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**EXPERIMENTAL ARTICLES**

# The Effect of Major Nutrient Elements on the Growth and Population Homogeneity of the R, S, and M Dissociants of *Pseudomonas aeruginosa* and the Glucose Oxidation and Fermentation Pathways

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**Abstract**—The population homogeneity of the stationary-phase monocultures of *Pseudomonas aeruginosa* dissociants was studied as a function of the initial content of major nutrient elements (C, N, and P) in the cultivation medium. The monocultures of the dissociants remained homogeneous during cultivation if the initial concentrations of the major nutrient elements were either sufficiently high or, conversely, very low, but became heterogeneous during cultivation in unbalanced (with respect to the major nutrient elements) media. At an initial concentration of nitrate in the medium equal to 0.07% or phosphate equal to 0.004–0.014%, the initially homogeneous population of R dissociant cultivated to the stationary growth phase turned out to contain 30–40% of S-type cells, whereas the initially homogeneous population of S dissociant was found to contain 50–80% of M-type cells. The population of M dissociant remained homogeneous throughout the cultivation period. R dissociant grew better at sufficiently high concentrations of glucose, nitrate, and phosphate in the medium, whereas M dissociant grew better when the initial concentrations of these nutrients were low. During the cultivation of R dissociant, the pH of the medium changed insignificantly, and the C/P ratio (the ratio of the carbon and phosphorus consumed during growth) was minimal (among the three dissociants), indicating that the R dissociant accomplishes the oxidative pathway of glucose metabolism. During the cultivation of the M dissociant, the pH of the medium dropped to 3.4–3.9, and the C/P ratio was maximal, indicating that this dissociant accomplishes the fermentative pathway of glucose metabolism. During the cultivation of the S dissociant, the pH of the medium and the C/P ratio exhibited variations, indicating that this dissociant triggers its pathways of glucose metabolism.

*Key words*: population homogeneity, dissociation, medium composition, growth dynamics, *Pseudomonas aeruginosa*.

Dissociation is one of the most important factors that make bacterial populations heterogeneous. Dissociation is a pleiotropic phenomenon caused by genomic rearrangements, which occur at frequencies several orders higher than that of random mutations. Dissociation is responsible for reversible changes in many morphological, physiological, and biochemical characteristics of cells, such as their degradative ability, synthesis of useful substances, resistance to unfavorable factors, and nutritional requirements.

Variable environmental conditions may change the proportion of dissociants in bacterial populations. This should be taken into account during scientific studies. Furthermore, the dissociation of bacterial populations may diminish the efficiency of microbiological technologies because of the appearance of inactive cell variants in industrial microbial populations. In nature, dissociation provides for the adaptation of bacterial populations to variable habitats and widens the survival boundaries of species [1].

Earlier, we investigated the effect of carbon, nitrogen, and phosphorus nutrition on the growth of the R, S, and M dissociants of *Pseudomonas aeruginosa* K-2 in the stationary phase [2], determined the mean required concentrations of C, N, and P in the medium for each of the dissociants, and found that insufficient phosphorus concentrations in the medium make the stationary-phase population of the *S* dissociant heterogeneous [3]. We also showed that the culture liquid of the M dissociant contains formic acid and that the glucose-6-phosphate dehydrogenase of this dissociant is two orders less active than in the two other dissociants [4]. This allowed us to suggest that the dissociants of *P. aeruginosa* may accomplish either the oxidative or the fermentative pathway of glucose metabolism. The predominance of the particular metabolic pathway can be indicated by specific changes in the pH of the cultivation medium and by the proportion between the amounts of carbon and phosphorus consumed by cells during their cultivation.

