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EXPERIMENTAL ARTICLES

The Role of Intercellular Contacts in the Initiation of Growth and in the Development of a Transiently Nonculturable State by Cultures of *Rhodococcus rhodochrous* Grown in Poor Media

S. A. Voloshin*,[1], M. O. Shleeva*, A. V. Syroeshkin, and A. S. Kaprelyants***

**Bach Institute of Biochemistry, Russian Academy of Sciences, Leninskii pr. 33, Moscow, 117071 Russia*

***State Institute of Oceanography, State Committee for Hydrometeorology, Moscow, Russia*

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Abstract—It was found that the growth of *Rhodococcus rhodochrous* cells in a modified Saton's medium strongly depends on the rate of culture agitation in the flask: agitation at 250 rpm in flasks with baffles stops cell multiplication, whereas slight agitation leads to pronounced culture growth. The growth retardation phenomenon was reversible and did not manifest itself in exponential-phase cultures or when the cells were grown in a rich medium; furthermore, it was not connected with the degree of culture aeration. When agitated at a moderate rate, the bacterial cells formed aggregates in the lag phase, which broke up into single cells in the exponential phase. The inhibitory effect of vigorous agitation was removed by the addition, to the medium, of the supernatant (SN) of a log-phase culture grown in the same medium with moderate agitation. Vigorous agitation is thought to interfere with cell contact, whose establishment is necessary for the development of an *R. rhodochrous* culture in a poor medium, which occurs in the form of (micro) cryptic growth. When grown in a modified Saton's medium, *R. rhodochrous* cells were capable of transition, in the prolonged stationary phase, to a resting and transiently nonculturable state. Such cells could be resuscitated by incubation in a liquid medium with the addition of the supernatant or the Rpf secreted protein. The formation of transiently nonculturable cells was only possible under the conditions of a considerable agitation rate (250–300 rpm), which prevented secondary (cryptic) growth of the culture. This circumstance indicates the importance of intercellular contacts not only for the initiation of growth but also for the transition of the bacteria to a dormant state.

Key words: aggregation, transiently nonculturable cells, *Rhodococcus rhodochrous*, resuscitation.

Until recently, it was thought that bacterial cultures are communities of independent cells capable of autonomic growth. However, a large body of facts has accumulated that testifies to the complexity of the processes occurring in a bacterial population, where the cells are in constant interaction. This complexity is reminiscent of that found in a multicellular organism [1]. Now, the role and mechanisms of bacterial intercellular communication via the secretion of signaling molecules have been clarified [2].

The physical interactions between cells and, especially, their functional role, remain to be studied in greater detail. Generally, the formation of cell aggregates is well known in microbiology. Thus, the appearance of specialized structures, namely, fruiting bodies, in myxobacteria can be considered as a differentiation stage for a culture experiencing a deficiency of nutrients [3]. Some species of bacteria form so-called consortia, within which different intermediate biochemical processes occur with the involvement of a certain type of cell, which provides for the effective formation of the end product (e.g., generation of methane and its oxidation) [4]. Biofilms and bacterial colonies can be regarded as an extreme case of intercellular interaction, when aggregates are so large that they can be seen with the naked eye. The occurrence of cells in biofilms endows them with certain advantages, e.g., increased resistance to antibiotics or to the defense systems of the host organism [5]. Numerous studies on the organization of microbial colonies provide evidence of the morphological and physiological heterogeneity of cell composition [6]. It is noteworthy that individual outer cell envelopes (capsules, extracapsular mucus, etc.) tend to merge, which results in the formation of a unified biopolymer matrix surrounding the colonies. The constituents of the matrix are acid polysaccharides, glycosylphosphate-containing biopolymers such as teichoic acid, and glycoproteins, as well as (in certain bacteria, e.g., bacilli) polyglutamic acid and other biopolymers [7]. Similarly to the intercellular matrix of animal tissues, the microbial matrix includes fibrillar elements [7]. Animal and microbial matrices also contain certain common chemical components (e.g., sialic acids). In a similar way to the eukaryotic cells in the tis-

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

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sues of animals and plants, prokaryotes establish intracolonial intercellular contacts, which probably aid in the propagation of signaling molecules in the population, especially those that do not diffuse in the medium. These intercellular contacts are established at the expense of diverse surface structures, including microfibrils, cone-shaped projections, cell wall evaginations, and glycocalyx [8].

The data cited show that, in bacterial colonies, there exists heterogeneity and the cells can perform different functions. Nevertheless, the biological significance of bacterial aggregations, including those formed in liquid media, still needs to be understood in greater detail. Microbiologists are well aware that aggregation often occurs when cells are grown under nonoptimal conditions. However, the significance of this phenomenon for the vital activity of the cells is unknown.

