Induction and resuscitation of viable but nonculturable bacteria from different taxonomic groups

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Abstract:
We obtained nonculturable cells of Escherichia coli M17, Salmonella Typhimurium 79, and Staphylococcus aureus FDA 209P under prolonged osmotic and trophic stresses. It was noted, that even in populations incubated under microaerophilic conditions the percentage of nonculturable cells exceeded 99% during long-term observation. The data gathered in the experiments showed that the combination of incubation of cells in normal saline and inoculation on a solid medium with a diluted nutrient base is necessary to get out of the nonculturable state. This combination of factors with a reduced level of nutrients provides gentle conditions for the growth of microorganisms that have been starving for a long time in conditions of increased osmotic pressure.
Keywords:*viable but nonculturable cells, stress, starvation, osmotic pressure, resuscitation*.

Introduction

Viable but nonculturable (VBNC) cells were first discovered in 1982 [1]. Viable pathogenic and saprotrophic microorganisms, being in natural and artificial systems (human and animal organisms, soil, natural reservoirs, plumbing, food products, medicines, laboratory substrates, etc.), can shift into an VBNC. This is caused by various stress factors and may be strain-specific [2,3,4]. Moving to the VBNC, rod-shaped microorganisms tend to round or shorten, decrease in size. Changes also occur in metabolism - the level of respiration, transmembrane transport and synthesis of macromolecules decreases [5]. An important factor leading to the formation of VBNCs is the oxidative potential of the environment due to the sensitivity of bacteria to oxidative stress [6].

However, scientists have discovered the properties of VBNC cells to switch out of this state. For the first time, the term “resuscitation” of viable but nonculturable cells was used to describe *Salmonella enteritidis* cells [7]. This is a phenomenon in which physiological and metabolic processes in the cells of a microorganism return to normal and they restore the ability to form colonies [8, 9, 10].

Resuscitation of the VBNC bacteria is possible only during a certain period, called the “resuscitation window” [11]. During this period, the number of resuscitated cells decreases to undetectable values [12]. The duration of the “resuscitation window” depends on the type of bacteria, the age of the culture, and the conditions created for the formation and resuscitation of the VBNC microbes [13]. However, the experiments should create conditions that exclude the growth of the undetectable part of cultured cells [5].

To prove true resuscitation of VBNC cells, the method of most probable numbers is used, for example, when the chance of the presence of active cells in the final dilutions is vanishingly small [14].

Many methods are used to achieve resuscitation of nonculturable cells, of which the most common are stress relief, such as temperature upshift or the addition of nutrients to VBNC microorganisms obtained under oligotrophic conditions (with nutritional deficiencies) [5]. For example, for *Vibrio parahaemolyticus* and *Vibrio vulnificus*, turning into VBNC state under conditions of incubation at low temperatures, incubation of VBNC cells at room temperature with subsequent inoculation of the culture on a rich medium will be sufficient [15]

The process of resuscitation of VBNC cells can be facilitated by the addition of substances that decompose hydrogen peroxide [16], since nonculturable cells experience a higher level of oxidative stress with a reduced activity of antioxidants.

In addition, it was revealed that the resuscitation of VBNC cells can occur under the influence of biological factors. For example, species such as *E. coli*, *Francisella tularensis*, and *Campylobacter jejuni* have been confirmed to return to a cultured state when co-grown with eukaryotic cells, mammalian cells, and embryonic eggs, respectively [5].

Prolonged exposure of the microbe in oligotrophic conditions leads to a slowdown in metabolism, as well as to the appearance of higher affinity transport proteins for better absorption of the remaining substrates from the environment [17]. When exposed to rich media in such cell, after the induction of metabolism, the accumulation of nutrients occurs in concentrations that disrupt the vital functions of the starving microorganism. This phenomenon is described in the literature under the term “substrate – accelerated cell death” [18].

The aim of our experiments was to study the effect of a diluted nutrient medium on the transition of nonculturable cells, which had been under stress for a long time, to a physiologically and metabolically active state.

