ISSN 0026-2617, Microbiology, 2009, Vol. 78, No. 5, pp. 585–592. © Pleiades Publishing, Ltd., 2009.
Original Russian Text © V.Yu. Gorshkov, O.E. Petrova, N.E. Mukhametshina, M.V. Ageeva, A.L. Mulyukin, Yu.V. Gogolev, 2009, published in Mikrobiologiya, 2009, Vol. 78, No. 5, pp. 647–655.

EXPERIMENTAL ARTICLES

Formation of "Nonculturable" Dormant Forms of the Phytopathogenic Enterobacterium *Erwinia carotovora*

V. Yu. Gorshkov[a], O. E. Petrova[a], N. E. Mukhametshina[a], M. V. Ageeva[a], A. L. Mulyukin[b], and Yu. V. Gogolev[a, 1]

[a] *Kazan Institute of Biochemistry and Biophysics, Kazan Research Center, Russian Academy of Sciences, ul. Lobachevskogo 2/31, Kazan, Tatarstan, 420111 Russia*

[b] *Winogradsky Institute of Microbiology, Russian Academy of Sciences, pr. 60-letiya Oktyabrya 7, k. 2, Moscow, 117312 Russia*

Received October 9, 2008

Abstract—Reversible transition of the phytopathogenic gram-negative bacterium *Erwinia carotovora*, subsp. *atroseptica*, strain SCRI1043, to a dormant state was demonstrated; it was associated with a complete loss of cell ability to form colonies on the standard medium, i.e., with acquiring "non-culturability". Entering of *Erwinia* cells to a nonculturable state occurred after long-term incubation (100–150 days) of the stationary-phase cell suspensions in either a fresh complete medium or in the carbon-free mineral medium or treatment with a chemical analogue of microbial anabiosis autoinducers (4×10^{-4} M of C_{12}-alkylhydroxybenzene, AHB). However, confocal laser microscopy of the cells stained with the Live/Dead BacLight kit revealed that the majority of *E. carotovora* cells (90%) from long-incubated suspensions retained membrane integrity. In these suspensions, round cells of smaller size prevailed, with the envelope, containing an electron-dense outer layer and an underlying layer of lower density; the cytoplasm was coarse-granulated. Detection of "nonculturable" *E. carotovora* cells by quantitative real-time PCR analysis (Q-PCR) with specific primers by using standard procedures of sample preparation was shown to be inefficient. A special procedure including cell washing from the incubation medium in the absence of growth stimulation was developed, which promoted recovery of the colony-forming ability of the cells (up to 10% of the initial CFU number) and improved cell detection by Q-PCR from the number of genomic copies. The results provided further insight into the ways of long-term survival of phytopathogenic bacteria under environmental changes and carbon starvation.

Key words: "nonculturable cells" of *Erwinia carotovora*, autoregulators, ultrastructure, resuscitation, quantitative PCR analysis.

DOI: 10.1134/S0026261709050099

The cell forms and mechanisms responsible for survival of nonspore-forming phytopathogenic gram-negative bacteria, whose natural populations are subjected to seasonal cycles, are still poorly studied. Gram-negative bacteria, mainly pathogens, were shown to form viable nonculturable cells (VNC) incapable of colony formation on standard media [1]. One of the main factors promoting the transition of bacteria to a nonculturable state is starvation stress, which is caused by transferring the cells into a nutrient-depleted medium [1–3]. The formation of VNC was observed in phytopathogenic bacteria *Erwinia amylovora* incubated in the nutrient-depleted medium with increased concentrations of copper ions [4] and in *Ralstonia solanacearun* under the same conditions as well as in soil microcosms [5]. It is suggested that VNC are similar to dormant forms of bacteria [1, 2, 5]; however, their ability to restore proliferative activity [2, 6] and the period of preserving their ability to resuscitate remain unclear. It is also assumed that the "nonculturable" state is an intermediate debilitation stage that precedes the cell death [6, 7].

It can be suggested that along with the ecological significance of VNC, the long-term survival of nonspore-forming bacteria in natural habitats is supported by formation of cyst-like resting cells (CRC), which exhibit prolonged preservation of viability under unfavorable conditions, resistance to damaging factors, and specific cell ultrastructure [8–11]. The CRC formation is promoted by increased concentrations of extracellular d_1 factors (anabiosis autoinducers) and their analogues, derivatives of alkyl hydroxybenzenes (AHB) [8, 10–12]. Formation of the structurally differentiated CRC in the cell cycle of microbial cultures requires special conditions and modified media including those simulating the natural environments [8, 9, 11]. However, the CRC of *Erwinia* and plant parasites responsible for long-term cell survival have not been described hitherto.

[1] Corresponding author; e-mail: gogolev21@mail.ru

Erwinia carotovora subsp. *atroseptica* SCRI1043 is a suitable object to study the survival of phytopathogenic bacteria since the synthesis of virulent factors in this species is controlled by the quorum-sensing autoregulatory system, which is switched on at a certain threshold cell number [13, 14]. In nature, fluctuations in the number and activity of phytopathogens may be influenced by, apart from trophic, climatic, and physicochemical factors, also by other biotic community components involving, for instance, the AHB widespread in bacteria and plants [10, 15]. To study long-term survival of *E. carotovora* in the laboratory, we imitated natural conditions such as interchanging of the substrate inflow and exhaustion (including the "plant–soil" host shift) and an increase in the level of extracellular AHB that may occur in the course of the plant's debris decay or soil drying. Since plating of microorganisms on standard solid media underscores the total cell numbers [1–5], to determine viable cells, a comprehensive approach was required, based on direct cell counting and special procedures for cell resuscitation.

