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EXPERIMENTAL ARTICLES

Survival Strategy of *Bdellovibrio*

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Abstract—The effects of cadmium and diuron, typical environmental pollutants, on the survival of predatory bacteria of the genus *Bdellovibrio* were studied. The adhesion and cohesion of bdellovibrios were shown to enhance cell resistance to xenobiotics. The viability of *Bdellovibrio* cells was shown to be higher at the stage of bdelloplasts. The obtained results confirm the concept of the surface-associated existence of *Bdellovibrio* in the natural environment and serve as a basis for the employment of predatory bacteria to solve the problems of public health, biological protection of ecosystems, and bioterrorism protection.

Key words: ecology of *Bdellovibrio*, survival strategy, bdelloplast resistance.

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The extent of anthropogenic impact on the biosphere has likely exceeded the capability of ecosystems for spontaneous self-purification; novel biotechnologies are therefore developed for the degradation of detrimental substances. At the same time, massive microbial contamination of the environment is possibly even more ecologically hazardous than chemical pollutants. At present, disinfection is the most conventional method for the removal of microbial pollution; it cannot, however, be applied everywhere. Moreover, some disinfectants, e.g., the most commonly used chlorine, are themselves pollutants and cause secondary contamination of the environment. The understanding that the most ecologically safe technologies are based on the mechanisms of self-recovery in nature has stimulated closer examination of these processes. It is known that self-purification of environments depends considerably on various physical and chemical factors, as well as on the physiological activity of natural microbial populations. Microbial communities possess specific mechanisms of self-regulation, in which predatory bacteria of the genus *Bdellovibrio* play a significant role in the preservation of microbial diversity and regulation of their population densities in nature [1]. Pathogenic bacteria occurring in water can promote the spread of infectious diseases, whereas predatory bacteria, being typical inhabitants of aquatic environments, are able to infect microbial pathogens and, therefore, diminish the risk of infections.

Why are bdellovibrios, which were isolated about 40 years ago [2], so remarkable? Their most significant feature is the ability to infect the other bacteria. Until recently, bdellovibrios were considered as inhabitants of aquatic ecosystems. However, this concept cannot explain the existence of *Bdellovibrio* in water bodies where the concentration of prey bacteria is much less than the critical level required for bdellovibrio reproduction (above 10^6 cells/ml). Therefore, another concept and new methods of investigations were required to solve this problem. As early as 1966, M. Shilo [3] asserted that in studies on the role of *Bdellovibrio* in nature, improper environments were investigated and incorrect suppositions were used. Since then, much time has been required to understand that bdellovibrios are inhabitants of solid surfaces rather than water bodies. Until now, the association of bdellovibrios with solid surfaces has been considered as a physical cell attachment; the issue of the predator–prey interaction under these conditions has not been discussed. Moreover, microbial colonization of solid surfaces was regarded as a strategy for protection against parasites, such as *Bdellovibrio* [4]. However, solid surfaces provide the most important condition for *Bdellovibrio* development, viz., the necessary density of the prey bacteria population, which is several orders of magnitude higher than that in the water phase. It is this idea that underlies our concept on the immobilization of *Bdellovibrio* cells as a strategy of their survival in the natural environment [5, 6]. According to our concept, solid surfaces are considered as renewable sources of the delivery of bdellovibrios into the water column; they regulate the density of predatory bacteria under conditions favorable for their development. This idea has received experimental support in our investigations and in the studies of other researchers.

The aim of this work was to compare the survival rates of free-living and surface-associated predatory bacteria of the genus *Bdellovibrio* in the presence of

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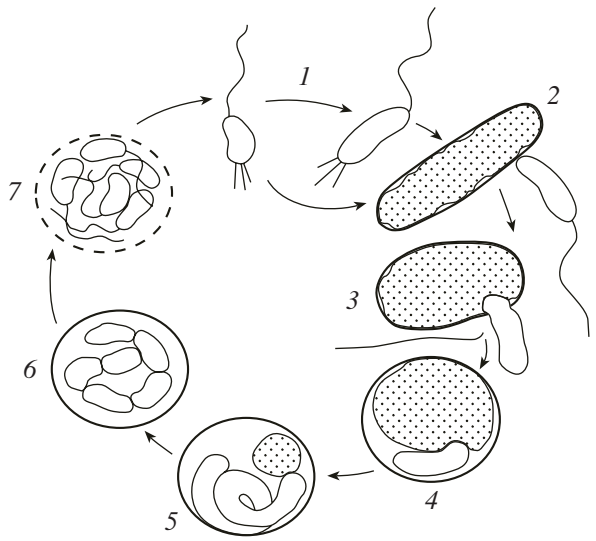

