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EXPERIMENTAL ARTICLES

Low-Molecular-Weight Autoregulatory Factors in the Haloalkaliphilic Bacteria *Thioalkalivibrio versutus* and *Thioalkalimicrobium aerophilum*

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Abstract—The haloalkaliphilic, lithoautotrophic, sulfur-oxidizing gram-negative bacteria *Thioalkalivibrio versutus* and *Thioalkalimicrobium aerophilum* were found to possess a special system for the autoregulation of their growth. The system includes the extracellular autoinducers of anabiosis (the d_1 factor) and autolysis (the d_2 factor). The principal components of the d_1 factor are alkylhydroxybenzenes, as evidenced by specific qualitative reactions. The principal components of the d_2 factor are free unsaturated fatty acids dominated by oleic acid isomers. Like the respective autoregulators of neutrophilic bacteria, the d_1 factor of haloalkaliphilic bacteria presumably controls their growth and transition to anabiotic state, while the d_2 factor controls autolytic processes. Alkylhydroxybenzenes of both microbial and chemical origin were found to influence bacterial respiration. The low-molecular-weight osmoprotectant glycine betaine enhanced the thermostability of trypsin. This suggests that both glycine betaine and the d_1 factor serve as chemical chaperones.

Key words: autoinducers of anabiosis, autoinducers of autolysis, haloalkaliphilic sulfur-oxidizing bacteria, glycine betaine, chemical chaperones.

There is increasing current interest in the study of low-molecular-weight adaptogenic substances providing for the preservation of microbial cells under varying environmental conditions. The osmotolerance of haloalkaliphilic bacteria of the genera *Thioalkalivibrio* and *Thioalkalimicrobium*, which inhabit soda lakes with high-salinity and alkaline water, is known to be provided by the low-molecular-weight cytoplasmic substance glycine betaine [1]. The mechanism of action of such osmoprotectants is related to their ability to bind to various cellular macromolecules, thereby decreasing their hydration and making them more tolerant to changes in osmotic pressure [2, 3]. Osmoprotectants can also enhance the thermostability of proteins [4], since the decreased extent of protein hydration and the interaction of osmoprotectant molecules with the side chains of protein molecules hinders the process of protein defolding [5, 6]. Baskakov and Bolen showed that trimethylamine oxide at denaturing concentrations increased protein stability five times [7].

These properties of osmoprotectants are similar to some properties of the so-called autoregulators of anabiosis (the d_1 factor) and autolysis (the d_2 factor), which form the coordinated system of autoregulation of growth and development in neutrophilic bacteria belonging to different physiological and taxonomic groups [8–13]. At low concentrations, the d_1 factor limits the growth of bacterial populations, while it induces their transition to anabiosis at high concentrations. This transition is associated with the formation of cystlike refractile cells (CRCs). The d_2 factor controls the germination of resting microbial forms in the lag phase and induces cell autolysis in the poststationary growth phase. In fact, the d_1 factor acts as a structural modifier of membranes, thereby altering their permeability and functional integrity and the water balance of cells [14]. The d_1 factor also serves as a chemical chaperone of proteins and enzymes, thereby changing their conformation and hence catalytic activity [15, 16]. All this influences the functional activity of cells and enhances the stability of cellular biopolymers. This suggests that the d_1 factor, together with the constitutively present

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Table 1. The biological activity of the d_1 factors of strains AL2 and AL3

Factor from strain	Activity, μl/μg protein		Specific activity, 10^3 units/g protein	Specific activity, 10^3 units/l	CRC-inducing activity, units
	towards AL2	towards AL3			
AL2	0.27	0.31	10.3	0.93	5.8
AL3	0.36	0.33	7.2	0.87	4.9

glycine betaine system, may provide for the defense of cells against severe osmotic stress.