The aim of this work was to study the strategies of *E. carotovora* survival under long-term incubation in complete or nutrient-depleted media as well as in the presence of AHB and to compare the efficiency of different methods of cell cultivation, electron microscopy, and molecular diagnostics for the detection of viable cells.

MATERIALS AND METHODS

Strain *Erwinia carotovora*, subspecies *atroseptica* SCRI1043 [16] was kindly provided by Dr. E.A. Nikolaichik (Belarus State University). Bacteria were grown in 2000-ml flasks with 400 ml of the Luria-Bertani (LB) medium [17] on a shaker (200 rpm) at 28°C. The media were inoculated with the stationary-phase cultures at the initial optical density of 0.1 units determined at 600 nm.

Obtaining of dormant cells. In preliminary experiments, 4-hexyl-1,3-dihydroxybenzene (C_{12}-AHB, Sigma, United State), the chemical analogue of microbial anabiosis autoinducers, was added to the stationary-phase culture at concentrations of 1×10^{-4}–5×10^{-4} M to study its effect on the maintenance of cell viability for 1.5 months.

The early stationary-phase cells at the concentration of $1–2 \times 10^9$ cells/ml were centrifuged (11000 *g*, 10°C, 10 min) and resuspended in an equivalent volume of (1) fresh LB medium; (2) fresh LB medium supplemented with C_{12}-AHB (4×10^{-4} M); (3) mineral carbon-free AB medium containing (g/l): NH_4Cl, 1; $MgSO_4 \cdot 7H_2O$, 0.62; KCl, 0.15; $CaCl_2 \cdot 2H_2O$, 0.013; $FeSO_4 \cdot 7H_2O$, 0.005; pH 7.5; (4) AB medium supplemented with 4×10^{-4} M C_{12}-AHB. The cell suspensions were incubated in sealed glass vials without aeration at 28°C for 6–9 months.

The number of viable cells was determined from the number of colony-forming units (CFU/ml) by plating serial tenfold dilutions of the cell suspensions onto LB agar (1.5%) and by the most probable number (MPN) method in liquid medium. The following procedures were applied for recovery of the cell's growth ability: (1) double washing of the cells from the incubation medium with subsequent suspending in the initial volume of AB medium; (2) cell "resuscitation" in liquid medium supplemented with the supernatant from the logarithmic-phase culture, as was described for mycobacteria [18, 19]; (3) heat and cold shocks; and (4) infecting of a specific host plant and addition of the plant tissue's extract to the bacterial culture [4, 5].

Index of phenotypic variation in *E. carotovora* populations was determined as a percentage of the colonies distinct from the dominant S-type in the total number of colonies grown on solid medium for 3–14 days of incubation. The variants were distinguished according to colony morphology (shape, size, and pigmentation). Typing of random colonies (10–12 for each variant type) was performed by the PCR method with three pairs of gene-specific probes according to the methods described below.

DNA Isolation and purification. DNA was isolated from *E. carotovora* cells by the standard phenol method [17]. The samples were incubated with proteinase K (Sigma, United States) at a concentration of 50 μg/ml for 1 h at 65°C and then were extracted with phenol. The deproteinized preparations were overlaid on the cesium chloride solution (1.7 g/ml) and ultracentrifuged according to [20]. The DNA concentration was determined by gel electrophoresis on a Gel-Doc device (Bio-Rad, United States) by means of the proprietary software package. The calibration was performed using the standard DNA solutions (Fermentas, Lithuania).

PCR analysis. Amplification of genomic DNA was carried out on a DNA Engine device (Bio-Rad, United States) using gene-specific primers (all the used oligonucleotides were purchased from Syntol, Russia): Eatr1F (5'-GATGATTCTTTTGAGTCATGTTTAC-3') and EG23R (5'-GACACTTTTCGCAGGCTACCACG-3') complementary to the spacer regions of the ribosomal operons; EcahAF (5'-GGCTTTAGGACTTTCTCAGGTTGCATCTC-3') and EcahAR (5'-GCTTTCGCCGCTTTCTGCCC-3') complementary to the encoding sequence of the *hrpA* gene; EcahLF (5'-TGGGTGTTCGGCATTGCTCTC-3') and EcahLR (5'-CCAGCATCTCATCGCCCATTTC-3') complementary to the encoding sequence of the *hrpL* gene. The primers and probes for quantitative analysis were constructed using the Vector NTI-9 software package (Invitrogen, United States). The PCR parameters (concentrations of oligonucleotides and $MgCl_2$, temperature, and the primer annealing time) were optimized in preliminary experiments according to recommendations [21]. The reaction mixture included a fivefold buffer (335 mM Tris–HCl, pH 8.8; 83 mM $(NH_4)_2SO_4$; 12.5 mM $MgCl_2$ and 10.5% Tween 20), 150 μM deoxyribonucleoside triphosphates; 5 μl DNA template (from 0.001 to 200 ng), 0.3 μM of each primer, and 0.04 U *Taq*-polymerase

(Syntol, Russia). The final volume of the reaction mixture was 25 μl. For preliminary denaturation of DNA samples, the reaction mixture was heated at 95°C for 30 s; then 30 temperature cycles were carried out: 94°C, 10 s; 60°C, 10 s; and 72°C, 25 s. The final elongation stage was performed at 72°C for 90 s.

