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EXPERIMENTAL ARTICLES

Development and Population Structure of Mixed (S + M) *Pseudomonas aeruginosa* Cultures in the Late Stationary Growth Phase

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Abstract—Population growth, the ratio between dissociants, pH, and levels of reducing sugars in the medium were monitored during prolonged (375 h) batch cultivation of *Pseudomonas aeruginosa* S and M dissociants on mineral medium with glucose. During the stationary growth phase (100–375 h), two scenarios were possible. The first one included extensive cell autolysis coupled to alkalinization of the medium and an increased ratio of the M dissociant. In the second case, acidification of the medium was coupled to the oscillating secondary growth, mostly of the M dissociant; the dynamics of cell numbers of this dissociant correlated with the dynamics of the culture optical density. In this scenario, periodical appearance of reducing sugars in the medium was detected; it was in the opposite phase with the changes of the M dissociant cell numbers. The differences between scenarios of *P. aeruginosa* growth in the late stationary phase were probably due to the physiological and biochemical characteristics of the S and M dissociants, including different pathways of glucose utilization (respiration or fermentation), resistance to acidification, as well as synthetic (proteolytic) activity and productivity of autoinducers.

Key words: late stationary phase, secondary growth, *Pseudomonas aeruginosa*, S and M dissociants, population structure of the culture.

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Oscillation of microbial populations in soil communities is well known [1, 2]. Oscillatory growth is supported, among other factors, by consumption of the products of microbial autolysis; this is a self-regulated process [3]. In laboratory cultures, it is determined as secondary growth in the late stationary phase. For example, *Pseudomonas fluorescens* batch cultures are known to undergo regular oscillations of viable cell numbers and of carbon released from the autolysing biomass, when maintained in batch mode for a long time (two months) without introduction of additional nutrients. The physiological and ecological factors initiating bacterial growth in the late stationary phase and the possible role of intrapopulational dissociation in this process are not known. It is known, however, that the population structure of a microbial culture changes in the course of its development [4]. Preferential growth of a certain dissociant (a variant in colony morphology) in a culture is determined by its nutrient requirements [5], as well as by resistance to varying physicochemical environmental factors (including pH) [6, 7] and to the concentration of extracellular autoregulators, which control the physiological activity of the cells [8]. By the end of the trophophase, in the stationary growth phase, the relative content of bacterial cells in mixed cultures (polycultures) has been shown to be higher, the lower their nutrient requirements are [9]. Nutrient requirements depend on the characteristic features of bacterial metabolism. Comparison of physiological and biochemical features of three *P. aeruginosa* K2 revealed different pathways of glucose utilization as an energy source. Respiration and fermentation with formate production were predominant in R and M dissociants, respectively; S dissociants could switch between these pathways of glucose utilization [10]. A variational model was developed of the changes in the population structure of mixed cultures of this strain (containing two or three dissociants) in the stationary phase, depending on initial concentrations of the major biogenic elements in the medium [11]. However, the possible role of dissociative transitions in the late stationary phase of a microbial culture in its secondary growth

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Proteolytic activity (PA) of *Pseudomonas aeruginosa* S and M dissociants at different pH values

Dissociant	pH	PA, U/ml	PA, U/cell
S	4.3	16.7	0.9
	6.7	24.1	1.3
	8.5	18.4	1.0
M	4.3	11.8	1.7
	6.7	23.1	3.3
	8.5	32.0	4.6

or an alternative variant of development (culture autolysis) was not considered.

The goal of the present work was the study of dynamics of the population structure in mixed cultures of *P. aeruginosa* S and M dissociants in the course of prolonged (375 h) cultivation in batch culture without introduction of additional nutrient sources.

