# Periodic Culturing of Nonculturable *Yersinia Pseudotuberculosis* Forms

**L. S. Buzoleva**

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 129, No. 4, pp. 444-447, April, 2000
Original article submitted November 15, 1999

*Yersinia pseudotuberculosis* survive no longer than 30 days during periodic culturing in nutrient medium at 37°C. Our experiments demonstrated transformation of these bacteria into resting (nonculturable) forms after one month in culture. Resting cells are more viable than dividing cells and under certain conditions can revert to the initial (proliferative) state.

**Key Words:** *Yersinia pseudotuberculosis; periodic culturing; nonculturable forms; reversion*

*Yersinia pseudotuberculosis* can live in both warm-blooded organisms and environmental objects and combine saprophyte and parasitic lifestyles. It should be noted that adaptive variability of these bacteria ensuring their survival are little studied. Recent studies showed that some pathogenic bacteria can survive in the environment in nonculturable forms, which do not grow in common nutrient media, but can revert to growing forms under the action of some factors. These resting, or nonculturable forms [8,12] are now identified in some pathogenic bacteria, including *Y. pseudotuberculosis*. However, most recent studies were focused on evaluation of the possibility of Yersinia conversion into nonculturable forms and the dynamics of their propagation in soil and plants by using polymerase chain reaction (PCR) [2]. This method can detect the agent in the medium when it cannot be detected by common bacteriological inoculations. However, PCR cannot be used to study the properties of nonculturable bacteria, and therefore new methods for obtaining nonculturable forms of bacteria and their revertants in liquid nutrient media in periodic culture for further studies are needed. We investigated the possibility of obtaining nonculturable *Y. pseudotuberculosis* in liquid nutrient medium in periodic culture at 37°C.

Institute of Epidemiology and Microbiology, Siberian Division of the Russian Academy of Medical Sciences, Vladivostok

## MATERIALS AND METHODS

*Y. pseudotuberculosis* strains 512, H-2781, 907, 282, 3515 were obtained from All-Russian Pseudotuberculosis Center (Institute of Epidemiology and Microbiology). The strains had typical morphological, cultural, biochemical, and serological characteristics.

*Y. pseudotuberculosis* were grown in periodic culture in 250 ml liquid nutrient broth prepared from Hottinger hydrolysate at 37°C. The bacteria were gradually added to nutrient broth, the density of bacterial cells was evaluated by turbidity standards. The initial concentration of bacteria was 1000 cells/ml medium. The dynamics of *Y. pseudotuberculosis* propagation during periodic culturing was evaluated by counting bacterial cells in the medium using different methods every hour during the first 48 h and then once a day for 3.5 months, and growth curves were plotted.

The total number of viable cells (colony-forming and resting-nonculturable cells) was evaluated using the inhibitory penicillin method by determining the relative content of elongated live cells and nonelongated dead cells [5] and by fluorescent microscopy of acridine orange-stained preparations (5 min, 0.05% in phosphate buffer saline, pH 6.0). Viable cells fluoresced bright emerald-green and dead cells were bright orange-red [7,13]. Additionally, the total counts of live and dead bacteria were determined on Gram-stained smears under a light microscope [4].

Bacterial cells were counted using a Gazhenko chamber and an ocular with a grid micrometer (×10);

0007-4888/00/0004-374$25.00 

at least 20 visual fields with the total area of 20,000 $\mu^2$ were counted. In each visual field four small squares on the diagonal were counted. Differences between the groups were analyzed using Student's *t* test.

For detecting live colony-forming cells, 0.1 ml liquid medium was inoculated in the known dilutions to Petri dishes with nutrient agar and cultured for 24 h at 37°C. In parallel, limiting dilution analysis was used for precise evaluation of the number of dividing viable cells in the studied nutrient media, and the results were recorded using standard Mac-Credi tables [3].

