Adaptation Variability of *Yersinia pseudotuberculosis* during Long-Term Persistence in Soil

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Adaptation changes in the morphological and biological characteristics of pathogenic *Yersinia pseudotuberculosis* inhabiting a model soil ecosystem for a long time were studied. Changes in cultural, biochemical, and morphological characteristics of Yersinia and a decrease or loss of virulence were observed under these conditions. The detected changes were phenotypical, *i. e.* several passages in common nutrient media restored the original characteristics of the strain.

Key Words: *Yersinia pseudotuberculosis; soil ecosystem; adaptation*

The life cycle of *Yersinia pseudotuberculosis*, an agent of sapronosis, includes, in addition to warm-blooded hosts, environmental objects, *e.g.* soil. Yersinia populations remaining in various types of soil for a long time are exposed to various abiotic and biotic environmental factors. The possibility of existence and multiplication of pathogenic bacteria under these conditions, so different from a warm-blooded host, suggests that they possess special adaptation mechanisms maintaining the population between epidemics.

We investigated adaptation potentialities of *Y. pseudotuberculosis* inhabiting a model soil ecosystem for a long time.

MATERIALS AND METHODS

Y. pseudotuberculosis strain H-2781 (serovar 1) was used in the study. This strain is characterized by typical cultural morphological, biochemical, and antigenic properties and was obtained from the collection of All-Russian Center for Yersiniasis and Pseudotuberculosis (Institute of Epidemiology and Microbiology, Vladivostok).

The variability of *Y. pseudotuberculosis* was studied in a model open-air macroecosystem (1-m^3 reservoir with nonsterile gardening soil) under natural meteorological conditions for 9 months. The soil was infected with bacterial suspension containing 10^9 CFU/ml (turbidity standard). The initial infective dose of bacteria in control inoculation from the soil was 6.2-6.5 Lg per ml soil suspension.

The bacteria were isolated from the soil using differential diagnostic growth media with rifampicin suppressing the growth of saprophytic soil microflora [3] and not modifying the multiplication of *Y. pseudotuberculosis* strain H-2781^{++} (serovar 1) carrying 4.4 and 48 MDa plasmids.

Changes in the bacterium number in the soil and in its biological characteristics were observed from March to November. Samples of soil infected with *Y. pseudotuberculosis* culture were collected monthly and the cultural, morphological, biochemical, and antigenic characteristics of soil strains and changes in their virulence were studied. The virulence was studied using Kerber's method on random-bred albino mice (14-16 g) infected intraperitoneally; LD_{50} and its confidence interval were calculated. Antigenic characteristics of the cultures were studied in the linear agglutination test with antisera prepared after rabbit immunization with the studied strains by the routine protocol. Enzyme activity was studied on solid Hiss' media, the reaction was evaluated by the size of color spot around the applied bacterial culture (in mm).

Final identification of modified Yersinia strains was carried out by PCR according to the Instruction

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on the Use of Diagnostic Kit for Detection of *Yersinia pseudotuberculosis* by DNA Amplification (NIARmedik, N. F. Gamaleya Institute of Epidemiology and Microbiology). Primers hybridizing with the invasiveness gene localized in the chromosome were chosen for the reaction: reverse primer 1: TAA GGG TAC TAT TAT CGC GGC and reverse primer 2: CGT CAA ATT AAC CGT CAC ACT, the size of the amplified fragment was 295 b. p.

RESULTS

The findings indicate that relatively high contamination of the soil with Yersinia (10^4-10^3 CFU/0.1 ml soil suspension) persisted during the entire spring and summer period (7 months) without changes in the morphological, cultural, and antigenic characteristics of the bacteria. However, the virulence of the culture inoculated in April 1993 (LD_{50} 1.2×10^4) was by one order of magnitude below the control (LD_{50} 1.1×10^3) and remained at this level until the end of September (Table 1).

Some changes in *Y. pseudotuberculosis* characteristics were detected at the end of September, when the mean daily temperature was 8-10°C (0°C at night). The rate of Yersinia isolation from the soil decreased 100-fold. The bacteria remaining in the soil for 7 months slower cleaved fructose, maltose, mannose, rhamnose, mannitol, esculine, and urea, did not cleaved xylose and salicin, but in contrast to the control strain acquired the capacity to cleave citrates (Kristensen, Simmons media) and sodium malonate (Table 2).