Electrophoretic separation of the amplicons was performed in agar gel using the standard DNA markers (Fermentas, Lithuania).

Quantitative PCR analysis in real-time (Q-PCR) was performed under the same conditions as PCR analysis except that the elongation stage was carried out at 60°C for 1 min. The reaction mixture was supplemented with 9.6 nM 6-carboxyfluorescein and 250 pm each of the fluorescent probes: EatFQ (5'-(Fam-t)GTGTCAA(BHQ1-t) GAGTCTCTCAAATAATCGCAGCGC-3'), EcahAFQ (5'-CGCC(Fam-t)GCTGCACGAG(BHQ1-t) CAGAGA ACCG, EcahLFQ (5'-(Fam-g)CAACTATT(BHQ1-t) TCAAGCAGGCACGACAGCG-3') at the intergenic spacer region of 16S–23S rRNA, *hrpA* and *hrpL* genes, respectively. The reaction continued for 35 to 45 cycles. The regimes of temperature, time, and fluorescence were monitored with an iCycler IQ4 amplifier (Bio-Rad, United States) equipped with an optical module. Data processing was performed using the software package developed by the manufacturer.

The calibration curves were constructed from the PCR results with the known concentrations of *E. carotovora* DNA accepting 5.5 fg (5064000 bp) DNA as a genomic copy [16]. To determine the number of genomic copies, the lysates of cell suspensions, diluted 10^N-fold in the TE buffer (10 mM Tris–HCl, pH 8.0 and 1 mM EDTA, pH 8.0), were used as targets in PCR-RT. To test the reaction efficiency, lysates of 1-day *E. carotovora* cultures with the known titer (CFU/ml) were used as a template. Lysis of the cells that were preliminary washed from the culture medium with the TE buffer was performed in 2% Triton X-100 at 100°C for 10 min [21].

Electron microscopy. Cells were fixed with a 2.5% solution of glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 12 h and then postfixed with a 0.1% solution of OsO_4 in the same buffer supplemented with 25 mg/ml of sucrose for 3 h at room temperature. The material was dehydrated with ethanol and acetone and embedded in Epon 812. Ultrathin sections were prepared using an LKB-8800 microtome (Sweden), mounted on nickel supporting grids, and stained with a water solution of uranyl acetate and with lead citrate [22]. Specimens were examined under a JEM-1200 EX electron microscope (Jeol, Japan) operated at 80 kV.

Confocal laser fluorescence microscopy. Cell suspensions were stained with a *Bac*Light Live/Dead dye kit (Molecular Probes Inc., United States) according to the manufacturer's instructions. Stained preparations were examined under an LSM 510 Meta confocal laser-scanning microscope (Carl Zeiss, Germany) at 488 and 543 nm; the cells that showed red and green fluorescence were counted.

The measurements were performed in triplicates from three independent series of experiments. The presented results show the averaged values. Statistical analysis was performed using the Student's test at the significance level $P < 0.05$.

RESULTS

The first method that was used to obtain the dormant cells of *E. carotovora* simulated a situation occurring in nature, i.e., an increase in the concentration of the regulatory factors; an analogue of microbial anabiosis autoinducers (C_{12}-AHB) at concentrations of 1×10^{-4}–5×10^{-4} M was added to the stationary-phase cultures grown in complete LB medium. One hour after the introduction of C_{12}-AHB at the concentration of at least 2×10^{-4} M, morphological changes were observed (reduced cell size and slight refractivity). However, after prolonged (45 days) incubation in the presence of 2×10^{-4} M C_{12}-AHB, the CFU number was lower by an order of magnitude than in the control or at lower autoinducer concentrations (table), while abundant cell autolysis was not observed. It can be suggested that the population of the dormant refractive *E. carotovora* cells formed under treatment with 2×10^{-4} M C_{12}-AHB was heterogeneous in its ability to grow on solid media, with most of the cells lacking this capacity. An increase in C_{12}-AHB concentration up to 5×10^{-4} M caused a complete loss of colony-forming ability; this may indicate either a transition to a nonculturable state [1] or formation of nonviable micromummies [10].

In the first transfer of the *E. carotovora* culture onto the solid LB medium, phenotypic variation with emergence of colonies distinct from the dominant S-type, occurred both in the control and the experiment (in the presence of C_{12}-AHB at 2×10^{-4} M). The phenotypic variants were mainly represented by (1) the T-type (translucent) with mucous colonies of usual size (2–3 mm in diameter); (2) the Sm1-type with small ($d < 0.3$ mm), transparent colonies; and (3) the Sm2-type with slowly growing (14 days) colonies. Index of phenotypic variation and predominant development of a certain phenotypic variant depended on both C_{12}-AHB concentration and incubation time (table). Thus, long-term incubated (dormant) *Erwinia* cells displayed an increased phenotypic variability much like the CRC of some other bacteria [10, 23].

