 and *Salmonella typhimurium* TA100 is induced by hexylresorcinol, an exogenous non-species-specific autoregulator of pleiotropic action, which is genotoxic for both pro- and eukaryotes. Nongenotoxic homoserine lactone, a chemical analogue of cell-density-responsive species-specific regulators, does not induce bacterial dissociation. The phage resistance of the S- and R-type variants of *S. typhimurium* TA100 induced by hexylresorcinol has been found to be the same as that of the S- and R-type salmonella variants obtained by the routine subculturing method.

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The adaptation of microorganisms to varying environmental conditions is determined by their innate and acquired reactions at the subcellular, cellular, and population levels. Exposure to stressful factors may give rise to phenotypically different microbial forms, such as the virulently different S and R dissociants of bacilli, pneumococci, and salmonella. In the course of laboratory subculturing, these bacteria isolated from afflicted patients tend to lose their virulence. This circumstance often leads to an underestimation of the role of these bacteria in the etiology and pathogenesis of animal and human diseases. There is presently no doubt that the phenotypic variability resulting from natural selection is a mechanism of adaptation to new environmental conditions. This idea is supported by the fact that phenotypic metastability in prokaryotes is genetically programmed and plays an important role in their ecology and evolution [1].

Microorganisms possess a specific system responsible for the regulation of their growth and development; this system includes various biochemical mechanisms associated with the accumulation of physiologically active autoregulatory molecules. For example, free unsaturated fatty acids stimulate autolysis. Alkyl hydroxybenzenes serve as inducers of anabiosis [2]. The recently discovered RPF factor in *Micrococcus luteus* accelerates the reactivation of resting bacterial forms [3]. The low-molecular-weight acyl derivatives of homoserine lactone serve as extracellular chemical communicative factors, or cell density autoregulators, which are used as signaling molecules in intraspecies interactions. These substances are produced by gram-negative bacteria and can induce the expression of their own genes (autoinduction) and some stationary- phase genes [4]. Mamson et al. showed that the low-molecular-weight thiolactone peptides produced by some gram-positive bacteria may also be involved in the regulation of cell density [5]. All these autoregulatory factors couple changes in the environment with intracellular reactions and serve as triggering elements of microbial adaptation.

This study was undertaken to evaluate the role of homoserine lactone and hexylresorcinol as triggering molecules in the adaptive reactions of gram-positive (*Bacillus subtilis* SK1) and gram-negative (*Salmonella typhimurium* TA100) bacteria. 4*n*-Hexylresorcinol is a chemical analogue of the bacterial autoregulatory factor d_1. Nonacylated homoserine lactone is a common structural element of bacterial cell-density-responsive regulators [6]. It is active at concentrations from 10 to 1000 μg/ml.

Experiments were performed in three variants. In experimental variant 1, 18-h-old stationary-phase bacterial cells were plated onto agar medium containing homoserine lactone or hexylresorcinol at different concentrations. In experimental variants 2 and 3, homoserine lactone and hexylresorcinol were added to cell suspensions immediately and 1 h before plating, respectively. The plates were incubated at 28°C (*B. subtilis* SK1) or 37°C (*S. typhimurium* TA100) for 3 days, and the percentage of colonies with a nondominant

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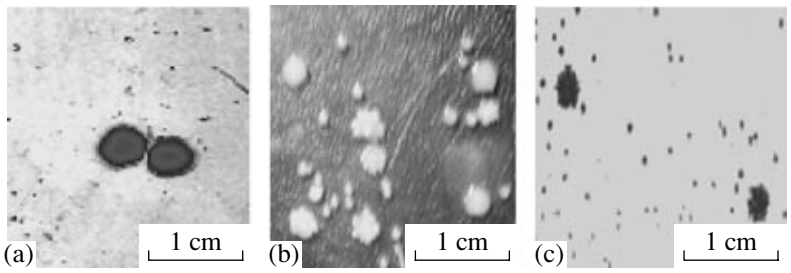

Fig. 1. Morphological changes induced by hexylresorcinol in bacteria: (a) colonies grown on the agar medium from the suspension of *S. typhimurium* TA100 cells to which hexylresorcinol was added at a concentration of 500 μg/ml immediately before plating (S-type colonies are small, R-type colonies are large); (b) colonies grown on the agar medium from *B. subtilis* SK1 cells treated with 500 μg/ml hexylresorcinol for 1 h (there are three colonial morphotypes: small, smooth colonies; large, smooth colonies; and large colonies with uneven edges); (c) colonies grown on the agar medium from *B. subtilis* SK1 cells treated with 100 μg/ml hexylresorcinol for 1 h (there are two morphotypes: small, smooth colonies and large colonies with uneven edges).

phenotype was determined. The data were statistically processed using the Microsoft Excel program.

It has already been shown that hexylresorcinol at concentrations of 10 and 50 μg/ml is not toxic to the bacterium *S. typhimurium* [7]. When added at these concentrations, both hexylresorcinol and homoserine lactone induced the formation of bacterial colonies of an unusual morphology with a rate of 0.1–0.2%, which did not differ from the frequency of spontaneous morphological changes. The bacterium *S. typhimurium* TA100 produced two colonial morphotypes. The dominant morphotype (S variant) produced small, round, smooth, dull colonies, which are typical of the normal growth of this strain. The other morphotype appeared as large, rough, yellowish, crater-shaped colonies typical of R variant (Figure 1a). At concentrations lower than 100 μg/ml, hexylresorcinol did not induce the transformation of S variant to R variant. When hexylresorcinol was added to bacterial suspensions at concentrations of 100 μg/ml or higher, it exerted a more toxic effect than when added to the agar medium. The preincubation of bacterial cells with hexylresorcinol for 1 h enhanced its toxic effect (see table). At toxic hexylresorcinol concentrations, the S-type colonies could not grow, whereas the number of R-type colonies increased. This observation suggests that the latter colonies are more resistant to unfavorable conditions.

