Microbiology, Vol. 73, No. 1, 2004, pp. 37–41. Translated from Mikrobiologiya, Vol. 73, No. 1, 2004, pp. 45–50.

90 8.01

EXPERIMENTAL ARTICLES

The Requirements of *Pseudomonas aeruginosa* Dissociants for Carbon, Nitrogen, and Phosphorus

P. V. Fursova[1], E. S. Mil'ko, I. A. Il'inykh, V. N. Maksimov, and A. P. Levich

Department of General Ecology, Faculty of Biology, Moscow State University, Vorob'evy gory, Moscow, 119992 Russia

Received December 20, 2002

Abstract—Quantitative data on the nutritional requirements of microorganisms are necessary to predict the behavior of bacterial populations and to control their cultivation. The requirements of the R, S, and M dissociants of *P. aeruginosa* for carbon, nitrogen, and phosphorus were derived from the results of 88 cultivation experiments. For each of the dissociants, we derived a coefficient that relates the optical density and the number of cells in the dissociant culture, determined the time when the cultures entered the stationary growth phase, studied cultural changes induced by transfer to the stationary phase, and determined what nutrients limit the growth of particular dissociants. The nutritional requirements of the dissociants are discussed in relation to our earlier data.

Key words: dissociation, *Pseudomonas aeruginosa*, requirement for major nutrients.

During their growth, many bacteria, including pseudomonads, can segregate (dissociate) into variants, which differ in an array of properties, such as colonial morphology, resistance to unfavorable environmental conditions, synthesis of particular substances, the ability to degrade xenobiotics and hydrocarbons, and nutritional requirements [1]. Some biochemical, morphological, and physiological properties of *Pseudomonas aeruginosa* were described in a number of earlier publications [2–5].

Of great interest are quantitative data on the nutritional requirements of microorganisms, which are necessary to predict the behavior of bacterial populations and to control their cultivation. This prompted us to study the requirements of *P. aeruginosa* dissociants for major nutrients.

MATERIALS AND METHODS

Eighty-eight experiments on the cultivation of the R, S, and M dissociants of *Pseudomonas aeruginosa* K-2 were performed in 1999 through 2001. The R and S dissociants were grown in a medium containing 2% glucose, 1.1% nitrate, and 0.055% phosphate, whereas the content of these nutrients in the medium for the M dissociant was reduced twofold. To investigate the dependence of bacterial growth on the initial concentration of carbon, nitrogen, and phosphorus in the cultivation medium, the initial concentrations of glucose, nitrate, and phosphate were varied from 0.03 to 2%, from 0.01 to 1.1%, and from 0.001 to 0.055%, respectively. Each of the dissociants was grown in 24 versions (some of which were replicated) of the respective nutrient medium. When investigating the effect of a nutrient, the two other nutrients were added to the medium at nonlimiting concentrations.

The bacteria were grown at 28°C with shaking (180 rpm) in 50-ml tubes with 10 ml of the nutrient medium for 1.5 to 3 days (to the stationary growth phase). The material for inoculation was 1-day-old cultures of the dissociants that were grown on MNBA (an agar medium containing malt extract and nutrient broth in equal amounts) slants. Bacterial cells grown on the slants were transferred to a tube with physiological saline solution. The densities of the dissociant inocula were adjusted to 10^9 cells/ml either nephelometrically or by using a turbidity standard. The inoculum size was 3 vol %.

During the cultivation of a dissociant, we controlled culture turbidity, pH, the appearance of cells of the other dissociants, and, in some experiments, the intermediate concentrations of the nutrients under study. Samples for analysis were taken after the first 14–16 h of cultivation and then after each 4–6 h until the stationary growth phase was reached. 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.

Culture turbidity was measured at 540 nm in a 0.5-cm cuvette by using an FEK-56M nephelometer equipped with a green light filter no. 6. When bacterial growth was poor, measurements were carried out with a 2-cm cuvette. In this case, the results of measure-

[1] Corresponding author. E-mail: polina_fursova@mail.ru

0026-2617/04/7301-0037 © 2004 MAIK "Nauka/Interperiodica"

ments were correspondingly recast. For convenience, the nephelometer readings were multiplied by 100.