Fig. 1. Stages of the life cycle of *Bdellovibrio*: *1*, free bdellovibrios; *2*, attachment of the bdellovibrio to the cell of prey bacterium; *3*, penetration of the bdellovibrio into the cell of prey bacterium (formation of bdelloplast); *4* and *5*, development of the bdellovibrio descendants inside the cell of prey bacterium (bdelloplast); *6*, division of the bdellovibrio cells; *7*, lysis of the cell of prey bacterium and liberation of daughter bdellovibrio cells.

cadmium and diuron, important environmental pollutants.

MATERIALS AND METHODS

Strain *Bdellovibrio bacteriovorus* 100 NCJB 9529 was kindly provided by Prof. H. Stolp (Germany) [7]. Strain *Pseudomonas fluorescens* VKM-1471 was used as a host bacterium. The cultivation of *B. bacteriovorus* and *P. fluorescens* and the study of their interactions were performed as described earlier [8, 9]. Commercial preparations of $CdCl_2$ (Sigma, Germany) and diuron (3-(3,4-dichlorophenyl)-1,1-dimethylurea) (Sigma, Germany) were applied as pollutants. $CdCl_2$, dissolved in distilled water and diuron dissolved in acetone were sterilized by filtration through 0.1-μm-pore-size filters (Nalgene, United States). Acetone-containing solutions were used as a control. The interactions of bdellovibrios and pseudomonads were studied in liquid media containing diuron or $CdCl_2$ (10 μg/ml each) at 25°C for 15 days with regular measurements of the number of predatory *B. bacteriovorus* and prey *P. fluorescens* cells per 1 ml of suspension. The number of *B. bacteriovorus* cells and bdelloplasts was calculated from the number of plaque-forming units by the double-layer agar method [7]. The number of *P. fluorescens* cells was determined by counting the colonies grown on tryptone–soybean agar (TSA) (BBL MS, Becton Dickinson, United States). The results were expressed in colony-forming units (CFU).

The effect of pollutants on the surface-grown cells of the binary bacterial system was studied by immobilizing cells of *B. bacteriovorus* and *P. fluorescens* on transparent plastic disks 12 mm in diameter (Fisher Scientific, United States) as described previously [8]. The disks with the immobilized binary cultures were immersed in 3 ml of distilled water containing either $CdCl_2$ (10 and 50 μg/ml) or diuron (10 and 50 μg/ml) and incubated at room temperature for 15 days. At one-day intervals, the number of bdellovibrio cells (both free and intracellular) and uninfected pseudomonad cells attached to the plastic surface was determined after cell staining with acridine orange as described earlier [8].

Microscopic observations were performed with the use of an Axioscope-40 light microscope (Zeiss, Germany) and a B×60 fluorescence microscope (Olympus Optical LTD, Japan).

The data are presented in the paper as the mean results of experiments performed in four replicates.

RESULTS AND DISCUSSION

The study of the effect of pollutants on the activity of *Bdellovibrio* is important for the elucidation of the protective role of solid surfaces in the ecology of bdellovibrios. The successive stages of the life cycle of *Bdellovibrio* cells in the course of their interaction with a prey bacterium are schematically shown in Fig. 1. At first, the bdellovibrio cell attaches itself to the cell wall of the prey bacterium; then it penetrates into periplasm, where its intracellular development occurs; the final stage involves lysis of the infected cell and liberation of numerous bdellovibrio descendants. In this case, the morphology of the infected cell changes from rod-shaped to spherical, named bdelloplast. According to our scheme (Fig. 2), the occurrence of intracellular bdellovibrios within bdelloplasts covered with the envelope of the dead prey cell is not a transient ecological niche, but rather the most important functional stage of the infectious cycle of the parasite. Surface-associated bdelloplasts either integrate into an already organized cell community or synthesize their own polymeric matrix, which can be colonized by other bacteria. The newly formed descendants of the parasites either continue to infect the prey cells incorporated into the polymeric matrix or migrate into a water system, where their fate will depend on the occurrence of prey bacteria. Bdelloplasts can exist in the surface-associated state until the concentration of prey cells becomes insufficient for the *Bdellovibrio* development. We revealed that when the concentration of prey bacteria is reduced below 10^5 cells/ml, or under other unfavorable conditions (extreme temperature, the presence of pollutants, etc.), the terminal stages of the infectious cycle is inhibited and the parasitic cells are sealed in the bdelloplast [6, 9, 10]. In this work, the effect of cadmium and diuron on the survival of predatory bacteria was studied by comparing the viabilities of both free-living