The minimal concentration of hexylresorcinol that induced atypical colonial morphotypes of *B. subtilis* was 50 μg/ml (table). At this hexylresorcinol concentration, the 1-h preincubation augmented the number of nondominant morphotypes. However, at higher hexylresorcinol concentrations, the preincubation diminished the number of nondominant morphotypes. At a

Relative number of the S and R morphotypes of *S. typhimurium* TA100 and *B. subtilis* SK1 grown on agar plates at different methods of cell treatment with hexylresorcinol

Hexylresorcinol concentration, μg/ml	Hexylresorcinol was added to agar medium	Hexylresorcinol was added to cell suspension used for plating	The same, but cells were plated after 1-h preincubation
The ratio of the number of S- and R-type colonies of *S. typhimurium*			
Control (0)	70 ± 5/0 (+1)	78 ± 6/0 (+1)	58 ± 7/0 (+1)
100	73 ± 7/5 ± 2	21 ± 3/3 ± 1	4 ± 1/1 ± 1
500	0/5 ± 1	0/10 ± 2	0/12 ± 2
1000	0/4 ± 1	0/8 ± 2	0/7 ± 1
The ratio of the number of S- and R-type colonies of *B. subtilis*			
Control (0)	200 ± 21/0 (+2)	150 ± 15/0 (+1)	150 ± 15/0 (+1)
50	190 ± 18/0 (+1)	137 ± 16/4 ± 1	142 ± 13/51 ± 5
100	176 ± 16/0 (+2)	134 ± 10/14 ± 2	140 ± 11/11 ± 3
500	89 ± 7/20 ± 4	73 ± 8/6 ± 2	38 ± 6/8 ± 1
1000	0/12 ± 2	0/3 ± 1	0/1(±1)

concentration of 100 μg/ml, hexylresorcinol induced the formation of three colonial morphotypes: (1) small smooth S-type colonies, which were dominant; (2) large smooth S-type colonies; and (3) large R-type colonies with uneven edges (Figure 1b). At 500 μg/ml, hexylresorcinol gave rise to only S-type colonies (subtypes 1 and 2) (Figure 1c).

It is known that hexylresorcinol promotes the transformation of gram-positive (both spore-forming and non-spore-forming) and gram-negative bacteria to a resting state [8, 9], exerts a mutagenic effect on prokaryotic cells [7], and induces an SOS response in bacteria [10]. In contrast, homoserine lactone fails to induce resting bacterial forms, does not exhibit mutagenic properties, and cannot induce SOS response (unpublished data). It should be noted that none of the concentrations of homoserine lactone used in this study could induce phenotypic dissociation in *B. subtilis* SK1 and *S. typhimurium* TA100.

Phenotypic variability is characterized by high frequencies of both dissociation and the reversion of dissociants to the original form, which is due to intragenomic rearrangements [11]. The functional significance of these processes is to provide for the adaptation of pro- and eukaryotic organisms to the current environmental conditions and their preadaptation to the environmental conditions possible in future. It can be suggested that intragenomic rearrangements underlie certain adaptive reactions and are controlled by trigger molecules, which serve as regulators of bacterial development. Although nongenotoxic cell density regulators such as acylated homoserine lactones, also couple external factors (for example, critical cell density) with intracellular reactions, their action is more species-specific [4, 5]. It is well-known that the R variants of gram-positive and gram-negative bacteria exhibit a higher resistance to stressful factors [12, 13]. However, it remains unclear whether the treatment of S- and R-type cells (which differ in antigenic properties) with hexylresorcinol (an inducer of morphogenesis) can change their properties, in particular, bacteriophage resistance.

In our experiments, we tested the resistance of the hexylresorcinol-induced R and S variants of *S. typhimurium* TA100 to the bacteriophage (lot 7, 2004) produced by ImBio (Nizhny Novgorod). This phage is similar to the type phage of *S. typhimurium.* The 18-h old stationary-phase S- and R-type cells of *S. typhimurium* TA100 (taken from the colonies grown in the presence of high concentrations of hexylresorcinol) were grown as lawns on agar plates. In the control experiment, the S- and T-type *S. typhimurium* TA100 cells used for plating were taken from the colonies grown in the absence of hexylresorcinol. Sterile filter-paper disks 1.5 mm in diameter were placed at the centers of the bacterial lawns and impregnated with a bacteriophage suspension (one drop per disk). After 1 day of incubation at 37°C, the zones of lawn lysis around the disks were measured. The measurements showed that, irrespective of whether hexylresorcinol was present in the growth medium of the bacterium (test experiments) or absent (control experiments), the test phage produced large lysis zones (4 cm in diameter) on the lawn of S-type cells. At the same time, no lysis zones were observed on the lawns of R-type cells in either of the experimental variants.

Thus, our experiments show that exogenous hexylresorcinol is able to induce phenotypic dissociation in gram-positive [14] and gram-negative bacteria. Irrespective of the presence of hexylresorcinol in the growth medium of *S. typhimurium*, the R-type cells of this bacterium exhibit a high phage resistance, while the S-type cells are phage sensitive.

It can be suggested that the autoregulatory trigger molecules produced by bacteria in response to stress can induce their phenotypic dissociation if these molecules are able to cause intragenomic rearrangements [7, 10]. Such autoregulatory molecules do not change the phage resistance of the original morphotypes.

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