Morphological characteristics of the culture also changed. Colonies in agar looked like colonies of transitional R forms (with uneven scalloped edges, granular, opaque white). They were smaller (1-2 mm) than in the control (2-3 mm) and were difficult to detach with a bacteriological loop, which could be explained by specific features of growth in agar. In Serov's medium the colonies also looked atypical for Yersinia in comparison with the control: they were large, lustrous, had uneven edges and poorly expressed center. Gram staining gave negative results, but the colonies were twice as large (2-3 μ) as in the control (0.5-1.0 μ).

Antigenic properties of the soil variants of cultures also changed: agglutination titer with hyperimmune rabbit serum decreased from 1:800 to 1:200, the virulence of the culture was lower (1.1×10^6) in comparison with the control (1.1×10^3, $p<0.05$). This was, presumably, due to elimination of 48 MD virulence plasmid playing the most important role in the pathogenicity of Yersinia [6-8]. The soil variant of the strain lost the virulence plasmid after 6-month stay in the soil (Fig. 1). High summer temperatures can promote

TABLE 1. Virulence, Morphological and Antigenic Characteristics of *Y. pseudotuberculosis* Staying in Soil Ecosystem for a Long Time

Characteristic	Control*	March-August	September	October	November	November (reversion)
Agglutination titer	1:3200	1:800	1:200	1:200	1:100	1:400
LD_{50} and its range	$1.1*10^3$	$1.2*10^4$	$1.1*10^5$	$8.8*10^6$	$2.3*10^6$	$1.2*10^6$
	$2.6*10^2$-$0.8*10^3$	$4.8*10^4$-$2.3*10^5$	$2.6*10^5$-$3.8*10^6$	$4.5*10^6$-$2.3*10^7$	$5.4*10^6$-$7.7*10^7$	$3.8*10^6$-$6.3*10^7$
Morphology of colonies	Typical in S shape, 1-2 mm	Typical in S shape, 1-2 mm	S-R variants, 2-3 mm	S-R variants, 2-3 mm	R shape, 2-3 mm	S shape, 2-3 mm
Morphology of bacteria	Gram-negative bacilli, 0.5-1.0 μ	Gram-negative bacilli, 0.8-1.0 μ	Gram-negative bacilli, 2-3 μ	Gram-negative bacilli, 2-3 μ	Gram-negative bacilli, 3-4 μ in chains	Gram-negative bacilli, 1-2 μ
Activity of enzymes***:						
DNase	20.0±7.2	80.0±9.5	150.0±20.1	220±35	—**	—
lipase	50.0±4.8	60.0±4.2	160±31	220±30	—	—
urease	80.0±9.5	80.0±5.2	130±20	350.0±21.4	—	—
amylase	10.0±2.1	15.0±1.4	25.0±7.1	55.0±9.3	—**	—
Plasmid spectrum	48, 4.4 MD	4.4 MD	4.4 MD	4.4 MD	4.4 MD	4.4 MD

Note. *Rifampicin-resistant strain; ** not determined; ***diameter of reaction field in molecular mass.

elimination of the plasmid, because more than 90% *Y. pseudotuberculosis* loose the virulence plasmid after 15-20 *in vitro* passages at 36-37°C [2].

More profound shifts towards saprophyte degeneration were observed in the soil strain isolated in November. Just solitary colonies of Yersinia with typical R shape were inoculated in differential diagnostic media with delayed growth (2-3 days). The colonies grew into agar and were poorly detached with a bacteriological loop. Light microscopy showed that these colonies were formed by 3-5-μ gram-negative bacteria (2-3-fold larger than in the control) forming chains of different length. Marked polymorphism of the studied bacteria was observed during this period. Table 1 shows that the agglutination titer of *Y. pseudotuberculosis* after 9 months in soil decreased to 1:100 in comparison with the initial strain and the culture remained slightly virulent (2.3×10^6).

Biochemical characteristics also changed in the 9-month soil strain. It did not cleaved the majority of carbohydrates which were cleaved by the parental strain, including monosaccharides, trisaccharides, sugar alcohols, and glycosides. However it cleaved sodium malonate and citrates and utilized them as the source of carbon, similarly to 6-7-month soil strains. It was therefore difficult to identify the species of the strain isolated from the soil by traditional bacteriological methods, and we used genetic analysis. PCR confirmed that the isolated soil strains are *Y. pseudotuberculosis*.

Since the majority of sugars were not fermented by the 9-month soil strain which however cleave the substrata previously not utilized by the initial *Y. pseudotuberculosis* strain, we hypothesized that different metabolic pathways were triggered in the course of biosynthesis, depending on the conditions of the bacterium habitation. The production of enzymes corresponding to a certain metabolic pathway was induced [5].

