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Correlation of Nitrogen Metabolism with Biosurfactant Production by *Pseudomonas aeruginosa*†

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A direct relationship between increased glutamine synthetase activity and enhanced biosurfactant production was found in *Pseudomonas aeruginosa* grown in nitrate and Proteose Peptone media. A chloramphenicol-tolerant strain showed a twofold increase in biosurfactant production and glutamine synthetase activity. Increased ammonium and glutamine concentrations repressed both phenomena.

Pseudomonas aeruginosa produces two types of rhamnolipid-type biosurfactants, R1 and R2, on either hydrocarbon or carbohydrate media (6). R1 contains two rhamnoses attached to β-hydroxydecanoic acid, whereas R2 consists of one rhamnose unit connected to the same hydroxy-fatty acid. Higher yields of these rhamnolipids must be realized to stimulate commercial interest. Although previous authors (5) have examined the effects of ammonium and nitrate as sources of nitrogen, the role of amino acids has not been investigated. Pseudomonads utilize nitrates, ammonia, and amino acids as nitrogen sources. Nitrates must be reduced to nitrite and then ammonia (9). Ammonia can then be assimilated either by glutamate dehydrogenase (GDH; EC 1.4.1.4) to form glutamate (2) or, with glutamate, by glutamine synthetase (GS; EC 6.3.1.2) to form glutamine. Glutamine and α-ketoglutarate are then converted to glutamate by L-glutamine 2-oxoglutarate aminotransferase (GOGAT; EC 1.4.1.13) (2, 7).

This paper describes the relationship of the activities of these enzymes to biosurfactant production with various nitrogen sources. Two strains of *P. aeruginosa*, a wild type, ATCC 9027, and a chloramphenicol-tolerant strain, ATCC 9027 var. RCII, are compared. The latter strain, derived from the wild type, was chosen for its ability to tolerate large doses of the antibiotic chloramphenicol (8b). The strains were maintained on *Pseudomonas* agar P (Difco Laboratories). The chloramphenicol-tolerant strain was isolated by repeated subculturing of the wild type in the presence of increasing amounts of chloramphenicol until a concentration of 150 μg/ml was achieved.

Kay minimal medium (10) was used for preculture, and Proteose Peptone-glucose salts (PPGS) medium (4) was used for biosurfactant studies. Nitrogen sources were varied for each experiment. Chloramphenicol (150 μg/ml) was added for experiments with the tolerant strain to maintain tolerance. All batch cultures were grown in a stirred-tank CHEMAP 3.7-liter fermentor under the following conditions: 30 h of incubation, agitation rate of 100 rpm, 15 ml of inoculum, aeration at 3.5 liters/min, 37°C, 1.5-liter working volume, and pH of 7.0. A CHEMAP Fundafom mechanical foam breaker was used. Growth was monitored turbidimetrically at 660 nm. One optical density unit is equivalent to 0.185 g (dry weight) of cells per liter. The surface tension, critical micelle concentration (CMC), and glucose, nitrate, and ammonium analyses were determined as described by de-Roubin et al. (4a). P_i and amino acid analyses and preparation of cell extracts by sonication for enzymatic assays were performed by the method of Mulligan et al. (8b).

Commercially available reagent-grade chemicals were obtained from local supply houses. All enzymes and substrates were purchased from Sigma Chemical Co. (St. Louis, Mo.). All enzyme assays were carried out at 37°C. GS activity is expressed in terms of nanomoles of γ-glutamyl hydroxamate formed per minute per milligram of protein (7). GDH and GOGAT were assayed spectrophotometrically by measuring NAD(P)H oxidation (7). Protein estimation was done by the Lowry method (8), with bovine serum albumin as standard.