Materials and methods

In our experiments we used strains *Escherichia coli* M17, *Salmonella enterica Typhimurium* 79 and *Staphylococcus aureus* FDA 209 P. Cultures were grown in nutrient broth for 18-20 hours at 37 ° C. The cells were then harvested by centrifugation at 10,000 rpm for 10 minutes. The sediment was transferred into flasks with 300 ml of 3% NaCl

(protein-free hypertonic medium) for long-term incubation and obtaining nonculturable cells. The suspensions were incubated at room temperature in the dark without stirring.Microaerophilic conditions were created by sealing the flasks with rubber stoppers with 2 mm glass aerator tubes with cotton wool in them. Samples were taken from the flasks to determine the parameters of viability and culturability of cell populations. The CFU / ml value was determined by plating on nutrient agar. The plates were incubated at 37 ° C for 24 hours, followed by counting the number of colonies. The total number of cells was counted in the Goryaev chamber. The proportion of living cells was assessed using a luminescent microscope after staining cell suspensions with a kit of luminescent DNA-tropic dyes Live/Dead® (Baclight ™). Based on the obtained value, the total number of living and dead cells in cultures was calculated. The percentage of VBNC cells was determined by comparing the values of the number of stained living cells and CFU / ml.

For the resuscitation of the obtained VBNC microbes, a series of eight consecutive tenfold dilutions of the suspension in normal saline(protein-free, isotonic environment) was prepared. This made it possible to achieve the absence of residual cultured cells in the final dilutions. The tubes were incubated at room temperature to maintain the temperature conditions of cell incubation for 48 hours. Platings were carried out immediately after dilution, as well as after 24 and 48 hours. A full-strength and ten times diluted 1.5% nutrient agar was used as a medium for determining the number of resuscitated cells.

The data were statistically processed with the calculation of the arithmetic mean and standard deviation of its ($\bar{S}\pm\sigma$) value.

Results and discussion

In our experiments we studied key population characteristics of studied microorganisms under stresses.

We used thee species of microorganisms *E. coli* M17, *S. enterica Typhimurium* 79, *S. aureus* FDA 209P that were incubated under trophic and osmotic stresses. Initial numbers of cells were 10^8 for *E. coli* M17 and *S. enterica Typhimurium* 79 and $8{,}96\pm0{,}98\times10^7$ for *S. aureus* FDA 209P Data obtained during long-term incubation are shown in tables 1-3.

Characterization of biological parameters in *E. coli* M17 population (Table 1) that was incubated in conditions of starvation osmotic stresses for 12.5 months (378 days) revealed dynamics of their variation. One of the characteristics of this population was decreasing of culturability by 1 order of magnitude. By day 56 of incubation percentage of nonculturable cells was 97.6%. However, viability of the population didn't reduce significantly (from 100 to 62%). After that culturable portion of the suspension, which was assessed by determination of CFU/ml value decreased from $(1{,}27 \pm 0{,}14) \times10^8$ CFU/ml to $(4{,}86 \pm 0{,}53) \times10^5$ CFU/ml by day 231. Although, we noted an increase in VBNC cells (from 21.6 to 99.3%). It is important that total cell counts didn't change significantly (from $1{,}62\pm0{,}18\times10^8$ to $1{,}06\pm0{,}12\times10^8$ cells per ml). At the end of observation all parameters did not change significantly and portion of VBNC cells remained at the 99% level.

Table 1: Parameters of total cell counts and culturability of *E. coli* M17 bacteria during long-term incubation under stressful conditions.

