Microbiology, Vol. 71, No. 2, 2002, pp. 133–138. Translated from Mikrobiologiya, Vol. 71, No. 2, 2002, pp. 160–165.

EXPERIMENTAL ARTICLES

Antagonistic Interactions between Stress Factors during the Growth of Microorganisms under Conditions Simulating the Parameters of Their Natural Ecotopes

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Received October 11, 2001

Abstract—Two stress factors, hypoxia (microaerobic conditions) and a high salt concentration, if applied simultaneously to aerobic microorganisms, display an antagonistic mode of interaction. As a result, the NaCl level that is usually optimal for moderate halophiles (5–6 %) becomes optimal for the growth of weak halophiles (*Rhodococcus erythropolis* and *Shewanella sp.* CN32); the halotolerant yeast *Yarrowia lypolytica* acquires halophilic properties (with a growth optimum at a NaCl concentration of 10%), and the growth rate of the extremely halophilic *Halobacterium salinarum* increases at supraoptimal salt concentrations (25–34%). This phenomenon is apparently due to multiple changes in metabolic reactions. In particular, high salt concentrations suppress respiration and the formation of enzymes (superoxide dismutase and catalase) that protect the cell from toxic oxygen species. Therefore, establishment of microaerobic conditions compensates for the loss of these protective mechanisms and enables cell growth at higher salt concentrations than under aerobic conditions. Of some importance can also be the increase in the intracellular concentrations of osmoprotectants caused by the suppression of their intracellular oxidation. The implications of this phenomenon for the ecophysiology of microorganisms (including oil-oxidizing species) and for the classification of weak and moderate halophiles are discussed.

Key words: multiple stress, hypoxia, halophiles, osmoprotection.

Researchers dealing with the ecophysiology of microorganisms often face the problem that the "optimum" cultivation parameters determined under laboratory conditions differ from those occurring in the natural ecotope from which the microorganism under study has been isolated. Numerous examples of this phenomenon have been described [1]. In such cases, the naturally occurring environmental conditions appear as stressfull for the microorganisms under consideration. This is exemplified by high or low salt concentrations with weak and extreme halophiles, respectively, by low partial oxygen pressure with obligate aerobes, by high temperature values with mesophiles, etc. Nevertheless, convincing evidence has been obtained that microorganisms in at least some such ecosystems do not merely endure stress but retain their metabolic activities and reproductive potential. One reason behind this apparent discrepancy is that, despite the inhibitory effects of each stressor per se on microbial metabolism and growth, antagonistic interactions occur between such stressors when applied in combination, and this mitigates their harmful influence. Evidence for this suggestion comes from a number of studies. For instance, an elegant method enabled Gorlenko *et al.* [2] to obtain compelling data that increasing the cultivation temperature increases the degree of halophily of anoxygenic phototrophic bacteria. Similar regularities have been revealed in psychrophilic bacteria [3]. It has also been shown that acid shock induces resistance to other stress factors (heat, osmotic, and oxidative stress) in a number of enteric and lactic acid bacteria [4, 5].

We did not find in the available literature any data on the effects caused by a combination of hypoxia and oxidative stress in aerobic microorganisms. We reported earlier on the antagonistic mode of interaction of these stress factors in some aerobic bacteria [6]. This work presents the results of a more detailed study of this phenomenon, performed with the use of a broader range of microbial species.

MATERIALS AND METHODS

Microorganisms and cultivation conditions. This study used both new and previously described strains of microorganisms. The strain of *Rhodococcus erythropolis* was isolated from the Bondyuzhskoe oil field in Tatarstan [7]. The salt content in the stratal water of this oil field reaches 272 g/l. These bacteria were cultivated on APY (acetate–peptone–yeast extract) medium containing (g per 1 l of distilled water) peptone from casein (Serva) or Casamino acids (Difco), 10; yeast extract (Serva), 5; sodium acetate, 5; and NaCl in amounts var-