In this study, we attempted to clarify the role of physical cell interactions (aggregation) in the development of a bacterial culture, or, more specifically, their role during the transition of bacteria to a resting (dormant) state and their exit from this state. The subject of this study was the gram-positive bacterium *Rhodococcus rhodochrous*, which is known to form aggregates at the initial stage of culture development.

MATERIALS AND METHODS

Conditions of cultivation of *Rhodococcus rhodochrous*. In order to obtain the inoculum, *Rhodococcus rhodochrous* NCIMB 13805 was grown in a rich Broth E medium (LabM) for 24 h. The inoculum was introduced in an amount of 1 ml into 750-ml Erlenmeyer flasks containing 125 ml of the rich Broth E medium (LabM) or 125 ml of a modified Saton's minimal nutrient medium with the following composition (g/l): $MgSO_4 \cdot 7H_2O$, 0.5; L-asparagine, 4; an iron–citrate–ammonium complex, 0.05; sodium citrate, 2; $K_2HPO_4 \cdot 3H_2O$, 7.75; $NaH_2PO_4 \cdot 2H_2O$, 4.25; glycerol, 60 ml; and $ZnSO_4 \cdot 7H_2O$ (1% solution), 0.1 ml. In some of the experiments, flasks with baffles were used. In other experiments, Twin-80, at a concentration of 0.05%, was added to the culture to act as a detergent. The initial amount of cells was 10^3 or 10^5 cells/ml. The cultures were grown at 37°C at the agitation rates indicated in the Results and Discussion section.

The optical density was measured on an SF-14 spectrophotometer at 600 nm in a 1-cm cuvette.

Obtaining transiently nonculturable *R. rhodochrous* forms. *R. rhodochrous* was grown in the rich Broth E medium for 18 h, and 1 ml of this culture was used to inoculate the flasks containing modified Saton's minimal nutrient medium. These flasks were incubated at an agitation of 200 rpm for 86–90 h.

Determination of cell viability from CFU numbers. Bacterial suspensions were serially diluted in a growth medium, and agar dishes with agarized Broth E were inoculated with 100 µl of each dilution. The colonies were counted after three to four days of growth. The detection threshold was 5 CFU/ml.

Determination of cell viability according to the serial tenfold dilution method (STDM). Serial tenfold dilutions were also used during resuscitation of dormant cells in liquid medium, and the number of resuscitated cells was determined simultaneously. The reactivation was carried out in 48-well plates, in which 12 wells were used per dilution. In the first four wells, containing 0.5 ml of Saton's medium with 0.5% yeast extract, 50 µl of the cell suspension from the dilution under study was introduced. In the following four wells, the protein Rpf was added to a concentration of 100 pM to promote cell resuscitation. In the remaining four wells, 0.5 ml of a supernatant of a log-phase (20-h) *R. rhodochrous* culture, supplemented with 0.5% yeast extract, was used as the resuscitation medium. The incubation was carried out at 37°C at an agitation rate of 150 rpm for 5–6 days. The cell viability was judged by visually determining the growth of the microbial culture in each of the wells.

The total cell number (TCN) was determined microscopically in a Helber chamber and was calculated according to the formula $n/5 \times 10^8$, where n is the average cell number in one square. For the counting process, dilutions providing for the occurrence of 50–200 cells per square were used.

Obtaining a recombinant factor Rpf. The recombinant protein Rpf was obtained according to the method described by Mukamolova *et al*. [9].

Obtaining the supernatant. The supernatant was obtained from log-phase (20-h) and stationary-phase (132-h) *R. rhodochrous* cultures by centrifugation at 10000 *g*, with subsequent filtration of the liquid phase through a filter possessing a 0.2-µm pore size (Watmann).

Quantitative determination of the cell distribution by size and shape was performed with a Malvern 3600Ec laser diffraction determiner of particle size and shape. This device includes a low-powered helium–neon laser, which emits a monochromatic light beam (λ_{max} = 633 nm) that passes through the experimental cell. The results, expressed as a percentage of each fraction in relation to the total cell number, were processed according to the method described by Syroeshkin *et al.* [10].

Measurements of the respiratory activity and membrane potential of the cells. Measurement of the respiratory activity of the cells was carried out, as described by Kaprelants *et al.* [11], with the use of 5-cyano-2,3-di-tolyl-tetrazolium chloride (CTC). Measurement of the membrane potential was carried out with the use of Rhodamine 123 as described by Kaprelants *et al.* [12].

