ISSN 0026-2617, Microbiology, 2008, Vol. 77, No. 2, pp. 192–197. © Pleiades Publishing, Ltd., 2008.
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EXPERIMENTAL ARTICLES

Cultivation of *Pseudomonas aeruginosa* Dissociants under Specified Limitation Conditions

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Received June 12, 2006

Abstract—The possibility of controlling the development of microbial communities was investigated on the basis of experimentally determined requirements for basic nutrients in R, S, and M dissociants of *Pseudomonas aeruginosa.* On media with the limitation conditions preset on the basis of the predictions of a mathematical model, exhaustion of glucose was experimentally confirmed for all monocultures and mixed cultures, as well as balanced consumption of glucose, nitrogen, and phosphorus by the R dissociant at the corresponding initial medium composition. The experimentally determined composition of mixed cultures was found to conform to the ones calculated using the mathematical model. The data obtained suggest the possibility of cyclic consumption of phosphorus by *P. aeruginosa.*

Key words: dissociation, *Pseudomonas aeruginosa*, nutrient requirements, limiting factors, community structure.

DOI: 10.1134/S0026261708020124

Prediction of the development of a multispecies microbial community and the possibility of controlling it are important problems with numerous practical applications. Varying the ratio of mineral nutrients in the medium is one of the approaches used to form the community structure, i.e., to modify the types and prevalence of species and to increase or decrease the ratio of certain microbial groups [1, 2].

A variational model of nutrient consumption and growth for ecological communities [3, 4] was used to describe microbial cultures for the purpose of prediction and control of their composition. From the known requirements of physiologically different microbial groups, this model allows calculation of both the limitation ranges for any combination of resources in the medium and the density of the populations within the community at the stationary growth phase as a function of the levels of limiting substrates.

Nutrient requirements of the organisms are the experimentally determined parameters. A requirement is understood as an amount of a resource per cell required for growth.

The carbon, nitrogen, and phosphorus requirements of *P. aeruginosa* were determined in our previous work [5]. The goals of the present work were as follows: (i) to determine the carbon, nitrogen, and phosphorus requirements of dissociants and to compare them with the previously obtained values [5]; (ii) to use the data obtained in order to compose media limited by each nutrient (according to the variational model of growth and consumption) and to obtain experimental confirmation of the specified limitation conditions; and (iii) to calculate the population structure of mixed (binary and ternary) cultures at the stationary growth phase on each medium and the optical density at the moment of growth termination caused by resource exhaustion, and to compare these data with experimental results.

MATERIALS AND METHODS

Cultivation of the R, S, and M dissociants of *P. aeruginosa* K-2 were tested in 65 experiments. Bacteria were grown on seven media with different initial concentrations of glucose, nitrates, and phosphates (Table 1) in monocultures and mixed cultures. The cultivation was carried out in shaken (180 rpm) 50-ml test tubes with 10 ml of the medium at 28°C; the stationary growth phase was achieved in two days. Inocula were grown for one day on the agarized media containing nutrient broth and wort (1 : 1). With a loop, bacterial biomass from slanted agar was transferred into physiological saline. Inoculum density was adjusted to 10^9 cells/ml using a nephelometer or turbidity standards. The inoculum ratio was 3% (vol/vol).

In the course of experiments, the values of pH, optical density, and nutrient concentrations were monitored, as well as the emergence of other dissociants in monocultures. The samples were taken at the beginning

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of the experiment and every four hours during the second day of cultivation.

Bacterial growth was assessed nephelometrically on a FEK 56 M colorimeter (green filter no. 6, green, transmission maximum at 540 nm) in a 0.5-cm cuvette. In cases of weak bacterial growth, 2-cm cuvettes were used and the values were accordingly recalculated. For convenience sake, the nephelometer readings were multiplied by 100.

For the stationary phase of each dissociant, a coefficient was obtained to calculate cell numbers from optical density [5].

A Checker micropotentiometer (HANNA Instruments) was used to determine the pH of the media.

Resource consumption was determined by express methods. Glucose content was determined by means of Diaglyuk indicator stripes (Biosensor AN, Russia); the range of glucose concentrations was 0–1000 mg% (0.0–55.5 mM). Express analysis of phosphate content was performed with the photometric phosphate test (Merck); the concentration range was 0.010 to 5.00 mg/l. Nitrate content was determined using the appropriate test stripes (Merck); the concentration range was 10–25–50–100–500 mg/l. Since these procedures are not very precise, the presence or absence of a component in the culture liquid was accepted as the principal parameter. At the stationary growth phase, such information on media composition was sufficient for our purposes.

Nutrient requirements were calculated from the equation $q_i^L = \frac{\Delta L}{\Delta n_i}$, where ΔL is the amount of the compound consumed, L is a nutrient component, and Δn_i is the number of cells formed during the time interval; i characterized the dissociants (for more details, see [5]). The standard Microsoft Excel 7.0 application was used to calculate the values of average requirements and confidence intervals.

