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Electrochemical investigation of the dynamics of *Mycobacterium smegmatis* cells' transformation to dormant, nonculturable form

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Abstract

Dynamics of transformation of *Mycobacterium smegmatis* cells by cultivation under nonoptimal conditions (partial starvation) to dormant, nonculturable form has been studied. For this aim, an electrochemical method was developed to detect both viable and 'viable but nonculturable' (VBNC) cells. The current produced by bacteria placed at the electrode surface was measured in the presence of 2,6-dichlorophenol indophenol (DCIP) at the applied potential of 350 mV. It has been established that electrochemical activity changes parallel with the growth of biomass. The transition of *M. smegmatis* to a dormant, nonculturable state goes along with the decrease of the detection current up to 20% of the maximum level. This means that nonculturable cells have rather high rest metabolic activity. The course of the CFU values has a complicated character during bacterial growth. The placement of the bacterial culture on the solid medium appears to cause a new stress that stops proliferation and stimulates aggregation. Both processes distort CFU measurement results. The quantitative estimation of the viable but nonculturable cells by counting colonies, measuring optical density and current produced by bacteria has been discussed.

Keywords: Bioelectrochemistry; Electrochemical approach; Dynamics of bacterial transformation; Nonculturable and dormant bacteria; *Mycobacterium smegmatis*; Partial starvation stress; Metabolism level; Computer processing; Revealing latent pathogens

1. Introduction

It has been demonstrated that numerous species of bacteria can be transformed to a 'viable but nonculturable' (VBNC) state in response to environmental stress. This phenomenon is widespread in nature. In particular, this state has been demonstrated for a number of human pathogens (*Esherichia coli*, *Salmonella enteritidis*, *Vibrio cholerae*, *Legionella pneumophila*, *Campylobacter jejuni* and *Mycobacteria tuberculosis*) [1], for some fish pathogens in the marine environment (*Streptococcus parauberis*) [2] and for plant-pathogenic bacteria (*Ralstonia solanacearum*) [3]. Some of these bacteria, as suggested, can persist in the host for long periods unrecognized by the immune system and then resuscitate later causing the disease [4,5].

The existence of dormant, nonculturable cells presents an extremely challenging problem to public health because the cells cannot be detected by traditional culturing methods (due to their inability to grow on solid or liquid media). Moreover, the cells remain potentially pathogenic under favorable conditions [6]. This phenomenon is to be thoroughly studied by express methods because some cells enter a dormant, nonculturable state within a short time interval.

The formation of nonculturable cells is considered to be a survival strategy or a genetically programmed physiological response of bacteria, analogous to the stress responses of the multiplying bacteria (e.g., spore formation). This process involves the induction of mechanisms that lead to sequentially regulated multiplication responses [5]. Ultimately, these cells should also have the ability to resuscitate when conditions become favorable for growth [7,8].

Numerous specific factors, synthesized in response to stress, provide evidence that the dormant state is a genetically programmed process. These include DNA binding protein from starved cells (Dps-like protein), histone-like proteins, and alkyl-substituted hydroxybenzenes (autoinducers or d1 factors). In addition, different changes in the cell morphology imply genetically programmed regulation,

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in particular, cell wall thickening, as well as association of bacteria in aggregates [9–13].

Conversely, there is an opinion that nonculturable state may be a state of moribund bacteria in which cells become progressively debilitated until cell death finally occurs. These cells show some of the same signs of senescence as aging organisms. Although they may maintain low metabolic activity or respiration for some time, ultimately, they are not able to resuscitate [14].

One of the problems to be elucidated is whether the dormancy represents a reversible state of low metabolic activity in which cells can persist for extended periods without division. To this end, it is of importance to determine the metabolic activity level of dormant bacteria. Numerous methods have been developed to detect dormant, nonculturable state of cells. These methods analyze viability by one of two criteria: demonstration of metabolic activity or maintenance of cellular structures. Indication of cellular metabolic activity is based on the measurement of inducible enzymatic activity as an indicator of de novo protein synthesis, the reduction of tetrazolium salts as an indication of an active electron transport chain [7] and on the measurement of the respiratory activity. However, there are limitations associated with the use of these methods.

Tetrazolium salt reduction assay requires nutrient addition and is dependent upon the ability of the organism to respond to the nutrient supplied. A recent report showed that the tetrazolium salt inhibits bacterial metabolism [7]. Measurements of respiration activity are applicable only for aerobic bacteria and they have low sensibility at low concentrations of oxygen.