Days of observation	Biological parameters($\bar{S}\pm\sigma$)			
	Total cell counts	CFU/ml	Portion of viable cells (%)	VBNC cells (%)
0	$1{,}62\pm0{,}18\times10^8$	$1{,}27\pm0{,}14\times10^8$	100	21,6
7	$1{,}68\pm0{,}18\times10^8$	$7{,}48\pm0{,}82\times10^7$	82	45,4
14	$1{,}35\pm0{,}15\times10^8$	$8{,}7\pm0{,}96\times10^7$	79	18,7
56	$1{,}36\pm0{,}15\times10^8$	$2\pm0{,}22\times10^7$	62	97,6
70	$1{,}29\pm0{,}14\times10^8$	$4{,}86\pm0{,}53\times10^7$	74	94,9
147	$1{,}11\pm0{,}12\times10^8$	$1\pm0{,}11\times10^6$	89	99
168	$1{,}46\pm0{,}16\times10^8$	$1\pm0{,}11\times10^6$	91	99.2
231	$1{,}06\pm0{,}12\times10^8$	$4{,}86\pm0{,}53\times10^5$	68	99,3
378	$1{,}23\pm0{,}14\times10^8$	$9{,}47\pm1{,}04\times10^5$	77	99

During examination of the population of *Salmonella enterica Typhimurium* 79 (table 2) we observed level of VBNC cells greater than 90% at day 14 which was faster than the other two species (tables 1 and 3). Total cell counts in that period decreased by 1.7 times. It is reasonable to notice that portion of cells shown as viable remained at 82%, which is similar to other enterobacterial species – *E. coli* M17 after7 days of incubation. Percentage of VBNC salmonella cells during the entire observation period was comparable with that of *E. coli* –did not exceed 99.3%

Table 2: Parameters of total cell counts and culturability of *Salmonella enterica Typhimurium* 79 bacteria during long-term incubation under stressful conditions.

Days of observation	Biological parameters($\bar{S}\pm\sigma$)			
	Total cell counts	CFU/ml	Portion of viable cells (%)	VBNC cells (%)
0	$1{,}82\pm0{,}2\times10^8$	$1{,}82\pm0{,}2\times10^8$	100	0

7	$1,34±0,15×10^8$	$7,29±0,8×10^7$	82	56,5
14	$1,08±0,12×10^8$	$7,62±0,84×10^7$	82	91,4
56	$1,21±0,13×10^8$	$3,2±0,35×10^7$	86	69,2
70	$1,43±0,16×10^8$	$8,75±0,96×10^7$	79	92,3
147	$1,16±0,13×10^8$	$1,96±0,21×10^6$	97	98,3
168	$1,44±0,16×10^8$	$8,2±0,9×10^6$	94	93,9
231	$1,04±0,11×10^8$	$4,86±0,53×10^5$	95	99,3
378	$1,2±0,13×10^8$	$1,06±0,12×10^6$	95	99,1

For *Staphylococcus aureus* (table 3) decrease in CFU/ml and stable increase of the portion of VBNC cells to the level of 90% was observed by day 49. This was close to *E. coli* M17 after 56 days and salmonella at day 50. At the same time portion of viable cells at day 231 decreased significantly and was 15% of total cell count. CFU/ml value at within this timeframe decreased by 177.3 times (from $5,1±0,56×10^7$ to $2,87±0,32×10^4$).It is possible that due to lysis of some amount of dead cells at the end of the experiment we observed some increase of the portion of viable cells and decrease of total cell counts. At the same time p by day 378 percentage of nonculturable cells did not change significantly. Portion of VBNC cells was 99.8% which is only 0.1% more than the same parameter at day 231. CFU/ml value at the end of observation was $5,1±0,56×10^5$.

Table 3: Parameters of total cell counts and culturability of *Staphylococcus aureus* FDA 209P bacteria during long-term incubation under stressful conditions.

Days of observation	Biological parameters($\bar{S}$±σ)			
	Total cell counts	CFU/ml	Portion of viable cells (%)	VBNC cells (%)
0	$8,96±0,98×10^7$	$5,1±0,56×10^7$	100	43
7	$9,1±1,0×10^7$	$6,83±0,75×10^7$	100	24,9
49	$7,96±0,87×10^7$	$3,21±0,35×10^6$	100	96
63	$3,76±0,41×10^7$	$1,0±,11×10^5$	100	99,7
140	$7,44±0,82×10^7$	$1,42±0,16×10^5$	30,8	99,3
161	$7,33±0,81×10^7$	$5,2±0,57×10^5$	33,6	97,9
231	$7,44±0,82×10^7$	$2,87±0,32×10^4$	15	99,7
378	$3,4±0,37×10^7$	$5,1±0,56×10^5$	36	99.8

Data showed that not only under such a well-known stress factor as the oxidative potential of the environment, there is a rapid transition to anonculturable state [6], but also under microaerophilic conditions. Bacteria, including gram-positive *Staphylococcus aureus*, formed a significant (more than 99%) number of VBNC cells. This indicates a possible underestimation of the levels of microbial contamination when it is assessed only by cultural methods. At the same time, a complete (100%) transition to VBNC state was not observed for any culture.

