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EXPERIMENTAL ARTICLES

A Comparative Study of the Effect of Certain Pollutants on Free-living and Immobilized *Bdellovibrio*

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Abstract—The paper deals with a comparative study of the growth of free-living and immobilized predatory bacteria of the genus *Bdellovibrio* in the presence of toxic concentrations of urea and phenol. It was found that the cell wall of bdelloplasts plays a protective role in the adaptation of bdellovibrios to xenobiotics. The attachment of bdellovibrios to solid surfaces allows them to survive under unfavorable environmental conditions.

Key words: *Bdellovibrio* ecology, *Bdellovibrio* association with surfaces, *Bdellovibrio* viability.

It is known that the self-reclamation of the environment is due to the activity of natural microbial populations and depends on a number of physical and chemical factors. The predatory bacteria of the genus *Bdellovibrio* are considered to be responsible for the maintenance of a microbial equilibrium in nature [1]. Liquid bdellovibrio cultures show a high susceptibility to various physical and chemical agents [2–4], which suggests that the pollution of the environment with xenobiotics may affect the processes of self-reclamation because of the suppression of the functional activity of bdellovibrios and the related disturbance of natural microbial populations. On the other hand, bdellovibrios were detected in the wastewater of an aeration station in St. Petersburg, where the concentrations of detergents, heavy metals, and other toxicants were fairly high [5]. Furthermore, the concentration of prey bacteria in many bodies of water is insufficient to provide for the development of bdellovibrios. All this made it necessary to reconsider the generally accepted mechanism of the survival and ecological role of *Bdellovibrio* in natural habitats. The recent concept of the bipartite predator–prey system occurring in an immobilized state seems to be the most adequate model of the life of *Bdellovibrio* in nature [6]. This concept agrees well with the experimental data showing a better survival of *Bdellovibrio* in agar layers than in liquid cultures [7]. It is possible that the bdellovibrios grown in agar layers not only have a higher resistance to stressful agents (pollutants) but can also better retain their virulence. Solid surfaces probably allow the attached bdellovibrios to maintain their populations and to control the populations of prey bacteria in the aquatic environment.

The aim of this work was to study comparatively the survival dynamics of free-living and solid surface–immobilized bacteria of the genus *Bdellovibrio* in the presence of such pollutants as urea and phenol.

MATERIALS AND METHODS

Experiments were carried out with the predatory bacterium *Bdellovibrio bacteriovorus* 100 (NCIB 9529) isolated from soil [8] and with the prey bacterium *Pseudomonas fluorescens* VKM B-1471. The bacteria were cultivated as described earlier [7].

Experiments to study the effect of pollutants were carried out with phenol (Fisher Scientific, United States) and urea (BDH, United Kingdom). Phenol and urea were dissolved in distilled water, and the solutions were sterilized by filtration through 0.1-μm-pore-size Nalgene TM filters (Nalgene, United States).

The effect of the pollutants on the interaction of bdellovibrios and pseudomonads was studied by incubating these bacteria in a liquid medium containing 0.5% urea and 0.01% phenol at 25°C for 15 days, with regular measurements of the number of predatory *B. bacteriovorus* and prey *P. fluorescens* cells per ml of the culture liquid. The number of *B. bacteriovorus* cells and bdelloplasts was determined in terms of the number of plaque-forming units (PFU) by the double-layer agar method [8]. The number of *P. fluorescens* cells was determined by counting the colonies grown on tryptone–soybean agar (BBL MS, Becton Dickinson, United States). The results were expressed in colony-forming units (CFU).

The effect of the pollutants on the surface-grown bdellovibrios and pseudomonads was studied by immobilizing the bacteria on transparent plastic disks 12 mm in diameter (Fisher Scientific) as described previously [6]. The disks with the immobilized bacteria were immersed in 3 ml of distilled water containing either 0.5% urea, 0.01% phenol, or 0.5% phenol and incubated at room temperature for 15 days. At one-day intervals, the number of intact bdellovibrios, bdelloplasts, and uninfected pseudomonads attached to the