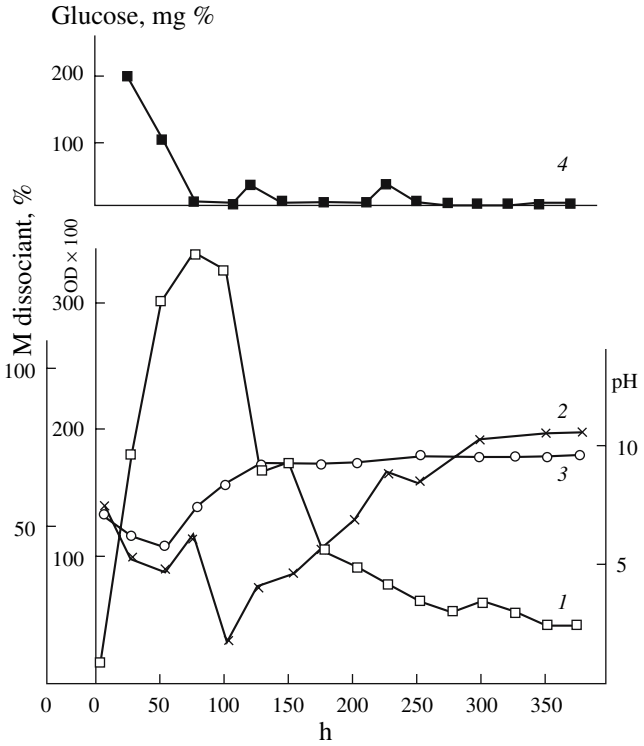

Fig. 1. Growth dynamics and population composition of a mixed culture of *P. aeruginosa* S and M dissociants in the variant of autolysis in the late stationary phase: optical density, OD × 100 (*1*); ratio of the M dissociant in the population, % (*2*); pH (*3*); and content of reducing sugars, mg % (as glucose) (*4*).

MATERIALS AND METHODS

Dissociant (S and M) of a widespread bacterium *P. aeruginosa* K2 were studied in this work. The strain was isolated from formation waters of a Siberian oil deposit. Bacteria were cultivated on a mineral medium containing the following (g/l): glucose, 20; sodium nitrate, 5; monosodium phosphate, 0.5; potassium chloride, 0.6; and magnesium sulfate, 0.2; pH 7.2. The cultures were grown for 375 h in 50-ml test tubes with 10 ml of the medium, with agitation (180 rpm), at 30°C. For inocula, one-day cultures of pseudomonad dissociants were used, grown on an agarized medium (nutrient broth + wort, 1 : 1) and resuspended to 10^9 cells/ml according to the turbidity standard. The inoculum content was 3 vol %.

Bacterial growth was determined by nephelometry (FEK-56M, filter no. 6). For convenience, the nephelometer readings were multiplied by 100. Cell numbers per 1 ml were determined as described in [4]. Dissociant ratios (%) in the population were determined by plating the samples on an agarized medium (nutrient broth + wort, 1 : 1). A Checker (HANNA Instrument) micropotentiometer was used to determine pH of the media. Reducing sugars were determined using Diaglyuk indicator stripes. The net extracellular proteolytic activity was determined by the modified Anson method [12] with casein (pH 6.7 and 8.5) or hemoglobin (pH 4.3) as substrates. Enzyme amount liberating 1 μg of tyrosine per minute was used as a unit of proteolytic activity (PA).

The graphs and tables present average values of three experiments (three repeats each).

RESULTS

Batch cultivation of mixed cultures of *P. aeruginosa* K2 S and M dissociants was carried out for 375 h without introduction of additional nutrients. The S : M ratio of the inoculum was 40 : 60. During the first 50 h (Figs. 1, 2), intense bacterial growth occurred (OD of the culture increased), the ratio of the slower growing M dissociant decreased, glucose consumption occurred, and pH decreased to 5.5. By 75 h, the highest biomass content was achieved, the ratio of dissociants reached 1 : 1 (in accord with the model calculations [11]), the medium became more alkaline (pH 7.0–7.3), and glucose was completely consumed.