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**Table 1.** The initial concentrations (in %) of glucose (C), nitrate (N), and phosphate (P) in various versions of media for the cultivation of the R, S, and M dissociants of *P. aeruginosa*

| Experimental series | Medium | R | | | S | | | M | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | C | N | P | C | N | P | C | N | P |
| I | 1 | 2.0 | 1.1 | 0.055 | 2.0 | 1.1 | 0.055 | 1.0 | 0.55 | 0.028 |
| II | 2 | 1.0 | 0.55 | 0.028 | 1.0 | 0.55 | 0.028 | 0.4 | 0.55 | 0.028 |
| | 3 | 2.0 | 0.44 | 0.028 | 2.0 | 0.44 | 0.028 | 2.0 | 0.12 | 0.028 |
| | 4 | 2.0 | 0.55 | 0.014 | 2.0 | 0.55 | 0.014 | 2.0 | 0.55 | 0.005 |
| III | 5 | 0.2 | 0.29 | 0.014 | 0.2 | 0.29 | 0.014 | 0.2 | 0.29 | 0.014 |
| | 6 | 0.8 | 0.07 | 0.014 | 0.8 | 0.07 | 0.014 | 0.8 | 0.07 | 0.014 |
| | 7 | 0.8 | 0.29 | 0.004 | 0.8 | 0.29 | 0.004 | 0.8 | 0.29 | 0.004 |
| IV | 8 | 0.07 | 0.07 | 0.004 | 0.07 | 0.07 | 0.004 | 0.07 | 0.07 | 0.004 |
| | 9 | 0.4 | 0.02 | 0.004 | 0.2 | 0.02 | 0.004 | 0.2 | 0.02 | 0.004 |
| | 10 | 0.4 | 0.07 | 0.001 | 0.2 | 0.07 | 0.001 | 0.2 | 0.07 | 0.001 |

The aim of this work was to study the effect of the initial concentrations of the major nutrient elements (C, N, and P) on the growth of the monocultures of *P. aeruginosa* dissociants, their population homogeneity in the stationary growth phase, and their pathways of glucose metabolism.

## MATERIALS AND METHODS

Experiments were carried out with the R, S, and M dissociants of *Pseudomonas aeruginosa* strain K-2, which was isolated from the formation water of an oil field in Siberia [5].

The dissociants were grown in a medium containing 0.06% KCl, 0.02% $MgSO_4$, and variable concentrations of glucose (from 0.07 to 2%), $NaNO_3$ (from 0.02 to 1.1%), and $NaH_2PO_4$ (from 0.001 to 0.055%). The composition of all the versions of the nutrient medium is shown in Table 1.

The bacteria were grown at 30°C with shaking (180 rpm) in 50-ml tubes with 10 ml of the nutrient medium. The material for inoculation was 1-day-old cultures of the dissociants that were grown on MNBA (a medium containing malt extract and nutrient broth in equal amounts and 1.5% agar). The densities of the inocula were adjusted to $10^9$ cells/ml. The inoculum size was 3 vol %.

Culture turbidity was measured with an FEK-56M nephelometer, whose readings were multiplied by 100 for the sake of convenience.

The proportion of the dissociants in cultures was determined by plating appropriate culture dilutions onto MNBA.

The pH of the medium was measured with a Checker micropotentiometer (Hanna Instruments). Glucose, nitrogen, and phosphorus were quantified with triphenyltetrazolium chloride [6], sulfophenol reagent [7], and by the Panush method [8], respectively.

Each of the experiments was repeated two times in triplicate. The results presented in the paper are the mean values of the experiments.

## RESULTS AND DISCUSSION

The population homogeneity of the monocultures of *P. aeruginosa* dissociants and the implemented pathways of glucose metabolism were studied by lowering stepwise the initial concentrations of the major nutrient elements (C, N, and P) in the cultivation medium, until it became unbalanced. The cultivation medium 1 of experimental series I (Table 1) contained glucose, nitrate, and phosphate (the sources of C, N, and P, respectively) in amounts that were two times smaller than the mean required values of these nutrients determined earlier for each of the dissociants [2, 3].