For each of the dissociants, we derived a coefficient that relates the optical density and the number of cells in the stationary-phase dissociant culture. For this purpose, the 48-h-old cultures of the R and S dissociants were diluted 100- and 10 000-fold, the 24-h-old culture of the M dissociant was diluted 10- and 100-fold, and the dilutions were examined under a Lyumam-I3 luminescence microscope, which was equipped with a ZhS-19 light filter, a × 4 eyepiece, and a × 90 L objective.

The number of microbial cells in ml of the original suspension was calculated by using the formula:

$$N = \frac{4an}{S} \times 10^{10},$$

where N is the number of cells in ml of the original suspension, a is the mean number of cells in a microscope field; S is the area of the microscope field (in the given case, 10000 μm^2), and n is the dilution factor [9]. The conversion coefficients (cell number to culture turbidity) for the R, S, and M dissociants were found to be 1×10^8, 0.3×10^8, and 0.17×10^8 cells/(ml × nephelometer units × 100), respectively.

The requirement of a dissociant for a nutrient was defined as the amount of this nutrient consumed from the medium in the course of cultivation to the stationary growth phase as measured per one grown cell. The requirement was calculated by using the formula: $q_i^L = \frac{\Delta L}{\Delta n_i}$, where ΔL is the amount of nutrient L consumed over a certain period, Δn_i is the number of cells of the ith dissociant grown during this period; L = glucose (C), nitrate (N), or phosphate (P); i = R, S, or M dissociant

Mean requirements and confidence intervals were calculated by using the Microsoft Excel 7.0 descriptive statistics analysis tool.

RESULTS AND DISCUSSION

In the experiments in which the dynamics of nutrients in the cultivation medium was monitored, nutritional requirements were calculated by the formula presented in the *Materials and Methods* section. In most of the experiments, however, the dynamics of nutrients in the cultivation medium was not monitored. For this reason, experimental design was based on the assumption that a nutrient which is investigated in a given experiment is growth-limiting. This implies that (1) the concentration of this nutrient in the stationary growth phase is zero, (2) the amount of this nutrient consumed by the stationary phase is equal to its original content in the medium, and (3) the number of cells grown at the expense of this nutrient should be measured in the stationary phase.

Such an experimental design posed the problem of recording the transfer of cultures to the stationary phase. This problem was solved by measuring culture turbidity for 2–2.5 days at intervals of several hours.

To make sure that the initial concentration of a nutrient in the medium is growth-limiting, we used three approaches. With the first approach, the initial concentration of this nutrient was reduced by two or more times as compared to its content in the medium described in the *Materials and Methods* section (this medium is likely to be balanced, as is evident from the practice of *P. aeruginosa* K-2 cultivation). The second approach was checking the proportionality between the initial concentration of the nutrient in the medium and the culture density in the stationary growth phase. With the third approach, the stationary phase culture was divided into four equal fractions (subcultures), one of which served as the control, and the three other were supplemented with glucose, nitrate, or phosphate. The subcultures were incubated for 4 h, and then their turbidities were measured relative to the control. An increase in the turbidity of the subculture to which, for instance, glucose was added served as a confirmation that glucose was the growth-limiting nutrient of the original culture.

The results of 26 experiments with the R dissociant, 34 experiments with the S dissociant, and 28 experiments with the M dissociant are presented in Table 1. The table summarizes the initial contents of the nutrient elements C, N, and P in the medium, culture turbidities (nephelometer readings multiplied by 100) and pH in the stationary growth phase (or at the end of the cultivation period). The original medium, whose composition is described in the *Materials and Methods* section, was numbered 1. In the media under increasing numbers, the initial concentrations of the nutrients under study were gradually lowered in order to reach their growth-limiting concentrations (as was mentioned above, this was checked by observing a proportionality between the culture turbidity and the initial concentration of the nutrient in the medium). The experiments with subcultures (the third approach) are marked in Table 1 by the asterisk symbol.

It should be noted that the results of some experiments were not used for further analysis. These are the experiments for which there was no certainty as to whether the culture reached the stationary growth phase or not and the experiments during which a monoculture of a dissociant was found to be contaminated with cells of either of the other dissociants [10]. Such experiments might give erroneous nutritional requirements. The results of the faultless experiments were processed to determine the requirements of the dissociants for the major nutrients (Table 2), the mean nutritional requirements, and their 95% confidence intervals (Table 3).