The next stage of research was the search for conditions for the reactivation of the VBNC of the studied microorganisms, since many bacteria are able to resuscitate from this state when stress conditions are removed [15]. We carried out experiments to relieve osmotic and trophic stresses in different periods of culture incubation. It is known that for long-term starved microorganisms a rich nutrient medium may turn out to be suboptimal as a resuscitating substrate [18], and on the contrary, be additional stress for microorganisms. Therefore, in addition to full strength nutrient agar, we tested an oligotrophic nutrient medium - agar with 10 diluted times base. The results of the experiments are shown in tables 4-9. To relieve osmotic stress, aliquots were taken from flasks with cultures that containedVBNC cells of microorganisms and a series of tenfold dilutions were made so that cultured cells were absent in the last dilution, according to calculations. This should have confirmed the truth of the process of resuscitation of the VBNC bacteria, and not the multiplication of the residual number of culturable cells.

As can be seen from Table 4, *E. coli* M17 cells incubated under trophic and osmotic stresses for 147 days fully recovered from the uncultivated state only after 48 hours of incubation innormal saline and only partially after 24 hours. The CFU / ml value for this microbe was $5.4 ± 0.59×10^8$. For *S. enterica Typhimurium* 79, a similar indicator ($1 × 10^8$/ml) was achieved after 24 hours of incubation, and then followed by an increase in the population by two orders of magnitude. For *S. aureus* FDA 209P set, a decrease in culturability of these bacteria was observed when plated on a full-strength medium.

Table 4: Resuscitation of VBNC microorganisms by incubating in normal saline and inoculating in full-strength nutrient agar after 147 days of stress.

Microorganism	CFU/ml($\bar{S}$±σ)		
	0 hours	24 hours	48 hours
*E. coli*M17	$1±0,11×10^6$	$6,8±0,75×10^7$	$5,4±0,59×10^8$
SalmonellaTyphimurium 79	$1,16±0,13×10^8$	$3,1±3,4×10^8$	$7,2±0,79×10^{10}$
*Staphylococcusaureus*FDA 209P	$1,42±0,16×10^5$	$3,3±0,36×10^4$	$3,15±0,34×10^4$

The data in Table 5 were obtained using a solid medium with a diluted nutrient base. They show that *E. coli* M17 VBNC cells massively resuscitated from the nonculturable state by 24 hours which is, earlier than after plating them on a full-strength medium. At the same time, the dynamics of the exit from the nonculturable state for *S. Typhimurium* 79 did not differ from the values for cells plated on a complete medium and reached $8.2 \pm 0.9 \times 10^{10}$ CFU/ml. At the same time, for *S. aureus* FDA 209P dilution of the medium, in contrast to the undiluted substrate, led to an increase in CFU/ml by one order of magnitude, that is, the inhibiting effect of the strength medium was removed.

Table 5: Resuscitation of VBNC microorganisms by incubating in normal saline and inoculating in diluted nutrient agar after 147 days of stress.