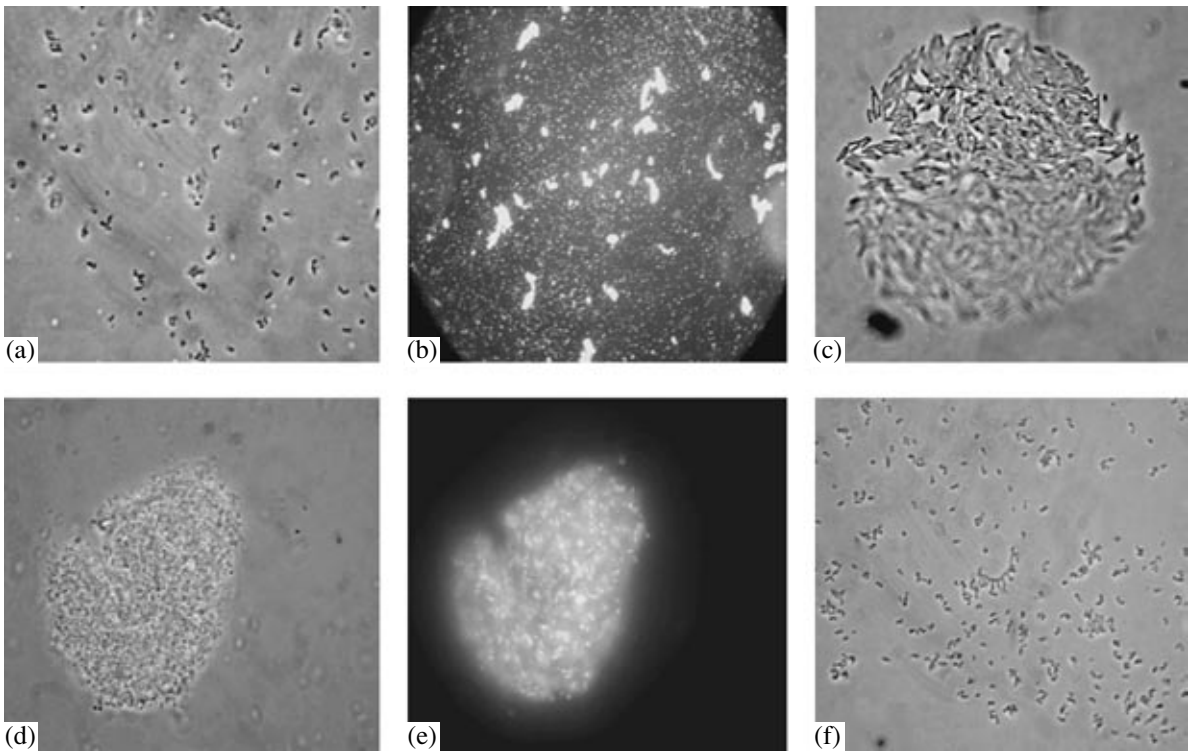

Fig. 1. Light and fluorescent microscopy of an *R. rhodochrous* culture: (a) inoculum (24 h); (b–e) midexponential growth phase; and (f) stationary growth phase. (e) Cells stained with propidium iodide and revealed with fluorescent microscopy. (c) A cell aggregate covered with transparent material. Magnification: (b) 100× and (a, c–f) 1000×.

RESULTS AND DISCUSSION

The formation of cell aggregates at the early stages of development of the *R. rhodochrous* culture in liquid media. Microscopic study of the development of the *R. rhodochrous* culture showed that, after 12 h of growth in a liquid medium, when the cell concentration was 5×10^6 cells/ml, virtually all the cells appear in the form of small aggregates (10–40 cells per aggregate) (Fig. 1). With an increase in the growth time, the aggregates become enlarged; after 18 h of incubation (the end of the exponential phase), they begin to disintegrate; and by the 24th hour (the beginning of the stationary phase), virtually the whole culture consists of single cells. The cell aggregates appear as accumulations of irregular form. Sometimes large aggregates are seen to be covered by transparent material, which probably stabilizes the cells in the aggregate (Fig. 1c). Staining the large aggregates with the fluorescent dye propidium iodide, which reveals bacteria only with damaged cytoplasmic membrane, helped to identify a considerable number of damaged cells within them (Fig. 1e).

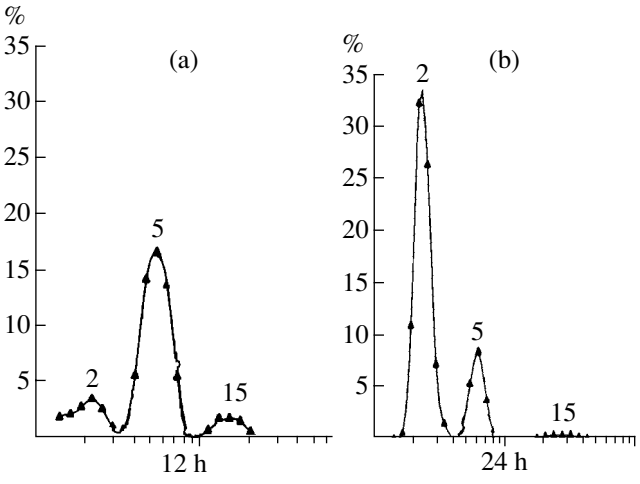

Fig. 2. Diffraction analysis of the size of the particles and their distribution in (a) a 12-h and (b) a 24-h culture of *R. rhodochrous*. Abscissa shows the size of the particles in μm. The sizes of the most frequently occurring particles are 2, 5, and 15.