In the second method of obtaining *E. carotovora* SCRI1043 dormant cells, we simulated stress conditions caused by a fresh medium or carbon starvation; the cells were resuspended either in fresh complete LB medium or in mineral carbon-free AB medium as described in Materials and Methods. During long-term cell incubation under static conditions, the time course of the number of colony-forming cells depended on the medium composition (Fig. 1). When the cells were

The number of viable cells (N, CFU/ml) and the frequency of variant occurrence during germination of *E. carotovora* cells incubated in LB broth for 45 days after addition of various concentrations of C_{12}-AHB

Concentration of C_{12}-AHB, M	Incubation time			
	7 days		45 days	
	N, CFU/ml	Frequency of variant occurrence**, %	N, CFU/ml	Frequency of variant occurrence**, %
0 (Control)*	$(4.2 \pm 1.9) \times 10^7$	S – 78% Sm2 – 22%	$(1.9 \pm 0.8) \times 10^7$	S – 45% Sm2 – 35% Sm1 – 20%
1×10^{-4}	$(1.0 \pm 0.3) \times 10^6$	S – 18% T – 82%	$(8.5 \pm 2.2) \times 10^6$	S – 48% Sm1 – 52%
2×10^{-4}	$(1.2 \pm 0.3) \times 10^6$	S – 85% T – 15%	$(1.1 \pm 0.3) \times 10^6$	S – 26% Sm1 – 74%
5×10^{-4}	0	–	0	–

Notes: * The number of viable cells in the stationary-phase cultures (3 days) prior to the addition of C_{12}-AHB was $(0.8 \pm 0.1) \times 10^9$ CFU/ml.
** Designations of the variants are given in the text.

incubated in rich medium (LB broth), the CFU number decreased by 1.5–2 orders of magnitude during the first 10 days, stabilized at the level of 4×10^7 CFU/ml within 45 days and dropped to zero by the 101st day of incubation. In AB medium, the CFU number decreased by 4 orders of magnitude within 10 days of incubation, remained almost unchanged ($3–6 \times 10^5$) during the next 3 months and dropped to an undetectable level by the 151st day. Thus, carbon starvation prolonged the preservation of the colony-forming ability in the studied bacteria.

In the third procedure for obtaining the dormant cells of *E. carotovora*, C_{12}-AHB at a concentration of 4×10^{-4} M was added to the cell suspensions incubated either in LB broth or in mineral AB medium. The used concentration of C_{12}-AHB, as distinct from the earlier applied concentration (2×10^{-4} M), provided for the development of physiologically homogeneous dormant cells (incapable of germination), which was important for carrying out molecular diagnostics of the culture. At the same time, the used AHB concentration should not exceed 5×10^{-4} M, which was shown to provoke irreversible lack of cell viability and transition to a mummified state in a number of gram-negative bacteria [10, 11, 23]. Cell incubation in the presence of C_{12}-AHB at 4×10^{-4} M in both nutrient-depleted and rich media resulted in a sharp drop in the CFU number to zero within 12 h (Fig. 2); therefore, AHB in this concentration accelerated the loss of colony-forming ability of *E. carotovora* cells.

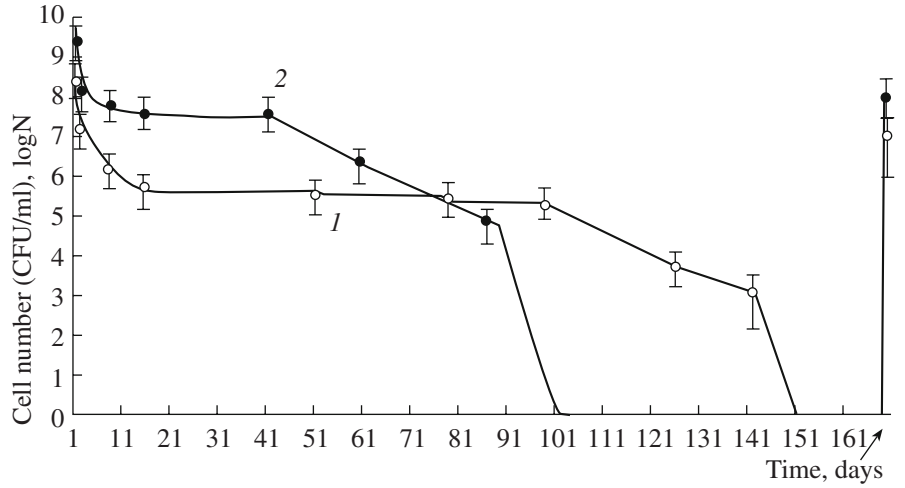

Fig. 1. Time course of the number of colony-forming cells (CFU/ml) in *E. carotovora* cultures incubated for a long time in mineral AB medium (*1*) and LB broth (*2*). Arrow indicates the CFU number of the corresponding cultures after their washing from the incubation medium.