Proliferating bacteria are larger than nonculturable cells [6,8,14], and therefore the latter were obtained by step-by-step filtration of *Y. pseudotuberculosis* culture grown at 37°C: 10 ml inoculate collected at the stage of bacterial death was filtered through a 0.45-μ Millipore filter (filtrate 1, F1); F1 was filtered through a 0.22-μ filter and filtrate 2 (F2) was then filtered through a 0.1-μ filter (F3). An aliquot of inoculate taken at the stage of bacterial death treated with 200 μg/ml chloramphenicol and inhibiting vegetative bacterial cells [8] was used as the control.

The number of colony-forming bacteria in F1, F2, and F3 and the ratio of live/dead cells was evaluated by the penicillin method and fluorescent microscopy. The presence of nonculturable forms in the filtrates was verified by restorative culturing at 37, 18-20, and 4-6°C [6,8]. Typical biological properties of revertants were restored during successive passages on nutrient agar. Morphology of Yersinia revertant colonies was determined on Serov's differential diagnostic medium. Bacterial morphology was studied on Gram-stained preparations. Biochemical properties were investigated using Hiss' sugar series. Serological properties were studied by agglutination test with specific sera.

## RESULTS

After periodic culturing in Hottinger broth at 37°C *Y. pseudotuberculosis* formed colonies during 30 days (Fig. 1). Fluorescent microscopy and penicillin method still detected live cells for the next 2 months. We assume that these cells were viable undividing resting *Y. pseudotuberculosis* cells.

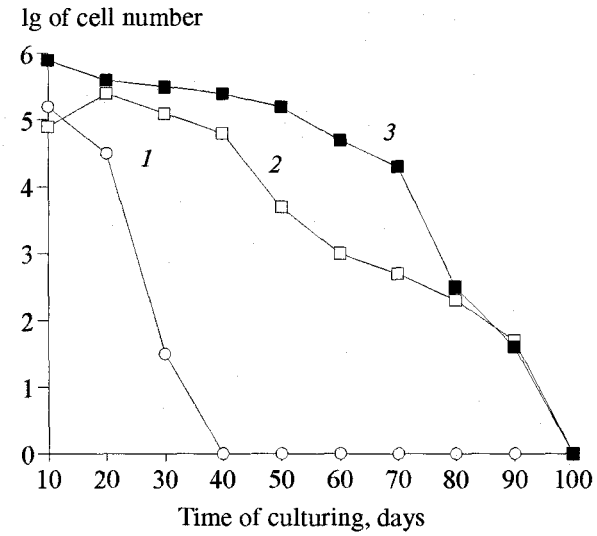


**Fig. 1.** Dynamics of *Y. pseudotuberculosis* (strain H-2781) multiplication in nutrient broth during periodic culturing at 37°C. *1*) CFU/0.1 ml; *2*) fluorescent microscopy (acridine orange staining); *3*) microcultural inhibitory penicillin method.

In further tests a 40-day culture was used, because this term (according to fluorescent microscopy data, Fig. 1) coincided with the stationary growth phase, when the number of dead and live cells was the same. Dead cells predominated from day 70 to day 90, while the culture died (all cells fluoresced orange) by the end of the 3rd month. Fluorescent microscopy data agree with the results of the microcultural inhibitory penicillin method. Hence, the culture remained viable for 3 months, but not for 30 days as was judged from the presence of colony-forming cells.

To confirm these findings, a 40-day bacterial culture was successively filtered through 3 filters with different pore sizes. F1, F2, and F3 were tested for the presence of colony-forming cells 3 times, and no live dividing cells were detected (by limiting dilution method and CFU count, Table 1). According to fluores-

**TABLE 1.** Isolation of Nonculturable *Y. pseudotuberculosis* Forms by Successive Filtration through Bacterial Filters of Different Size (*M±m*)

| Test samples | Acridine orange staining, $10^4$ green cells | Penicillin treatment, $10^4$ elongated cells | CFU/0.1 ml reversed cells at 20°C, $\times 10^4$ |
|---|---|---|---|
| Initial culture in stationary phase (40-day) | 100±22 | 120±15 | — |
| F1 | 88±12 | 100±20 | 64±11 |
| F2 | 60±6 | 49±3 | 38±6 |
| F3 | 0 | 0 | 0 |

**Note.** *Y. pseudotuberculosis* were not detected in samples during estimation of CFU/0.1 ml and by the limiting dilutions method.

cent microscopy, F3 contained no bacteria. Evidently, these bacterial cells sized 0.45-0.22 μ.