The cells' size distribution in the population of the growing *R. rhodochrous* culture, deduced from the diffraction of the laser beam passing through a cell suspension, confirmed that, at the early stages (12 h) in the development of *R. rhodochrous*, most of the cells occur in small (5 μm in diameter) and medium-sized (15 μm in diameter) aggregates (Fig. 2). At the late stages of development (24 h), the bulk of the culture consists of single cells (2 μm in diameter).

The role of intercellular interactions in the initiation of *R. rhodochrous* growth in Saton's synthetic medium. In order to clarify the role played by the

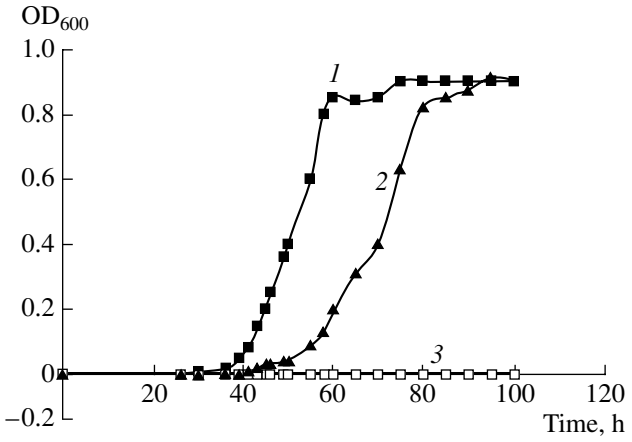

Fig. 3. Relationship between the growth of *R. rhodochrous*in Saton's liquid medium and the intensity of agitation. Erlenmeyer flasks containing 150 ml of Saton's medium were inoculated (10^3 cells/ml) with an *R. rhodochrous* culture grown in a rich medium (Broth E, 24 h). (*1*) Agitation at 250 rpm in an ordinary flask; (*2*) bubbling of the medium without agitation; and (*3*) agitation at 250 rpm in a flask with baffles.

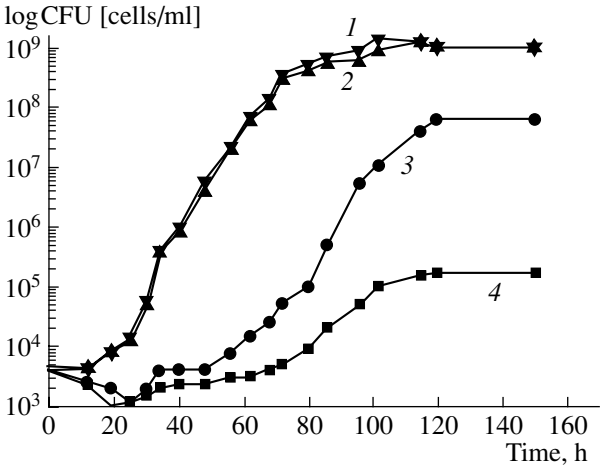

Fig. 4. Growth of *R. rhodochrous* cultures under different agitation conditions in rich and poor media. The growth conditions were as described in the caption to Fig. 3. In experiments 1 and 2, yeast extract (YE) was added to the Saton's medium, adjusting it to a final concentration of 0.5%. In experiment 3, the Saton's medium was replaced by the supernatant (SN) of a log-phase *R. rhodochrous* culture grown in this medium. (*1*) Saton's medium + YE, an ordinary flask; (*2*) Saton's medium + YE, a flask with baffles; (*3*) SN, a flask with baffles; (*4*) Saton's medium, a flask with baffles.