The present work is an attempt to reveal the d_1 and d_2 factors in the haloalkaliphilic bacteria *Thioalkalivibrio versutus* and *Thioalkalimicrobium aerophilum* and to compare their protective effects with that of glycine betaine.

MATERIALS AND METHODS

Experiments were carried out with the type strains, $AL2^T$ and $AL3^T$, of the haloalkaliphilic, strictly autotrophic, sulfur-oxidizing gram-negative bacteria *Thioalkalivibrio versutus* and *Thioalkalimicrobium aerophilum* isolated recently from soda lakes [17]. The strains were grown in a mineral medium containing (g/l) Na_2CO_3, 24; NaCl, 10; K_2HPO_4, 1; and KNO_3, 1. The pH of the medium was adjusted to 10.2 with $NaHCO_3$ before sterilization. Solutions of trace elements [18], $MgCl_2 \cdot 6H_2O$, and $Na_2S_2O_3 \cdot 5H_2O$ were sterilized separately. The trace element solution was added to the mineral medium in amounts of 1 ml/l (strain AL2) and 2 ml/l (strain AL3). The solution of $MgCl_2$ was added to the medium at concentrations of 0.5 mM (strain AL2) and 1 mM (strain AL3), and the $Na_2S_2O_3$ solution, at concentrations of 40 and 80 mM, respectively. The strains were grown at 28°C in either 250-ml flasks with 50 ml of the medium or 2-l flasks with 450 ml of the medium with shaking (140 rpm). The growth medium was inoculated with stationary-phase cells in an amount of 10–15 vol %.

Agar medium was prepared by mixing aseptically equal volumes of the mineral medium and 3.5–4% agar solution, both heated to 50–60°C. The agar medium was supplemented with the same amounts of trace elements, $MgCl_2$, and $Na_2S_2O_3$ as indicated above.

Bacterial viability was determined by the serial dilution method. The results obtained were expressed in colony-forming units (CFU) per one milliliter of the culture.

Cell morphology was analyzed under an Amplival phase-contrast microscope (Germany).

To determine the protein content of cells, they were separated from the medium by centrifugation, washed with 0.6 M NaCl, and suspended in 1 ml of 1 N NaOH. Further manipulations were carried out according to the protocol of the Lowry *et al.* method.

The d_1 and d_2 factors were obtained by extracting the stationary-phase culture liquids of strains AL2 and AL3 with two volumes of *n*-butanol. The cultures were preliminarily acidified to pH 5.5. After the removal of *n*-butanol in a rotary evaporator, the residue was extracted with a chloroform–methanol–water (2 : 2 : 1.8) mixture. The lower chloroform–methanol phase was also evaporated, and the residue was extracted with another chloroform–methanol–water (2 : 2 : 1) mixture. The chloroform–methanol and methanol–water phases thus obtained were evaporated separately, and each of the residues was dissolved in 1 ml of ethanol. The resultant preparations were tested for the activity of the d_1 and d_2 factors as described in the earlier publications [8–13].

The effect of these factors on cell respiration in the presence of $Na_2S_2O_3$ was evaluated using a Clark-type oxygen electrode with a temperature-controlled 5-ml measuring cell. One unit of activity of the factors was defined as the amount inhibiting cell respiration by 50%. In these experiments, the AL2 and AL3 cultures were diluted with the mineral medium to cell concentrations of 75 and 49 μg protein/ml, respectively. The respiration rates of the control (i.e., untreated with the d_1 and d_2 factors) AL2 and AL3 cells were 530 and 1380 nmol O_2/(mg protein min), respectively.

To induce the formation of cystlike refractile cells, the ethanol solution of the microbial d_1 factor or its chemical analogue (alkylhydroxybenzenes, AHBs) were added to the stationary-phase AL2 and AL3 cultures (48 h of growth) in amounts such that the concentration of ethanol in the cultures did not exceed 5 vol %. The control samples were supplemented deliberately with the same amount of ethanol.