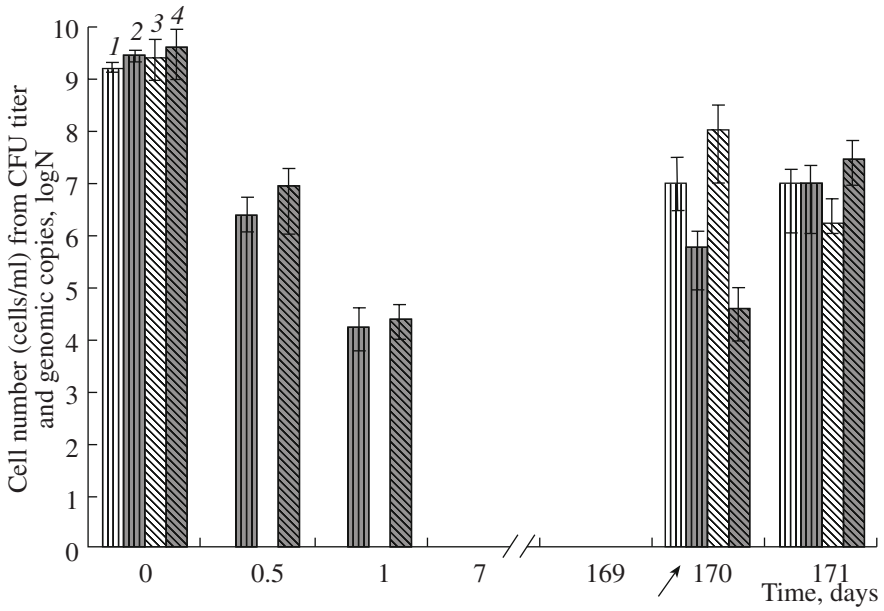

Fig. 2. Time course of the number of cells determined from the CFU titer (*1, 3*) and number of genomic copies (*2, 4*) in *E. carotovora* cultures incubated in mineral AB medium (*1, 2*) and LB broth (*3, 4*) in the presence of C_1-AHB (4×10^{-4} M). Arrow indicates the time of the "resuscitation" procedure.

As revealed by laser-scanning confocal microscopy, cell suspensions of *E. carotovora* long-term incubated (up to 150 days) in rich or nutrient-depleted media with and without C_{12}-AHB had a high content (90% and more) of cells emitting green fluorescence (determined by the Live/Dead staining test), i.e., retaining intact membranes, whereas the level of dead and damaged cells with red fluorescence did not exceed 10% of the total cell number.

The cells incubated in AB medium for 170 days differed considerably from the stationary-phase vegetative cells; the latter were rods (0.7×2.0 μm) with easily distinguishable outer and cytoplasmic membranes; and the electron-dense cytoplasm had fine-granular structure with an evenly distributed nucleoid (Fig. 3a). In starving cultures, most cells were spherical of less than 0.7 μm in diameter; the cell envelopes had a clearly distinguishable electron-dense outer coating and an underlying electron-transparent layer; and the cytoplasm had a coarse-granular structure with a poorly distinguishable nucleoid (Figs. 3b and 3c). Some cells were characterized by plasmolysis (Fig. 3b), which is possibly associated with their dehydration. Similar changes in the cell structure were revealed in the course of long-term cell incubation in LB broth as well as in the presence of C_{12}-AHB.

The presence of intact cells with an undisturbed barrier membrane function in the suspensions of cells lacking the colony-forming capacity after prolonged incubation (150–170 days) indicated a transition of the cells to a nonculturable state rather than the death of the major part of the population. To prove the reversible recovery of colony-forming ability of cells or their ability to grow in liquid medium, we tried procedures 1–4 described in Materials and Methods

Washing twice with a fresh mineral AB medium (procedure 1) promoted the recovery of the colony-forming ability (from 0.5 to 10% of the initial CFU number) (Figs. 1 and 2). It should be emphasized that the AB medium did not support growth and propagation of *E. carotovora* cells; when AB medium was inoculated with vegetative cells at the ordinary initial titer of 1.9×10^6, no increase in the CFU number (9.6×10^5) was observed the next day. Moreover, the CFU number was higher when the cells were inoculated 30 min after washing than after 24 h. Thus, the effect of washing with AB medium on the recovery of the number of colony-forming cells was not due to the cryptic growth and cell propagation. The other procedures (2–4) showed no effect on cell "resuscitation".

Since a discrepancy was observed between the CFU number and the total number of viable cells, additional experiments were performed with the use of quantitative PCR analysis to estimate the cell number from the titer of genomic copies. For *E. carotovora* suspensions incubated in LB broth or in carbon-depleted AB medium supplemented with C_{12}-AHB (4×10^{-4} M), a decrease in the CFU number was shown to correlate with a decreased number of genomic copies. The washing of cells from the incubation medium allowed their detection with the use of PCR-RT (Fig. 2). A decrease in the CFU number of cell suspensions inoculated 24 h after washing, as distinct from those inoculated after 30 min was not accompanied by a change in the number of cells detected by molecular diagnostics.

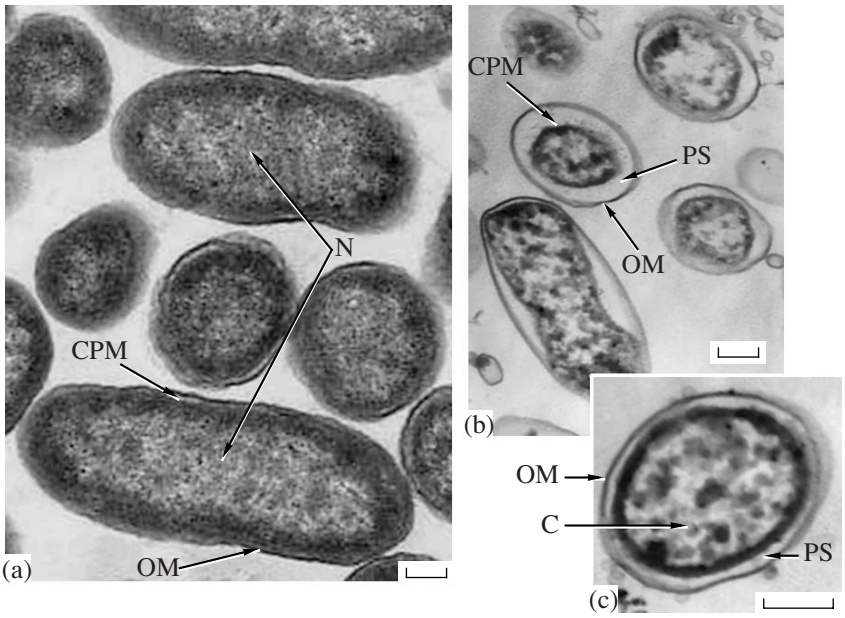

Fig. 3. Electron micrographs of the sections of *E. carotovora*: vegetative cells (a); cells incubated in carbon-free AB medium for 150 days (b, c). The designations are as follows: OM, outer membrane; PS, periplasmic space; CPM, cytoplasmic membrane; C, cytoplasm; N, nucleoid. Bars – 200 nm.