aggregation of rhodococcal cells at the early stages of population development, the culture growth was studied under conditions in which the intercellular contacts were destroyed mechanically. For this purpose, the bacterial cells were grown at different agitation rates. Enhanced agitation of the culture (250 rpm) appeared to impair cell growth, and agitation at 250 rpm in flasks with bafflers completely stopped visible cell growth (Fig. 3). The observed effect was not related to increased aeration of the culture, since the bubbling of air under moderate agitation conditions did not affect the growth rate (Fig. 3). In addition, the *R. rhodochrous*cells showed a virtually 100% survival rate on a solid medium, where oxygen availability is virtually unlimited. If the intensity of the agitation was decreased or the flask was allowed to stand, so that the culture could grow without agitation, very soon, an increase in turbidity indicated cell multiplication (data not shown). An Increase in the agitation rate at the moment when the culture was already in the logarithmic phase of development did not affect the growth rate (data not shown). A more detailed study of the culture development under conditions resulting in growth retardation revealed that, after inoculation (in this experiment, the inoculum dose was 10^3 cells/ml), the cells underwent several cycles of division, attaining a density of 10^5 cells/ml, and then stopped dividing; however, their concentration did not decrease (Fig. 4). It is noteworthy that the addition of the yeast extract to the Saton's medium at a concentration of 0.5% completely abolished the negative effect of vigorous agitation (Fig. 4). It should also be noted that this effect was observed only when the cells were grown with agitation in the Saton's minimal medium; in the rich Broth E medium, the cell growth curves did not differ at different agitation rates (data not shown). These observations demonstrate that vigorous cell agitation in flasks with baffles did not cause lethal mechanical cell damage. Thus, when the stationary-phase cells were transferred to a vigorously agitated fresh liquid nutrient medium, they retained their viability. We assume that, in a culture with a very small amount of inoculum, especially if its quality is nonoptimal, other conditions for intercellular communication are important.

The formation of transiently nonculturable *R. rhodochrous* cells in Saton's minimal medium. The above-described experiments demonstrated that intercellular interactions in the *R. rhodochrous* culture manifest themselves in a poor medium, i.e., under unfavorable conditions. In order to find out whether this conclusion can be generalized, we staged experiments to study the role of intercellular contacts for rhodococcal cells experiencing another type of stress, namely, transition to the dormant state and resuscitation from the dormant state. To model this process, we used an approach, which we earlier employed for *Micrococcus luteus* cells, consisting in prolonged incubation of the cells in the stationary phase [11]. For the transiently nonculturable *R. rhodochrous* cells to be obtained, the culture was initially grown in the rich Broth E (LabM) medium for 18–19 h and then used to inoculate (10^5 cells/ml) the modified Saton's minimal medium (the increased phosphate concentrations in this medium stabilized the medium pH throughout the growth period). After 24 h of growth, the stationary phase of the culture growth was observed under these condi-

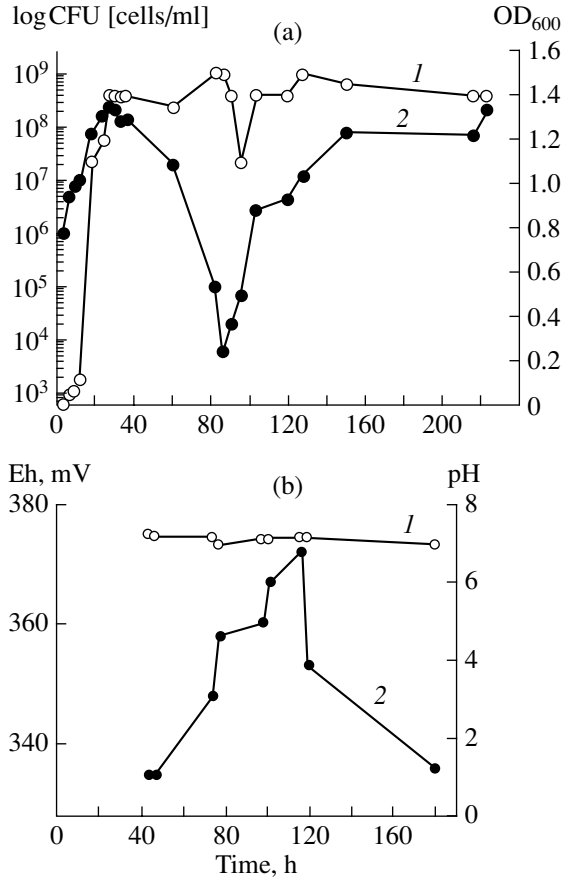

Fig. 5. Dynamics of development of the cell population of *R. rhodochrous* in a poor medium in prolonged stationary phase. The inoculum was grown in the rich Broth E medium for 18 h, and the poor medium was inoculated at a dose of 10^6 cells/ml. During incubation, the optical density (a, curve *1*), CFU number (a, curve *2*), pH (b, curve *1*), and redox potential (b, curve *2*) were measured.