AHBs were assayed by a modified colorimetric method based on the interaction of AHBs with *p*-diazo-2,5-diethoxy-*N*-benzoylaniline (Fast Blue BB salt) purchased from Sigma. The method has a sensitivity of 1–2 μg AHBs per ml [11].

To determine the chemical composition of the d_2 factor, a 40-μl aliquot of the crude chloroform–methanol preparation of this factor was dried at 80°C in a flow of an inert gas. The residue was silylated at 80°C for 15 min in 20 μl of bis(trimethylsilyl)trifluoroacetamide (BSTFA) and diluted to 100 μl with hexane. One microliter of this solution was analyzed by combined gas chromatography–mass spectrometry using a Hewlett–Packard HP-5973 gas chromatograph–mass spectrometer (United States) equipped with an HP-5 capillary (25 m × 0.25 mm ID) quartz column. The resolving power of the mass spectrometer was 0.5 amu

Table 2. The chemical composition of the extracellular d_2 factors of strains AL2 and AL3

Compounds	Content, %	
	Strain AL2	Strain AL3
FUFAs		
16 : 1	1.29	–
18 : 1 7	–	3.00
18 : 1 9	6.56	6.15
18 : 1	14.32	0.85
Σ18 : 1	3.06	11.46
18 : 2	23.94	21.46
19 : 1	0.76	–
Σof FUFAs	–	1.38
Other unsaturated compounds	**25.99**	**22.84**
18 : 1 alcohol	3.36	8.97
monoglyceride C18:1	1.76	–
Σof total unsaturated compounds	**31.11**	**31.81**
Saturated FAs		
10 : 0	0.82	–
12 : 0	1.77	–
14 : 0	1.18	1.26
16 : 0	26.26	23.62
17 : 0	–	0.59
18 : 0	9.35	11.78
19 : 0	2.00	–
22 : 0	1.16	–
24 : 0	0.64	–
Σof saturated FAs	43.18	37.25
4-Hydroxybenzoic acid	0.34	0.53
Phosphoric acid	5.46	–
Abietic acid	0.83	–
Monoglyceride C16:0	1.33	–
16 : 0 alcohol	–	6.89
18 : 0 alcohol	–	4.39
2-OH-C20 alcohol	–	1.18
4-OH-C21 alcohol	–	0.71
26 : 0 alcohol	3.53	–
28 : 0 alcohol	3.18	–
Σof C22–29 *n*-hydrocarbons	11.04	10.65
Other substances	–	6.59
Total	100.00	100.00

Table 3. The content of alkylhydroxybenzenes in the d_1 factors of strains AL2 and AL3 as determined with Fast Blue BB salt

No.	Species	AHB content	
		mg/g protein****	mg/l medium
1	*T. versutus*, strain AL2	0.53	0.048
2	*T. aerophilum*, strain AL3	0.29	0.034
3	*Pseudomonas aurantiaca**	0.35	0.10
4	*Arthrobacter globiformis* B-1112*	0.017	0.014
5	*Arthrobacter globiformis* 245**	0.20	0.20
6	*Micrococcus luteus****	4.76	5.00
7	*Micrococcus roseus***	1.86	1.20
8	*Micrococcus* sp.**	3.14	2.00

* Unpublished data.

** Data from Mulyukin *et al.* [20].

*** Data from Mulyukin *et al.* [11].

**** The content of AHBs per g protein for items 3 through 8 was calculated under the assumption that dry cells contain 70% protein.

within a working range of 2 to 1000 amu. Ionization was performed by electron impact (70 eV). The sensitivity of the device with respect to methylstearate was 0.1 ng. The initial temperature of the column was 120°C. After injecting the sample, the column temperature was raised to 280°C at a rate of 5°C/min. The injector and the interface were kept at 280°C. Separated substances were identified using the mass-spectral databases nbs75k and wiley275.