It is known that AHB homologues can interact with DNA and its supramolecular complexes [10, 23]. In additional experiments, we revealed that the introduction of C_{12}-AHB (10^{-3} M) into the reaction mixture with DNA-templates from *E. carotovora* vegetative cells had no effect on the yield of amplification products in PCR with three pairs of gene-specific primers (see Materials and Methods). However, no DNA amplification was observed in "nonculturable" cells incubated for 7 or more days in the presence of C_{12}-AHB (Fig. 2). Thus, it can be assumed that "nonculturable" cells contained inhibitors of the PCR reaction, which seemed to differ from AHB.

The development of the Sm1-phenotypic variants was revealed when long-term incubated suspensions of *E. carotovora* were inoculated (30 min or 24 h after washing) onto solid LB medium. Gene typing of random colonies of the variants by PCR (see Materials and Methods) showed that the variants did not differ in the number and size of amplicons from the dominant type (electrophoregrams are not shown); this result rules out cell contamination, which becomes more probable with increasing incubation time.

DISCUSSION

It was shown that the phytopathogenic gram-negative bacterium *E. carotovora* subsp. *atroseptica* strain SCRI1043 was capable of spontaneous or induced (by analogues of anabiosis autoinducers) reversible transition to a dormant state when the cells completely lost their ability to form colonies on standard media and became "nonculturable". Substrate limitation was not the main factor promoting the transition of *E. carotovora* cells to a reversible "nonculturable" state when the cells restored their colony-forming ability after a short-term washing from the incubation medium. Earlier, the formation of similar viable but nonculturable forms in *Erwinia* was explained by starvation and/or toxic action of copper ions [4]. In our experiments, the rate of *E. carotovora* transition to the nonculturable state was shown to depend rather on the presence of an analogue of extracellular autoregulators of cell growth [10], C_{12}-AHB, at a concentration of 4×10^{-4} M than on the stress caused either by transfer into fresh LB broth or by the carbon starvation in a mineral AB medium (Figs. 1 and 2). In long-term incubated cultures of *E. carotovora*, much like other bacteria [11], the effect of C_{12}-AHB on the preservation of the colony-forming ability depended on its concentration (table).

No cell growth was observed when concentrated *E. carotovora* suspensions (10^9 cells/ml) were inoculated into fresh rich medium (LB broth) (Fig. 1), which can indicate the occurrence of the regulation of cell density involving the factors diffusing from the cells into the medium, which fulfilled the function of the cell number's restriction. This function is characteristic of autoregulatory factors d_1, AHB, which have been revealed in a number of microorganisms [9]; however, the occurrence of such a regulatory system in erwinia needs to be confirmed by further research. Factors of quorum-sensing control represented by acylated homoserine lactones were revealed in *E. carotovora* [13, 14]; however, it remains unclear whether they show direct inhibitory effect on the producer growth. It can be assumed that the transition of *E. carotovora* cells

to a "nonculturable state involves two or several regulatory components.

The long-term incubated cells of erwinia may be considered an example of a dormant, tentatively "noncultarable" state [2] due to the following characteristics: loss of ability to form colonies on standard medium and, most importantly, reversibility of this state; the absence of cell division; preservation of the barrier function of cell walls; and specific structural characteristics of cells. The reversion of strain SCRI1043 to colony formation (up to 10% of the initial CFU number) after a special procedure of "resuscitation" by washing the cells with mineral medium was demonstrated. An increase in the CFU number occurred due to the recovery of the colony-forming ability of nonculturable forms rather than due to growth of some part of the population; this is confirmed by the following: (1) using cell suspensions with the zero CFU number which did not contain cells capable of growth; (2) short-term cell incubation (30 min); and (3) inability of the vegetative cells to grow in AB medium.

"Nonculturable" cells of strain SCRI1043 (Figs. 3b and 3c) differed in their ultrastructural characteristics (by the cell envelope's structure) from the CRC of a number of gram-negative bacteria [9, 11]. It can be assumed that under the incubation conditions applied, immature cyst-like cells with an increased periplasmic space were formed. Long-term incubation of *E. carotovora* in either carbon-depleted or rich media did not promote the formation and maturing of culturable and morphologically different CRC of *Erwinia*; special conditions are probably required to obtain these forms.

The essential novelty concerns a revealed correlation between culturability of *Erwinia* cells and the efficiency of the PCR reaction performed with the samples of the relevant cultures by using standard procedures (Figs. 1 and 2). In earlier studies, a decrease in the efficiency of PCR amplification was shown for only several genes of the "nonculturable" cells of *Acholeplasma laidlawii* [24] and *Vibrio vulnificus* [25]. Inefficient detection of "nonculturable" *E. carotovora* cells by PCR can be due to the presence of PCR inhibitor(s) of an unknown nature as well as to the imperfect DNA isolation by the standard methods. The occurrence of inhibitors is important not only for molecular diagnostics but also for the elucidation of the possible mechanisms for the preservation of proliferative dormancy in "nonculturable" cells. Low efficiency of PCR may be also associated with changes in the properties of the DNA template or with its specific structural characteristics in starved and dormant cells that resulted in the nucleoid compaction [8–12, 23]. PCR data can be used as a criterion of changes in the structure of the supramolecular complexes of bacterial chromosomes.