For experimental determination of the limiting resource, experiments with supplementation were carried out [6]. At the beginning of the stationary phase, the culture was subdivided into seven subsamples. Six of these contained supplements (glucose, nitrate, or phosphate, combinations of two compounds, and all three of them); the seventh was used for control. The amount of the supplements was equal to their initial concentration in the medium. The cultures were incubated for 12 h; afterwards, optical density was measured. A resource was considered limiting if addition of it resulted in cell growth. When the supplement did not cause growth, it was considered not to limit the development of the community.

The model calculations of the limitation (stratification) zones and of the number of dissociants at the stationary growth phase were carried out according to the algorithms developed by the authors [2, 6, 7].

Table 1. Composition of the media at the beginning of experiments

Medium no.	Carbon, mg/ml	Nitrogen, mg/ml	Phosphorus, mg/ml
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

Table 2. Average values of requirements ($\times 10^{-12}$ mg/cell), considering the 95% confidence intervals (A) as compared to the average values obtained earlier (B) [5]

Dissociant	Resource					
	Carbon		Nitrogen		Phosphorus	
	A	B	A	B	A	B
R	129 ± 22	134	7.0 ± 2.5	8.7	1 ± 0.5	1.1
S	409 ± 55	399	17.5 ± 3.0	21.8	4 ± 1.5	3.4
M	525 ± 114	679	31 ± 3	37	6 ± 2.5	5.6

RESULTS AND DISCUSSION

The nutrient requirements calculated from the results of our experiments correspond well to those obtained earlier (Table 2). The requirements of M dissociants in carbon and of R and S dissociants in nitrogen do not fit into the 95% confidence intervals. These differences, however, are not significant from the point of view of the sensitivity of model [8]; the possible corrections could not have affected the stratification of the resource space and the predicted community structure. The same values of the model parameters (requirements) as in our previous experiments were therefore used.

The reasons for the application of the seven media used were that (i) their nutrient content was adjusted to ensure an experiment duration of about two days and (ii) the nutrient ratios were adjusted to achieve limitation by different resources. On the basis of the data on the borders of the limitation zones corresponding to the given requirements values [6], the following media were composed: a balanced medium (no. 1) with the concentration of each resource approximately proportional to the requirements of all dissociants (the monocultures were grown in this medium); a carbon-limited medium (no. 2) for combinations of monocultures and mixed cultures; a nitrogen-limited medium for monoc-

Table 3. Results of experiments with supplementation (optical density is in the nephelometer readings multiplied by 100; media numbers are the same as in Table 1)

	Optical density at the moment of supplement addition	Optical density after growth with supplemented nutrients							Optical density after growth without supplements	Compound in medium at the stationary growth phase			pH at the moment of supplement addition
		C	N	P	CN	CP	NP	CNP		Glucose, mg%	Nitrogen, mg%	Phosphorus, mg%	
Medium no. 1													
R	61	69	62	64	73	64	51	89	59	0	–*	–	7.6
S	47	50	51	48	60	38	80	70	47	80	–	–	4.7
M	Dissociation												
Medium no. 2													
R	58	91	38	43	–	–	–	72	37	0	2.1	–	8.6
S	70	99	44	58	–	–	–	81	58	0	2.1	–	7.8
M	62	94	47	57	–	–	–	83	51	0	3.4	–	8
RS	71	110	49	58	101	102	55	106	50	0	5.7	1.3	8.1
RM	64	96	45	52	95	109	–	109	48	0	5.7	1.3	8.4
RM	67	121	53	60	128	104	59	90	52	0	5.7	1.6	8
SM	65	95	48	58	105	135	46	132	49	0	5.7	1.5	8.3
RSM	65	109	49	44	102	109	50	102	43	0	5.7	1.3	8
R	60	102	50	62	98	107	48	107	48	–	–	–	8.2
R	51	105	46	55	92	97	54	97	44	–	–	–	8.7
S	60	115	54	65	96	113	65	118	54	–	–	–	8.4
S	55	73	49	64	108	68	63	90	52	–	–	–	8.6
M	60	109	54	62	102	104	59	95	49	–	–	–	7.9
M	44	73	48	56	92	69	60	66	49	–	–	–	8.3
RSM	58	100	45	54	100	115	51	99	45	–	–	–	8.4
RSM	48	101	49	55	–	–	–	–	49	–	–	–	8.5
Medium no. 3													
R	42	43	39	38	–	–	–	40	40	>300	0	1.5	4.5
S	45	49	46	46	–	–	–	45	39	>300	0	1.6	4.5
M	49	57	55	55	–	–	–	51	51	>300	0	1.9	4.7
RSM	45	55	54	55	48	50	51	52	50	>300	0	1.7	4.9
Medium no. 4													
RS	41	40	40	39	41	31	36	32	42	120	0	0.5	4.8
RM	42	45	39	40	39	33	30	43	38	120	0	0.4	4.4
RM	58	63	66	53	60	57	61	55	58	–	–	–	5.6
SM	50	59	52	59	50	54	58	56	60	120	0	0.6	4.1
RSM	42	41	38	49	37	37	35	43	37	120	0	0.6	4.2
RSM	57	56	62	51	61	56	60	58	57	–	–	–	5.5
Medium no. 5													
R	88	135	100	105	115	129	77	165	96	0	0	0.02	6.8
R	115	116	116	110	95	114	90	185	110	0	0	0.01	7.8
S	55	59	82	69	70	58	82	104	60	40	0	0.01	6.6
S	67	87	73	79	83	65	69	72	72	–	–	–	5.6
M	68	74	79	60	83	77	77	78	74	40	0	0.02	3.7
M	88	78	77	78	79	78	75	96	78	–	–	–	3.6
RS	84	105	82	85	79	96	89	95	88	0	0	0.02	6
RS	96	97	110	106	110	105	91	107	95	0	0	0.01	7.6
RM	105	130	113	126	143	120	79	149	115	0	0	0.02	7
RM	133	117	95	104	185	123	80	185	118	0	0	0.02	7.6
SM	99	110	101	108	106	92	100	127	99	0	0	0.02	4.3
SM	105	89	100	100	125	118	105	144	115	0	0	–	7.6
RSM	50	66	62	66	85	63	60	72	62	0	0	0.02	5.2
RSM	70	88	65	72	96	60	62	140	70	–	–	–	6.7