Figure 1a shows the growth dynamics of the dissociants and the associated decline of C, N, and P in medium 1. As can be seen from this figure, the stationary-phase cultures of the R and M dissociants had the maximal and minimal turbidities, respectively. The most intense consumption of C, N, and P from the medium was observed between 16 and 24 h of cultivation, when the growth rate of all three dissociants was at a maximum. By the end of the cultivation period, the culture liquids of all the dissociants still contained the nutrients in sufficiently large amounts. The pH of the culture liquids of the R and S dissociants during their cultivation first decreased to, respectively, 6.3 and 5.3 (Fig. 2a) and then increased to reach, respectively, 8.2 and 8.5 in the stationary growth phase. The increase in the pH was likely due to cell lysis. The rapid transition (after about 20 h of cultivation) of the M dissociant to the stationary growth phase (Fig. 1a) and the drastic acidification of the cultivation medium by this dissociant to pH 3.3 (Fig. 2a) were probably associated with the fermentation of glucose to formic acid. This assumption is in agreement with the earlier observation

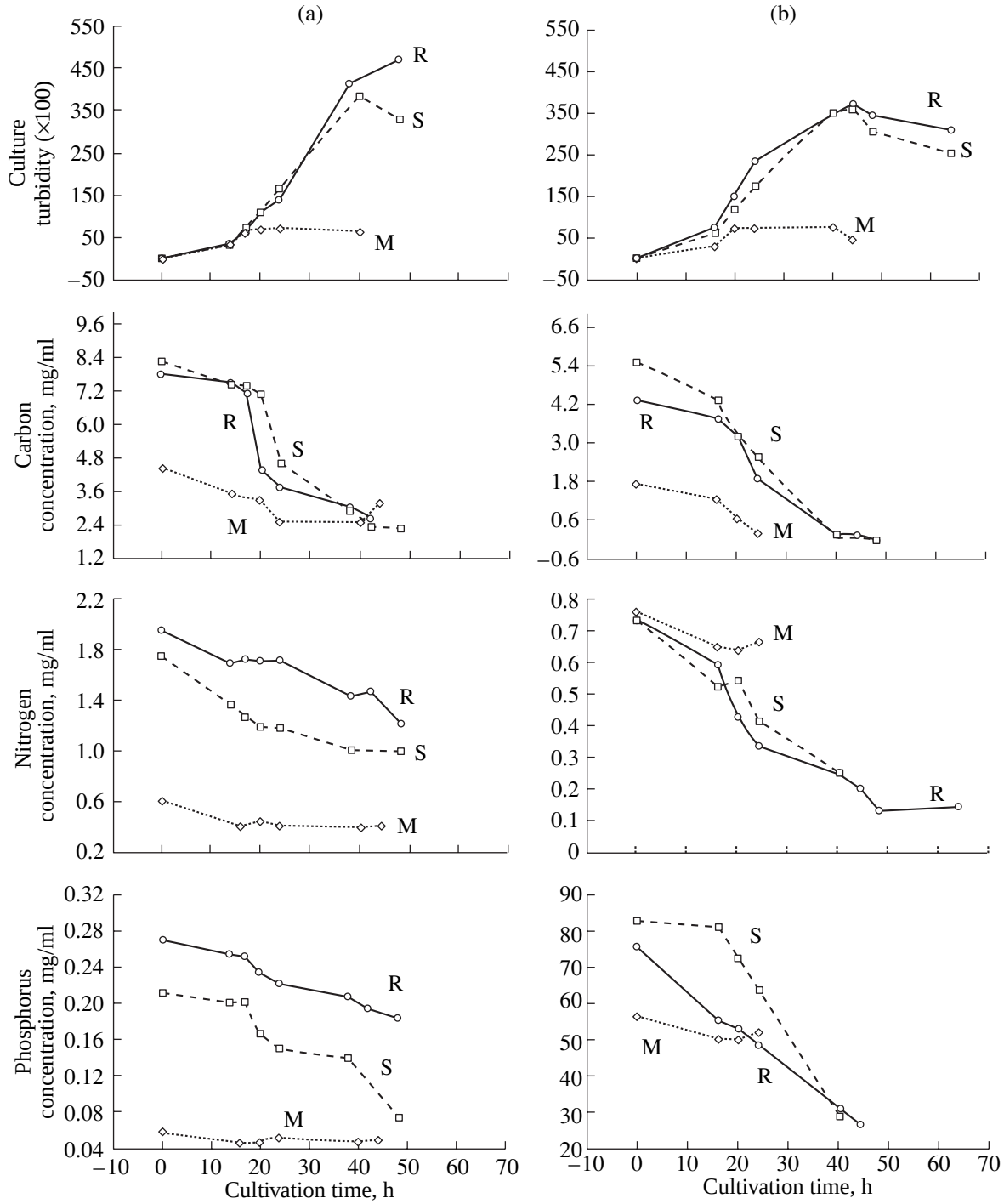


**Fig. 1.** Growth of the R, S, and M dissociants of *P. aeruginosa* and the dynamics of carbon, nitrogen, and phosphorus in (a) medium 1 and (b) medium 2 with a reduced content of carbon.

that the buffering of the cultivation medium with chalk greatly improves the growth of the M dissociant [4]. The relatively high decrease in the pH of the culture liquid of the S dissociant suggests that it fermented glucose too.