0026-2617/02/7102-0133$27.00 © 2002 MAIK "Nauka / Interperiodica"

ied depending on the experimental conditions (the original medium contained 0.1–0.2% Na^+ (calculated as NaCl) originating from admixtures to other medium components). The GPY (glucose–peptone–yeast extract) medium contained 5 g/l glucose instead of acetate. Two percent agar was added to obtain solid medium. The cultivation temperature was 28–30°C, and the pH value was 7.0–7.2. Strain S9 of the extremely halophilic *Halobacterium salinarum* was obtained from the laboratory of Prof. D. Oesterhelt (Max-Planck-Institut für Biochemie, Germany). It was grown on a modified peptone medium [8] containing (g per l of distilled water) peptone (Oxoid, United Kingdom), 10; $MgSO_4 \cdot 7H_2O$, 20; KCl, 2; trisubstituted sodium citrate, 3; and NaCl, 20 to 34%. The cultivation temperature was 37–38°C, and the pH value was 7.0–7.2. The yeast *Yarrowia lypolytica*, which, like the rhodococci, was isolated from the stratal water of the oil field, was grown on the GPY medium supplemented with the antibiotic benzylpenicillin (2.5 mg/ml) to prevent occasional contamination with bacteria. The cultivation temperature was 28–30°C, and the pH value was adjusted to 5.4 using a phosphate buffer (0.03 M). Bacteria of the genus *Shewanella* were obtained from A.S. Belyaev (Nat. Lab., Oak-Ridge, United States). *Shewanella* sp. CN32 was cultivated on LML medium modified by us [9] that contained (g/l) meat peptone (Serva), 0.5; yeast extract (Serva), 1; glucose, 3.6; and HEPES, 2.4. The cultivation temperature was 28–30°C, and the pH value was 7.4. Aerobic cultivation was carried out in flasks closed with cotton plugs either using a shaker (180 rpm) or without shaking (stationary conditions). Microaerobic conditions were created as described earlier [6], using rubber stoppers penetrated with syringe needles with an internal diameter of 0.6–0.8 mm. The oxygen content in the gas phase did not exceed 1–2% at the initial cultivation stage.

Culture growth was estimated from optical density, including both light absorption and scattering by microbial cells (KFK-2 nephelometer, $\lambda = 540$ nm), and by determining the weight of dry biomass and protein content. The dry biomass of halobacteria, which lyse if washed with distilled water, was determined by a method developed by us: their cells were stabilized by acidification to a pH value of 3.0 [10]. Protein was determined by the method of Lowry *et al.* [11]. Intracellular soluble substances (including amino acids) were extracted by heating the cell suspension in distilled water at 90°C for 30 min. Amino acids were determined with an amino acid analyzer, and the total content of organic compounds in the extract was measured as described by Panikov *et al.* [12]. The oxygen content was determined using a CS 3300 gas chromatograph. The sample volume was 200 μl; air (20.9% O_2) served as the standard. Superoxide dismutase activity was measured using a modified method of Beauchamp and Fridovich [13]: by measuring the difference between the rates of superoxide anion radical ($^{\cdot}O_2^-$) formation in the xanthine/xanthine oxidase system before and after the addition of cell lysate. Catalase activity was determined manometrically from the volume of oxygen evolved [14].

The figures represent the results of typical experiments (3–4 replicates of each experiment were done).

RESULTS AND DISCUSSION

Although data on the degree of halophily of the microorganisms under study are available in the literature, it was expedient to determine this parameter more precisely for the tested strains under our experimental conditions. The results are shown in Fig. 1. The growth dynamics of each culture was monitored; the plot represents data obtained for the phase of growth deceleration (when the maximum biomass yield was attained) This stage occurred after 48–96 h of cultivation with different cultures. Most of the results obtained were consistent with the data available in the literature: based on the generally accepted classification [1], *Shewanella* sp. CN 32 and *Rh. erythropolis* are to be regarded as weak halophiles (the optimum NaCl concentration for their growth is 1–3%); *Y. lypolytica* is a halotolerant species; and *H. salinarum* is an extreme halophile (the optimum NaCl concentration is 20%).