Table 3. (Contd.)

	Optical density at the moment of supplement addition	Optical density after growth with supplemented nutrients							Optical density after growth without supplements	Compound in medium at the stationary growth phase			pH at the moment of supplement addition
		C	N	P	CN	CP	NP	CNP		Glucose, mg%	Nitrogen, mg%	Phosphorus, mg%	
Medium no. 6													
R	125	76	84	84	84	105	95	80	87	0	13	0.02	4.2
R	93	72	80	138	90	139	–	60	105	0	0	0	4.4
S	76	102	58	60	66	56	68	64	51	0	13	0	3.9
S	66	90	62	57	53	69	63	88	95	0	13	0.04	4.6
M	98	45	42	47	44	48	43	40	48	0	13	0.05	3.9
M	55	41	40	43	39	44	75	37	45	–	–	–	4.3
RSM	134	59	125	99	57	50	75	45	60	–	–	–	3.9
RSM	76	61	59	90	50	68	65	50	60	0	13	0	4.2
Medium no. 7													
R	54	58	39	42	92	58	43	89	43	0	0	0.03	7.9
R	46	59	40	40	57	51	40	80	39	0	0	0	8.3
S	39	79	50	50	110	78	50	102	52	0	0.7	0.04	7.2
S	63	70	48	46	95	72	49	92	47	0	0	0	8.2
M	52	87	50	49	102	90	50	98	50	0	1.4	0.09	7.4
M	63	89	52	50	108	79	46	109	50	0	1.4	0.09	7.7
RS	25	35	51	41	58	34	56	62	43	0	0	0.02	7
RS	48	50	45	50	52	45	46	85	50	0	0	0	7
RM	30	34	50	33	71	33	50	67	39	0	0.3	0.02	7.1
RM	50	53	45	48	61	48	45	80	49	0	0	0	7.3
SM	28	43	60	50	90	43	59	78	53	0	0	0.22	7
SM	53	57	54	55	62	55	50	80	49	0	0	0.07	7.5
RSM	55	64	42	45	97	66	45	87	45	0	0	0	7.9
RSM	58	61	44	45	88	61	42	83	43	0	0	0	8.3

* Characteristics was not measured.

ultures (no. 3) and for mixed cultures (no. 4); and a phosphorus-limited medium for all the combinations of monocultures and mixed cultures (no. 5) and for monocultures and the mixture of three dissociants (no. 6). The composition of medium no. 7 is balanced for the monoculture of R dissociants (resource concentrations are proportional to its requirements) and phosphate-limited for mixed cultures and for monocultures of S and M dissociants. The media numbers are the same as in Table 1. Experiments with supplementation were carried out for each cultivation medium. In order to "catch" the beginning of the stationary phase (the best moment for the introduction of supplements), the nutrients were added at two ages of the culture (e.g., 30 and 34 h). The results are presented in Table 3.