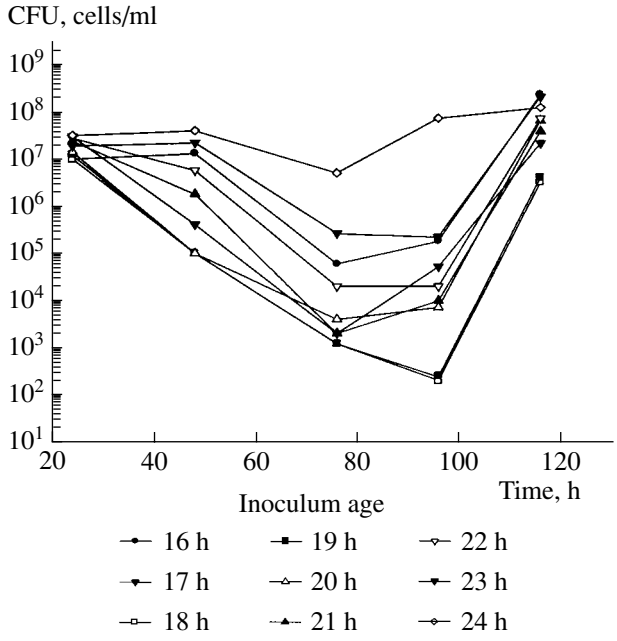

Fig. 6. Influence of the inoculum age on the formation of nonculturable *R. rhodochrous* cells in the stationary phase. Inocula of different ages were grown in the rich Broth E medium for the purpose of inoculating modified Saton's medium (see the caption to Fig. 5a). The cells were inoculated at 200 rpm.

tions, but further incubation led to a sharp decrease in the number of viable cells (CFU numbers) 30 h after inoculation (Fig. 5a). The minimal CFU value was recorded between the 80th and 100th hours of incubation. With further cultivation, the CFU number gradually increased until it attained the value observed in the stationary phase (in the 24th hour). With further cultivation, the total cell number (data not shown) and the optical density of the culture remained virtually the same (Fig. 5a).

The time at which the CFU number attained its minimal value differed from one experiment to another, but the CFU minimum coincided with the moment of increase in the culture redox potential (Fig. 5b) (the culture pH barely changed, Fig. 5b). The minimal CFU value attained in the stationary phase depended on the inoculum age. The greatest CFU decrease was observed when the inoculum was aged 18–19 h (Fig. 6). Microscopy of the bacteria that were incapable of producing colonies on the solid media revealed that most of the cells in this phase were coccoid or ovoid and measured 0.6 μm, whereas ordinary cells were rod-shaped and measured 2–4 μm (not shown). Approximately 10% of the cells in this growth phase were metabolically active (according to fluorescent microscopy with the use of rhodamine 123 as a membrane potential indicator and CTC as an indicator of respiratory activity). When using propidium iodide as a dye, 80–90% of the cells did not stain, which is indicative of membrane integrity [13].

Thus, at a certain stage of prolonged cultivation, *R. rhodochrous* cells were obtained that exhibited minimum colony-forming capacity but retained their structural integrity. Such properties allow the assignment of the rhodococcal cells occurring during this period of loss of colony-forming capacity (80–85 h) to transiently nonculturable proliferative–dormant forms [14]. For the transition of *R. rhodochrous* cells to the transiently nonculturable state, the rate of culture agitation appeared to be an important factor: it had to be no less than 200 rpm to ensure a quick and complete transition. At a lesser agitation rate, no CFU decrease was observed (data not shown). Under these conditions, the cells formed aggregates of varying size, where, evidently, nonviable cells with a damaged membrane barrier were present (not shown).

Activation of transiently nonculturable *R. rhodochrous* cells. In order to prove the ability of *R. rhodochrous* cells to form transiently nonculturable dormant forms, it is essential to demonstrate their ability to

restore their metabolism and the capacity for division. We have earlier shown that a special procedure, the activation of dormant cells, is necessary for the reversal of growth [11]. Similarly to the activation of *M. luteus* cells [15], the activation of transiently nonculturable cells of *R. rhodochrous* (Fig. 5a) implies cultivation in a specially selected liquid medium. The serial tenfold dilution method was used for quantitative assessment of the reversal of transiently nonculturable cells to the dividing state [15]. The incubation of transiently nonculturable cells in the Saton's medium with the addition of 0.5% yeast extract led to an increase in viability by three orders of magnitude as compared to that determined according to the CFU number (table). Different changes in the medium composition did not result in a further increase in the STDM-determined value. When resuscitation was carried out in a rich medium (Broth E) (table) or more than 0.5% yeast extract was added to the Saton's medium, a decrease in the STDM-determined cell titer was observed as compared to the resuscitation in the Saton's medium without yeast extract (data not shown). The addition of catalase (3 mg/ml) or pyruvate (0.5 mM) did not contribute to cell resuscitation either in liquid or solid media. However, a substantial increase in the number of dividing cells determined by the STDM was observed after four to five days of incubation of transiently nonculturable cells in the supernatant obtained by centrifugation of log-phase cultures of *R. rhodochrous* (table). The supernatant obtained from the cultures aged 18–20 and 118–132 h revealed resuscitating activity, whereas the supernatant obtained from 45- to 70-h cultures had an inhibitory effect (Fig. 7). The latter supernatant also suppressed the development of the *R. rhodochrous* culture in the logarithmic growth phase (data not shown). The resuscitating activity of the supernatants depended on their concentration: the maximum effect was observed when it constituted 50% or more of the medium volume (Fig. 7). The addition of the recombinant protein Rpf, which is secreted by *M. luteus* and aids in the activation of its dormant cells in Saton's medium with 0.5% yeast extract, also increased the effectiveness of activation of transiently nonculturable *R. rhodochrous* cells, but the effect was less than that observed in the supernatant (table). The intensity of the culture agitation proved to be equally important for both passing into and out of the transiently nonculturable state. However, in the latter case, vigorous agitation of the cells in the Saton's medium negatively affected the efficiency of cell activation. When the cells suspended in the supernatant were activated, the agitation rate was not crucial (data not shown). This result also agrees with the supposition that, under favorable conditions of growth, the cells behave independently of each other in any physiological process.