The methods, based on the investigation of maintenance of stable cellular structure (flow cytometry and methods of direct microscopic analysis using laser tweezers and traps [15]), as well as methods based on the analysis of DNA and mRNA [16], are time consumed and are difficult for employment. None of these methods is suitable for the study of fast processes and express analysis.

Obviously, several methods should be employed simultaneously to obtain more detailed and reliable information on the transition of bacteria into the dormant, nonculturable state; it is also necessary to find a method to continuously monitor cell activity. This paper reports a combination of three measurements employed to accomplish this task. Estimation of biomass was made by optical density at 600 nm (OD_{600}). The quantity of culturable bacteria was determined by the plate count method (CFU). The cell activity was measured by electrochemical methods. A discussion of this combinatory method for investigation and monitoring the metabolic state of bacteria is also presented.

The success of this combinatory method is dependent on the contribution of its component parts. The electrochemical method is an important component and is based on the unique property of viable cells to exchange electrons with an electrode [17–19]. With rare exceptions, there is no biochemical mechanism currently available to account for such a transfer of electrons [20–22]. Nevertheless, there is the fact that a biological electron transport chain can be coupled with the electrochemical circuit. Information about the quantity of cells and their activity can be obtained by voltammetry or by the steady-state current measurements in such a coupled circuit [18,19,23]. In most cases, the bacteria do not secrete any redox-active mediator, metabolite or protein electron carrier. Only direct contact of bacteria with the electrode surface facilitates the electron transfer and consequently provides the change of electrode charge, potential and generation of current in the circuit [17].

Adsorption of bacteria and biofilm adhesion proved to be an essential process when potential and current measurements are performed in such a system. The equilibrium or steady-state condition in the biofilm is approached after a long period, about 20–30 min [24,25]. The measured values of metabolic activity can be significantly distorted because of involvement of these slow processes. Hence, it is clear that the investigation of a fast alteration in the metabolism requires the use of a redox-mediator that can significantly accelerate the electron transfer and can shorten the response time [26–28]. In this paper, 2,6-dichlorophenol indophenol (DCIP) was employed. This mediator is able to penetrate quickly into the cell; it is electrochemically active and its reduced form is quite resistant against oxygen.

This paper reports on the study of the transition dynamics of *Mycobacterium smegmatis* cells to dormant, nonculturable form by cultivation under nonoptimal conditions. It also presents an approach for estimating the quantity of nonculturable bacteria. The elaboration of an electrochemical method, which was the essential task for this investigation, is also presented.

2. Experimental

2.1. Organism and medium

M. smegmatis (155 mc^2) was obtained from the culture collection of Institute of Biochemistry RAS [1]. It was kept on a nutrient agar plate at 4 °C. Experimental cultures were grown aerobically at 37 °C in shake flasks by agitation at 200 rpm. A flask of 750 ml contained 150 ml of medium with nutrient broth E (5.0 g of peptone, 5.0 g of KCl, 1.5 g of meat extract and 1.5 g of yeast extract/l). An amount, 1 ml, of such experimental culture in second generation was taken for obtaining of the nonculturable form. Formation of nonculturable form was observed in stationary phase while cultivating bacteria at the same conditions but in unfavorable modified Hartman's de Bont medium. The medium contained 950 ml of citrate–phosphate buffer, 10 ml of 5% Tween-80, 20 g of $(NH_4)_2SO_4$, 30 ml of glycerol and 10 ml of solution of microelements: 1 g of EDTA, 10 g of $MgCl_2 \cdot 6H_2O$, 0.1 g of $CaCl_2 \cdot 2H_2O$, 0.04 g of $CoCl_2 \cdot 6H_2O$, 0.1 g of $MnCl_2 \cdot 2H_2O$, 0.02 g of $NaMoO_4 \cdot 2H_2O$, 0.2 g of $ZnSO_4 \cdot 7H_2O$, 0.02 g of $CuSO_4 \cdot 5H_2O$ and 0.5 g of $FeSO_4 \cdot 7H_2O$/l, pH 7.0. Citrate–

phosphate buffer was prepared using 200 ml 0.05 M $Na_2HPO_4 \cdot 12H_2O$ by justifying with 0.025 M citric acid (about 200 ml) up to pH 7.0.