After 75 h, two different scenarios for development of *P. aeruginosa* mixed cultures were possible. In the first case (Fig. 1), pH increased further, the medium became alkaline, and the culture entered the dying off phase without a noticeable stationary phase. The dying off phase started with autolysis of the M dissociant cells (75–100 h); their ratio decreased to 10–15%, and the S dissociant became predominant (85–90%). After 100 h of growth, pronounced autolysis occurred. After 100–125 h, OD decreased twofold; the medium was

then further alkalinized by autolysis products, and pH 9.0–9.5 was achieved by 125th hour. Later, the rate of autolysis decreased and pH stabilized. This intense dying off of the culture was mainly due to autolysis of the M dissociant cells; the ratio of the M type cells increased to 75–85%. After 275 h, the culture stabilized; since practically no changes of the registered parameters occurred during the last 100 h (275–375 h), the experiment was terminated. The remaining intact cells were probably in an anabiotic state; their poliferative capacity (CFU number) was not affected.

In another series of experiments, development of *P. aeruginosa* mixed cultures in the late stationary phase was radically different in that oscillating secondary growth of bacteria occurred (Fig. 2). During the trophophase, no differences in the growth characteristics were revealed between these two series of experiments (Figs. 1, 2).

Pronounced differences were detected in the beginning of the dying off phase. In the cultures following the second scenario (Fig. 2), in the period from 100 to 150 h the medium became acidified (in the first variant, it became alkalinized); accumulation of reducing sugars began at the same time (up to 150–170 mg %). The dying out rate was substantially lower (OD decreased by 30%) than in the first variant, when OD decreased by 50%. During the period of intense autolysis (100–150 h), mostly the M type cells died off, unlike the S type cells in the first variant. In the period from 150 to 225 h, pH remained low (4.0–4.5) and the first wave of secondary growth occurred; OD increased due to an increase in the M dissociant cells. The next two waves of secondary growth followed the same pattern. Since the changes in OD and in the ratio of the M dissociant cells coincided in time and amplitude, both the secondary growth and periodical dying off of the cells in the 150–375 h time interval were due to the M dissociant. Prior to resumption of growth (150–200, 225–275, and 325–375 h of cultivation), reducing sugars appeared in the medium due to autolysis of some of the cells within the population (100–125, 200–225, and 275-325 h, respectively).

One more block of data is required to explain the results obtained in this work. Since *P. aeruginosa* developed in batch mode without introduction of additional nutrients, their secondary growth could be supplied only by the nutrients due to the nutrients from the autolysed cells within the population. Proteinases play the triggering role in autolytic cell destruction; their productivity may be different in different dissociants [4, 6, 13, 14], and their activity depends on environmental physicochemical parameters, including pH.

Planning our experiments, we considered the fact that pH differences are important characteristics of *P. aeruginosa* growth strategies in the late stationary phase (the dying off phase). We determined extracellular proteolytic activity of the S and M dissociants in the

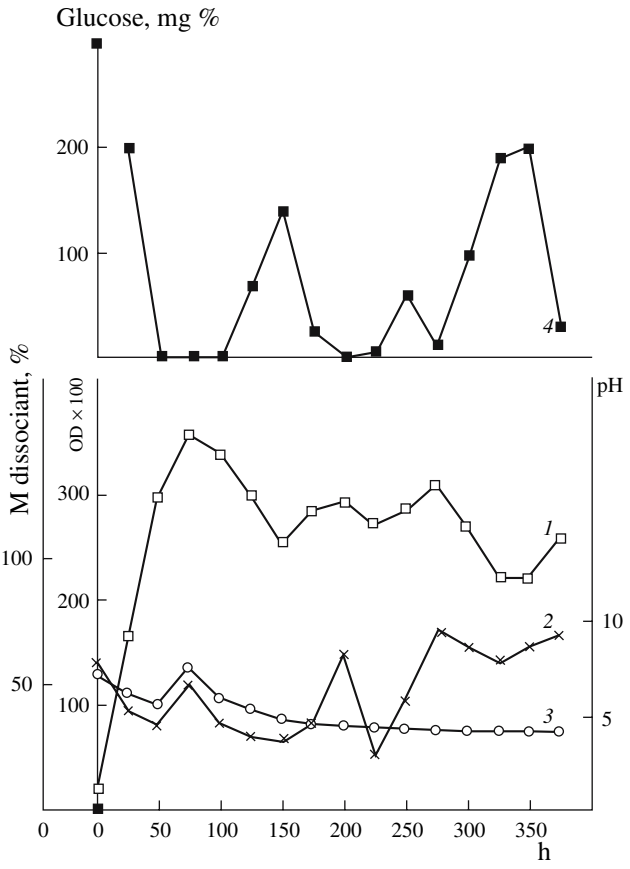

Fig. 2. Growth dynamics and population composition of a mixed culture of *P. aeruginosa* S and M dissociants in the variant of oscillation of secondary growth. The designations are as in Fig. 1.