The effect of a nitrogen source on surfactant production by *P. aeruginosa* was investigated in PPGS medium for both

TABLE 1. Effect of nitrogen source on enzyme activities and biosurfactant production by *P. aeruginosa*

Strain and medium	Enzyme activity (nmol/min per mg of protein)				CMC^{-1}[a]
	GS[b]	GDH (NADP)	GDH (NAD)	GOGAT	
ATCC 9027					
20 mM NH_4^+ + PPGS	272	61	15	16	8
20 mM NO_3^- + PPGS	362	16	25	33	10
PPGS	1,199	8	30	13	13
ATCC 9027 var. RCII					
20 mM NH_4^+ + PPGS	705	34	6	18	15
20 mM NO_3^- + PPGS	859	61	5	38	17
PPGS	2,468	32	8	39	25
6 mM glutamine + PPGS	139	9	9	37	2

[a] The CMC is determined by measuring the surface tension of the medium at various dilutions, and the logarithm of the dilution is plotted as a function of surface tension. The CMC is the point of abrupt surface tension increase, and CMC^{-1} increases with surfactant concentration and is an indication of relative concentration.
[b] Total GS activity.

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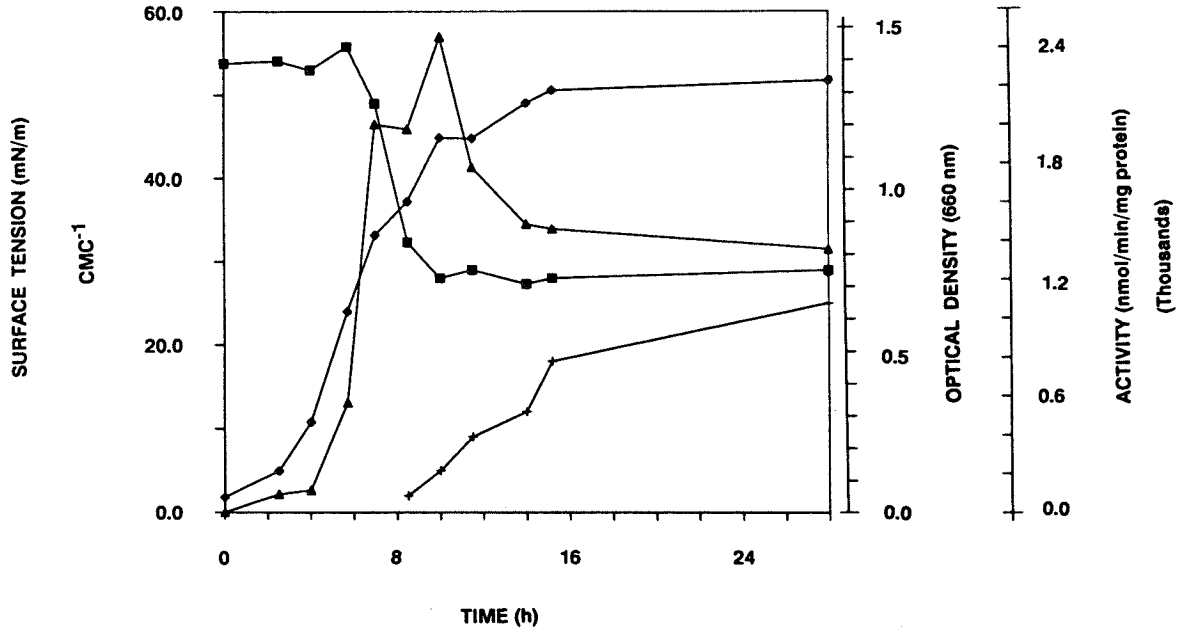

FIG. 1. Growth and biosurfactant production by *P. aeruginosa* var. RCII. Symbols: ◆, optical density; ■, surface tension; +, CMC^{-1}; ▲, GS activity.

strains (Table 1). The production of biosurfactant was highest when Proteose Peptone was the sole nitrogen source. In the supplemented media, ammonium ions and nitrates were used at slightly higher rates by the chloramphenicol-tolerant strain than by the wild type (22 and 40%, respectively, compared with 7 and 10% for the wild type). The preferred nitrogen source was glutamic acid from the peptone (80% utilization). Growth without Proteose Peptone was very poor (optical density of <0.3).