This hypothesis is confirmed by the fact that activity of some enzymes in the 9-month soil strain was higher than in the initial strain. For example, lipase activity in the soil strain was 3-fold higher that in the

TABLE 2. Changes in Biochemical Characteristics of *Y. pseudotuberculosis* Staying in Nonsterile Artificially Infected Soil for a Long Time

Substrate	Control	Strain H-2781 inhabiting the soil				
		March-August	September	October	November	November (after reversion)
Glucose	+	+	+	+(7)	+(8)	+
Xylose	±	±	—	—	—	±
Fructose	+	+	+(4)	+(7)	—	+
Maltose	+	+	+(5)	+(8)	—	+
Salicin	+	+	—	—	—	+
Mannose	+	+	+(4)	+(7)	—	+
Arabinose	+	+	+	+(6)	—	+
Melibiose	±	±	±	—	—	±
Rhamnose	+	+	+(7)	+(8)	—	+
Mannitol	+	+	+(5)	+(7)	—	+
Sorbitol	—	—	+	—	—	—
Sodium malonate	—	—	+	+	+	—
Kristensen citrate	—	—	+	+	+	—
Simmons citrate	—	—	+	+	+	—
Lactose	—	—	—	—	—	—
Arginine	—	—	—	—	—	—
Sucrose	—	—	—	—	—	—
Lysine	+	—	—	—	—	+
Esculine	—	+	+(3)	+(6)	—	+(2)
Dulcite	—	—	—	—	—	—
Inositol	—	—	—	—		—
Urea	+	+	+(2)	+(5)		+

Note. "-" no reaction; "±" weak reaction; "+" pronounced reaction. Day of the reaction manifestation is shown in parentheses.

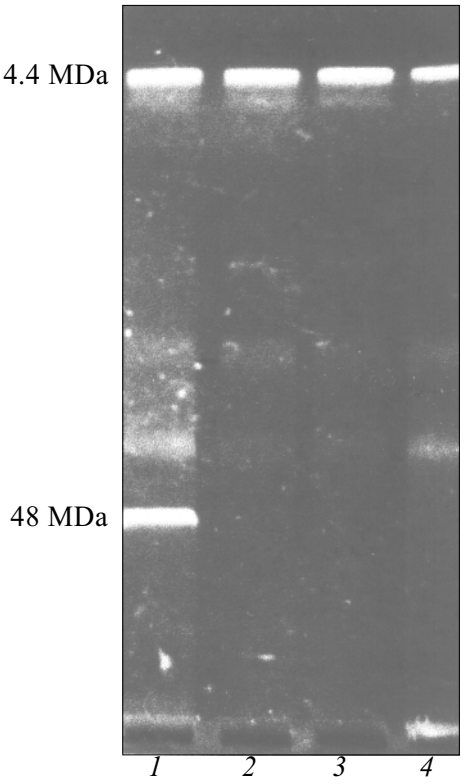

Fig. 1. Changes in the plasmid spectrum of *Yersinia pseudotuberculosis* (strain H-2781^{++}) after long-term stay in nonsterile soil under conditions of open ecosystem. *1*) control (plasmids with molecular weights 4.4 and 48 MDa); *2-4*) months 6, 7, 9 (plasmid with molecular weight 4.4 MDa), respectively.

control (Table 1), which correlates with the data on utilization of citrates by soil bacteria (in contrast to the initial strain); citrates are obligatory stimulators of fatty acid and lipid synthesis. DNase activity in soil strain was 6-fold higher than in the original strain, which can be due to the involvement of this enzyme in cleavage of soil nucleic acid and their utilization by the cell in anabolic processes.

Hence, *Y. pseudotuberculosis* acquires the characteristics of a saprophyte after long-term stay in nonsterile soil. Further studies showed that all lost characteristics of the bacterium, typical of the original strain, were restored after 7 passages of the 9-month culture in nutrient fish agar at 6-8°C, but the virulence of the strain was not restored. In this case we observed modification variability of *Y. pseudotuberculosis* caused by alteration of ecological conditions.

It is noteworthy that saprophytic transformation of *Y. pseudotuberculosis* was paralleled by its dissociation into R form, which represent microbial adaptation to unfavorable conditions [1]. This was not true R form, because it reversed into the initial S form after 4-7 passages at 6-8°C. Moreover, 9-month stay in soil did not abolish, but only decreased agglutination capacity of this strain.

Hence, Yersinia gradually adapted to changing environmental conditions during 9-months stay in the soil. New phenotypical characteristics of laboratory *Y. pseudotuberculosis* strain appearing under novel ecological conditions were first labile, but later became stable in more and more individual bacteria.

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