stationary phase at different pH values (table). At low pH, the S dissociant had the highest proteolytic activity per volume (1 ml); under alkaline conditions, the M dissociant was most active. The calculated productivity (per cell) followed the same pattern. Thus, in the first variant of *P. aeruginosa* mixed culture (M + S) development (Fig. 1), high proteinase activity and the ratio of the M cells increasing at the onset of the dying off phase were possibly responsible for triggering uninterrupted unidirectional autolysis. According to our earlier results [7], the M dissociant cells had selective advantages under alkaline conditions; they remained intact and viable (CFU), and the M dissociant predominated.

In the cultures following the second scenario (Fig. 2), at low pH proteinase activity of both dissociants was lower than under alkaline conditions; autolysis was therefore less rapid. The more resistant M cells with lower growth rates [10] were capable of secondary growth on autolysis products, including reducing sugars liberating via decomposition of cellular biopolymers.

It is worth mentioning that the M dissociants of other bacteria (*Bacillus cereus, Rhodococcus rubroperctinctus*, and *P. aeruginosa* PAO1) also exhibited high proteolytic activity [6, 13,14].

Thus, development of mixed (S + M) cultures of *P. aeruginosa* K2 in the late stationary phase may follow two scenarios: unidirectional autolysis (mostly of the S type cells) and alkalinization of the medium with autolysis products (pathway I) or, with oscillating secondary growth (mostly due to development and autolysis of the M type cells), consumption of reducing sugars liberated from the autolysed cells and acidification with the products of incomplete sugar oxidation (pathway II).

DISCUSSION

Prolonged microbial growth in batch culture without nutrient supply may follow alternative scenarios for no obvious physicochemical or ecological reasons. Analysis of our results suggests that differences in the biochemical and physiological characteristics of *P. aeruginosa* dissociants, primarily in the pathways of carbohydrate assimilation, may be the reason for this variability of mixed (S + M) cultures in the late stationary phase [10]. Investigation of physiological and biochemical characteristics of the variants of the full *P. aeruginosa* K2 dissociative spectrum revealed differences in the pathways of glucose oxidation. The R cells, with the minimal nutrient requirements and high proliferation rates, have respiratory metabolism. The M cells, with the maximal nutrient requirements and low growth rate, ferment carbohydrates to form with acidification of the medium to pH 3.3. The S dissociant can switch between respiration and fermentation [10]. The nutrient requirements of dissociant cells are in inverse ratio to their numbers in mixed cultures during the stationary growth phase. For example, we have previously demonstrated that the R variant prevailed in *P. aeruginosa* mixed cultures (R + S + M, R + S, R + M) at the end of the trophophase–stationary phase [4]. In the case of prolonged cultivation, however, activity of the proteolytic enzymes, levels of the autoregulatory factors, and medium pH are important, rather than the trophic relations.

During the late stationary phase of *P. aeruginosa* K2 mixed (R + M) cultures, the ratio of the M dissociant increased, pH increased steadily to 9.5, and no secondary growth occurred [4]. In the present work, the S dissociant was among the components of a mixed (M + S) culture; since it is able to switch between glucose respiration and fermentation, these alternative pathways were equally probable for the S cells in the stationary phase–early dying off phase. This choice determined the direction of pH changes, i.e., either acidification with slow autolysis of some of the cells or alkalinization and quick irreversible dying off of the population. Activity of the extracellular proteinases, which was different in different dissociants, affected autolysis rates considerably (table).