When the initial concentration of carbon in the medium was reduced twofold (Table 1, experimental series II, medium 2), the biomass of the R dissociant decreased by 100 units and became close to the biomass of the S dissociant (Fig. 1b). In this medium, the biomass of the M dissociant did not change. In the stationary phase, the culture liquids of the dissociants contained residual glucose in a sufficient amount. The maximum consumption rate of C, N, and P was observed between 16 and 24 h of cultivation, i.e., within the same cultiva-

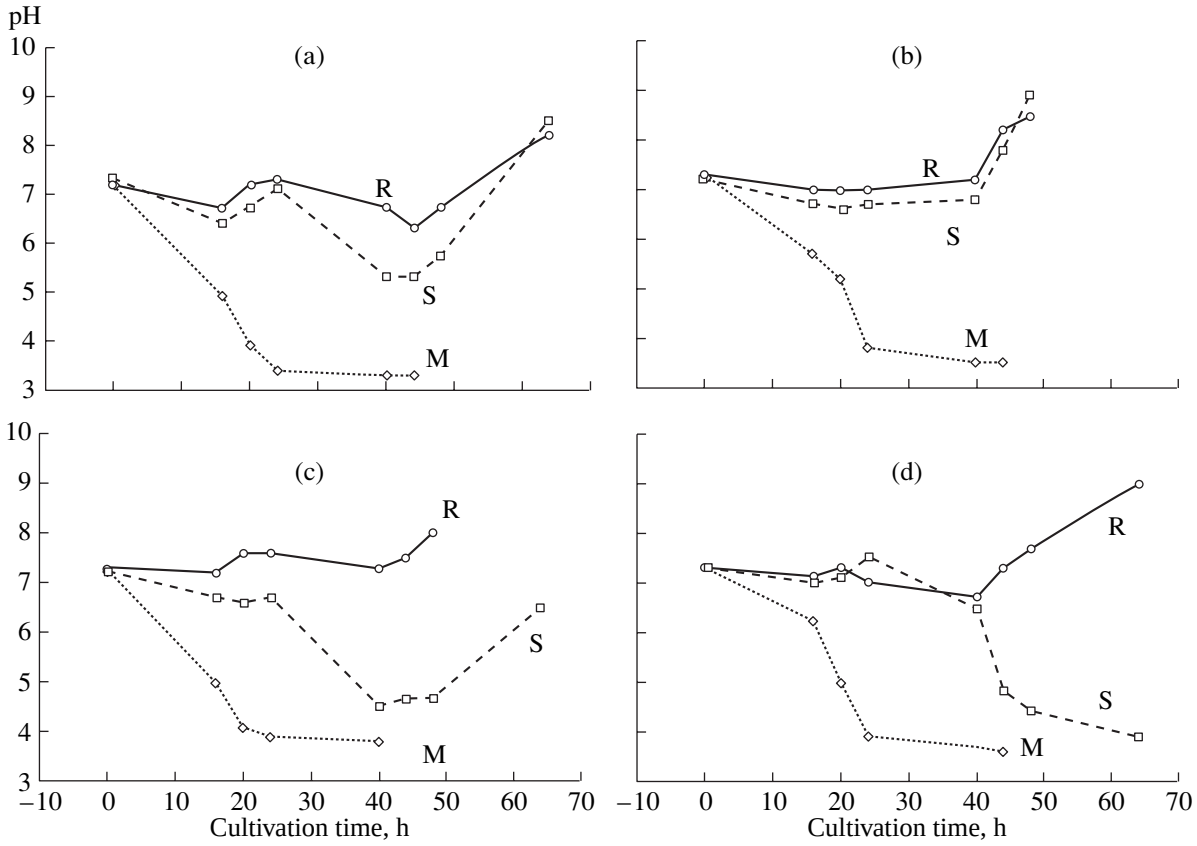


**Fig. 2.** Dynamics of pH during the growth of the R, S, and M dissociants of *P. aeruginosa* in (a) medium 1, (b) medium 2 with a reduced content of carbon, (c) medium 3 with a reduced content of nitrogen, and (d) medium 4 with a reduced content of phosphorus.