Since some of the microorganisms under study often occur in natural ecotopes with a low oxygen content and are usually cultivated in a laboratory under aerobic conditions, we investigated the influence of various aeration levels on the degree of halophily (halotolerance) of these cultures. The results are given in Figs. 2–5.

Shewanella sp. CN32 is a facultative anaerobe, and its growth under aerobic conditions with agitation is followed by cell lysis. Without agitation, the culture reaches the stationary phase and can persist for a sufficiently long time. The growth rate under microaerophilic conditions is 1.5–2 times lower than that obtained with agitation. However, after 72–96 h, the culture reaches the same density level as in an intensely aerated system. The degree of halophily increases under these conditions to such an extent (the optimum NaCl concentration is 5–6%, see Fig. 2) that the culture can be regarded as a moderate halophile [1].

Rh. erythropolis is an obligatory aerobe, and it does not grow under anaerobic conditions. The transition from an agitated culture to a stationary culture markedly decreases the growth rate, and the growth of the culture is nearly 10 times slower under microaerobic conditions than in an agitated system. However, the optimum NaCl concentration increases under microaerobic conditions (as compared to an intensely aerated system) so significantly (from 1 to 5%, Fig. 3) that this culture, like *Shewanella* sp., should be considered a moderate, not a weak, halophile.

The yeast *Y. lypolytica* is a typical aerobe and a halotolerant organism. This culture can grow with agita-

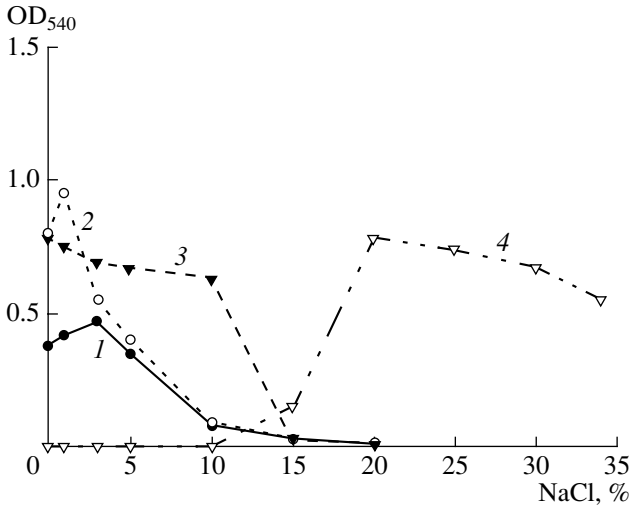

Fig. 1. Growth of microorganisms under aerobic conditions with agitation at various NaCl concentrations: (*1*) *Shewanella* sp. CN32; (*2*) *Rhodococcus erythropolis*; (*3*) *Yarrowia lypolytica*; (*4*) *Halobacterium halobium* S9. The data for *Y. lypolytica* were divided by 10 to make the curve fit the diagram.

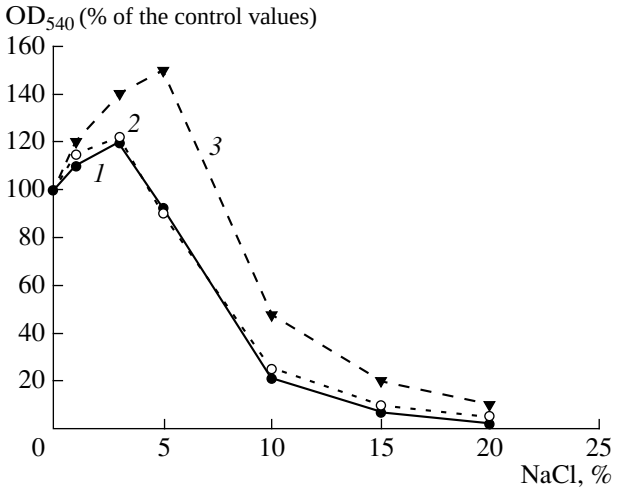

Fig. 2. Growth of *Shewanella* sp. CN32 at various NaCl concentrations and aeration levels: (*1*) aerobic conditions with agitation; (*2*) aerobic conditions without agitation (stationary); (*3*) microaerobic conditions. Control cultures were grown under the specified conditions but without NaCl addition (the same in Figs. 3–4).