According to the concept of the experiments with supplementation, growth on balanced media should resume after addition of all three nutrient components. This was precisely the case for R dissociants. For S dissociants, limitation by nitrogen and phosphorus occurred (introduction of this combination of resources resulted in more pronounced resumed growth). However, apart from the errors in determination of requirements (Table 2) and optical density, errors of media composition exist; their role becomes especially important for attempts at multifactor limitation. It is therefore difficult to establish the initial cultivation conditions resulting in growth cessation after simultaneous exhaustion of all the nutrient components. Thus, the results of S dissociant cultivation on medium no. 1 can be considered not contradictory to the values predicted by the variational model.

The results of experiments with supplementation for monocultures and mixed cultures on carbon-limited media completely confirm the specified character of limitation; in all cases, growth resumed after addition of glucose or of a combination of chemical containing glucose.

Experimental confirmation of nitrogen limitation was not obtained in our experiments. In all cases, the

Table 4. Composition of mixed cultures (as a percent) and their optical density (in nephelometer readings multiplied by 100) at the stationary growth phase (media numbers are the same as in Table 1)

Culture	Community composition		Total optical density	
	experimental	calculated	experimental	calculated
Medium no. 2				
R : S	57 : 43	69 : 31	71	61
R : M	74 : 26	73 : 27	64	75
R : M	66 : 34	73 : 27	67	75
S : M	72 : 28	54 : 46	65	58
R : S : M	54 : 37 : 12	62 : 23 : 15	65	66
R : S : M	38 : 23 : 39	62 : 23 : 15	65	66
R : S : M	61 : 22 : 17	62 : 23 : 15	58	66
Medium no. 4				
R : S	62 : 38	65 : 35	41	68
R : M	71 : 29	74 : 26	58	69
S : M	47 : 53	60 : 40	50	76
R : S : M	53 : 20 : 27	60 : 29 : 11	57	70
Medium no. 5				
R : S	35 : 65	72 : 28	96	55.5
R : M	54 : 46	78 : 22	133	60
S : M	65 : 35	57 : 43	105	53.1
R : S : M	56 : 24 : 20	68 : 22 : 10	76	56.1
Medium no. 6				
R : S : M	–*	68 : 22 : 10	134	57
Medium no. 7				
R : S	67 : 33	72 : 28	50	54
R : M	–	78 : 22	50	60
S : M	62 : 38	57 : 43	56	54
R : S : M	76 : 9 : 15	68 : 22 : 10	58	55.5

* Characteristics was not measured.

medium was acidified, which probably caused bacterial death, since addition of any combination of resources did not result in resumed growth.

Phosphorus limitation was also not observed in our experiments. This can be seen both from the results of experiments with supplementation (growth did not resume after addition of phosphorus) and from the values of phosphate content in the medium. Although the relative phosphate content decreased (due to increased absolute values of carbon and nitrogen content), this resource was not exhausted completely. Moreover, analyses performed in the course of experiment revealed that phosphate content not only did not decrease, but sometimes even exceeded the initial value. Analysis of these results suggested the probability of repeated phosphorus consumption by *P. aeruginosa* dissociants. A similar concept was suggested earlier on the basis of the biochemical research on phosphorus metabolism in microorganisms [9]. The paucity of nutrient media used in the experiments is probably important for this phenomenon, since both phosphorus exhaustion and resumed growth upon addition of phosphorus were recorded some earlier experiments; in fact, this was a prerequisite for the calculation of phosphorus requirements [5].

In experiments with polycultures, the population structure was determined in the stationary growth phase. The results are presented in Table 4.

In all the seven experiments on carbon-limited medium (no. 2), the difference between the calculated and experimental values of optical density did not exceed 17%. In two cases out of seven, the differences between the predicted and experimental composition of the mixture of dissociants exceeded 12%. On nitrogen-limited medium (no. 4), all the calculated values of optical density were somewhat higher than the experimental ones. This is possibly because the culture did not reach the maximal possible density due to acidification of the medium (no. 4). The differences in dissociant structure on this medium did not exceed 13%.

In five out of seven mixed cultures grown on phosphorus-limited media, the predicted population structure differed from the one experimentally recorded by no more than 12%. In two other cases, the difference was 27 and 24%. The results on optical density provide a confirmation for the hypothesis of phosphorus turnover. Only on medium no. 7, which is relatively close to the balanced one (analyses of the medium composition revealed consumption of all the resources), was good correspondence between the calculated and experimental values observed (the error was 8, 20, and 4%). When bacteria were grown in media nos. 6 and 7, the model predicted on average an optical density half that of the experimental data. According to the phosphate test, this resource was not exhausted. Assuming that the phosphorus of the media was used twice (from the point of view of the model, this is equivalent to a twofold increase in the initial phosphorus concentration), the calculated and experimental values of culture density are close.

ACKNOWLEDGMENTS

The work was supported by the Russian Foundation for Basic Research, project 05-04-49238.

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