2.2. Cell viability

Bacterial suspensions were serially diluted in distilled water and 100 μl samples were plated duplicate onto agar-solidified nutrient broth E. The number of microorganisms was measured by counting colonies that appeared after incubation at 37 °C. The correction of possible overlapping colonies was obtained from the following derived equation

$$N = 0.503 \frac{S}{2q}\left[1 - \sqrt{1 - \frac{4N_{\text{obs}}q}{0.503S}}\right] \quad (1)$$

where S is the square of a plate and q is that of a colony spot in cm^2, N is the total number of colonies and N_{obs} the number of colonies observed. The maximum fractional area covered by nonoverlapping disks was assumed equal to 0.503 as by random placing [29].

2.3. Bacterial growth

The change in the concentration of the bacterial suspension during the time of cultivation was characterized by OD_{600} values obtained on spectrophotometer Hitachi-557 (Japan).

2.4. Electrochemical measurements

For the determination of dormant bacterial state, an electrochemical analysis method was developed. The BAS CV-50W (USA) voltammetry analyzer was used for current and potential measurements. A biomass sample of 2 ml was taken when required, filtered through Schleicher and Schüll filter (Germany), 4 cm in diameter and with pore diameter 0.45 μm. This filter with bacteria was placed on the bottom of the electrochemical cell thermostabilized at 37 °C. Then, 0.75 ml of the citrate–phosphate buffer containing 0.1-mM mediator DCIP was introduced into the bacterial sample being on the filter. The working glassy-carbon electrode (BAS) was lightly pressed to the filter together with the salt bridges of the calomel reference electrode and auxiliary platinum electrode [27,30]. The potential of the electrode was measured by open circuit for 5 min. Then, the potential of 350 mV (vs. SCE) was applied, and the current was recorded for 200 s. Currents by the end of recording were close to steady-state ones. The current value corresponds to the level of the electrochemical activity of the bacteria.

These operations were always repeated two or three times. Fig. 1 illustrates the scheme of an experiment and shows the course of the measured currents as well as the potentials measured and applied. The second and third scans were made under the similar initial conditions; that is, preliminary, the potential of 350 mV was applied for 200 s

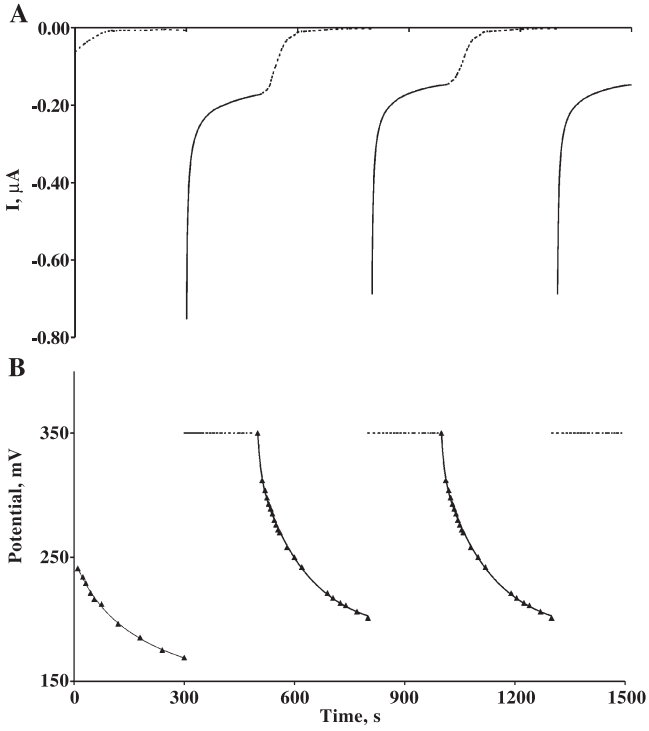

Fig. 1. Scheme of electrochemical measurements. (B) After the electrode was just pressed to the filter with bacteria, the measurement of the electrode potential was performed by open circuit for 300 s. (A) During the time interval 300–500 s, the potential of 350 mV (SCE) was applied and the current was recorded. Then the circuit was opened again and the operations were repeated during the same time intervals. Solid lines are the measured currents, and the potentials measured by open circuit. (—) Applied potentials and currents calculated as described in the text.

and then the circuit was open for 5 min. Therefore, these measurements can be compared to establish the extent of retaining the intact state of the bacteria being close to the electrode during the electrochemical analysis. The current decreased by 5% when the third scan was performed. The value can characterize this extent. In this paper, the second scan current recorded by 200 s was used to analyze the metabolism level and the quantity of bacteria. Reproducibility of experiments is 15% if they are repeated starting with the filtration stage of the same sample. The current deviation is about 20% if the experiments are compared from the start of cultivation of bacteria.