The effect of glycine betaine on the activity of trypsin was studied as follows: a solution (2 ml) containing 0.002% trypsin in phosphate buffer (pH 7.8) was mixed with 1 ml of a solution containing 0.02 to 0.5% glycine betaine and preincubated at 40°C for 30 min. Then the mixture was supplemented with a substrate (2 ml of a 2% casein solution) and incubated at 40°C for the next 20 min. The reaction was stopped by adding trichloroacetic acid (TCA) to a concentration of 5%. After the removal of the casein fragments that are insoluble in 5% TCA, the soluble hydrolysis products of casein were determined spectrophotometrically at 280 nm. The activity of casein was determined from the difference between the absorbances of the experimental and control samples (TCA was added to the latter before the addition of the substrate).

In the experiments in which the effect of glycine betaine on the thermostability of trypsin was investigated, the mixture of trypsin and glycine betaine was first preincubated as described above and then incubated at 60°C for 15 or 30 min. The substrate was added after the mixture had been cooled to 40°C. The subsequent manipulations were the same as above.

RESULTS AND DISCUSSION

The extremely haloalkaliphilic, lithoautotrophic, sulfur-oxidizing gram-negative bacteria *T. versutus* strain AL2 and *T. aerophilum* strain AL3 differ from one another in the maximum specific growth rate, bio-

Table 4. The inhibitory effect of AHBs of microbial and chemical origin on the cell respiration of various bacteria

Substance	Half-inhibition concentration, μg/μg protein		
	Strain AL2	Strain AL3	*M. luteus**
d_1 from AL2	0.051	0.059	–
d_1 from AL3	0.043	0.040	–
d_1 from *M. luteus*	–	0.056	0.015**
4-*n*-hexylresorcinol	0.40	0.36	0.015
4-*n*-decylresorcinol	–	0.11	0.095
5-*n*-decylresorcinol	0.68	0.075	0.012
2,5-dibutylresorcinol	–	0.089	0.027
2-nonyl-5-decylresorcinol	>1.66	0.45	0.10

* Data from Mulyukin *et al*. [20].
** Unpublished data.

Table 5. The viability of the cells of strains AL2 and AL3 after 2 h of incubation in the presence of C_6-AHB and after 1 month of storage under conditions promoting cell autolysis

C_6-AHB concentration, μg/μg protein	Viability, CFU/ml and % of the control			
	Strain AL2		Strain AL3	
	2 h	1 month	2 h	1 month
0 (control)	2.3×10^9	2.3×10^7	2.3×10^7	4.5×10^1
	(100.0)	(100.0)	(100.0)	(100.0)
8.083	0	0	0	0
	(0)	(0)	(0)	(0)
0.81	2.5×10^1	0	0	0
	(10^{-6})	(0)	(0)	(0)
0.16	6.0×10^6	0	1.3×10^2	0
	(0.24)	(0)	(6.5×10^{-4})	(0)
0.081	–	–	1.5×10^4	0
			(5.6×10^{-2})	(0)
0.016	5.8×10^8	7.2×10^3	2.5×10^6	3.0×10^1
	(23.2)	(0.031)	(10.9)	(66.7)
0.0081	6.0×10^8	1.4×10^4	2.5×10^6	3.3×10^1
	(24.0)	(0.061)	(12.6)	(73.3)

mass yield, cell respiration, and sensitivity to unfavorable environmental conditions [19]. The extraction of the stationary-phase culture liquids of these strains with *n*-butanol and the separation of the extract with chloroform–methanol–water mixtures by the procedures described in the *Materials and Methods* section showed that the cells of both strains contain activity typical of the d_1 factor (this activity concentrated in the methanol–water fraction of the butanol extract). The addition of this factor to the linear-growth-phase cells inhibited their metabolic activity and promoted the formation of cells with a refractile appearance, as is evident from observations under the phase-contrast microscope. The specific activities of the d_1 factors were judged from the inhibition of exogenously stimulated respiration. For the d_1 factors from the culture liquids of strains AL2 and AL3, 1 unit of biological activity corresponded to 20.0 and 16.2 μl/ml, respectively. To compare the activ-