The optimal temperature for recovery of nonculturable bacteria was 18-20°C, at which liquid medium became turbid on days 1-2. At 4-6°C turbidity was observed on day 10 of culturing, and at 37°C no growth was observed during 30 days. The number of revertant colonies on nutrient agar coincided with the number of fluorescing green cells in F1 and F2 and the number of elongated cells detected by the penicillin method (Table 1). Revertant colonies in Serov's medium differed by morphology and included S and R variants. Only 10-15% were S colonies (smooth with pronounced center and even edges), while 85-90% were R variants (rough granular colonies with scalloped peripheral zone or flat lacy edges of varying width). At this stage of recovery, the culture was polymorphic and presented by bacterial cells of different shape and size. No revertants was found in control samples treated with chloramphenicol.

Biochemical characteristics of revertants did not differ from the control (fresh broth culture), but exhibited weak agglutination (1:200 *vs.* 1:1600 in the control). Repeated second and third passages on nutrient agar completely restored agglutination. Morphological study showed that the revertants were more uniform, the bacteria became smaller approximating the control size; culturing in Serov's medium yielded S-form colonies.

During periodic culturing in nutrient broth at 37°C *Y. pseudotuberculosis* survive for 15-30 days depending on the strain [1,9,10]. We revealed that some cells do not die under these conditions, but transform in nonculturable resting forms with a longer viability in comparison with dividing cells, which should be taken into account in experimental microbiological studies and isolation of cultures from environmental objects.

The study was supported by the Russian Foundation for Basic Research (grant No. 97-4-48534).

## REFERENCES

1. L. S. Buzoleva, *Ecology of Pathogenic Bacteria* [in Russian], Novosibirsk (1994), pp. 144-150.
2. E. N. Emel'yanenko, M. Yu. Aksenov, A. L. Gintsburg, and V. Yu. Litvin, *Pathogenic Bacteria in Communities* [in Russian], Moscow (1994), pp. 135-139.
3. L. E. Korsh and T. Z. Artemova, *Rapid Methods for Sanitary Bacteriological Studies* [in Russian], Moscow (1978), pp. 110-111.
4. A. S. Labinskaya, *Microbiology and Technique of Microbiological Studies* [in Russian], Moscow (1978), pp. 47-53.
5. K. A. Lusta and B. A. Fikhte, *Methods for Evaluating Microorganism Viability* [in Russian], Pushchino (1990), pp. 51-52.
6. G. V. Mukamolova, *Formation and Biochemical Characteristics of Resting Forms of Nonsporulating Micrococcus Luteus*, Abstract of Cand. Biol. Sci. Dissertation, Moscow (1995).
7. D. M. Novogrudskii, *Microbiology of Soil* [in Russian], Alma-Ata (1956), pp. 225-229.
8. Yu. M. Romanova, *Nonculturable State of Pathogenic Bacteria as Exemplified by Salmonella typhimurium: Phenomenon and Genetic Control*, Abstract of Doct. Biol. Sci. Dissertation, Moscow (1997).
9. G. P. Somov, *Far-Eastern Scarlatiniform Fever* [in Russian], Moscow (1979), p. 31.
10. G. P. Somov, T. N. Varvashevich, and N. F. Timchenko, *Psychrophility of Pathogenic Bacteria* [in Russian], Novosibirsk (1991), pp. 26-27.
11. R. Steinier, E. Edelgerg, and J. Ingram, *World of Bacteria* [in Russian], Moscow (1979), vol. 2, pp. 40-43.
12. R. R. Colwell, P. R. Brayton, D. J. Grimes, *et al., Biotechnology,* **3,** 817-820 (1985).
13. Z. Darzynkiewicz, M. Andreeff, and G. Traganos, *Exp. Cell. Res.,* **115,** 31-35 (1978).
14. T. V. Votyakova, A. S. Kaprelyants, and D. B. Kell, *Apl. Environ. Microbiol.,* **60,** No. 9, 3284-3291 (1994).