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Carbon and Nitrogen As Resources Limiting the Growth of Mono- and Mixed Cultures of *Pseudomonas aeruginosa* Dissociants

P. V. Fursova[a], E. S. Mil'ko[b], and A. P. Levich[c]

[a] *Department of Biophysics*
[b] *Department of Microbiology*
[c] *Department of General Ecology, Moscow State University, Moscow, 119991 Russia*
e-mail: fursova@biophys.msu.ru

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Abstract—New experiments for detection of resources limiting the growth of mono- and mixed cultures of *Pseudomonas aeruginosa* dissociants were carried out. The results were analyzed on the basis of the consumption and growth variational model in accordance with the data on the dissociant metabolism special traits. In 83% of cases, the theoretical calculation was confirmed by the experimental results.

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The work is the continuation of the study aimed at detection of resources limiting the growth of *Pseudomonas aeruginosa* dissociant cultures. The previous publication (Fursova et al., 2004a) showed the theoretical possibilities of predicting limiting substances on the basis of the consumption and growth variational model for biocenoses (Levich, 1980; Levich et al., 1997; Levich, 2000). Experiments with additives aimed at detection of limiting resources confirmed the model calculations in most cases. However, new data on the special traits of the metabolism of *P. aeruginosa* dissociants made it necessary to reconsider the obtained results.

MATERIAL AND METHODS

Mono- and mixed cultures of R, S, and M dissociants of bacterial strain *Pseudomonas aeruginosa* K-2 were cultivated, without replenishment of resources, on media with various initial concentrations of glucose, nitrates, and phosphates. The total number of experiments neared 200.

The bacteria were cultivated in 50-ml tubes on 10 ml of the media on a shaker (180 rpm) at a temperature of 28°C until they reached the stationary growth phase (Tables 1–3). One-day cultures of pseudomonade dissociants cultivated on solid medium containing meat-peptone broth and wort in the ratio of 1 : 1 (BSA) were used for inoculation. The bacteria from agar slant were transferred with a loop to a tube with physiological solution. The inoculum density of each dissociant in all experiments was adjusted with the help of a nephelometer or according to opacity standards until the cell concentration was 10^9 (for mixed cultures, 10^7) in 1 ml. The inoculum was administered at a rate of 3% of the total volume. The bacterial growth was estimated on the basis of culture density using the nephelometer. The dissociant correlation in a population was determined by sifting on BSA on the basis of colony morphology.

Table 1. Initial concentration of carbon, nitrogen, and phosphorus in media for monocultures (mg/ml) (Fursova et al., 2004a)

Medium number	R dissociant			S and M dissociants		
	carbon	nitrogen	phosphorus	carbon	nitrogen	phosphorus
1	0.78	0.4	0.028	0.78	0.4	0.028
2	3.18	0.1	0.028	3.18	0.1	0.028
3	3.18	0.4	0.007	3.18	0.4	0.007
4	0.282	0.1	0.008	0.282	0.1	0.008
5	1.6	0.03	0.008	0.78	0.03	0.008
6	1.6	0.1	0.002	0.78	0.1	0.002

Table 2. Initial concentration of carbon, nitrogen, and phosphorus in media for mono- and mixed cultures (mg/ml) (Fursova et al., 2004a)

Medium number	Carbon	Nitrogen	Phosphorus
1	0.4	0.035	0.01
2	1.62	0.14	0.04
3	0.4	0.015	0.01
4	1.6	0.06	0.04
5	0.12	0.035	0.01
6	0.48	0.14	0.04
7	0.12	0.015	0.01
8	0.48	0.06	0.04
9	0.78	0.1	0.01
10	3.24	0.4	0.04
11	1.6	0.2	0.01
12	6	0.8	0.04
13	1.2	0.035	0.01
14	4.8	0.14	0.04

For pH measurements of the medium, a Checker micropotentiometer (HANNA Instruments) was used. Glucose concentration was determined with the help of triphenyltetrazolium chloride (*Chemistry of Carbohydrates*, 1967), nitrogen was determined with sulfophenol reagent (Polyakov, 1950), and phosphorus was determined by the Panush method (*Practical Course of Biochemistry*, 1979). In subsequent experiments, express methods of substance determination were used: Diagluc test strips for semiquantitative determination of glucose in blood (the range of determined concentrations is 0.0–1000 mg% (0.0–55.5 mM)); Merck photometric phosphate test (concentration determination is possible within the range from 0.010 to 5.00 mg/l); Merck analytic test strips for determination of nitrate content (the range of determined concentrations is 10–25–50–100–500 mg/l).