The level of bacterial electrochemical activity was also characterized by the descent rate of the potential (E) measured by open circuit just after the potential of 350 mV was switched off. Fig. 1B shows the course of potential with time by open circuit (—) and then when the potential of 350 mV is applied (---). The course of potential with time in the beginning allows for the determination of the steady-state current by Eq. (2). In this case, the charging current will be equal to

$$I = C\frac{dE}{dt}. \quad (2)$$

Here, C is the electrode capacitance and it is equal to 6.5 μF (measured by CV method).

The current values calculated by this method are coincident in the joint points with the measured current I_{200}. Fig. 1A demonstrates these charging currents taking place when circuit is opened (---) and the currents I_{200} (—). Thus, the steady-sate current was determined by these two methods.

3. Results and discussion

Fig. 2 shows the experimental results when the bacteria *M. smegmatis* grow during the first 60 h although the medium is unfavorable and nutrient deficient and when they enter into a dormant, nonculturable state. Parallel measurements were made of optical density at 600 nm (OD_{600}), quantity of colonies (CFU) and the current recorded 200 s after a 350-mV potential was applied (I_{200}). The optical density measurements indicate the amount of the cell or biomass growth, the quantity of colonies data informs on the concentration of the viable culturable cells, while the current characterizes the total metabolic activity of both culturable and nonculturable cells. All the experimental data were computer fitted to sigmoid or exponential equations. The model curves were reliable enough because, on the one hand, proliferation follows an exponential or logistic law and, and on the other hand, the consistency criterion χ^2 is well minimized by the curve-fitting process.

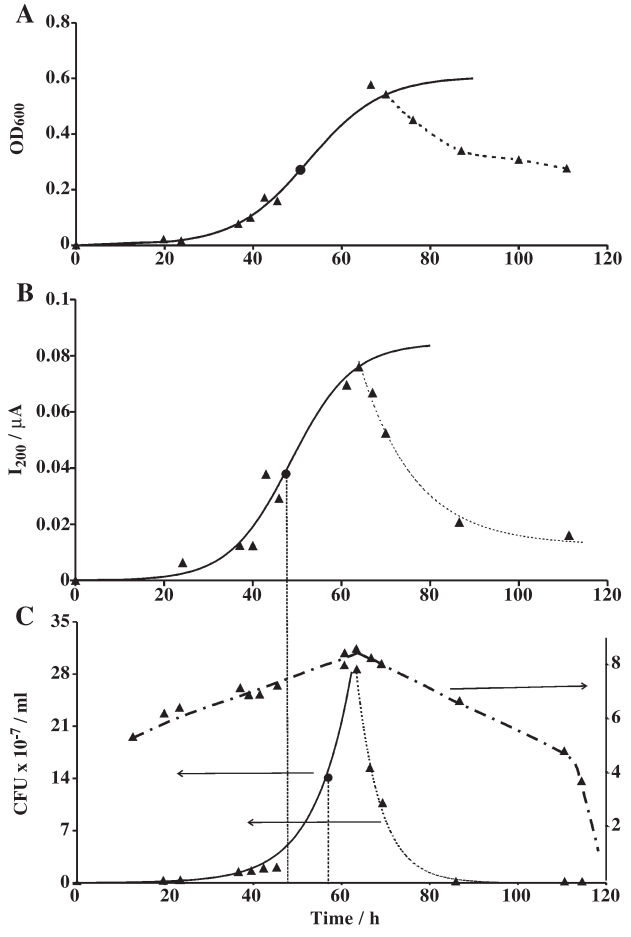

Fig. 2. The time course of optical density at 600 nm (OD_{600}; A), steady state current (I_{200}; B), and CFU values (scale on the left) and their logarithm (scale on the right; C) during the growth and formation of 'nonculturable' forms of *Mycobacterium smegmatis*. (—) Sigmoid in panels A and B and exponential in panel C; curves obtained by fitting the initial part of experimental data. (---) The exponential curves obtained by fitting the second part of experimental data. (- · - · -) The logarithmic curve of CFU values. (▲) Experimental data.