In this study, we found that cells of the bacterium *R. rhodochrous* cultivated in a poor medium depend on the presence of physical intercellular contacts. This conclusion is based on the fact that vigorous agitation, which evidently destroys intercellular contacts established in the lag phase of population development, prevents the bacterium from initiating growth (Fig. 1). The possibility of culture growth under conditions of aeration by air bubbling (Fig. 3) and the activation of growth after a decrease in the intensity of agitation preclude attributing the effects observed to excessive oxygenation or cell damage by agitation. This behavior of the bacterial culture is reminiscent of the requirements of some eukaryotic cell cultures, which need weak agitation to establish the intercellular contacts necessary for growth in the form of a tissue monolayer. Many bacteria are known to exhibit cell aggregation when grown in a liquid medium [6]. Moreover, it is well known that such "multicellular" organisms can be stabilized by special supracellular structures, i.e., secreted polymers that envelop the cell aggregates (examples are the orga-

Table

Method of study	Number of cells per ml
TCN	7.39×10^8
CFU	6.7×10^2
STDM	
Broth E	4.5×10^4
Saton's	1.5×10^5
Saton's + Rpf	1.4×10^6
SN-1	3.7×10^7
SN-2	1.6×10^8

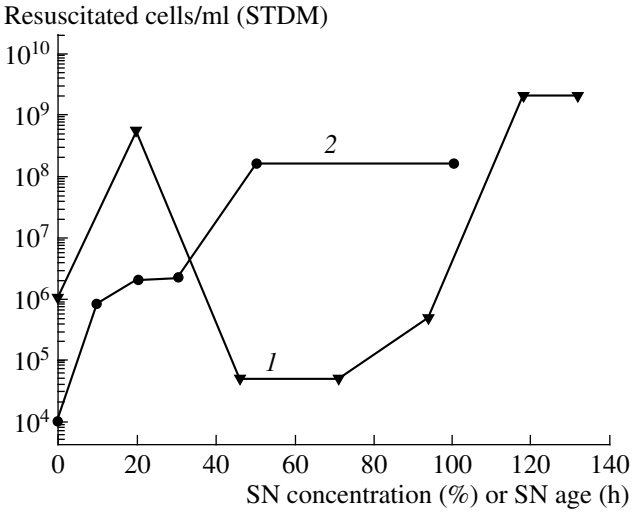

Fig. 7. Influence of the supernatant concentration and age on the resuscitation of nonculturable forms. An *R. rhodochrous*culture occurring in the nonculturable state was inoculated into (*1*) supernatants of different age and (*2*) media containing different amounts of the supernatant of a log-phase *R. rhodochrous* culture. In order to obtain the SNs, 1 ml of a 24-h inoculum grown in a rich medium was introduced into 125 ml of Saton's medium.

nization of streptococcal chains [16] and colonies of *E. coli, St. aureus*, or *M. bovis*, covered by several polymer layers [17]). The extracellular material found around the *R. rhodochrous* aggregates (Fig. 1) may also be involved in aggregate stabilization.