Table 6. The effect of glycine betaine on the thermostability of trypsin

Glycine betaine, wt %	Trypsin activity, % of the control		
	before heating	after 15-min heating at 60°C	after 30-min heating at 60°C
0 (control)	100.0	46.5	10.3
0.007	100.8	104.5	52.9
0.017	104.9	108.4	80.6
0.033	108.1	107.1	68.4
0.067	98.4	105.8	78.7
0.17	90.03	103.2	76.1

ities of the factors more exactly, we recalculated activity units per μg protein (Table 1). The d_1 factors exhibited cross-reactivity, i.e., the d_1 factor isolated from strain AL2 was active towards strain AL3 and vice versa, although the activities towards the producer strains were higher. Strain AL2 seems to produce the d_1 factor in a greater amount (as calculated per μg protein) than strain AL3, although this difference may be related to the fact that the accumulation maxima of the d_1 factor in the two strains do not coincide. The activity of the d_1 factor of strain AL3 with respect to the induction of CRCs was higher than that of strain AL2 (Table 1).

The chloroform–methanol fractions of the butanol extracts of cells exhibited activity typical of the d_2 factor. At low concentrations (below 0.031 μl/μg protein), the d_2 factors of both strains stimulated cell respiration, while they inhibited it at high concentrations. The 50% inhibition of the respiration of strains AL2 and AL3 corresponded to the concentrations of the d_2 factor equal to 0.35 and 0.30 μl/μg protein, respectively. When added in an amount of 4–5 units of biological activity, the d_2 factor induced the rapid autolysis of cells.

Taking into account the fact that free unsaturated fatty acids (FUFAs) are the principal components of microbial d_2 factors [10–13], we determined the chemical composition of the d_2 factors of strains AL2 and AL3, with emphasis on the content of fatty acids (FAs). As is evident from the results presented in Table 2, the d_2 factors of both strains virtually did not differ in the content of FUFAs and other unsaturated compounds, which is in agreement with the observation that the biological activities of the both d_2 factors are close. The FUFAs of both strains were dominated by oleic acid isomers and contained hydrocarbons, organic acids, and alcohols (including unsaturated ones). It should be noted that unsaturated alcohols were not detected in the d_2 factors of the microorganisms studied earlier.

Analysis of the d_1 factors of strains AL2 and AL3 for the presence of alkylhydroxybenzenes (the principal components of the d_1 factor of some microorganisms [9, 10, 13]) with Fast Blue BB salt showed that they contained AHBs in amounts of 190 and 120 μg/ml, respectively. These values correlate with the productivity levels (expressed in units of biological activities) of the d_1 factors of the strains (10.3 and 7.2 units/g protein, respectively) (Table 1). A comparative analysis of the content of extracellular AHBs in bacteria of various physiological and taxonomic groups showed that the gram-negative bacteria *Thioalkalivibrio versutus, Thioalkalimicrobium aerophilum,* and *Pseudomonas aurantiaca* produce extracellular AHBs in close amounts, whereas the content of extracellular AHBs in the gram-positive bacteria of the genus *Micrococcus* was higher by an order of magnitude (Table 3). At the same time, the inhibition of the endogenous respiration of cells by the d_1 factor of *Micrococcus luteus* was almost the same as by the d_1 factors of strains AL2 and AL3 (Table 4).

Determination of AHB concentrations in the obtained d_1 fractions allowed us to quantitatively compare their effect on the electron-transport chain with the effect of chemical analogues (AHBs with varying position and length of alkyl radicals, Table 4).