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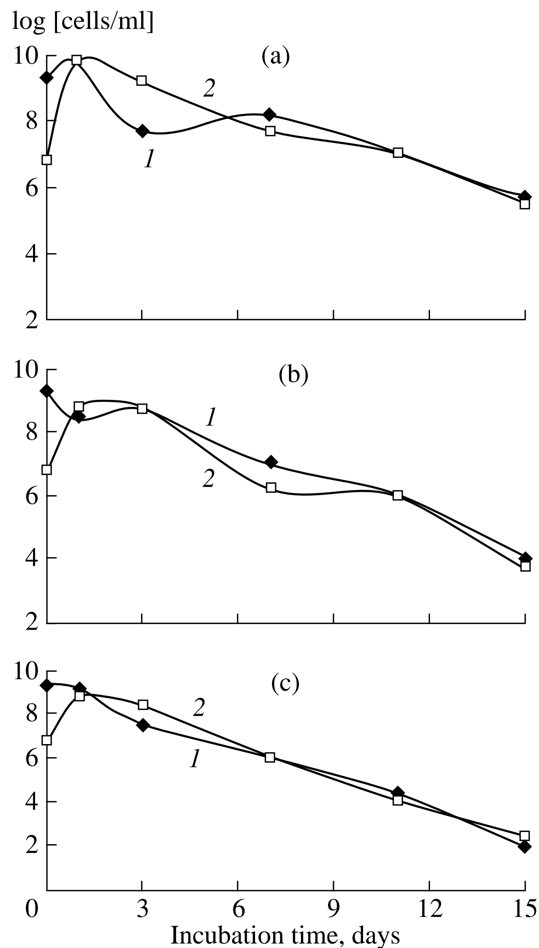

Fig. 1. The population dynamics of (*1*) uninfected *P. fluorescens* cells and (*2*) *B. bacteriovorus* free-living cells and bdelloplasts in (a) the control liquid culture and in the presence of (b) 0.5% urea and (c) 0.01% phenol.

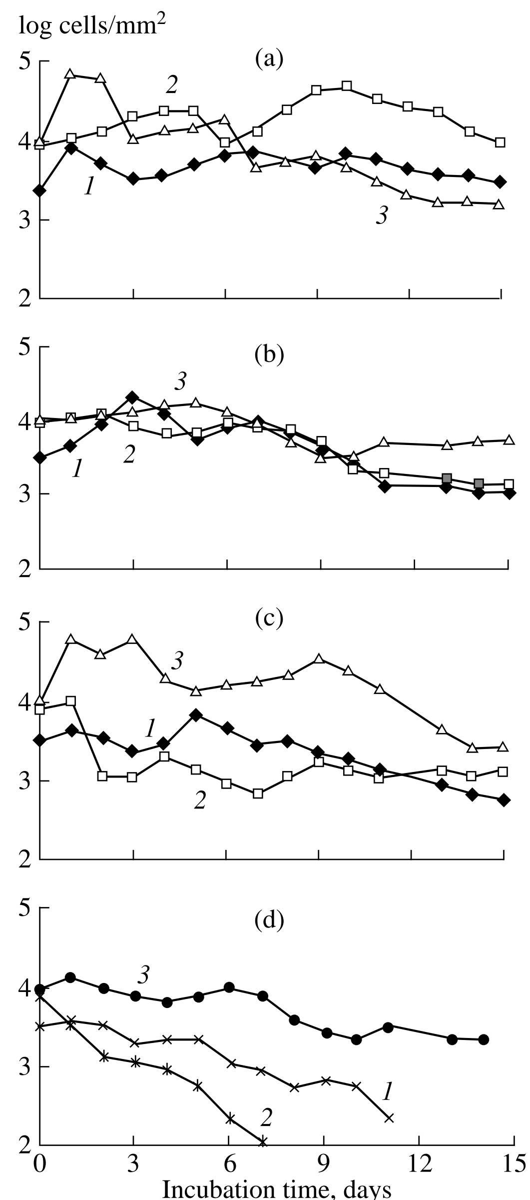

Fig. 2. The population dynamics of (*1*) uninfected *P. fluorescens* cells, (*2*) intact *B. bacteriovorus*, and (*3*) bdelloplasts immobilized on the plastic surface and incubated in (a) water and in the presence of (b) 0.5% urea, (c) 0.01% phenol, and (d) 0.5% phenol.

plastic surface was determined by the epifluorescence microscopy of cells stained with acridine orange [6].

