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EXPERIMENTAL ARTICLES

The Effect of Lowered Concentrations of Carbon, Nitrogen and Phosphorus Sources on the Growth Dynamics of the R, S, and M Dissociants of *Pseudomonas aeruginosa*

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Abstract—The effect of lowered concentrations of carbon, nitrogen, and phosphorus sources in the medium on the specific growth rate μ of the R, S, and M dissociants of the hydrocarbon-oxidizing strain *Pseudomonas aeruginosa* K-2, culture pH, and the population composition was studied. Within the first 16 hours of cultivation in all of the four media tested, the R, S, and M dissociants have virtually identical μ. The maximal values of μ were reached by the 20th h of growth in the basal medium (R and S dissociants) and in the carbon-deficient medium containing 0.4% glucose (M dissociant). The R and M dissociants showed the most rapid decrease in μ in the nitrogen-deficient medium containing 0.55% $NaNO_3$. By the end of cultivation in the basal medium, the pH of the R, S, and M cultures decreased to 6.3, 5.3, and 3.3, respectively. In the case of the carbon-deficient medium, the drop in the culture pH was lower. After a 2.5-day incubation of the S dissociant in the phosphorus-deficient medium containing 0.028% $NaH_2PO_4 \cdot 2H_2O$ and of the M dissociant in the basal medium supplemented with chalk powder, these dissociants were completely displaced from the media.

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

Bacteria of the genus *Pseudomonas* are widely used in biotechnology, particularly for wastewater treatment and decontamination of oil pollution [1]. However, the efficiency of the industrial application of bacteria, including pseudomonads, can be limited by the formation of inactive dissociants in bacterial populations.

The dissociation or phase variation of bacteria are due to rearrangement of their genome with a frequency of 10^{-2} to 10^{-4} per one cell division. This rearrangement possesses a pleiotropic effect and causes reversible changes in the morphological, physiological, and biochemical properties of cells, such as the ability to degrade and synthesize biologically active substances, resistance to external factors, and nutritional requirements. It is assumed that environmental influences as selective factors may alter the proportion of dissociants in bacterial populations [2].

To the best of our knowledge, there is no data in the literature concerning the dissociation of pseudomonads [3] and the effect of the medium composition on the growth of dissociants [4].

The investigation of the effect of carbon, nitrogen, and phosphorus nutrition on the stationary-phase growth parameters of the three dissociants of *Pseudomonas aeruginosa* K-2 grown in mono- and mixed cultures showed that the main factor determining the proportion of dissociants in bacterial populations is the initial concentration of phosphate [5, 6].

The aim of the present investigation was to study the effect of lowered concentrations of essential biogenic elements in the medium on the culture pH, population composition, and the growth dynamics of the R, S, and M dissociants of the hydrocarbon-oxidizing strain *Pseudomonas aeruginosa* K-2.

MATERIALS AND METHODS

The effect of glucose, nitrate, and phosphate on the stationary-phase growth parameters of the R, S, and M dissociants of *P. aeruginosa* was earlier studied using the $3 \times 3 \times 3$ factorial design of experiments with the concentrations of the essential biogenic elements taken at the upper, middle, and lower levels [5].

In the present work, the concentrations of glucose, nitrate, and phosphate, except for phosphate for the M dissociant, were decreased by 2 times as compared to the middle level taken as the control. The basal medium contained (%) $NaNO_3$, 1.1; $NaH_2PO_4 \cdot 2H_2O$, 0.055; KCl, 0.06; $MgSO_4 \cdot 7H_2O$, 0.02; and glucose, 2 (R and S dissociants) or 1 (M dissociant). The carbon-deficient medium (designated – C) contained 1% glucose for the R and S dissociants and 0.4% glucose for the M dissociant. The nitrogen-deficient medium (designated –N) contained 0.55% $NaNO_3$. The phosphate-deficient medium (designated –P) contained 0.028% $NaH_2PO_4 \cdot 2H_2O$ for the R and S dissociants and 0.005% of this salt for the M dissociant.