Table 3. Initial concentration of carbon, nitrogen, and phosphorus in media for mono- and mixed cultures under specified limitation conditions (mg/ml) (Fursova et al., 2007)

Medium number	Carbon	Nitrogen	Phosphorus
1	0.9	0.05	0.008
2	0.76	0.165	0.02
3	2.4	0.04	0.02
4	1.6	0.04	0.006
5	1.6	0.08	0.006
6	4.8	0.25	0.006
7	0.76	0.04	0.006

The technique of the experiments with additives aimed at detection of limiting resources was improved. At the estimated time of entering the stationary phase, the culture was divided into seven tubes. Six of them were filled with additives (glucose, nitrate or phosphate, their paired combinations, and all three substances) and one was left unchanged (control). The amount of the additives was equal to their initial concentration in the medium. The culture was left for 12 more hours to grow, and then the optical density was measured. If the added nutritional component caused the renewal of cell division, the resource (or combination of resources) was considered to be limiting. If administering of substances did not lead to culture growth, it was assumed that the respective factors did not limit the community development. As compared to the previously conducted experiments (Fursova et al., 2004a), the time of culture exposure to the medium with additives was increased, thus excluding the lag phase effect. Moreover, using combinations of resources, as opposed to addition of separate nutritional components, enabled joint limita-

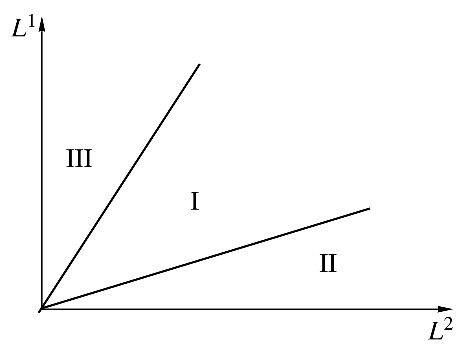

Fig. 1. Spatial stratification of two consumed resources. In Area I, both factors are limiting; in Area II, L^1; in Area III, L^2.

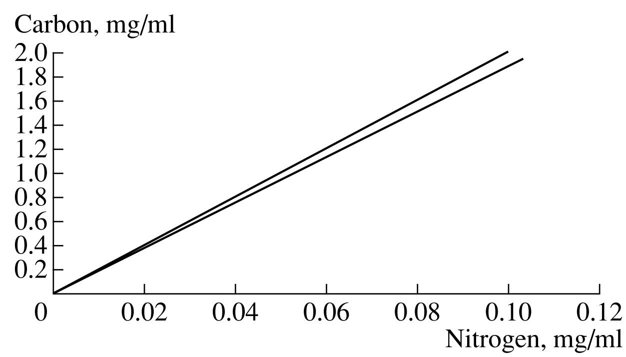

Fig. 2. Spatial stratification of two resources for the mixture of R, S, and M dissociants of *P. aeruginosa*.

tion of bacterial growth by several substances to be fixed.

According to the results of studying the consumption and growth mathematical model (Levich et al., 1904; Fursova, 2003), the space of initial resource stocks falls into areas where one of the possible combinations of consumed substances (including each individual substance) is limiting. The boundaries of these areas are calculated on the basis of the experimentally determined requirements of organisms in environmental resources (Fursova et al., 2004b).

RESULTS AND DISCUSSION

In the cited work of Fursova et al. (2004a), the analysis of the experimental results allowed one to suppose that the main resources that can limit the growth of mono- and mixed cultures of *P. aeruginosa* dissociants are carbon, nitrogen, and phosphorus. It was shown that the resource vectors defining the experimental media at the given stage of research (Tables 1, 2) belong to strata with single-factor limitation. Limitation of the culture growth by the model-predicted substance was confirmed in 75% of the analyzed experiments.

Next, a series of experiments with preset limitation conditions was conducted. Seven media (Table 3) were chosen so that they would be limited by various resources: a balanced medium (no. 1) where the concentration of each resource on average is proportional to the respective requirements of all dissociants (three monocultures were cultivated on this medium); a medium limited by carbon for all possible combinations of mono- and mixed cultures (no. 2); a medium limited by nitrogen for monocultures (no. 3) and mixed cultures (no. 4); a medium limited by phosphorus for all possible combinations of mono- and mixed cultures (no. 5); for monocultures and a mixture of three dissociants (no. 6). The composition of medium no. 7 was balanced for the monoculture of R dissociant (the concentration of resources was proportional to the requirements of the dissociant) and limited by phosphorus for mixed cultures and S and M dissociant monocultures.

The analysis of the data obtained in the above-mentioned series of experiments resulted in the assumption that phosphorus is repeatedly consumed by *P. aeruginosa* dissociants (Fursova et al., 2007).