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

RESULTS AND DISCUSSION

The effect of urea and phenol on bdellovibrios was studied by comparing the viabilities of bdellovibrio cells living free in liquid bicultures and those immobilized on the plastic surface.

Figure 1a shows the population dynamics of *P. fluorescens* and *B. bacteriovorus* cells incubated in the control liquid biculture without pollutants. During 7–10 days of incubation, the population of predatory *B. bacteriovorus* cells showed a typical oscillatory behavior, whereas the population of prey *P. fluorescens* cells gradually decreased. In the course of further cultivation, the number of both predatory and prey cells tended to decrease. In the test bicultures, which were incubated in the presence of 0.5% urea (Fig. 1b) or 0.01% phenol (Fig. 1c), the predator and prey populations tended to decrease beginning from the 3rd day of incubation, the effect of phenol being more profound than that of urea. Urea and phenol considerably diminished the physiological activity of bdellovibrios, so that the predator–prey interactions in the biculture were getting weaker after 6 days of incubation, which is in agreement with the data available in the literature [2–4, 7].

The effect of immobilization on the interaction dynamics of predatory and prey bacterial cells was studied earlier within 24 h [6]. Extending the time of measurements to 15 days in current experiments

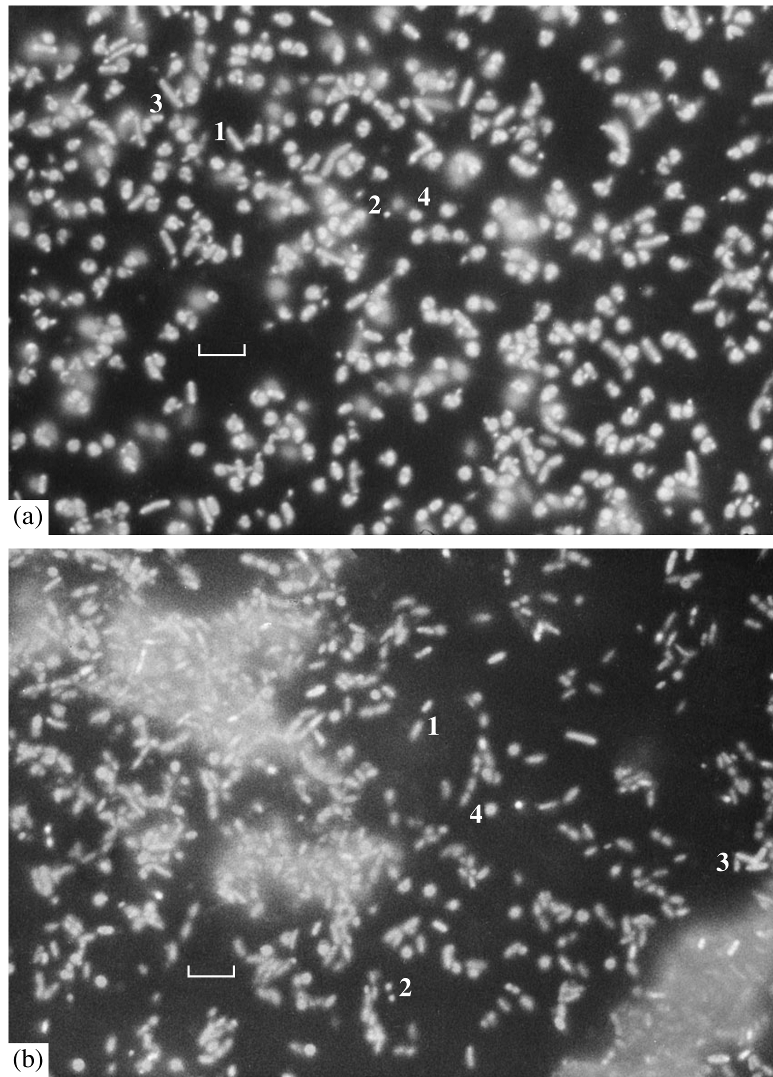

Fig. 3. The epifluorescence microscopy of the *P. aeruginosa–B. bacteriovorus* biculture immobilized on the plastic surface and incubated (a) in the absence of pollutants and (b) in the presence of 0.01% phenol: (1) intact *B. bacteriovorus* cells, (2) uninfected *P. fluorescens* cells, (3) interacting *B. bacteriovorus* and *P. aeruginosa* cells, and (4) bdelloplasts. Cells were stained with acridine orange.

allowed me to study the long-term dynamics of the predator–prey interactions of immobilized *B. bacteriovorus* and *P. fluorescent* cells. The employment of transparent plastic substrates and epifluorescence microscopy made it possible to visually observe the dynamics of the interactions on the plastic surface throughout the cultivation period.