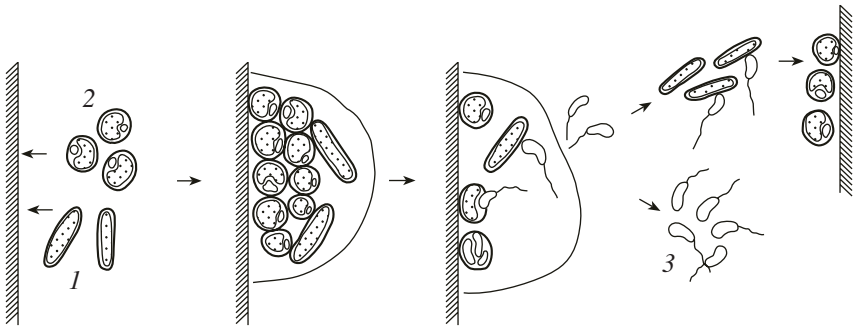

Fig. 2. Model of the co-existence of predatory bacteria and their prey in ecosystems: association of bdellovibrios and their prey with solid surface; predator–pray interaction within polymeric matrix; liberation of bdellovibrios; searching for new prey and attachment of bdelloplasts to other surfaces. *1*, prey bacteria; *2*, bdelloplasts; *3,* predatory bacteria.

and immobilized bdellovibrio cells in the presence of these pollutants.

The interaction of free-living bdellovibrios and *P. fluorescens* cells was studied by measuring the dynamics of cell numbers in binary liquid cultures grown for 15 days with and without pollutants. As can be seen from Fig. 3, the growth curves of both bacteria in the mixed predator–prey culture showed oscillatory behavior, which was more pronounced in the control than in the presence of pollutants. In the control (Fig. 3a), the population of predatory bacteria remained constant for 7–10 days, whereas the concentration of prey cells decreased; however, in the course of further cultivation (up to 15 days), the number of bdellovibrio cells dropped more rapidly than that of pseudomonads. In the experimental variants (Figs. 3b and 3c), both pollutants (cadmium and diuron) caused a gradual decrease in the cell number of both bacteria beginning from the third day of their interactions. Thus, it can be stated that xenobiotics considerably decreased the physiological activity of bdellovibrios and the predator–prey interactions dropped after 3–4 days of cultivation; this observation is in line with our previous results and the data available in the literature [6, 9–11].

According to our concept of the surface-associated pattern of predatory bacteria existence, the method of cell immobilization allows the study of the bdellovibrio survival under conditions close to those in their natural environments [8]. The interaction of bdellovibrios with pray bacteria immobilized on solid surface was studied by measuring their numbers in binary bacterial systems for 15 days after their attachment to the solid surfaces (Fig. 4).

The time courses of the cell numbers of free bdellovibrios, bdelloplasts, and intact pseudomonads cultivated in the absence of pollutants (Fig. 4a) indicated that the interactions between the predator and prey cells proceeded throughout the whole period of observation. The interactions between surface-associated cells of *B. bacteriovorus* and *P. fluorescens* in the presence of different concentrations of $CdCl_2$ (Fig. 4b) and diuron (Fig. 4c) differed considerably from the pattern