Analysis of the inhibitory activity of AHBs in relation to their structure confirmed earlier observations [14, 21] that AHBs with one alkyl radical at position 5 are relatively active and that their activity increases with the length of the carbon chain. The presence of a second radical at position 2 diminished the inhibitory activity of AHBs to a degree dependent on the length of this radical (AHBs with shorter radicals were more active than those with longer radicals). The fact that all of the individual chemically synthesized AHBs tested were less active than the d_1 factors of strains AL2 and AL3 suggests that these factors may contain AHBs with longer alkyl radicals than the tested AHBs, as was observed for the d_1 factors of pseudomonads and azotobacters [8, 21]. Presumably, long alkyl radicals facilitate the interaction of AHB molecules with membrane lipids and proteins.

The modification of the conformation of protein molecules by the d_1 factor may enhance the stability and catalytic activity of enzymes [15, 16] and increase the resistance of cells to unfavorable environmental conditions. This suggestion is confirmed by the data presented in Tables 1, 3, and 4, which show that strain AL3, less resistant to various adverse effects, produces the d_1 factor in lower amounts and that the respiration of this strain is more susceptible to the action of AHBs than that of strain AL2. Similarly, 4-*n*-hexylresorcinol, a chemically synthesized analogue of the d_1 factor, stimulated the formation of refractile cells in strain AL3 at a lower concentration (0.65 μg/μg protein or 1.8 units of biological activity) than in strain AL2 (0.81 μg/μg protein or 2 units of biological activity). The refractile cells resembled CRCs in such properties as suppressed metabolism (as follows from respiration measurements) and the preservation of cell integrity for long periods of time (more than 12 months) but differed from CRCs in that they have a relatively low viability (Table 5). The results of the study of the resting forms

of strains AL2 and AL3 will be considered in more detail in our next publication.

Thus, the haloalkaliphilic bacteria *T. versutus* and *T. aerophilum*, like the microorganisms studied earlier, possess the universal system of regulation of growth and development, which includes extracellular autoinducers of anabiosis (presumably, alkylhydroxybenzenes) and autolysis (presumably, free unsaturated fatty acids).

To gain insight into the mechanism of action of the d_1 factor on the resistance of cells to unfavorable environmental conditions, we compared the chaperone activity of the d_1 factor [15, 16] and osmoprotectants [3–7]. Our earlier studies showed that C_6-AHB at a concentration of 0.05% efficiently prevented chymotrypsin from thermoinactivation during heating at 60°C for 15 min, whereas this enzyme was totally inactivated by such a heating in the control mixture without C_6-AHB. At a concentration of 0.1%, which is higher than the critical micelle-forming concentration of C_6-AHB, this compound preserved the activity of the heated chymotrypsin at a level of 60% [16].

In the present study, a similar protective role was shown for glycine betaine, which considerably enhanced the thermostability of trypsin (Table 6). Unlike C_6-AHB, glycine betaine at elevated concentrations exhibited the same or an even more profound protective effect than at low concentrations. The difference in the concentration dependence of the protective effects of C_6-AHB and glycine betaine can be explained by the higher reactivity of AHBs in their interactions with macromolecules in comparison with glycine betaine. Furthermore, hydrophilic glycine betaine differs from hydrophobic AHBs in the type of molecular dispersion in solutions [15] and in the dependence of this dispersion on pH and ionic strength.

Even when accumulated in cells in great amounts, osmoprotectants (in our case, glycine betaine) do not suppress the metabolic activity of the cells at any stage of microbial culture development. At the same time, the d_1 factor at low concentrations serves as a protectant, whereas it acts as an autoinducer of anabiosis at high concentrations. The content of the d_1 factor in actively growing microbial cultures is low, but its accumulation in the stationary-phase cultures induces the formation of resting (dormant) cells. Therefore, the d_1 factor controls the transition of microbial populations from the strategy of growth to the strategy of survival.

To conclude, the autoregulatory d_1 factors of microorganisms are polyfunctional compounds and their effect should be considered in a complex with the effect of other low-molecular weight regulators, osmoprotectants in particular.

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