tion period as in the case of medium 1. Within the first 16 h of cultivation, the R and S dissociants showed similar growth, although the S dissociant consumed more carbon and less phosphorus than the R dissociant did. Nitrogen was gradually consumed by all the dissociants. The pH of the culture liquid of the R dissociant did not change until the stationary growth phase (Fig. 2b). The S dissociant slightly acidified the medium at the early terms of cultivation. The moderate acidification of medium 2 was likely to be related to a lower concentration of glucose in this medium and hence lower production of acid. In the growth retardation phase (44 h of cultivation), the pH of the culture liquids of the R and S dissociants increased to 8.5 and 8.9, respectively. The M dissociant markedly acidified the medium throughout the cultivation period. The monocultures of all the dissociants cultivated in medium 2 to the stationary growth phase remained homogeneous.

The about twofold lower concentration of nitrate in medium 3 (see Table 1) virtually did not affect the growth of the dissociants (Fig. 3a). The stationary-phase culture liquids of the dissociants still contained residual nitrogen. As in the case of medium 2, with the reduced carbon content, the growth of the S dissociant in medium 3 during the first 16 h of cultivation was accompanied by rapid consumption of carbon and slow consumption of phosphorus. The pH dynamics during the cultivation of the R and M dissociants in medium 3 (Fig. 2c) was the same as in the case of medium 2. In contrast, the growth of the S dissociant in medium 3 was accompanied by a greater decrease in pH (by the 40th h of cultivation, pH was 4.5) and less profound alkalinization of the medium in the growth retardation phase (pH 6.5) than in the case of medium 2. The monocultures of all the dissociants cultivated in medium 3 to the stationary growth phase remained homogeneous.

The twofold lower initial concentration of phosphorus in medium 4 (see Table 1) insignificantly influenced the growth (Fig. 3b) and the pH dynamics (Fig. 2d) of the R and M dissociants. It should be noted that the S dissociant continued to grow in medium 4 even after 64 h of cultivation. By this time, the proportion of S-type cells in the culture decreased to 55%, the rest (45%) being M-type cells. It is likely that once the phosphorus concentration in the cultivation medium of the S dissociant has decreased to a certain value, M-type cells begin to grow at a rate so high as to gradually exclude the original S-type cells from the population. As this occurs, the pH of the medium decreases (in our case, it decreased to 3.9, which is typical of M-type cells). The monocultures of the R and M dissociants