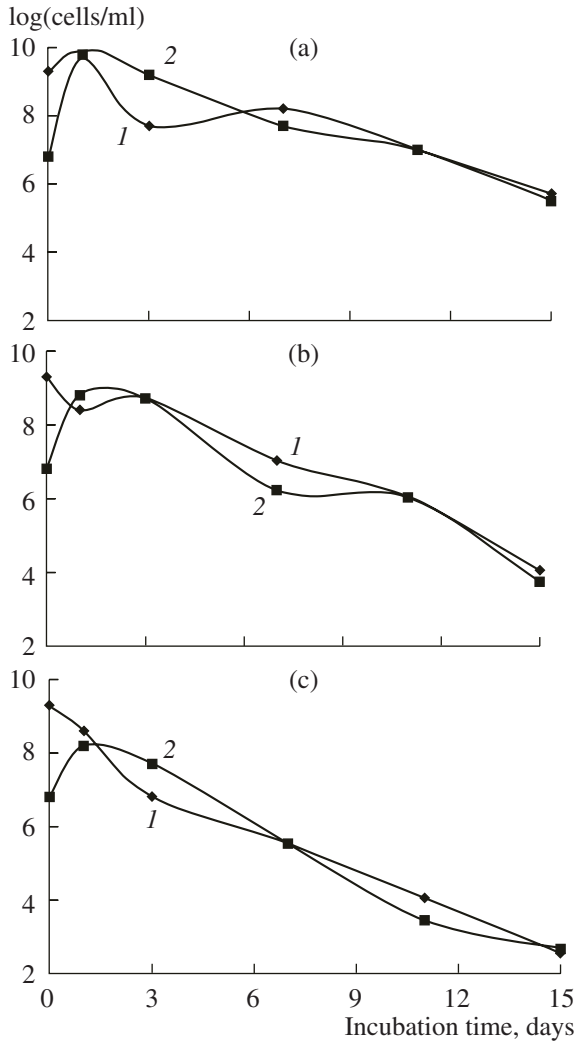

Fig. 3. Effect of cadmium and diuron on the viability of free-living *B. bacteriovorus* (in the binary culture with *P. fluorescens*). Dynamics of interaction of *P. fluorescens*) (*1*) and *B. bacteriovorus* (*2*) in the absence of xenobiotics (control) (a); in the presence of 10 μg/ml of $CdCl_2$ (b); in the presence of 10 μg/ml of diuron (c).