The data analysis process began with the comparison of bacteria growth dynamics, the course of the current and the CFU values with time. The initial parts of the experimental curves (OD_{600} and I_{200}) for growing bacteria (y) correspond well with the time-dependent equation for a sigmoid curve

$$y = \frac{y_\infty}{1 + be^{-kt}}. \tag{3}$$

Here, the y_∞ term represents the maximum value of OD_{600}, I_{200} or CFU, t is the time in minutes and $b = (y_\infty/y_0) - 1$, where y_0 is the initial values of OD_{600}, I_{200} and CFU at $t = 0$ respectively. The value of k characterizes the rate of proliferation. The computer fit of the experimental data to this model equation produced values for all of these parameters. Eq. (3) was derived by assuming that bacterial growth is proportional to the quantity of the viable cells (y) and the part of cell quantity ($1 - y/y_\infty$), characterizing the reserve for the bacterial growth if there is some limit (y_∞) for the cell reproduction. Thus, the differential equation for bacterial growth rate, dy/dt, to be solved is:

$$\frac{dy}{dt} = ky\left(1 - \frac{y}{y_\infty}\right) \tag{4}$$

Bacterial growth lag-periods were calculated analytically for the OD_{600} and I_{200} sigmoid curves using Eq. (3). The tangent to the sigmoid curves was drawn in the point of $y_\infty/2$, and the point of intersection of tangent with abscissa axis gave the value T_l assumed as lag-period. The equation for the calculation of lag-period can be written:

$$T_l = \frac{(\ln b - 2)}{k} \tag{5}$$

The course of the ascending CFU values (y) for bacteria growing and entering into the dormant state was better described by an exponential curve

$$y = y_{0a}\exp(k_a t) \tag{6}$$

and for descending values as

$$y = y_{\max}\exp[-k_d(t - T_{\max})] + y_{\lim} \tag{7}$$

In this case, the time (T_{max}) of the peak value (y_{max}) and the half height times (T_a) of the ascending part (found from graphics) and the descending part ($T_d = T_{max} - \ln 2/k_d$) of the CFU curve and other curves were analyzed. Here, the y_{lim} term is a limit value of y after nonculturable form has been formed. It is equal to zero only for CFU values. The values of y_∞, y_{max}, y_o, y_{0a}, y_{lim}, b, k, k_a, k_d and T_l, T_{max}, T_a, T_d for the OD_{600}, I_{200} and CFU curves are given in Table 1.

Table 1 and Fig. 2 indicate the similarity in the initial parts of the OD_{600} and I_{200} curves before the maximum values are obtained. Indeed, the respective multiplication constant values k (0.12/h and 0.13/h), k_a (0.098/h and 0.094/h), apparent lag-periods T_l (36.2 and 34.5 h) and half-height times T_a (50.7 and 47.5 h) are close. This similarity indicates that increased electrochemical activity is parallel to the growth of biomass and the ratio I_{200}/OD_{600} is constant.

Information can also be gleamed from the relative rates of the appearance of the maxima for all three curves. Although they have their respective maximum at about 64 h, the growth of CFU values starts 8 h later ($T_a = 57$ h) than that of OD_{600} and I_{200} values (51 and 48 h, respectively). Significant growth of OD_{600} and I_{200} values occurred in the 35–45 h time interval, during which time the CFU level remained low. The metabolic activity (I_{200}) and biomass (OD_{600}) reached the half-maximum value by 50 h, while the CFU value came only to the end of the lag-period. This phenomenon was observed in four experiments and this fact is expected to be reliable.

Obviously, these data are not compatible. To find explanation for this observation, it is necessary to take into account microscopic studies of bacterial transition into the dormant state [31]. By the arrival at the 40–50 h time interval, the essential change in bacterial morphology was observed. Moreover, the 40–50 h interval represents a period of intensive aggregation. Tens of microorganisms join to form aggregates. During this period, the method of counting colonies could not be correct because it only reflects the quantity of aggregates. In addition, the placement of bacteria on the solid medium with an excess of nutrient may cause a new, second stress situation. Reorganization of bacterial metabolism after the first stress continues for a long period of time, during which bacteria may become more sensible to the subsequent stresses. Proliferation apparently stops when the second metabolic reorganization occurs. This reorganization process can also initiate a new stage of aggregation. The result of all these phenomena is the distorted measurement of the true dynamics of cell multiplication and subsequent conclusion about the apparent significant decrease in reproductive activity.