It should be noted that the conditions applied in this study (medium replacement and increased concentration of an analogue of regulatory molecules) that promoted the transition of *E. carotovora* cells to a "nonculturable" state imitated environmental conditions in the habitats of these phytopathogenic bacteria. Since most of bacterial populations in nature are represented by the forms difficult to obtain in a culture, a combination of direct microscopic and molecular methods for their detection and cultivation under standard and specially selected conditions allowed more complete enumeration of potentially viable cells. On the whole, our results on reversible transition of phytopathogenic bacteria to a "nonculturable" state provide further insight into the mechanisms of their survival in nature, although laboratory conditions cannot reveal completely the whole potential of microbial adaptation to environmental changes.

ACKNOWLEDGMENTS

We are grateful to E.A. Nikolaichik for methodical help, G.I. El'-Registan for valuable consultations, and A.G. Daminova for assistance in the experiments.

This work was supported by the Russian Foundation for Basic Research (project nos. 08-04-01518-a and 09-04-00915-a), grant of the President of the Russian Federation "Leading Scientific Schools" (NSh-5492.2008.4), and the Presidium of the Russian Academy of Sciences, Fundamental Research Program "Molecular and Cellular Biology".

REFERENCES

1. Roszak, D.B. and Colwell, R.R., Survival Strategies of Bacteria in the Natural Environment, *Microbiol. Rev.*, 1987, vol. 51, no. 3, pp. 365–379.
2. Kell, D.B., Kaprelyants, A.S., Weichart, D.H., Harwood, C.R., and Barer, M.R., Viability and Activity in Redilly Culturable Bacteria: a Review and Discussion of the Practical Issues, *Antonie van Leeuwenhoek*, 1998, vol. 73, pp. 169–187.
3. Oliver, J.D., The Viable But Nonculturable State in Bacteria, *J. Microbiol.*, 2005, vol. 43, pp. 93–100.
4. Ordax, M., Marco-Noales, E., López, M.M., and Biosca, E.G., Survival Strategy of *Erwinia amylovora* against Copper: Induction of the Viable-but-Nonculturable State, *Appl. Environ. Microbiol.*, 2006, vol. 72, no. 5, pp. 3482–3488.
5. Grey, B.E. and Steck, T.R., The Viable but Nonculturable State of *Ralstonia solanacearum* May Be Involved in Long-Term Survival and Plant Infection, *Appl. Environ. Microbiol.*, 2001, vol. 7, no. 9, pp. 3866–3872.
6. Bogosian, G. and Bourneuf, E.V., A Matter of Bacterial Life and Death, *EMBO Rep*, 2001, vol. 2, no. 9, pp. 770–774.
7. Joux, F., Lebaron, P., and Troussellier, M., Succession of Cellular States in a *Salmonella typhimurium* Population During Starvation in Artificial Seawater Microcosms, *FEMS Microbiol. Ecol.*, 1997, vol. 22, pp. 65–76.
8. Mulyukin, A.L., Lusta, K.A., Gryaznova, M.N., Kozlova, A.N., Duzha, M.V., Duda, V.I., and El'-Registan, G.I., Formation of Resting Cells by *Bacillus cereus* and *Micrococcus luteus, Mikrobiologiya*,

1996, vol. 65, no. 6, pp. 782–789 [*Microbiology* (Engl. Transl.), vol. 65, no. 6, pp. 683–689].

9. Suzina, N.E., Mulyukin, A.L., Kozlova, A.N., Shorokhova, A.P., Dmitriev, V.V., Barinova, E.S., Mokhova, O.N., El'-Registan, G.I., and Duda, V.I., Ultrastructure of Resting Cells of Some Non-Spore-Forming Bacteria, *Mikrobiologiya*, 2004, vol. 73, no. 4, pp. 516–529 [*Microbiology* (Engl. Transl.), vol. 73, no. 4, pp. 435–447].

10. El'-Registan, G.I., Mulyukin, A.L., Nikolaev, Yu.A., Suzina, N.E., Gal'chenko, V.F., and Duda, V.I., Adaptogenic Functions of Extracellular Autoregulators of Microorganisms, *Mikrobiologiya*, 2006, vol. 75, no. 4, pp. 446–456 [*Microbiology* (Engl. Transl.), vol. 75, no. 4, pp. 380–389].

11. Mulyukin, A.L., Suzina, N.E., Duda, V.I., and El'-Registan, G.I., Structural and Physiological Diversity among Cystlike Resting Cells of Bacteria of the Genus *Pseudomonas, Mikrobiologiya*, 2008, vol. 77, no. 4, pp. 512–523 [*Microbiology* (Engl. Transl.), vol. 77, no. 4, pp. 455–465].

12. Osipov, G.A., El'-Registan, G.I., Svetlichnyi, V.A., Kozlova, A.N., Duda, V.I., Kaprel'yants, A.S., and Pomazanov, V.V., On the Chemical Nature of the d Regulatory Factor of *Pseudomonas carboxydoflava, Mikrobiologiya*, 1985, vol. 54, no. 2, pp. 186–190.