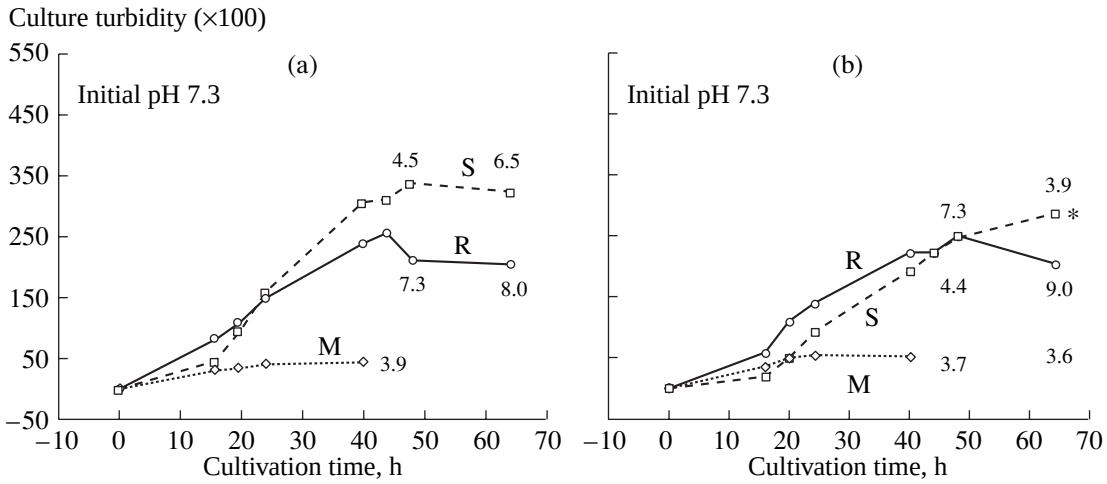


**Fig. 3.** Growth of the R, S, and M dissociants of *P. aeruginosa* in (a) medium 3 with a reduced content of nitrogen and (b) medium 4 with a reduced content of phosphorus. The asterisk symbol marks the culture whose cell population became heterogeneous during cultivation (see text for explanation). Numerals at the curves indicate pH values of the media.

cultivated in medium 4 to the stationary growth phase remained homogeneous. In the stationary phase, the pH of the culture liquid of the R dissociant drastically increased, whereas the pH of the culture liquid of the M dissociant remained very low. By the end of the cultivation period, the culture liquids of all the dissociants still contained an amount of residual phosphorus.

The pH dynamics in the culture liquid of the S dissociant grown in media 1, 2, and 3 indicates that cells of this dissociant ferment glucose, forming an acid. It is known that the energetic efficiency of glucose fermentation is lower than that of glucose oxidation, so the C/P ratio (the ratio of the carbon and phosphorus consumed during growth) is higher upon glucose fermentation than upon its oxidation. Table 2 summarizes data on the C/P ratios of all the dissociants grown in media 1–3 either to the early logarithmic phase or to the stationary phase. The C/P ratio of the R dissociant was minimal for all three cultivation media and for the two growth phases, strongly suggesting that this dissociant accomplishes the oxidative pathway of glucose metabolism, although the possibility of glucose fermentation cannot be completely excluded. The S dissociant grown in media 1–3 showed high C/P ratios in the early logarithmic phase and 3 to 8 times lower C/P ratios in the stationary growth phase, which indicates that this dissociant can trigger the pathways of glucose metabolism during its cultivation. The M dissociant showed relatively high C/P ratios for all three media throughout the cultivation period, which is an indication of the predominance of glucose fermentation in this dissociant. The possibility of glucose oxidation in the M dissociant cannot be completely excluded because of the occurrence of glucose-6-phosphate dehydrogenase, albeit with two orders lower activity than in the two other dissociants [4].

**Table 2.** The C/P ratios for the R, S, and M dissociants of *P. aeruginosa* cultivated in different media either to the early exponential phase or to the stationary growth phase

| Growth phase | Medium | Dissociants | | |
|---|---|---|---|---|
| | | R | S | M |
| Early exponential phase | Medium 1 | 3 | 60 | 46 |
| | Medium 2 | 7 | 60 | 22 |
| | Medium 3 | 2 | 202 | 17 |
| Stationary phase | Medium 1 | 12 | 18 | 30 |
| | Medium 2 | 14 | 16 | 56 |
| | Medium 3 | 2 | 26 | 65 |

Note: C/P is the ratio of the carbon and phosphorus consumed by cells during their growth.

In medium 5, with the tenfold lower initial concentration of glucose (see Table 1, experimental series III), the maximum biomass in the stationary growth phase was accumulated by the M dissociant (Fig. 4a-1). In this case, the pH of the medium decreased to 6.3 by the 24th h of cultivation and then tended to increase, reaching 8.5 in the stationary phase (40 h of cultivation). This is in agreement with the supposition that the poor growth of the M dissociant at relatively high glucose concentrations is associated with the acidification of the culture medium by acid produced from glucose. The monocultures of all the dissociants cultivated in medium 5 to the stationary growth phase remained homogeneous. In contrast, reducing the initial concentration of nitrate in the medium to 0.07% (Table 1, medium 6) greatly influenced the growth of the dissociants (Fig. 4a-2) and their population homogeneity. Namely, by the end of the cultivation period, the initially homogeneous population of the R dissociant was found to contain 44% of S-type cells, whereas the initially homogeneous population of the S dissociant was found to contain up to 80% of M-type cells. The