Microorganism	CFU/ml($\bar{S}\pm\sigma$)		
	0 hours	24 hours	48 hours
*E. coli*M17	$8,6\pm0,94\times10^5$	$1,08\pm0,12\times10^8$	$1,56\pm0,17\times10^8$
*SalmonellaTyphimurium*79	$2,7\pm0,29\times10^6$	$4,8\pm0,53\times10^8$	$8,2\pm0,9\times10^{10}$
*Staphylococcusaureus*FDA 209P	$2,05\pm0,23\times10^5$	$7,4\pm0,81\times10^5$	$9,06\pm0,99\times10^6$

With an increase in the observation period for the suspensions to 231 days, individual changes in the parameters of resuscitation were noted in comparison with 147 days, depending on the nutritional value of the nutrient agar used during these periods (Tables 6-7). Plating *S. aureus* FDA 209P on a full-strength medium showed a strong inhibition of colony-forming activity after 24 and 48 hours, which manifested itself as the growth of singular colonies on plates with a complete medium. At the same time, no significant resuscitation of cells was observed for this microbe on a diluted nutrient medium. During this period, when Enterobacteria were inoculated on full and diluted nutrient agar, a slower cell recovery was observed in both cases compared to the previous sample. Comparison of the number of CFU/ml of staphylococcus on diluted agars on days 147 and 231 (Tables 5 and 7) showed that the tendency towards a decrease in the ability to resuscitate in staphylococcus persisted as the duration of stress was prolonged. At the same time, in this period, individual changes in the parameters of resuscitation were noted in comparison with 147 days, depending on the nutritional value of the agar used during these periods (Tables 6-7). So, for *E. coli* M17 at 231 days, when plated on full-strength agar, the number of CFU / ml at 0 h decreased 1.6 times; at 24 hours by 53 times, and at 48 hours by 3 times (Tables 4 and 6). It can be seen that the most significant decrease in the number of CFU / ml in *E. coli* M17 was recorded at 24 hours (53 times). Apparently, as a result of incubation, which lasted from 147 to 231 days, the cells decreased their proliferative activity. A similar trend towards a decrease afterprolongedincubationup to 231 days was noted for *S. enterica Typhimurium* 79 on diluted agar at 0; 24 hours (2 and 5 times, respectively), (Tables 5 and 7). But by 48 hours, this trend did not persist, and on the contrary, a 2.4-fold increase in CFU / ml was noted.

Comparison of our and literature data on day 231 for complete and diluted agar at the indicated resuscitation hours explained this by the fact of the opposite effect (Tables 6 and 7), since it is known that for a metabolically inactive population, diluted nutrient agar is more gentle and conducive forreversion of cells to normal state [18].This also explains the suppression of the growth of cultured staphylococcal cells and the decrease in CFU/ml as a result of plating on full-strength nutrient agar.

A process of decrease in CFU/ml similar to day 147 was noted for the other member of *Enterobacteriaceae* - *Salmonella enterica Typhimurium* 79 (tables 4 and 6). At 0, 24 and 48 hours on full-strength medium (by 22.6, 64, and 4.5 times). We also noted that maximum increase in CFU/ml (64 times) was at 24 hours like for *E. coli* M17. Analysis of quantity of CFU/ml at day 231 on diluted agar showed (tables 5 and 7) that at 0- and 24-hours *Salmonella* significantly (9 and 16.5 times) decreased their concentration, but had statistically insignificant differences at 48 hours.

Comparison of the colony-forming activity of salmonella on day 231 between complete nutrient agar and diluted agar under the conditions of our experiment (Tables 6 and 7) again confirmed the fact of an increase in the level of resuscitation on diluted agar known for many microbes [6].

Table 6:Resuscitation of VBNC microorganisms by incubating in normal saline and inoculating in full-strength nutrient agar after 231 days of stress.

Microorganism	CFU/ml($\bar{S}\pm\sigma$)		
	0 hours	24 hours	48 hours
*E. coli*M17	$6,4\pm0,7\times10^5$	$1,3\pm0,15\times10^6$	$1,82\pm0,2\times10^8$
SalmonellaTyphimurium 79	$4,68\pm0,51\times10^6$	$4,8\pm0,53\times10^6$	$1,6\pm0,18\times10^{10}$
*Staphylococcusaureus*FDA 209P	$1,86\pm0,2\times10^4$	singular colonies	singular colonies

Table 7:Resuscitation of VBNC microorganisms by incubating in normal saline and inoculating in diluted nutrient agar after 231days of stress.