Table 1
Parameters of curves obtained by sigmoid and exponential fittings[a]

		OD_{600}	I_{200}	CFU
Sigmoid	y_∞	0.61	87	–
	b	565	780	–
	y_o	1.1×10^{-3}	0.11	–
	k	0.12	0.13	–
	T_l	36	35	–
Exponential ascending part	y_{0a}	0.75×10^{-3}	0.14	1.8×10^5 CFU/ml[b]
	k_a	0.099	0.094	0.12
	T_a	50.7	47.5	56.9
Exponential descending part	y_{max}	0.58	81.4	2.8×10^8 CFU/ml
	T_{max}	66	64	63
	k_d	0.026	0.093	0.18
	T_d	93	71	67
	y_{lim}	0.28	16.3	0 CFU/ml

[a] Dimension of k, k_a, k_d values is h^{-1}, that of T_l, T_a, T_d is h and dimension of y_∞, y_o, y_{0a}, y_{max}, y_{lim} values for I_{200} is nA.

[b] The culture of bacteria in the quantity of 4.1×10^5 CFU/ml was sowed.

Stabilization of metabolism and then turning on the stop mechanism of the multiplication takes place after 60 h. The stop of multiplication is accompanied with the significant decrease of metabolism but some metabolic activity remains (Fig. 2B). The I_{200} value decay reaches 20% of the maximum value. This value corresponds to the real metabolic activity of the dormant bacteria of *M. smegmatis*. Dead bacteria, obtained by treatment with glutardialdehyde, were electrochemically inactive (not shown).

The decay of reproductive activity (CFU curve) is rather sharp and begins earlier ($T_d = 67.2$ h) than the smooth decay of electrochemical activity (I_{200} curve: $T_d = 71.4$ h). When bacteria transform to dormant, nonculturable form the CFU value reaches zero. Thus, dynamics of the CFU value may be distorted by both the second stress response and the additional aggregation of bacteria, but the full conversion of them in the dormant form can be exactly determined.

The I_{200} value depends on aggregation to a lesser extent and corresponds well to the total activity of both culturable and nonculturable bacteria. Though permeation of the DCIP molecules is reduced in aggregates, the rate of electron transport can stay high due to the fast intermolecular electron transfer between molecules of DCIP in different parts of the bacterium and the aggregate. There is another advantage of electrochemical investigation of the bacterial transformations. The measurement of current was performed in the same medium where the bacteria were sowed. Therefore, the second stress was not expected for the bacteria. The effect, caused by the presence of DCIP during electrochemical measurement, was not revealed markedly. Overall, the electrochemical method proved to be suitable for the adequate identification of the level of metabolism and quantity of bacteria.

Morphological changes of cells such as wall thickening and aggregation [1] alter absorbance and scattering of light. Therefore, the second part of the OD_{600} curves may be distorted. In addition, the smooth decrease of the optical density can be caused by cell lyses. Survival strategy of bacteria appears to include all the processes: morphology changes, reducing metabolism, aggregation and wall thickening. The biological role of aggregation may be to save the population at the expense of some aggregate cell lysis to produce needed nutrients for the remaining cells.

In summary, three experimental observations, CFU, OD_{600} and I_{200}, constitute a combined method to follow the bacterium transformation dynamics into a dormant, nonculturable state. The course of proliferation for the first 50 h is better followed by measuring both optical density and currents produced by bacterium metabolism. The next stage is to determine the maximum position of the CFU value and then the time when it becomes equal to zero. By this moment, the conversion is completed. The measured current corresponds to the activity of nonculturable bacteria. Taking into account that this current equals to 20% of the maximum, and using the relation CFU/I_{200} for viable bacteria, it is possible to estimate the quantity of nonculturable bacteria. In this experiment, the quantity is 4.4×10^7 cells/ml. In this paper, the term ''dormant, nonculturable'' has been used for bacteria with significantly reduced level of metabolism. However, this level proved to be much higher than it was expected earlier [1].