The activities of enzymes involved in nitrogen metabolism were examined to deduce a regulatory role during rhamnolipid production (Table 1). All results shown are for single experiments. All assays were performed in duplicate and were reproducible to ±5%. For both strains, total GS activity was significantly higher in PPGS medium. In the nitrate medium, there was more GS synthesis than in the ammonium medium. GDH (NADP) repression was also evident. GOGAT activity did not differ significantly in the

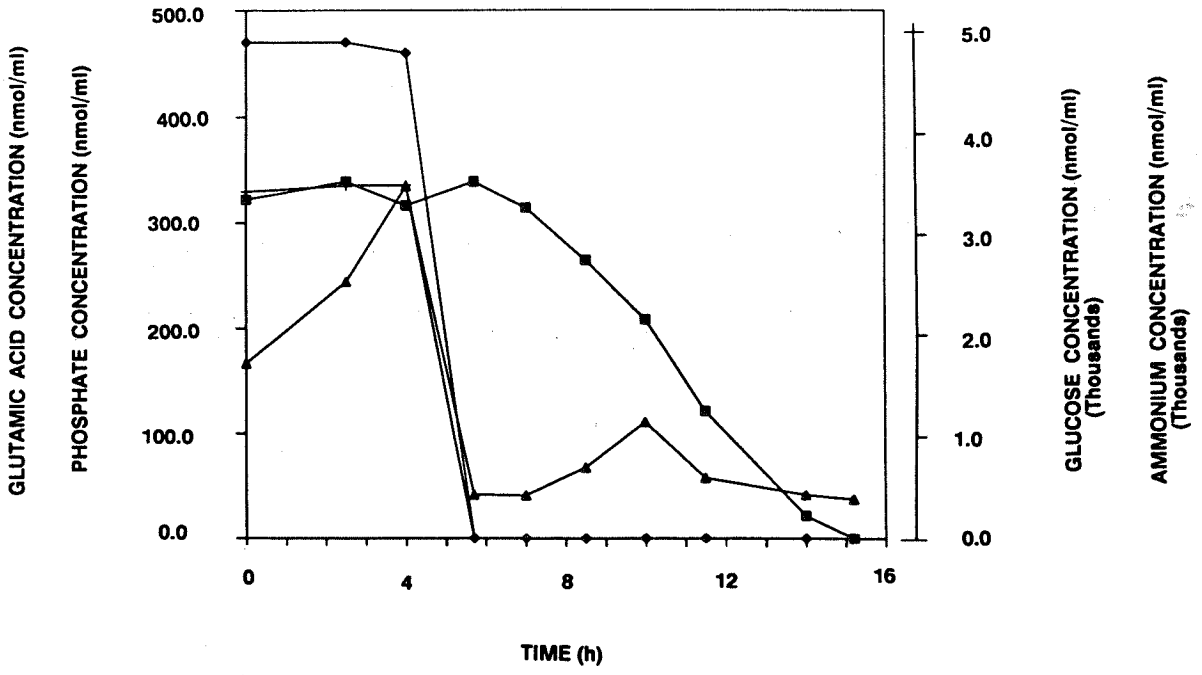

FIG. 2. Concentration of medium components during the growth of *P. aeruginosa* var. RCII. They include phosphate (+), glutamic acid (▲), glucose (■), and ammonium (◆).