The behavior of curves showing the number of intact bdellovibrios, bdelloplasts, and uninfected pseudomonads in the control immobilized biculture (Fig. 2a) indicated that *B. bacteriovorus* and *P. fluorescens* cells interacted throughout the observation period. In the presence of the same concentrations of urea (Fig. 2b) and phenol (Fig. 2c) as were used in the case of liquid bicultures (Fig. 1), the predator–prey interactions in the immobilized biculture also persisted throughout the observation period (15 days), with the populations of predatory and prey cells showed a typical oscillatory behavior. Interestingly, in the presence of the pollutants, the number of bdelloplasts exceeded the number of intact bdellovibrios, which can be explained by the ability of predator bacteria to block the terminal stages of their life cycle in order to survive extreme environmental conditions inside the host cells. This speculation is in line with the supposition that bdellovibrios can survive unfavorable conditions inside killed prey cells, i.e., in the state of stable bdelloplasts [7, 9].

It should be noted that bdellovibrios occurring in the state of stable bdelloplasts in the immobilized biculture were able to survive exposure to 0.5% phenol, although this concentration of phenol completely suppresses the typical predator–prey interactions between *B. bacteriovorus* and *P. fluorescens* (Fig. 2d). After 3 days of incuba-

tion of this immobilized biculture, the number of intact bdellovibrios and uninfected pseudomonads drastically fell, but the number of bdelloplasts remained at a sufficiently high level (Fig. 2d). These results provide further evidence that the cell wall of bdelloplasts acts as a protective barrier to defend the intracellular bdellovibrios against xenobiotics, while the immobilization of bdelloplasts on the solid surface adds to the protective effect.

Figure 3 illustrates the effect of 0.01% phenol on the conversion of pseudomonads into bdelloplasts 24 h after the addition of bdellovibrios. A comparison of Figs. 3a (control) and 3b shows that 0.01% phenol did not prevent the formation of bdelloplasts but changed the distribution of cells on the plastic surface. Specifically, 0.1% phenol induced the aggregation of cells (predominantly bdelloplasts) (Fig. 3b). The cohesion of bdelloplasts can be considered to be another protective response (in addition to the very formation of bdelloplasts) of bdellovibrios to the xenobiotic. It can be suggested that the attachment of bdelloplasts to each other or to a solid surface provides for a better survival of the intracellular bdellovibrios. Earlier studies [10] showed that bdelloplasts possess a high adhesive capacity and dominate cell aggregates produced by predator and prey bacteria under unfavorable conditions. Cell cohesion is accompanied by the secretion of polysaccharides or glycoproteins from bdelloplasts, the secreted substances sticking together the bdelloplasts. It is known that microbial adhesion and cohesion are governed by the same mechanisms [11]. Consequently, the aggregation of bdellovibrios and the formation of cell conglomerates dominated by bdelloplasts in immobilized predator–prey systems in the presence of xenobiotics may serve as the protective responses of predatory bacteria.

To conclude, the experiments demonstrated that surface-immobilized predatory bacteria are less susceptible to the toxic pollutants urea and phenol than free-living bacteria, which suggests that immobilization on solid surfaces plays a protective role. The attachment of bacteria to surfaces can influence the expression of some bacterial genes and the behavior of the immobilized bacteria [12]. It is also known that bacteria occurring in biofilms are more resistant to antimicrobial agents than the same free-living bacteria [13]. The results of the relevant laboratory experiments agree well with the in situ experiments of Williams *et al.* [14], who showed that bdellovibrios attached to solid surfaces in the aquatic environment multiply several orders more intensely than the bdellovibrios occurring in the main water mass. The good agreement between the in situ and laboratory experiments supports the supposition that immobilization on solid surfaces provides for a better survival of bdellovibrios and contributes to our understanding of the ecology of *Bdellovibrio.*

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