Based on the data presented in Table 3, we determined the composition of a balanced medium, which must contain 0.225% glucose, 0.03% nitrate, and

Table 1. The initial content of carbon, nitrogen, and phosphorus in the medium, the optical density of stationary-phase cultures expressed in nephelometer readings multiplied by 100, and the pH of the medium in the stationary phase (or at the end of the experiment)

R dissociant

Medium number	Initial content (mg/ml) of nutrients			Optical density (×100)	pH
	Carbon	Nitrogen	Phosphorus		
1.	7.98	1.81	0.11	473	
2.	4	0.9	0.056	430	
3.	4	0.9	0.056	342	8.5
4.	7.8	0.72	0.056	224	7.3
5.	7.8	0.72	0.056	276	8
6.	7.8	0.9	0.056	249	7.7
7*.	0.78	0.4	0.028	87	8.8
8*.	3.18	0.1	0.028	89	4.1
9*.	3.18	0.4	0.007	95	4
10*.	0.282	0.1	0.008	21	8.4
11*.	1.6	0.03	0.008	37	4.4
12*.	1.6	0.1	0.002	26	4.1
13.	0.4	0.035	0.01	31	8.1
14.	1.62	0.14	0.04	120	7.5
15.	0.4	0.015	0.01	22	6.8
16.	1.6	0.06	0.04	110	7.6
17.	0.12	0.035	0.01	17	7.1
18.	0.48	0.14	0.04	31	7.2
19.	0.12	0.015	0.01	14	7.5
20.	0.48	0.06	0.04	52	8.4
21.	0.78	0.1	0.01	63	8.8
22.	3.24	0.4	0.04	200	5.3
23.	1.6	0.2	0.01	120	7.6
24.	6	0.8	0.04	225	8.4
25.	1.2	0.035	0.01	49	7.1
26.	4.8	0.14	0.04	100	4.1

S dissociant

Medium number	Initial content (mg/ml) of nutrients			Optical density (×100)	pH
	Carbon	Nitrogen	Phosphorus		
1.	7.98	1.81	0.11	400	
2.	7.98	1.81	0.11	372	
3.	4	0.9	0.056	376	
4.	4	0.9	0.056	334	7.7
5.	7.98	0.72	0.056	350	6.7
6.	7.98	0.72	0.056	340	6.2
7.	7.98	0.9	0.056	320	8.6
8.	7.98	0.9	0.056	243	4.4
9*.	0.78	0.4	0.028	51	8.8
10*.	0.78	0.4	0.028	74	8
11*.	3.18	0.1	0.028	111	4
12*.	3.18	0.1	0.028	109	4
13*.	3.18	0.4	0.007	106	3.7
14*.	3.18	0.4	0.007	90	3.6
15*.	0.282	0.1	0.008	22	8.3
16*.	0.78	0.03	0.008	60	7.4
17*.	0.78	0.1	0.002	34	6.6
18*.	0.282	0.1	0.008	25	8.2

S dissociant

Medium number	Initial content (mg/ml) of nutrients			Optical density (×100)	pH
	Carbon	Nitrogen	Phosphorus		
19*.	0.78	0.03	0.008	63	7.3
20*.	0.78	0.1	0.002	30	6.6
21.	0.4	0.035	0.01	30	8.4
22.	1.62	0.14	0.04	123	8.7
23.	0.4	0.015	0.01	28	7.6
24.	1.6	0.06	0.04	136	8.8
25.	0.12	0.035	0.01	9	7.7
26.	0.48	0.14	0.04	30	6.9
27.	0.12	0.015	0.01	14	8.2
28.	0.48	0.06	0.04	52	8
29.	0.78	0.1	0.01	74	8.2
30.	3.24	0.4	0.04	177	8.3
31.	1.6	0.2	0.01	99	8.6
32.	6	0.8	0.04	220	8.9
33.	1.2	0.035	0.01	68	7.3
34.	4.8	0.14	0.04	160	3.2