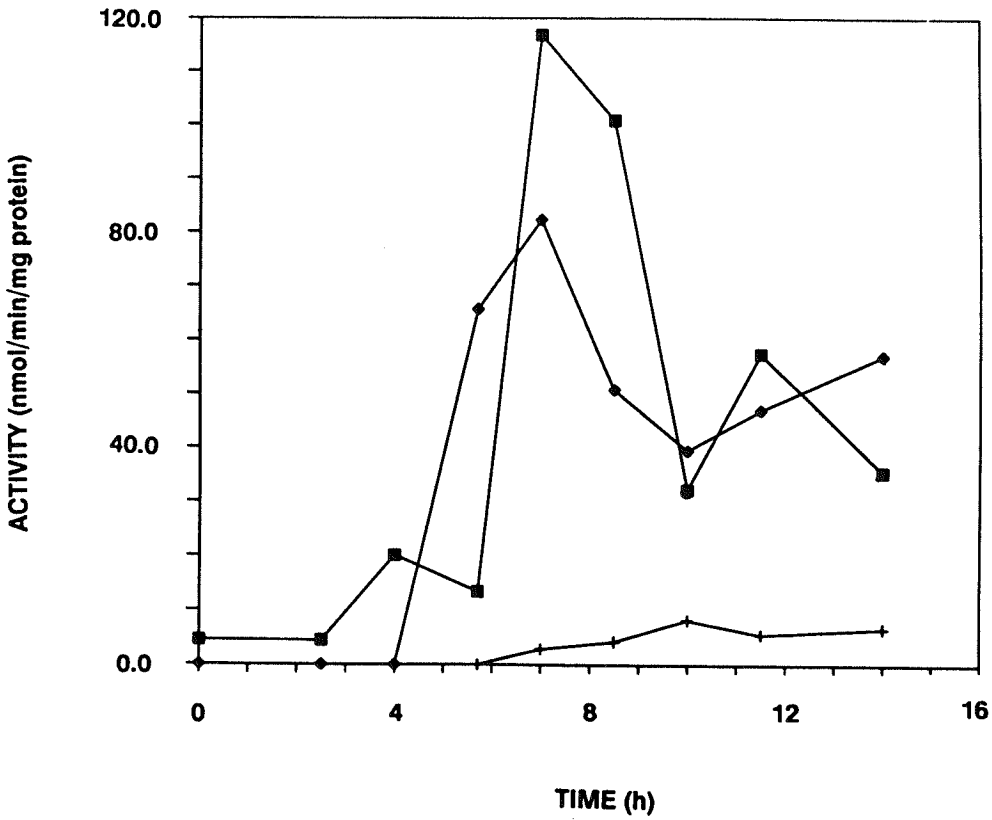

FIG. 3. Intracellular enzyme activities of *P. aeruginosa* var. RCII during growth. Enzymes include GDH (NADP) (■), GDH (NAD) (+), and GOGAT (◆).

various media. GDH activity (NAD specific) was evident in all types of media but was not as affected by the medium as was the NADP-dependent form. The addition of glutamine, an end product in the GS reaction, inhibited both GS activity and biosurfactant production by the tolerant strain. Glutamine was taken up quickly (4 h) by the bacteria. It has been reported that an elevated amount of glutamine has a detrimental effect on GS activity in *Klebsiella aerogenes* (1).

In PPGS medium, as the growth of *P. aeruginosa* var. RCII slowed (Fig. 1), surface tension started to decrease (at 7 h) until a value of 28 mN/m was reached. Subsequently, the CMC^{-1} and GS activity increased significantly. Similar trends were observed for the wild type, with a reduction of 50% for both phenomena. Ammonium and phosphate levels (Fig. 2) were depleted at 6 h. After an initial increase in free glutamic acid concentration, significant amounts were utilized as the growth rate increased (4 h). As the growth rate slowed, however, excess glutamic acid was secreted into the medium (9 h) and there was simultaneous glucose consumption. Carbon was not exhausted until 15 h.

Both GDH (NADP) and GOGAT reached maxima at 7 h and then declined (Fig. 3). Increased GS levels (Fig. 1) coincided with decreased GDH (NADP) activity, indicating a preference for the GS-GOGAT pathway over the GDH pathway during biosurfactant production. This condition can be obtained in an organic nitrogen medium with a small amount of ammonium ions and by the specific selection of strains with elevated GS activity. NAD-dependent GDH increased as the glutamic acid levels decreased. During rhamnolipid biosynthesis, lipid, not sugar, formation is the rate-determining factor (3). Nutrient limitation (via nitrogen) may promote lipid accumulation.

In this paper, we have shown with both strains that GS activity and biosurfactant production are regulated by glutamate, glutamine, and ammonia levels. As growth slows, glutamate and ammonia are utilized while GS activity increases. Glutamine inhibits this process. Cell metabolism is then switched from nitrogen as it becomes limiting (glutamic acid) to glucose, resulting in rhamnolipid production.

To produce biosurfactants economically, increased yields are necessary. We have recently shown that a mutant of *Bacillus subtilis* could produce significantly higher amounts of biosurfactant than the wild-type strain (8a). In the present case, since a quick method for obtaining rhamnolipid producers is not known, screening for mutants of *P. aeruginosa* which have increased levels of GS activity could lead to enhanced production of biosurfactants and promote commercial interest.

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