If we accept the hypothesis about cyclic consumption of phosphorus during culture growth, it is necessary to reconsider the results of the experimental analysis. Let us assume that the growth of *P. aeruginosa* dissociants can actually be limited only by carbon and nitrogen, and all other substances necessary for their growth, including phosphorus, are present in sufficient amount in the media. In this case, the boundaries of limitation areas are defined by two rays coming from the center of coordinates and positive semiaxes of the Cartesian plane (Fig. 1) (Levich et al., 1994). These rays coincide for monocultures. Stratification for the

Table 4. Reaction to additives in monocultures (%) (Fursova and Levich, 2007) (see explanations in the text)

Experiment number	Model-predicted limiting resource	Additive	R	S	M
1	C	C	**121**	**220**	**110**
		N	91	102	96
2	N	C			101
		N			**131**
3	C	C			117
		N			119
4	C	C	**220**	**140**	**217**
		N	97	80	74
5	C	C		**227**	
		N		73	
6	N	C	93		
		N	93		
7	C	C	94	62	100
		N	95	100	68
8	N	C	95	100	90
		N	**143**	**112**	100
9	C	C		**122**	**160**
		N		100	90
10	C	C	212		97
		N	162		66
11	C	C	**129**	**129**	**143**
		N	86	100	86
12	C	C	**131**	**101**	**119**
		N	76	68	81
13	C	C			**112**
		N			91
14	C	C	**246**	**171**	**184**
		N	103	76	92
		CN			
		C1			
		N1			
		CN1	**195**	**140**	**163**
15	C	C	**213**	**213**	**222**
		N	104	100	110
		CN	**204**	**178**	**208**
		C1	223	209	212
		N1	100	120	120
		CN1	**223**	**219**	**194**
16	CN	C	105		
		N	105		
		CN	86		
		C1	104		
		N1	82		
		CN1	**168**		
17	CN	C	135	149	178
		N	91	102	104
		CN	**214**	**202**	**216**
		C1	135	153	158
		N1	100	104	92
		CN1	**207**	**196**	**218**

Table 5. Additives in mixed cultures (%) (Fursova and Levich, 2007) (see explanations in the text)

Experiment number	Model-predicted limiting resource	Additive	RS	RM	SM	RSM
1	C	C		82	89	87
		N		59	84	98
2	N	C	92	96	104	96
		N	**104**	**104**	**116**	**128**
3	C	C	**142**	**160**	**160**	**150**
		N	75	100	90	71
4	C	C			**121**	
		N			79	
5	C	C	**120**	**120**		**113**
		N	100	93		75
6	C	C	**103**	**143**		
		N	81	102		
7	C	C	**103**	**109**		**129**
		N	88	81		91
8	C	C	**220**	**200**	**194**	**254**
		N	98	94	98	114
		CN	**202**	**198**	**214**	**237**
		C1	**204**	**227**	**276**	**254**
		N1	110	114	94	116
		CN1	**212**	**227**	**270**	**237**
9	C	C		**233**		
		N		102		
		CN		**246**		
		C1		**200**		
		N1				
		CN1		**173**		
10	C	C				**222**
		N				100
		CN				**222**
		C1				**256**
		N1				113
		CN1				**220**
11	CN	C	102	99	77	126
		N	116	81	87	93
		CN	116	**157**	**107**	**137**
		C1	111	104	103	86
		N1	96	68	91	89
		CN1	113	**157**	**125**	**200**
12	CN	C	100	108	116	142
		N	90	92	110	102
		CN	104	**125**	**127**	**205**
		C1	90	98	112	142
		N1	92	92	102	98
		CN1	**170**	**163**	**163**	**193**

mixed culture of three *P. aeruginosa* dissociants is shown in Fig. 2 (for double mixtures, the stratification patterns are the same).

According to such division of the resource space, the limiting factor remained the same for all media that were limited by carbon or nitrogen (media nos. 1, 2, 4, 5 in Table 1; all media except nos. 11 and 12 in Table 2; media nos. 1–4 in Table 3). Media nos. 3 and 6 from Table 1 and nos. 11 and 12 from Table 2, initially limited by phosphorus, became limited by carbon according to the new conditions. Cultivation conditions on media nos. 5–7 from Table 3 set combined limitation by carbon and nitrogen.

The results of the experiments with additives are summarized in Tables 4 and 5. The optical density of the culture without additives was taken to be 100% (the presence of phosphorus in the added resources is marked by 1). The characteristics pointing to the growth renewal as a result of introduction of the model-predicted limiting nutrient are given in bold. Thus, in 83% of cases, the theoretical and experimental data coincide.

It should be noted that the corrections to the experimental procedure (see Materials and Methods) made on the basis of the work by Fursova et al. (2004a) allowed the number of experiments to be increased substantially, the results of which are suitable for analysis in terms of a variational model.

Account of the metabolic peculiarities of *P. aeruginosa* dissociants in the model approach and improvement of the experimental techniques made it possible to describe the experimental results more precisely. The previous model predictions were confirmed in 75% of cases.

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