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Bacteria were cultivated at 30°C in 50-ml tubes containing 10 ml of the medium on a shaker (180 rpm). One-day-old cultures of the dissociants grown on an agar-solidified nutrient broth–wort mixture (1 : 1) (NBWA) were used as the inocula.

Bacterial growth was evaluated nephelometrically. The density of the inocula was equalized. The proportion of the dissociants in bacterial populations was determined by estimating the percentage of respective colonial morphotypes on the NBWA plates. The pH of the medium was measured using a Checker micropotentiometer.

The data presented in the table are the average of duplicate or triplicate measurements.

RESULTS AND DISCUSSION

The growth dynamics of the dissociants of *P. aeruginosa* K-2 in the basal medium containing glucose, nitrate, and phosphate at the middle level was studied in comparison with that in the media deficient in one of the three biogenic elements tested. The specific growth rate μ expressed in h^{-1} was evaluated based on the nephelometric data. The pH of the medium was measured throughout the incubation period (64 h). The proportion of the dissociants in bacterial populations was determined after the culture growth had ceased (see table).

Within the first 16 h of cultivation in all of the four media tested, the μ of all three dissociants was almost the same, making up 0.12–0.13 h^{-1}.

All dissociants showed the maximal values of μ by the 20th h of growth in the basal medium. The maximal values of μ for the R and S dissociants were 0.18 and 0.2 h^{-1}, respectively. After 40–44 h of growth, these two dissociants reduced the pH of the medium to 6.3 and 5.3, respectively. The growth of the R and S dissociants ceased by the 44th and 64th h of cultivation, respectively. After the growth had ceased, the pH of the medium increased to 8.2–8.5 (presumably because of the autolysis of cells), and the culture liquid turned bright green–blue. Throughout the cultivation period, the pH of the culture liquid of the R dissociant was higher than that of the S dissociant.

By the 24th h of cultivation, the M dissociant acidified the medium to pH 3.4 (likely due to the production of formic acid [7]), which resulted in the growth cessation. The pH of the culture then remained constant, and its color did not change. The addition of chalk powder to the basal medium resulted in the culture pH decreasing only to 5.3 after 24 h of cultivation. In this case, the μ of the M dissociant increased by several times. By the 40th h of cultivation, the specific growth rate decreased and then again increased. At the time, the pH of the culture slightly increased. Analysis of the M dissociant culture by plating it onto NBWA showed that M cells comprise, respectively, 98, 45, and 10% of the culture population after 20, 44, and 64 h of growth, the rest being S cells. Earlier studies showed that the S dissociant is more acidotolerant than the other two dissociants [8]. Therefore, S cells, which grow faster at low pH than M cells, must displace the parental M dissociant from the population.

Like in the basal medium, the maximal specific growth rate of all three dissociants in the carbon-deficient medium was observed by the 20th h of cultivation. The growth rates of the R and S dissociants in the carbon-deficient medium were lower and that of the M dissociant was higher (0.21 h^{-1}) than in the basal medium. In this case, the culture pH of the M dissociant increased. The decrease in the carbon concentration in the medium resulted in the growth cessation of the R and S dissociants by 20 h earlier and of the M dissociant by 4 h earlier than in the basal medium.

The deficiency of nitrogen in the medium resulted in a rapid decrease in the μ of the R and M dissociants in the course of their growth. Namely, the specific growth rates of these dissociants declined between the 16th and the 20th h of cultivation by 1.6 and 3 times, respectively. Like in the carbon-deficient medium, the growth of the R and M dissociants in the nitrogen-deficient ceased by the 44th and 40th h of cultivation, respectively. In this case, the growth of the S dissociant ceased by the 64th h of cultivation, i.e., as in the basal medium.

For M cells grown in the phosphate-deficient medium, μ and pH decreased more slowly than in the nitrogen-deficient medium, whereas the opposite was true for the S dissociant. The specific growth rate declined by the 44th h of cultivation and then again rose with a simultaneous decrease in pH to 3.9. The population of the S dissociant contained M cells, whose content by the 64th h of cultivation reached 45%. Due to their minimal requirement for phosphorus [5, 6], M cells grow in the phosphate-deficient media faster than S cells and thereby displace them from the population.

In all other experimental variants, the parental cells made up 100% of the culture population by the end of the incubation period.