The second block of results deserving comments is domination of the M dissociant in the late stationary phase (after 200 h) in both variants of mixed (S + M) *P. aeruginosa* culture development. Apart from the differences in productive biosynthetic activity, dissociants differ in their cell structure (the size and chemical structure of the cell wall and capsule, as well as in the composition of the cytoplasmic membrane). Thus, the cells of dissociants have different resistance to physical, chemical, and biological environmental factors [6]. The cells of the slowly growing M dissociant are known to have higher resistance to alkaline pH (8.5) [7] and other deleterious factors. This is possibly the reason for the M dissociant domineering during the dying off stage in mixed (S + M) *P. aeruginosa* cultures when nonoptimal stress conditions are established. Moreover, growth dynamics and the ratio of S and M dissociants in the late stationary phase are controlled by the concentration and activity of extracellular autoregulatory factors, primarily of anabiosis autoinducers; synthesis of these compounds have been demonstrated for pseudomonads [15]. Anabiosis autoinducers of these bacteria belong to alkylhydroxybenzenes (AHB) of the alkylresorcinol class [15]. In the *P. aeruginosa* strain under study, the M variant produced the highest amount of extracellular AHB [4]. Adaptogenic activity of AHB is important for analysis of our results; these compounds enhance stress resistance in both prokaryotes and eukaryotes [16, 17] and induce intragenomic rearrangements associated with dissociative transitions (phase variations), i.e., induce phenotypic dissociation [8]. To explain the domineering of the M dissociant by the end of cultivation (375 h in both series of experiments), these features of AHB should be considered.

The study of *Escherichia coli* population structure under prolonged batch cultivation without nutrient addition yielded results similar to those reported in the present paper [18, 19]. In some experimental variants, after the lag phase (6 h), exponential growth phase (6 h), stationary phase (36 h), and phase of intense dying off (2 days), the period termed the late stationary phase followed (10 days), when reliable oscillations of bacterial numbers were detected. Viable cell numbers periodically increased by an order of magnitude at the expense of death of another part of the population [19]. In the course of subsequent observations (four years), the number of viable cells (approx. 10^6 cells/ml) did not change significantly; bacteria were probably in an anabiotic state. In other parallel experimental variants, dying off of the cells was unidirectional and their numbers steadily decreased. Plating of bacteria responsible for secondary growth revealed changes in their cell and colony morphology. These cells were described as a specific phenotype which prevails during secondary growth in the late stationary phase, GASP (growth

advantage in stationary state) [19]. The GASP phenotype cells were resistant to signals preventing division of the remaining cells of the population [18]. A broad set of "stress" genes is possibly expressed in GASP cells; these genes are responsible for alternative metabolic pathways of amino acid metabolism and thus enable growth on autolysates. The possible role of dissociation (phase variation) in the oscillation of cell numbers in the late stationary phase was not discussed in the article. However, this phenomenon possibly occurred at prolonged cultivation of *E. coli*, since the patterns of this experiment were similar to those reported in the present work (pathway II). The GASP phenotype is probably a subtype of the M dissociant and is manifested only via emergence of the M phenotype. The fact that the GASP phenotype was not expressed in some *E. coli* strains [18, 19] does not contradict this hypothesis. Investigation in this field is of high importance, especially to microbial ecology and clinical medicine.

Thus, our investigation revealed that two scenarios are possible for the late stationary batch culture of *P. aeruginosa*, viz., intense cell autolysis with alkalinization of the medium and oscillating secondary growth with acidification of the medium. Dissociative transitions within the population were found to be responsible for the choice between developmental programs. Mixed culture of *P. aeruginosa* S + M dissociants was used to demonstrate that the differences between scenarios of *P. aeruginosa* growth in the late exponential phase were possibly caused by the physiological and biochemical features of the S and M dissociants, viz., their different pathways of glucose utilization (respiration or fermentation), resistance to acidification, and synthetic activity (proteolytic and AHB synthesis). AHB act as autoregulators controlling stress resistance and induction of dissociative transitions (phase variations) in bacterial populations.

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