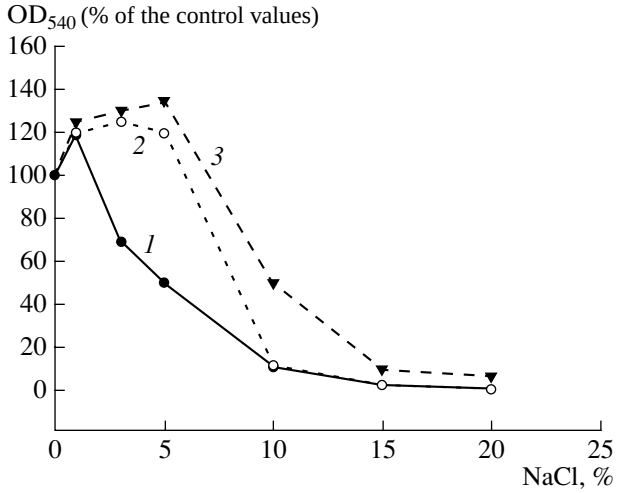

Fig. 3. Growth of *Rhodococcus erythropolis* at various NaCl concentrations and aeration levels: (*1*) aerobic conditions with agitation; (*2*) aerobic conditions without agitation (stationary); (*3*) microaerobic conditions.

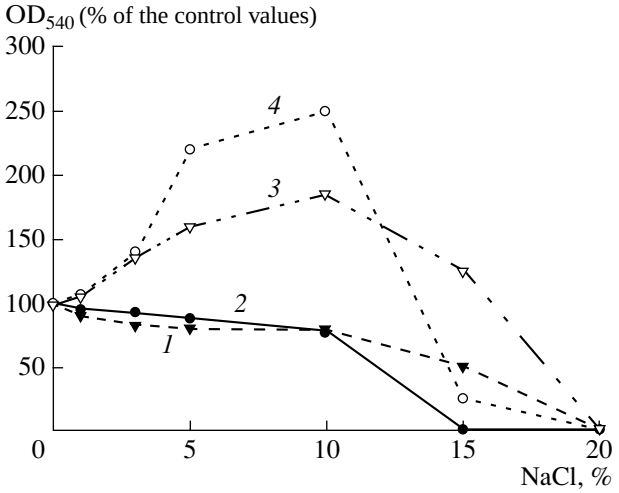

Fig. 4. Growth of *Yarrowia lypolytica* at various NaCl concentrations and aeration levels: (*1, 2*) aerobic conditions with agitation; (*3, 4*) microaerobic conditions; (*1, 3*) 9th day of cultivation; (*2, 4*) 4th day of cultivation.

tion at NaCl concentrations of up to 15%. Under stationary and particularly under microaerobic conditions its growth rate is significantly decreased (up to 10-fold). Apart from an increase in the halotolerance degree (growth occurs at NaCl concentrations of up to 20%), the culture under these conditions is characterized by a dependence of its growth on the salt concentration; i.e., it becomes halophilic (with a growth optimum at 10% NaCl), irrespective of the culture age (Fig. 4).