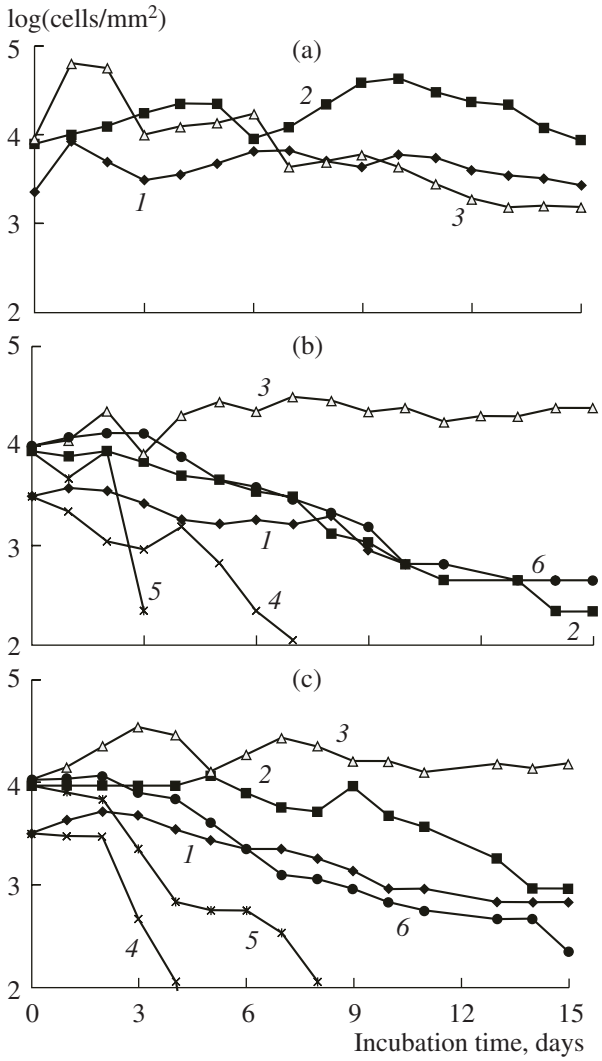

Fig. 4. Effect of cadmium and diuron on the viability of the surface-immobilized binary *B. bacteriovorus–P. fluorescens* culture. (a) Dynamics of bacterial interactions in the absence of xenobiotics (control): *P. fluorescens*) (*1*); *B. bacteriovorus* (*2*); bdelloplasts (*3*). Dynamics of bacterial interactions in the presence of 10 and 50 μg/ml of (b) $CdCl_2$ and (c) diuron. Curve designations in the presence of 10 μg/ml of xenobiotics: *P. fluorescens* (*1*); *B. bacteriovorus* (*2*); bdelloplasts (*3*). Curve designations in the presence of 50 μg/ml of xenobiotics: *P. fluorescens* (*4*); *B. bacteriovorus* (*5*); bdelloplasts (*6*).

observed in liquid culture (Fig. 3). Unlike liquid culture, the interactions between surface-associated cells of predatory and prey bacteria in the presence of the same concentrations of $CdCl_2$ and diuron (10 μg/ml) occurred throughout the whole period of 15-day cultivation (Figs. 4b and 4c). The oscillations in the cell number of bdellovibrios, *P. fluorescens*, and bdelloplasts indicate repeated cycles of predator–prey interactions on the plastic surface. It is noticeable that in the presence of pollutants, the number of bdelloplasts exceeded the cell number of free bdellovibrios due to the ability of predatory bacteria to block the terminal stages of their own infectious cycle and subsist for a long time inside the host cells under unfavorable conditions. This finding confirms our earlier supposition [9, 10] that bdellovibrios survive unfavorable conditions inside dead prey cells in the stage of bdelloplasts. It should be noted that bdellovibrios survived in the form of bdelloplasts even at concentrations of xenobiotics as high as 50 μg/ml, although in this case no interactions between predatory and prey bacteria was observed. As is seen from Figs. 4b and 4c, the cell number of free bdellovibrios and pseudomonads dropped drastically after three days of cultivation, whereas the number of bdelloplasts decreased gradually; this finding also confirms the ability of *Bdellovibrio* to survive inside dead host cells. Therefore, the cell walls of bdelloplasts performed a protective function in the adaptation process and shielded predator cells from the damaging action of xenobiotics. Moreover, the bdelloplast capacity to attach to surfaces also contributed to the survival of predatory bacteria.

The employment of transparent plastic disks and epifluorescence microscopy allowed us to visually observe the dynamics of the intercellular interactions on the solid surface and to calculate the cell numbers in the course of the experiment. The conversion of the pseudomonad cells into bdelloplasts was revealed after 48 h of their interaction with bdellovibrios in the absence of xenobiotics (Fig. 5a) and in the presence of $CdCl_2$ (10 and 50 μg/ml) (Figs. 5b and 5c). In all cases, the bacterial systems contained three components, viz., intact prey cells and bdellovibrios, both free and intracellular. In the absence of xenobiotics, the distribution of cells on the solid surface was uniform, whereas cadmium chloride (10 μg/ml) induced the aggregation of the cells (predominantly bdelloplasts) (Fig. 5b); the number of these aggregates increased with an increase in $CdCl_2$ concentration up to 50 μg/ml. The observed cohesion of bdelloplasts can be considered as a protective response of bdellovibrios to the addition of xenobiotics; this phenomenon confirms both the role of bdelloplasts in bdellovibrio survival and the protective functions of adhesion and cohesion, since surface-associated or aggregated cells survive better under unfavorable conditions. Earlier, we demonstrated that bdelloplasts exhibited more pronounced adhesion properties than cells of prey bacteria and free bdellovibrios [6, 9, 10]. This is why in the predator–prey bacterial cultures, bdelloplasts prevailed in the cell conglomerates under unfavorable conditions, when the number of prey bacteria became low. The cohesion of cells was accompanied by excretion of polysaccharides or glycoproteins from bdelloplasts; the excreted substances promoted the bdelloplast sticking (Fig. 2). It is known that microbial adhesion and cohesion are governed by the same mechanisms [4]. Consequently, the aggregation of *Bdellovibrio* cells and the formation of cell conglomerates dominated by bdelloplasts in immobilized predator–prey systems in the presence of pollutants may