M dissociant

Medium number	Initial content (mg/ml) of nutrients			Optical density (×100)	pH
	Carbon	Nitrogen	Phosphorus		
1.	4	0.9	0.056	72	3.3
2.	4	0.9	0.056	75	3.3
3.	1.62	0.9	0.056	86	5.5
4.	1.62	0.9	0.056	76	3.5
5.	7.8	0.2	0.056	37	4
6.	7.8	0.2	0.056	42	3.9
7.	7.8	0.9	0.01	67	4.1
8.	7.8	0.9	0.01	44	4.2
9*.	0.78	0.4	0.028	111	8.5
10*.	3.18	0.1	0.028	95	4.1
11*.	3.18	0.4	0.007	77	3.8
12*.	0.282	0.1	0.008	27	8.3
13*.	0.78	0.03	0.008	58	7.4
14*.	0.78	0.1	0.002	31	3.4
15.	0.4	0.035	0.01	31	8.5
16.	1.62	0.14	0.04	109	8.3
17.	0.4	0.015	0.01	29	7.7
18.	1.6	0.06	0.04	129	8.8
19.	0.12	0.035	0.01	10	8.5
20.	0.48	0.14	0.04	35	7.3
21.	0.12	0.015	0.01	15	7
22.	0.48	0.06	0.04	69	8.6
23.	0.78	0.1	0.01	71	8.8
24.	3.24	0.4	0.04	142	3.3
25.	1.6	0.2	0.01	90	7.8
26.	6	0.8	0.04	220	8.8
27.	1.2	0.035	0.01	69	7.2
28.	4.8	0.14	0.04	155	3.1

Note: The asterisk symbol marks the experiments with subcultures (see text for explanation).

Table 2. The calculated requirements of the dissociants for carbon, nitrogen, and phosphorus (in 10^{-12} mg/cell)

R dissociant				S dissociant				M dissociant			
Medium number	q_R^C	q_R^N	q_R^P	Medium number	q_S^C	q_S^N	q_S^P	Medium number	q_M^C	q_M^N	q_M^P
2.	96		0.7	3.	342		2.3	1.			4.2
3.	120			4.	384		5.6	2.	630		3.2
4.	156			5.			5.1	7.	384		9
6.			1.8	6.	384		3.1	9.	432		
7.	90			7.	432		6.5	11.			5.3
10.	148		1.2	9.	540			12.	642		
11.		8	1.1	10.	384			13.		31	
12.			0.8	15.	433			14.			4
14.	144			16.		19		17.		32	
15.		6		17.			2	18.		28	
18.	156			18.	381			21.	480		
21.	120			19.		18		22.	420		
22.			2	20.	243		2	23.	684		
24.			1.8	23.		18		25.			6.7
25.		7		24.		15		26.			10.6
				25.	450			27.		31	
				28.	348						
				29.	384						
				30.	612						
				32.			6				

Note: q_i^C, q_i^N, and q_i^P are the requirements of the ith dissociant ($i = R, S$, and M) for carbon, nitrogen, and phosphorus, respectively. The medium numbers correspond to those in Table 1.

0.004% phosphate. Additional experiments on cultivation in this medium allowed the requirements of the R and S dissociants for the nutrient elements C, N, and P to be estimated as, respectively, 122×10^{-12} and 345×10^{-12} mg C/cell, 5.75×10^{-12} and 24×10^{-12} mg N/cell, and 0.85×10^{-12} and 3.5×10^{-12} mg P/cell. A comparison of these values and those presented in Table 3 shows that they are close.

It should be noted that the earlier estimates of the nutritional requirements of the dissociants for glucose, nitrate, and phosphate [11] were obtained by using one conversion coefficient (cell number to culture turbidity) for all three dissociants. Their nutritional requirements recast with due consideration of the different conversion coefficients obtained in this study are presented in Table 4. As can be seen from a comparison of Tables 3 and 4, the earlier and current estimates of the nutritional requirements of the R and S dissociants are in agreement (taking into account that the error of the earlier estimates reach 50% [11]). The discrepancy of the ear-

Table 3. The mean requirements (with standard errors) of the R, S, and M dissociants for carbon, nitrogen, and phosphorus (in 10^{-12} mg/cell)

Dissociant	Nutrient element		
	Carbon	Nitrogen	Phosphorus
R	129 ± 22	7.0 ± 2.5	1.0 ± 0.5
S	409 ± 55	17.5 ± 3.0	4.0 ± 1.5
M	525 ± 114	31 ± 3	6.0 ± 2.5