Earlier study of the carbon metabolism of the dissociants showed that only M cells produce formic acid. The maximum activity of formate dehydrogenase was observed for the M dissociants and minimum for the R dissociant [7]. The data presented in the table demonstrate that the culture pH of the R dissociant and especially S dissociant grown in the basal medium gradually decreased. These data suggest that the R and S dissociants can also produce formic acid, albeit in lower amounts than the M dissociant. It should be noted that *Escherichia coli* produces acetic acid and excretes it into the medium in the stationary growth phase [9].

The results presented suggest that the R dissociant of *P. aeruginosa* possesses a more aerobic, whereas the M dissociant a more anaerobic, type of glucose metabolism than the S dissociant. Similar results were earlier reported for the R and S cells of *Escherichia coli* and *Staphylococcus albus* [2].

The specific growth rate μ (h^{-1}), culture pH, and the population composition (as a percentage of the R, S, and M dissociants) of *Pseudomonas aeruginosa* grown in the media with lowered concentrations of essential biogenic elements (C, N, and P)

Cultivation time, h		R							
		Basal medium		−C		−N		−P	
		μ	pH	μ	pH	μ	pH	μ	pH
0			7.2		7.3		7.3		7.3
16		0.13	6.7	0.13	7.0	0.13	7.2	0.12	7.1
20		0.18	7.2	0.16	7.0	0.08	7.6	0.15	7.3
24		0.12	7.3	0.07	7.0	0.03	7.6	0.06	7.0
40		0.05	6.7	0.01	7.2	0.04	7.3	0.03	6.7
44		0.03	6.3	0	8.2	0	7.5	0	7.3
48		0.01	6.7		8.5		8.0		7.7
64		0	8.2						9.0
Dissociant, %	R	100		100		100		100	
	S	0		0		0		0	
	M	0		0		0		0	

Cultivation time, h		S							
		Basal medium		−C		−N		−P	
		μ	pH	μ	pH	μ	pH	μ	pH
0			7.3		7.2		7.2		7.3
16		0.14	6.4	0.13	6.7	0.13	6.7	0.12	7.0
20		0.20	6.7	0.15	6.6	0.18	6.6	0.18	7.1
24		0.14	7.1	0.06	6.7	0.13	6.7	0.11	7.5
40		0.10	5.3	0.04	6.8	0.04	4.5	0.03	6.5
44		0.04	5.3	0	7.8	0.02	4.7	0.01	4.8
48		0.02	5.7		8.9	0.01	4.7	0.04	4.4
64		0	8.5			0	6.5	0.01	3.9
Dissociant, %	R	0		0		0		0	
	S	100		100		100		55	
	M	0		0		0		45	

Cultivation time, h		M									
		Basal medium		−C		−N		−P		Basal medium + chalk	
		μ	pH	μ	pH	μ	pH	μ	pH	μ	pH
0			7.2		7.3		7.3		7.3		7.2
16		0.13	4.9	0.12	5.7	0.12	5.0	0.13	6.2	0.13	6.2
20		0.15	3.9	0.21	5.2	0.04	4.1	0.09	5.0	0.21	5.7
24		0.03	3.4	0.02	3.8	0.03	3.9	0.06	3.9	0.17	5.3
40		0.01	3.3	0	3.5	0	3.8	0.02	3.7	0.02	6.5
44		0	3.3		3.5			0	3.6	0.08	7.7
48										0.07	8.0
64										0	
Dissociant, %	R	0		0		0		0		0	
	S	0		0		0		0		90	
	M	100		100		100		100		10	

Experiments on the addition of formic acid to the basal medium showed that the concentrations of formic acid equal to 0.05, 0.1, and 0.5% did not affect the growth of the dissociants, and 1% formic acid inhibited their growth only by 2 times. These data suggest that the rapid cessation of the growth of the M dissociant as compared to two other dissociants is determined by the sensitivity of this dissociant to low pH values of the medium, rather than by the inhibiting action of formic acid.

The data presented in the given paper demonstrate that the initial concentration of phosphate and the pH of the medium are important factors influencing the growth of the *P. aeruginosa* dissociants.

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