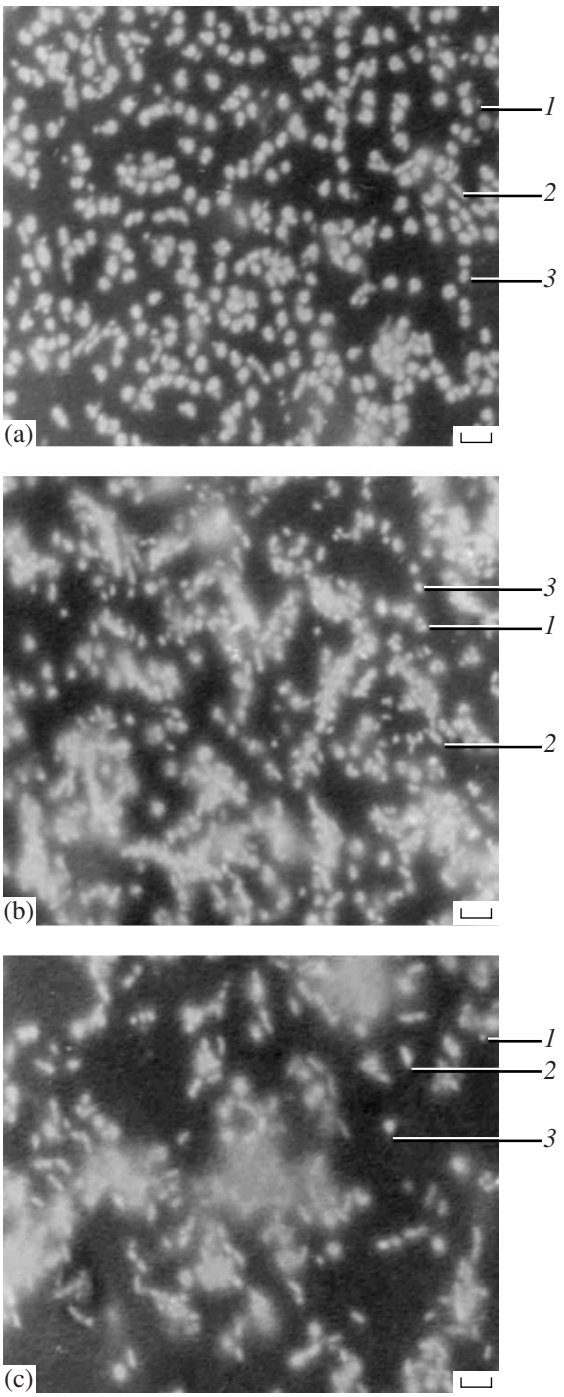

Fig. 5. Surface-immobilized cells of *B. bacteriovorus* and *P. fluorescens* after 48 h of incubation: (a) in the absence of pollutants uniform cell distribution of bdellovibrios (predominantly in the stage of bdelloplasts) and prey bacteria was observed; (b) in the presence of 10 μg/ml of $CdCl_2$, the formation of cell conglomerates was observed; (c) in the presence of 50 μg/ml of $CdCl_2$, formation of cell conglomerates intensified. *B. bacteriovorus* (*1*); *P. fluorescens* (*2*); bdelloplasts (*3*). The cells were stained with acridine orange. The bar represents 10 μm.

serve as a protective response of predatory bacteria to unfavorable conditions.

Thus, the results obtained demonstrate that free-living and immobilized cells of *Bdellovibrio* exhibit different susceptibility to detrimental factors; this is an indication of the protective role of attachment to solid surfaces for the survival of bdellovibrios. It is known that the attachment of bacteria to surfaces can influence the expression of some bacterial genes and affect cell behavior, enhancing cell resistance to antimicrobial agents [12, 13]. Our results are in good agreement with the data of Williams et al. [14], who showed that in the natural environment, *Bdellovibrio* cells attached to solid surfaces grow more intensely. The number of bdellovibrios is several orders of magnitude more intensively than in a water layer.

To conclude, our results and the data obtained recently by other authors [14–16] completely alter the present concept of the life and ecology of predatory bacteria of the genus *Bdellovibrio* and can be used as a basis for the application of bdellovibrios to the solution of problems of medicine, food conservation, and bioprotection of ecosystems from microbial pollution.

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