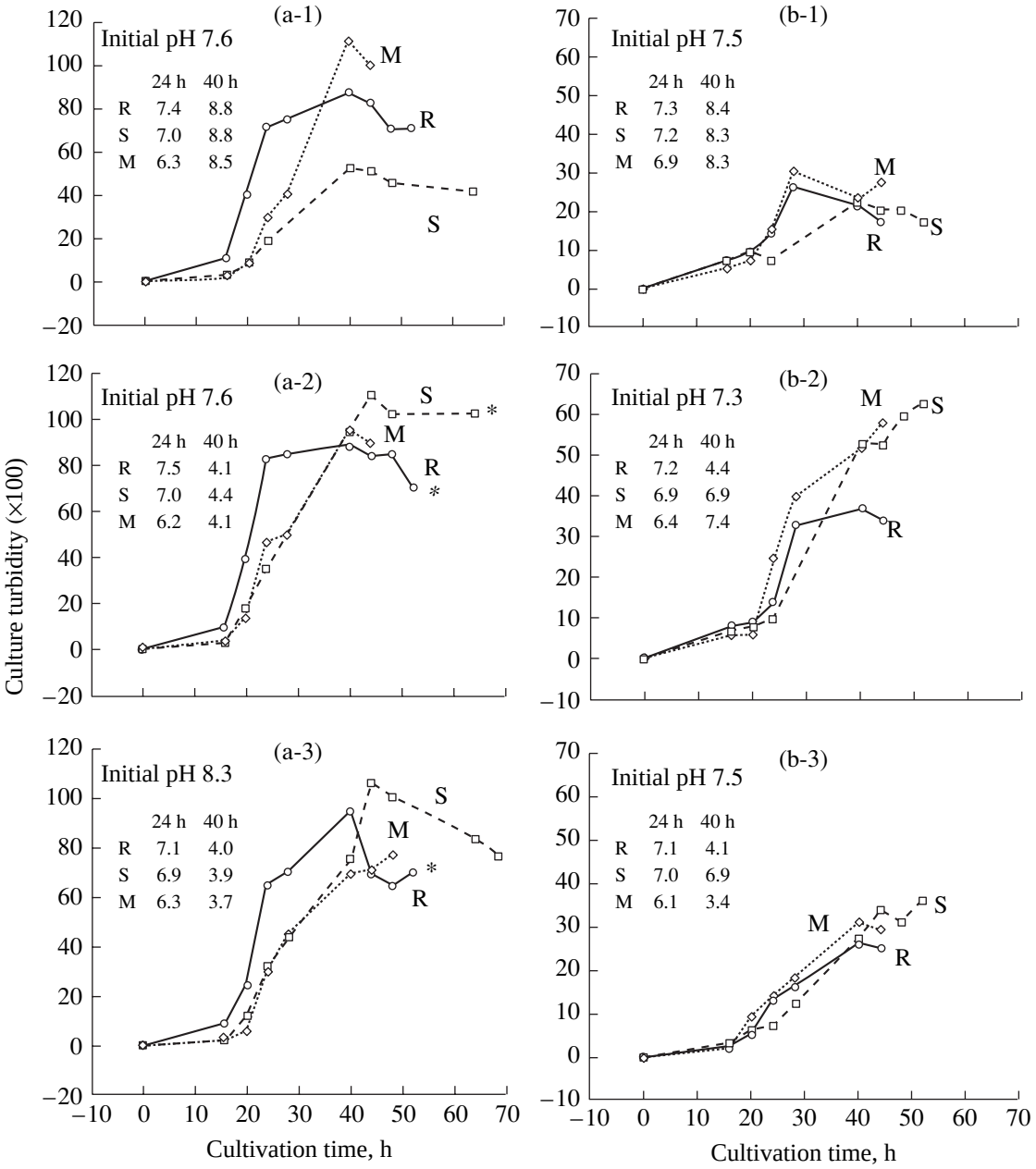


**Fig. 4.** Growth of the R, S, and M dissociants of *P. aeruginosa* in (a) experimental series III (1, medium 5 with a reduced content of carbon; 2, medium 6 with a reduced content of nitrogen; 3, medium 7 with a reduced content of phosphorus) and (b) experimental series IV (1, medium 8 with a reduced content of carbon; 2, medium 9 with a reduced content of nitrogen; 3, medium 10 with a reduced content of phosphorus). The asterisk symbols mark the cultures whose cell populations became heterogeneous during cultivation (see text for explanation). Tabulated numerals are pH values of the media.