When speculating on the role of cell aggregation in the growth of *R. rhodochrous* cultures, it is essential to remember that their growth was independent of intercellular contacts in a rich medium or after the exponential growth phase had been attained by the culture. The latter fact is obviously connected with the accumulation, in a moderately agitated culture, of certain metabolites that promoted its growth when the agitation rate was increased; accumulation of such metabolites is shown by the stimulating effect of the culture fluid (Fig. 4). We suggest that the products of cell degradation (lysis), which is probably undergone by some of the cells in the culture during the lag phase, may well be these metabolites. Thus, the growth of rhodococcal cells, at least at the beginning of the exponential phase, is likely to be stimulated by cell degradation products and actually represents so-called cryptic growth. An alternative explanation may be connected with the accumulation of specific growth-stimulating factors, such as protein Rpf, in the growth medium, as we earlier revealed. However, this assumption seems to be less probable, since the stimulants released into the growth medium were insensitive to autoclaving (in contrast to Rpf). Obviously, at the early stages of culture development, cryptic growth does take place within the aggregates. We suggest that the cells multiply or persist in the maintenance state in aggregates at the expense of microcryptic growth, utilizing the products of damaged or destroyed neighboring bacteria. Since this process has a local character, the amounts of such substances are sufficient to afford several cycles of division of the cells remaining within the aggregate (Figs. 1d, 1e). With an increase in the level of nutrients, transition to the exponential phase begins. If the suggested scenario for the development of events in the lag phase of *R. rhodochrous* is correct, then the inability of a culture begin exponential growth is easily explained by the destruction of cell aggregates in the lag phase under conditions of vigorous agitation. The cell communities in the lag phase can be regarded as a kind of incubator for the formation of viable cells when the initial cell concentrations are small and the medium composition is too poor for autonomic growth. Such a type of cell growth in liquid media is analogous to the growth of a colony on solid media.

Although the phenomenon of cryptic growth has long been known [18], until now it was considered to be characteristic of the stationary phase, when the cell concentration is high and that of the nutrients is low. It cannot be ruled out that the lysis of bacterial cells is controlled genetically and is an analogue of programmed death (apoptosis) in eukaryotes [19]. Interestingly, cell death and lysis within microcolonies during the formation of a *Pseudomonas aeruginosa* biofilm leads to the dispersion of a local film fragment and dissemination of viable bacteria into the environment [20].

We believe that the features of *R. rhodochrous* growth in a poor medium may represent a special strategy for the survival and development of the bacteria in poor media, where they would never grow autonomously; nevertheless, it is social behavior that makes such growth of the bacterial cells possible.

The role of intercellular contacts was also manifested when the *R. rhodochrous* culture passed into the proliferative–dormant state, which was characterized by the formation of transiently nonculturable cells in the prolonged stationary phase. Such a transition to the dormant state was only possible when the culture was vigorously agitated (Fig. 5a); at the same time, no decrease in cell viability was observed under slight agitation. This result would seem to be linked to the effects discussed above; i.e., under conditions permitting intercellular contacts to be established (slight agitation of the culture), the cells aggregate and, possibly, release factors of a chemical nature that sustain their viability. Under more vigorous agitation, the social survival of the cells is interfered with, which induces their transition to the transiently nonculturable state.

The phenomenon of nonculturability of bacterial cells is the subject of heated debate [14]. Many publications supporting the existence of viable but nonculturable bacteria, including several pathogenic species, are available. However, only in relatively few cases has the reversibility of the nonculturable state been clearly demonstrated [21]. If such reversibility cannot be produced, the corresponding cells should be considered as nonviable. It was earlier found that, in the prolonged stationary phase, a culture of *M. luteus* contains a large number of nonculturable cells, which remain in the dormant state for several months [11]. In the present investigation of the *R. rhodochrous* culture, a similar approach enabled us to obtain a population of nonculturable cells; however, as distinct from *M. luteus*, the occurrence of the cells in the nonculturable state was transient and lasted for about 2–4 h (Fig. 5a). It appears that, for such a transfer to occur, it is necessary to ensure a number of specific cultivation conditions (the composition of the growth medium and the inoculum age). This behavior of the *R. rhodochrous* culture is similar to the formation of dormant *Mycobacterium tuberculosis* cells in the prolonged stationary phase under aerobic conditions [22].

The possibility of resuscitating transiently nonculturable cells of *R. rhodochrous*, as assessed by the serial tenfold dilution method (table), indicated the reversibility of their dormant state. However, as in the case of *M. luteus* cultures, cell resuscitation was successful if the SN obtained from a log-phase *R. rhodochrous* culture of a certain age was added to the medium (Fig. 7). It is possible that the primary agent conducive to cell resuscitation is a protein similar to the protein Rpf, which we previously discovered in the SN of *M. luteus.*

Indeed, the immunoenzyme method and immunoblotting revealed proteins immunologically close to Rpf in the SN of *R. rhodochrous* (unpublished data). Interestingly, the nonpathogenic bacterium *R. rhodochrous* is close to *M. luteus* in relation to its capacity for resuscitation and the dependence of resuscitation on exogenous SN or Rpf, while nonculturable cells of the pathogenic bacterium *M. tuberculosis* can spontaneously resuscitate in liquid medium [22]. This property of the tuberculosis pathogen is probably somehow linked to its capacity for long-term persistence in a host body and the reactivation of tuberculosis.

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