Of considerable interest are the results obtained with a *H. salinarum* S9. This extremely halophilic microorganism can grow in media with near-saturation NaCl concentrations (34%, Fig. 1). However, the culture halophily is somewhat increased under stationary and microaerobic conditions. Although the NaCl concentration of 20% remains optimum for growth, the difference between the growth rate at NaCl concentrations of 20 and 25% becomes less significant, and the growth rate under microaerobic conditions is higher than that

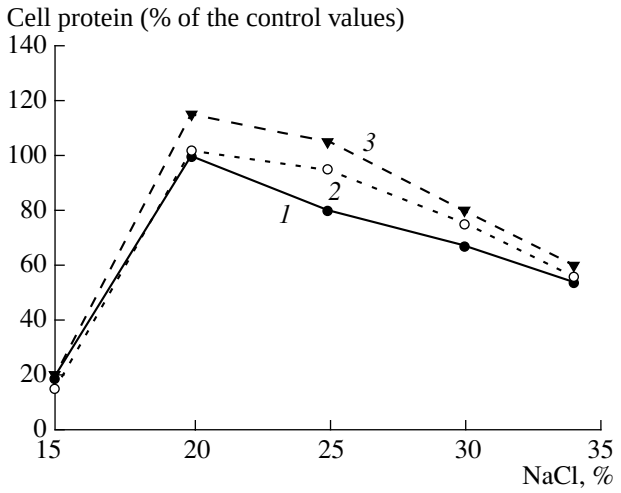

Fig. 5. Growth of *Halobacterium salinarum* S9 at various NaCl concentrations and aeration levels: (*1*) aerobic conditions with agitation; (*2*) aerobic conditions without agitation (stationary); (*3*) microaerobic conditions. The control culture was grown at a NaCl concentration of 20%.

in the agitated system (up to a NaCl concentration of 30%) (Fig. 5). In these experiments, the growth rate was estimated not only from the optical density but also from the protein content, because the effect under study was relatively small in its extent and the changes in the suspension turbidity caused by changes in the cell volume could result in erroneous data. Analogous control studies on the content of dry biomass and cell protein were also conducted in some experiments with other tested cultures. In all of the cases, the data obtained confirmed the data from optical density measurements.

From these results we drew the conclusion that the dependence of the degree of microbial halophily or halotolerance on the partial pressure of oxygen is a phenomenon widespread among microorganisms. This is in line with our earlier data [6]. This phenomenon manifests itself both in weakly and extremely halophilic bacteria, as well as in representatives of halotolerant yeasts.

The phenomenon revealed by us seems to be too complex to be caused by a single biological mechanism; such a regulatory system typically involves numerous metabolic processes. We earlier put forward some suggestions concerning the possible biological mechanisms of this phenomenon [6]. The supplementary data obtained in the present work allow us to discuss these mechanisms in more detail.

A high salt concentration rather than a low oxygen level may represent the crucial factor that determines the degrees of aerophily and halophily of the microorganisms in ecotopes with a low partial pressure of oxygen and high salinity. As we showed earlier, NaCl at a concentration of 10–15% drastically suppresses respiration in *Rh. erythropolis* [15] and switches its metabolism to "microaerobic" pathways, although the oxygen level remains high (the decrease in oxygen solubility in water in the presence of salt does not exceed 10–15%). The effect of NaCl is sufficiently specific, since KCl produces a much lesser inhibitory effect when added at the same concentrations. Switching the metabolism to pathways less dependent on aerobic respiration results, as a rule, in a decrease in the synthesis of protective enzymes (catalase, superoxide dismutase, etc.). Under these conditions, decreasing the partial pressure of oxygen (creating stationary or microaerobic conditions) should stimulate microbial growth. Indeed, data have been obtained that NaCl suppresses superoxide dismutase and catalase synthesis in *H. halobium (salinarum)* [16]. Unfortunately, this work only dealt with NaCl concentrations not exceeding 25%, and, therefore, we conducted analogous studies in the 20–34% concentration range. A precise quantitative assessment of the results of these studies presents difficulties, because *H. salinarum* S9 has several types of superoxide dismutases. However, from the general pattern of changes in this system, it is evident that the activities of these enzymes drop by 50–80% with an increase in salt concentration. Although salt suppresses superoxide dismutase synthesis both under aerobic and microaerobic conditions, the microorganism retains the ability to grow microaerobically.