13. Jones, S., Yu, B., Bainton, N.J., Birdsall, M., Bycroft, B.W., Chhabra, S.R., Cox, A.J., Golby, P., Reeves, P.J., Stephens, S., Winson, M.K., Salmond, G.P.C., Steward, G.S.A.B., and Williams, P., The Lux Autoinducer Regulates the Production of Exoenzyme Virulence Determinants in *Erwinia carotovora* and *Pseudomonas aeruginosa, EMBO J.*, 1993, vol. 12, no. 6, pp. 2477–2482.

14. Chatterjee, A., Cui, Y., Hasegawa, H., Leigh, N., Dixit, V., and Chatterjee, A.K., Comparative Analysis of Two Classes of Quorum-Sensing Signaling Systems That Control Production of Extracellular Proteins and Secondary Metabolites in *Erwinia carotovora* Subspecies, *J. Bacteriol.*, 2005, vol. 187, pp. 8026–8034.

15. Kozubek, A. and Tyman, J.H.P., Resorcinolic Lipids, the Natural Non-Isoprenoid Phenolic Amphiphiles and Their Biological Activity, *Chem. Rev.*, 1999, vol. 99, no. 1, pp. 1–29.

16. Bell, K.S., Sebaihia, M., Pritchard, L., Holden, M.T.G., Hyman, L.J., Holeva, M.C., Thomson, N.R., Bentley, S.D., Churcher, L.J.C., Mungall, K., Atkin, R., Bason, N., Brooks, K., Chillingworth, T., Clark, K., Doggett, J., Fraser, A., Hance, Z., Hauser, H., Jagels, K., Moule, S., Norbertczak, H., Ormond, D., Price, C., Quail, M.A., Sanders, M., Walker, D., Whitehead, S., Salmond, G.P.C., Birch, P.R.J., and Toth, I.K., Genome Sequence of the Enterobacterial Phytopathogen *Erwinia carotovora* subsp. *atroseptica* and Characterization of Virulence Factors, *Proc. Natl. Acad. Sci. USA*, 2004, vol. 101, no. 30, pp. 11 105–11 110.

17. Sambrook, J., Fritsch, E.F., and Maniatis, T., *Molecular Cloning: A Laboratory Manual. 2nd ed.*, New York: Cold Spring Harbor Lab. Press, 1989.

18. Shleeva, M.O., Mukamolova, G.V., Telkov, M.V., Berezinskaya, T.L., Syroeshkin, A.V., Biketov, S.F., and Kaprel'yants, A.S., Formation of Nonculturable Cells of *Mycobacterium tuberculosis* and Their Resuscitation, *Mikrobiologiya*, 2003, vol. 72, no. 1, pp. 76–83 [*Microbiology* (Engl. Transl.), vol. 72, no. 1, pp. 64–70].

19. Kana, B.D., Gordhan, B.G., Downing, K.J., Sung, N., Vostroknutova, G., Machowski, E.E., Tsenova, L., Young, M., Kaprelyants, A., Kaplan, G., and Mizrahi, V., The Resuscitation-Promoting Factors of *Mycobacterium tuberculosis* Are Required for Virulence and Resuscitation from Dormancy but Are Collectively Dispensable for Growth *in vitro*, *Mol. Microbiol.*, 2008, vol. 67, no. 3, pp. 672–684.

20. Osterman, L.A., *Metody issledovaniya belkov i nukleinovykh kislot: Elektroforez i ul'tratsentrifugirovanie* (Methods of Investigation of Proteins and Nucleic Acids: Electrophoresis and Ultracentrifugation), Moscow: Nauka, 1981.

21. Lee, J.L. and Levin, R.E., Use of Ethidium Bromide Monoazide for Quantification of Viable and Dead Mixed Bacterial Flora from Fish Fillets by Polymerase Chain Reaction, *J. Microbiol. Methods*, 2006, vol. 67, no. 3, pp. 456–462.

22. Reynolds, E., The Use of Lead Citrate at High pH as an Electron Opaque Stain in Electron Microscopy, *J. Cell Biol.*, 1963, vol. 17, pp. 208–212.

23. Mulyukin, A.L., Vakhrushev, M.A., Strazhevskaya, N.B., Shmyrina, A.S., Zhdanov, R.I., Suzina, N.E., Duda, V.I., Kozlova, A.N., and El'-Registan, G.I., Effect of Alkylhydroxybenzenes, Microbial Anabiosis Inducers, on the Structural Organization of *Pseudomonas aurantiaca* DNA and on the Induction of Phenotypic Dissociation, *Mikrobiologiya*, 2005, vol. 74, no. 2, pp. 157–165 [*Microbiology* (Engl. Transl.), vol. 74, no. 2, pp. 128–135].

24. Chernov V.M., Gogolev Y.V., Mukhametshina N.E., Abdrakhimov F.A., Chernova O.A., Adaptive reactions of mycoplasmas *in vitro*: "viable but unculturable forms" and nanocells of *Acholeplasma laidlawii, Microbiologiya*, 2005, vol. 74, no. 4, pp. 498–504. [*Microbiology* (Engl. Transl.), vol. 74, no. 4, pp. 428–433]

25. Smith, B. and Oliver, J.D., In situ and in vitro Gene Expression by *Vibrio vulnificus* During Entry into, Persistence within, and Resuscitation from the Viable but Nonculturable State, *Appl. Environ. Microbiol.*, 2006, vol. 72, no. 2, pp. 1445–1451.