4. Conclusions

The proposed combination technique introduces a new analysis approach of importance for medicine and epidemiology applications as well as environmental monitoring. The identification of latent pathogens that exist in a dormant, nonculturable state is the key element of the technique, and the electrochemical procedure reported here is an essential element for the determination of reliable information on the transition of bacteria into the dormant, nonculturable state. The I_{200} information combined with CFU analysis and the optical density measurements reveals the state of bacteria and determines the activity of dormant, nonculturable bacteria. The CFU measurement alone does not adequately reflect the dynamics of bacterial transformation but gives exact time of full absence of proliferation. The optical density values are distorted in the stationary phase of bacterial growth. However, the measurement of optical density provides insight into in the initial part of the growth phase. The I_{200} data established that the activity of *M. smegmatis* cells in the dormant, nonculturable state remained but at a level that was 20% of the activity of viable bacteria. This property is important for revealing the metabolic activity of cells in such states when the CFU measurement is not able to determine nonculturable state. This property can be employed also for the quantitative determination of dormant, nonculturable form of bacteria.

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References

[1] A.S. Kaprelyants, G.V. Mukamolova, T.V. Votyakova, H.M. Davey, D.B. Kell, Dormancy in non-sporulating bacteria: its significance for environmental monitoring, Rapid Methods for Analysis of Biological Materials in the Environment, Kluwer Academic Publishers, 2000, pp. 49–65.
[2] M. Curras, B. Magarinos, A.E. Toranzo, J.L. Romalde, Dormancy as a survival strategy of the fish pathogen *Streptococcus parauberis* in the marine environment, Dis. Aquat. Org. 52 (2) (2002) 129–136.
[3] B.E. Grey, T.R. Steck, The viable but nonculturable state of *Ralstonia solanacearum* may be involved in long-term survival and plant infection, Appl. Environ. Microbiol. 67 (9) (2001) 3866–3872.
[4] M.O. Shleeva, K. Bagramyan, M.V. Telkov, G.V. Mukamolova, M. Young, D.B. Kell, A.S. Kaprelyants, Formation and resuscitation of 'non-culturable' cells of *Rhodococcus rhodochrous* and *Mycobacterium tuberculosis* in prolonged stationary phase, Microbiology 148 (2002) 1581–1591.
[5] G.V. Mukamolova, A.S. Kaprelyants, D.B. Kell, M.Y. Young, Adoption of the transiently 'non-culturable state—a bacterial survival strategy? Adv. Microb. Physiol. 47 (2003) 65–129.
[6] T. Lillebaek, A. Dirksen, I. Baess, B. Strunge, V.O. Thomsen, A.B. Andersen, Molecular evidence of endogenous reactivation of *Mycobacterium tuberculosis* after 33 years of latent infection, J. Infect. Dis. 185 (2002) 401–404.
[7] D. McDougald, S.A. Rice, D. Weichart, S. Kjelleberg, Nonculturability: adaptation or debilitation? FEMS Microbiol. Ecol. 25 (1) (1998) 1–9.
[8] M. Bar, J. von Hardenberg, E. Meron, A. Provenzale, Modelling the survival of bacteria in drylands: the advantage of being dormant, Proc. R. Soc. Lond., B Biol. Sci. 269 (1494) (2002) 937–942.
[9] S. Gupta, S.B. Pandit, N. Srinivasan, D. Chatterji, Proteomics analysis of carbon-starved *Mycobacterium smegmatis*: induction of Dps-like protein, Protein Eng. 15 (6) (2002) 503–512.
[10] B.H. Lee, B. Murugasu-Oei, T. Dick, Upregulation of a histone-like protein in dormant *Mycobacterium smegmatis*, Mol. Gen. Genet. 260 (5) (1998) 475–479.
[11] V.V. Yeremeev, T.K. Kondratieva, E.I. Rubakova, S.N. Petrovskaya, K.A. Kazarian, M.V. Telkov, S.F. Biketov, A.S. Kaprelyants, A.S. Apt, Proteins of the Rpf family: immune cell reactivity and efficacy against tuberculosis in mice, Infect. Immun. 71 (8) (2003) 4789–4794.
[12] A.F. Cunningham, C.L. Spreadbury, Mycobacterial stationary phase induced by low oxygen tension: cell wall thickening and localization of the 16-kDa alpha-crystallin homolog, J. Bacteriol. 180 (4) (1998) 801–808.
[13] A.I. Kolpakov, O.N. Ilyinskaya, M.M. Bespalov, F.G. Kupriyanova-Ashina, V.F. Galchenko, B.I. Kurganov, G.I. El-Registan, Stabilization of enzymes by anabiosis autoinducers as a possible mechanism of resistance of resting microbial forms, Microbiology 69 (2) (2000) 224–230.
[14] T. Nystrom, Not quite dead enough: on bacterial life, culturability, senescence and death, Arch. Microbiol. 176 (2001) 159–164.
[15] M. Ericsson, D. Hanstorp, P. Hagberg, J. Enger, T. Nystrom, Sorting out bacterial viability with optical tweezers, J. Bacteriol. 182 (19) (2000) 5551–5555.
[16] S.R. Pai, J.K. Actor, E. Sepulveda, R.L. Hunter, C. Jagannath, Identification of viable and non-viable *Mycobacterium tuberculosis* in mouse organs by directed RT-PCR for antigen 85B mRNA, Microb. Pathog. 28 (6) (2000) 335–342.
[17] M.J. Allen, Electrochemical aspects of metabolism, Bacteriol. Rev. 30 (1) (1966) 80–93.
[18] T. Matsunaga, I. Karube, S. Suzuki, Electrochemical determination of cell populations, Eur. J. Appl. Microbiol. Biotechnol. 10 (1980) 125–132.
[19] R.G. Compton, S.J. Perkin, D.P. Gamblin, J. Davis, F. Markin, A.N. Padden, P. John, *Clostridium isatidis* colonised carbon electrodes:

voltammetric evidence for direct solid state redox processes, New J. Chem. 24 (2000) 179–181.
[20] B.H. Kim, T. Ikeda, S. Park, H.J. Kim, M.S. Hyum, K. Kano, K. Takagi, H. Tatsumi, Electrochemical activity of an Fe(III)-reducing bacterium, *Shewanella putrifaciens* IR-1, in the presence of alternative electron acceptors, Biotechnol. Tech. 13 (1999) 495–498.
[21] C.R. Myers, J.M. Myers, Localization of cytochromes to the outer membrane of anaerobically grown *Shewanella putrefaciens* MR-1, J. Bacteriol. 174 (11) (1992) 3429–3438.
[22] C.A. Pham, S.J. Jung, N.T. Pung, J. Lee, I.S. Chang, B.H. Kim, H. Yi, J. Chun, A novel electrochemically active and Fe(III)-reducing bacterium phylogenetically related to *Aeromonas hydrophila*, isolated from a microbial fuel cell, FEMS Microbiol. Lett. 223 (2003) 129–134.
[23] H.N. Li, Y.X. Ci, Electrochemical method for analyzing intracellular redox activity changes of the etoposide-induced apoptosis in HL-60 cells, Anal. Chim. Acta 416 (2000) 221–226.
[24] R. Armon, J. Starosvetsky, M. Dancygier, D. Starosvetsky, Adsorption of *Flavobacterium breve* and *Pseudomonas fluorescens P*17 on different metals: electrochemical polarization effect, Biofouling 17 (2001) 289–301.
[25] J.P. Busalmen, S.R. de Sanchez, Adhesion of *Pseudomonas fluorescens* (ATCC 17552) to nonpolarized and polarized thin films of gold, Appl. Environment. Microbiol. 67 (7) (2001) 3188–3194.
[26] L. Karube, E. Tamiya, H. Matsuoka, Electrochemical estimation of protoplast population, J. Biotechnol. 1 (1984) 197–204.
[27] C. Xu, G. Li, H. Cai, Microbial sensor for on-line determination of microbial population in a fermenter, Sens. Actuators, B 12 (1993) 45–48.
[28] N.J. Richardson, S. Gardner, D.M. Rawson, A chemically mediated amperometric biosensor for monitoring eubacterial respiration, J. Appl. Bacteriol. 70 (1991) 422–426.
[29] L. Finegold, J.T. Donnell, Maximum density of random placing of membrane particles, Nature 278 (5703) (1979) 443–445.
[30] S. Han, X. Li, G. Guo, Y. Sun, Z. Yuan, Voltammetric measurement of microorganism populations, Anal. Chim. Acta 405 (2000) 115–121.
[31] M. Shleeva, G. Mukamolova, M. Young, H. Williams, A. Kaprelyants, Formation of non-culturable cells of *Mycobacterium smegmatis* in stationary phase in response to growth under sub-optimal conditions and their Rpf-mediated resuscitation, Microbiology UK 150 (2004) 1687–1697.