The data on the catalase activity in *Y. lypolytica* and *H. salinarum*S9 cells are presented in Fig. 6. Similar results were obtained with *Rh. erythropolis* (data not shown). It is evident that catalase synthesis is drastically suppressed with the increase in the NaCl concentration both under aerobic and microaerobic conditions.

Hence, the mitigation of the toxic effects of oxygen can be one of the factors causing an increase in the degree of halophily of microorganisms upon their transfer to microaerobic conditions, which are less challenging in respect to the protective enzymes required.

As we suggested earlier [6], the increase in the degree of halophily caused by microaerobic conditions can also result from an increase in the intracellular pool of low-molecular-weight metabolic products (which can serve as osmoprotectants) due to the retardation of their oxidation at low oxygen levels. According to our experimental data, the content of soluble organic substances in *Rh. erythropolis* and *Y. lypolytica* is 1.3–1.5 times higher under microaerobic than under aerobic conditions, and the most significant increase occurs in the content of amino acids (primarily aspartic acid and also proline and lysine). It was shown earlier that glycine betaine and the amino acids glutamic acid and serine are the main osmoprotectants in rhodococci under aerobic conditions [17]. Glycerol and the amino acids proline and alanine are the main osmoprotectants in *Y. lypolytica* [18]. However, this mechanism apparently does not work in extreme halophiles, in which K^+ ions are the main osmoprotectants, whereas organic

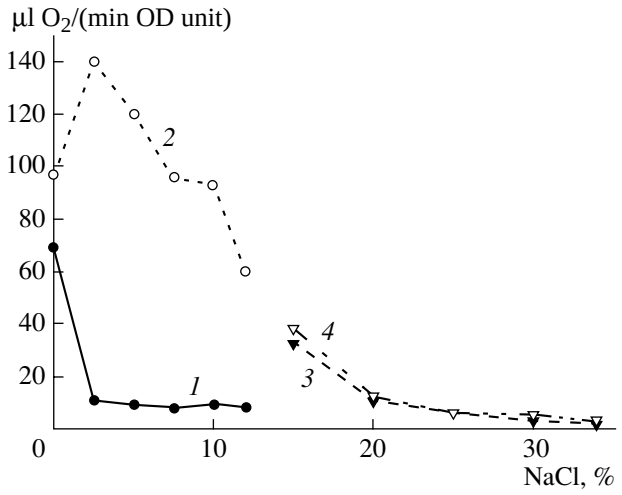

Fig. 6. Catalase activity at various NaCl concentrations and aeration levels: (*1*, *2*) *Y. lypolytica*; (*3*, *4*), *H. halobium* S9; (*1*, *3)* microaerobic conditions; (*2*, *4*) aerobic conditions with agitation.

osmolytes (glycine betaine) only occur in trace amounts [19].

In summary, the increase in the degree of halophily of microorganisms under microaerobic conditions is of considerable importance in ecophysiological terms. Knowledge of this phenomenon allows predictions to be made concerning potential manifestations of the metabolic activities of aerobic microorganisms under simultaneous influence of two stressors, a low partial pressure of oxygen (hypoxia) and high medium salinity. This is of particular value with respect to the assessment of the metabolic activities of microorganisms inhabiting oil fields, where they encounter such a combination of stressors. Importantly, weakly halophilic aerobes often form a significant part of the microorganisms isolated from such ecotopes [7].

The discussed phenomenon is also of relevance to the classification system used for aerobic halophilic microorganisms. From our findings it follows that cultivation conditions should be taken into account when deciding to which group (in terms of the degree of halophily) a microorganism is to be assigned.

ACKNOWLEDGMENTS

This work was supported by the Russian Foundation for Basic Research (project no. 01-04-48275) and, in part, was financed within the framework of the subproject “Biotechnology for the Enhancement of Oil Recovery” of the Federal Central Science and Technology Program “Research and Development in the Priority Directions of Civil Science and Technology.”

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