Microorganism	CFU/ml($\bar{S}\pm\sigma$)

	0 hours	24 hours	48 hours
*E. coli*M17	$4{,}09\pm0{,}45\times10^{5}$	$2{,}8\pm0{,}31\times10^{6}$	$3{,}75\pm0{,}41\pm10^{8}$
SalmonellaTyphimurium 79	$2{,}97\pm0{,}32\times10^{5}$	$2{,}9\pm0{,}32\times10^{7}$	$9{,}6\pm1{,}06\times10^{10}$
Staphylococcusaureus FDA209P	$2{,}6\pm0{,}29\times10^{5}$	$4{,}66\pm0{,}51\times10^{5}$	$2{,}63\pm0{,}29\times10^{5}$

At the end of the observation (day 378, Tables 1 - 2, and 8 - 9), the ability of the cells to resuscitate significantly decreased. For Salmonella, no population growth was observed above the total number of cells counted in the counting chamber on the second day after transferring the cells to saline. For Enterobacteriaceae, the CFU/ml value, even after 48-hour incubation in saline, did not reach the values of the total number of living cells (Tables 1 and 2). For staphylococcus, no significant increase in the CFU/ml parameter was noted either on a full-strength or on a diluted nutrient medium. On the full-strength medium, as well as in the rest of the samples, inhibition of the growth of colonies was present.

Таблица 8:Resuscitation of VBNC microorganisms by incubating in normal saline and inoculating in full-strength nutrient agar after 378 days of stress.

Microorganism	CFU/ml($\bar{S}\pm\sigma$)		
	0 hours	24 hours	48 hours
*E. coli*M17	$9{,}5\pm1{,}05\times10^{5}$	$9{,}6\pm1{,}6\times10\times^{5}$	$4{,}0\pm0{,}44\times10^{7}$
SalmonellaTyphimurium 79	$1{,}06\pm0{,}12\times10^{6}$	$6{,}6\pm0{,}73\times10^{5}$	$1{,}7\pm0{,}19\times10^{7}$
StaphylococcusaureusFDA 209P	$5{,}08\pm0{,}56\times10^{5}$	$1{,}2\pm0{,}13\times10^{4}$	$8\pm0{,}88\times10^{4}$

Таблица 7:Resuscitation of VBNC microorganisms by incubating in normal saline and inoculating in diluted nutrient agar after 378 days of stress.

Microorganism	CFU/ml($\bar{S}\pm\sigma$)		
	0 hours	24 hours	48 hours
*E. coli*M17	$2{,}46\pm0{,}27\times10^{5}$	$8{,}4\pm0{,}92\times10^{5}$	$2{,}06\pm0{,}23\times10^{6}$
SalmonellaTyphimurium 79	$2{,}47\pm0{,}27\times10^{5}$	$1{,}75\pm0{,}19\times10^{6}$	$1{,}29\pm0{,}14\times10^{7}$
Staphylococcus aureus FDA 209P	$1{,}7\pm0{,}19\times10^{4}$	$7{,}21\pm0{,}79\times10^{4}$	$5{,}8\pm0{,}64\times10^{4}$

The results obtained in the experiment confirm the hypothesis that rich media may be unfavorable for the resuscitation of long-term starved cells. This is most clearly demonstrated in the experiment with staphylococcus, for which the inhibition of colony growth of on trophically rich nutrient agar was observed during the entire observation period, while inoculation on a diluted nutrient medium caused an increase in the CFU/ml value.

Conclusion

Experiments were carried out to obtain uncultivated cells of three genera of microorganisms under the action of 2 stresses in a hyperosmotic and trophically starved environment.After a long incubation, more than 1 year, under these conditions, the portion of VBNC cells for all bacteria was more than 99%.Experiments showed that the use of resuscitation methods based, for example, on the removal of one or several stresses affecting the population of microorganisms, can contribute to obtaining more accurate data on the microbial contamination of the materials under study. The stimulating effect of oligotrophic media on the resuscitation of the VBNC microorganisms to a physiologically active state has been shown. A decrease in the efficiency of the resuscitation procedure was noted during incubation of microorganisms for 378 days, which is also consistent with the literature data on the limited period of transition of nonculturable cells to a physiological active state.

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