Table 4. The mean requirements (with standard errors) of the R, S, and M dissociants for carbon, nitrogen, and phosphorus (in 10^{-12} mg/cell), which are estimated from the results of the earlier experiments [11]

Dissociant	Nutrient element		
	Carbon	Nitrogen	Phosphorus
R	110 ± 8	8.5 ± 3.1	1.0 ± 0.3
S	366 ± 23	38.3 ± 10.3	3.8 ± 1.0
M	1706 ± 941	144.0 ± 63.5	11.8 ± 5.8

lier and current estimates of the nutritional requirements of the M dissociant can be explained as follows: The stationary-phase culture turbidities of the M dissociant in the earlier and present experiments are nearly the same, although the concentrations of the nutrients in the earlier experiments were 5 to 10 times higher. Conversely, at the same contents of the nutrients, the culture turbidities of the M dissociant that were reached in the present experiments are several times greater than those reached in the earlier experiments. It can be suggested that the growth of the M dissociant in the earlier experiments was limited by the formation of formic acid from glucose [5] and the concurrent decrease in the pH of the medium, but not by the exhaustion of the nutrients from the medium. In other words, the nutrients were not consumed completely, and the earlier estimates of the nutritional requirements of the M dissociant were overestimated.

ACKNOWLEDGMENTS

We are grateful to L.M. Polyanskaya for her help with luminescence microscopy and to N.G. Bulgakov for his help and fruitful discussion of the manuscript.

This work was supported by grant nos. 02-04-48085 and 03-04-06044 from the Russian Foundation for Basic Research.

REFERENCES

1. Mil'ko, E.S. and Egorov, N.S., *Geterogennost' populyatsii bakterii i protsess dissotsiatsii* (Heterogeneity of Bacterial Populations and Their Dissociation), Moscow: Mosk. Gos. Univ., 1991.
2. Mil'ko, E.S. and Martynkina, L.P., Morphological, Physiological, and Biochemical Properties of the *Pseudomonas aeruginosa* Dissociation Variants, *Mikrobiologiya*, 1996, vol. 65, no. 5, pp. 352–356.
3. Mil'ko, E.S., Survival of Dissociants of a Hydrocarbon-oxidizing *Pseudomonas aeruginosa* Strain during Storage, *Mikrobiologiya*, 1998, vol. 67, no. 1, pp. 102–105.
4. Mil'ko, E.S. and Nikitenko, L.A., The Effect of Physical and Chemical Environmental Factors on the Growth of *Pseudomonas aeruginosa* Dissociants, *Prikl. Biokhim. Mikrobiol.*, 1998, vol. 34, no. 2, pp. 171–174.
5. Mil'ko, E.S. and Krasil'nikova, E.N., Peculiarities of the Carbohydrate Metabolism of the R, S, and M Dissociants of *Pseudomonas aeruginosa*, *Mikrobiologiya*, 1999, vol. 68, no. 2, pp. 211–214.
6. *Khimiya uglevodov* (Carbohydrate Chemistry), Kochetkov, N.K. *et al.*, Eds., Moscow: Khimiya, 1967.
7. Polyakov, G., *Posobie po gidrokhimii dlya rybovodov* (Handbook of Hydrochemistry for Fish Breeders), Moscow: Pishchepromizdat, 1950.
8. *Malyi praktikum po biokhimii* (Minor Laboratory Manual on Biochemistry), Moscow: Mosk. Gos. Univ., 1979.
9. *Metody pochvennoi mikrobiologii i biokhimii* (Methods of Soil Microbiology and Biochemistry), Zvyagintseva, D.G., Ed., Moscow: Mosk. Gos. Univ., 1991.
10. Mil'ko, E.S. and Il'inykh, I.A., 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, *Mikrobiologiya* (in press).
11. Maksimov, V.N., Mil'ko, E.S., and Levich, A.P., The Requirements of *Pseudomonas aeruginosa* Dissociants for Glucose, Nitrate, and Phosphate: The Growth-limiting Concentrations of These Nutrients during Batch Cultivation, *Izv. Akad. Nauk, Ser. Biol.*, 2001, no. 5, pp. 607–612.

Spell: Ok