appearance of the S- and M-type cells in the dissociant populations was probably responsible for the acidification of the medium to 4.1 and 4.4, respectively. At the same time, the monoculture of the M dissociant cultivated in medium 6 to the stationary growth phase remained homogeneous. The reduction of the initial concentration of phosphate in medium 7 to 0.004% (Fig. 4a-3) led to the stationary-phase population of the R dissociant containing 30% of S-type cells. By the end of the cultivation period (40 h of growth), the pH of the culture liquids of all the dissociants decreased to 3.7–4.0. In the case of the R dissociant, the pH drop was likely to be due to the formation of acid from glucose by the segregated S-type cells.

In experimental series IV (Table 1, media 8–10), the initial concentrations of glucose, nitrate, and phosphate were further reduced down to 0.07, 0.02, and 0.001%, respectively. In these media, the monocultures of the R, S and M dissociants remained homogeneous throughout the cultivation period. The growth of all the disso-

ciants was poor and differed insignificantly, except that the biomass of the S and M dissociants cultivated in the medium with 0.04% nitrate was twofold higher than in the other media studied.

It should be noted that the populations of the M dissociants remained homogeneous during cultivation in all ten media tested, irrespective of whether they were balanced (with respect to the major nutrient elements) or not. In the media with reduced concentrations of C, N, and P, the M dissociant had an advantage over the other dissociants and accumulated a higher biomass. On the other hand, the R dissociant had an advantage in the nutritionally reach media or at the early terms of cultivation, when the concentration of the major nutrient elements remained sufficiently high.

To conclude, this work is the first attempt to study the population homogeneity of the stationary-phase monocultures of *Pseudomonas aeruginosa* dissociants as a function of the initial concentration of the major nutrient elements C, N, and P in the cultivation medium. The monocultures of the dissociants remained homogeneous during cultivation if the initial concentrations of the major nutrient elements were either sufficiently high (experimental series I and II) or, conversely, very low (experimental series IV), when the number of grown cells was small. The monocultures of the dissociants became heterogeneous during cultivation in unbalanced (specifically, with a reduced content of nitrogen or phosphorus) media. At an initial concentration of nitrate in the medium equal to 0.07% or phosphate equal to 0.004–0.014%, the initially homogeneous population of *R* dissociant cultivated to the stationary growth phase was found to contain 30–40% S-type cells, whereas the initially homogeneous population of S dissociant was found to contain 50–80% M-type cells. This led to a more complete utilization of the nutrients whose levels were limiting. The monoculture of bacteria capable of dissociation can be considered as a mixed culture of bacterial dissociants in which the equilibrium between the dissociants is drastically shifted toward the prevalence of one of the dissociants.

In batch cultures, the ability of bacteria to dissociate is markedly profound in the stationary growth phase, when nutrients are partially or completely exhausted. Changes in the composition of the stationary-phase bacterial populations can be associated with the activation of the global stress response system [9] or with the action of the autoregulatory factors of anabiosis (alkyloxybenzenes) [10, 11].

The data presented in this paper can be compared with those obtained during the investigation of *P. aeruginosa* and *P. fluorescence* bacteria grown in glucose-limited chemostat cultures [12]. Aminov showed that, at a small feeding rate, the number of viable cells decreased, the consumption of glucose increased, and the amount of oxygen consumed was sufficient to oxidize only a few percent of the available glucose. In our opinion, such data indicate that glucose was utilized primarily through fermentation with the excretion of the respective acidic products. Unfortunately, changes in the cell population and the pH of the medium were not investigated by the author. Nevertheless, it is tempting to suggest that the S-type cells originally present in the cultivation medium were substituted by the slow-growing M-type cells with the fermentative pathway of glucose metabolism.

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