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Cyanide Production by *Pseudomonas fluorescens* and *Pseudomonas aeruginosa*

RONALD A. ASKELAND†* AND SUMNER M. MORRISON

Department of Microbiology, Colorado State University, Fort Collins, Colorado 80523

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Of 200 water isolates screened, five strains of *Pseudomonas fluorescens* and one strain of *Pseudomonas aeruginosa* were cyanogenic. Maximum cyanogenesis by two strains of *P. fluorescens* in a defined growth medium occurred at 25 to 30°C over a pH range of 6.6 to 8.9. Cyanide production per cell was optimum at 300 mM phosphate. A linear relationship was observed between cyanogenesis and the log of iron concentration over a range of 3 to 300 μM. The maximum rate of cyanide production occurred during the transition from exponential to stationary growth phase. Radioactive tracer experiments with [1-^{14}C]glycine and [2-^{14}C]glycine demonstrated that the cyanide carbon originates from the number 2 carbon of glycine for both *P. fluorescens* and *P. aeruginosa*. Cyanide production was not observed in raw industrial wastewater or in sterile wastewater inoculated with pure cultures of cyanogenic *Pseudomonas* strains. Cyanide was produced when wastewater was amended by the addition of components of the defined growth medium.

Bacterial cyanogenesis has been demonstrated among *Pseudomonas* species and by *Chromobacterium violaceum* in complex and defined media (11). Iron has a stimulatory effect on cyanogenesis, and the majority of cyanide is produced in exponential and early stationary growth phases (7, 9, 18, 19). Cyanide synthesis from glycine by *Pseudomonas aeruginosa* (8) and *C. violaceum* (3, 15) has been demonstrated, and the effects of temperature, pH, and phosphate concentrations on cyanide production have been reported (7, 13, 18, 23). The purpose of bacterial cyanide production has not been determined.

A major source of cyanide in aquatic habitats is discharge as an industrial by-product; however, substantial amounts of cyanide have been found in rivers in nonindustrial areas (11a). Bacteria may contribute to cyanide production in the environment since the species which have been shown to be cyanogenic are native to soil and water (5). The possible role of bacterial cyanogenesis in aquatic environments has not been previously investigated.

In this study, the distribution of cyanogenic bacteria in aquatic habitats was examined. The effects of temperature, pH, phosphate concentration, iron concentration, and growth phase on cyanide production by *Pseudomonas fluorescens* in a defined medium were determined. Studies were conducted with ^{14}C-labeled glycine as a substrate for cyanide biosynthesis by *P. fluorescens* and *P. aeruginosa*. Cyanide metabolism in an industrial wastewater was also investigated.

MATERIALS AND METHODS

Isolation and identification. Water samples were collected from several sources in north central Colorado, and serial dilutions were made in sterile phosphate buffer (2). Samples were plated on standard methods agar by the spread plate method and incubated at 10 or 25°C. Isolated colonies were removed and streaked twice to ensure that pure cultures were obtained. Cyanogenic bacteria were lyophilized for long-term storage and routinely maintained on standard methods agar slants at 4°C.

Isolates were screened for cyanide production by heavily inoculating standard methods agar plates and incubating them in an inverted position at 10 or 25°C with picric acid indicator papers (20) placed inside the lids. Hydrogen cyanide caused the indicator paper to turn from yellow to brown. Production of cyanide was confirmed by the modified Aldridge procedure (16) and the formation of Prussian blue (19).

Cyanogenic isolates were identified by the following characteristics: Gram stain, cellular morphology, flagellum stain (10), pigment production, denitrification, motility, gelatin hydrolysis, oxidase and catalase tests, growth at 4 and 41°C, oxidation and fermentation of glucose, arginine dihydrolase (12), and utilization of organic substrates as sole carbon sources. A Colorado State University stock culture of *P. aeruginosa* 1-73 was used in radioactive tracer studies.

Cyanide determination. The Aldridge procedure (1) as modified by Nusbaum and Skupeko (16) and Row-

† Present address: Department of Chemistry, Colorado State University, Fort Collins, CO 80523.

ley (P. G. Rowley, Ph.D. thesis, University of New South Wales, Australia, 1976) was used to quantitatively determine thiocyanate, free cyanide, cyanogen chloride, and labile complex cyanides. The lower limit of detection was 1.5 nmol in a 5-ml sample (8 μg/liter). To differentiate between thiocyanate and cyanide, 25-ml samples were acidified with phosphoric acid, placed in an 85°C water bath, and air sparged for 15 min with cyanide-free air. Nonvolatile thiocyanate remained in the sample, whereas free cyanide, cyanogen chloride, and labile complex cyanides were collected in 10 ml of 0.1 M NaOH.

Medium and growth conditions. The defined growth medium contained monosodium glutamate, 20 mM; glycine, 12.5 mM; L-methionine, 5 mM; Tris buffer, 20 mM; sodium citrate, 2 mM; $MgSO_4 \cdot 7H_2O$, 2 mM; K_2HPO_4, 1 mM; and $FeCl_3 \cdot 6H_2O$, 30 μM. The medium was adjusted to pH 8.0 and sterilized by autoclaving.

Cultures (24 h) grown in defined medium were centrifuged at 10,000 × *g* for 15 min at 4°C, washed twice in sterile phosphate buffer, suspended in the appropriate growth medium, and diluted to a cell density of 5×10^7 viable cells per ml to make up the inoculum. For the temperature, pH, phosphate, and iron experiments, 1 ml of inoculum was added to 9 ml of medium and incubated for 12 h. In the growth-phase experiments, 200 ml of medium was incubated in 500-ml Erlenmeyer flasks with shaking at 100 rpm for 14 h. Filter-sterilized, cyanide-free air was passed over the surface of the medium, and HCN was collected in 0.1 M NaOH. All experiments were conducted in triplicate; distilled water was used for all procedures, and sterile growth medium was used as a control. An incubation temperature of 30°C was used unless otherwise specified.

Radioactive tracer studies. Three test tubes, each containing 25 ml of defined growth medium, were inoculated with a washed suspension of a 24-h culture. A 0.3-ml volume of [1-^{14}C]glycine (100 Ci/ml) was added to the first test tube, 0.3 ml of [2-^{14}C]glycine (100 Ci/ml) was added to the second, and the third test tube was used as a non-radioactive control. Cultures were incubated for 12 h at 100 rpm, filter-sterilized, cyanide-free air was passed over the surface of the medium, and HCN and CO_2 were collected in 10 ml of 0.1 M NaOH. At the end of the incubation period, cultures were acidified with 2 ml of phosphoric acid (10% [wt/vol]), and cyanide and CO_2 were collected for 15 min. Cyanide was separated from CO_2 by the addition of 2 ml of $BaCl_2 \cdot 2H_2O$ (1% [wt/vol]) to 2 ml of the NaOH solution. A $BaCO_3$ precipitate was allowed to form, and the solution was centrifuged at 15,000 × *g* for 15 min at 4°C. A portion of the supernatant was added to 10 ml of biocount cocktail (Beckman Instruments Inc., Mountainside, N.J.) and counted on a Beckman LS-133 liquid scintillation counter. Counts per minute were converted to disintegrations per minute by using an external standard versus counting efficiency curve constructed with [^{14}C]toluene, with chloroform as a quenching agent. Radioactive [1-^{14}C]glycine, 47.4 mCi/mmol; [2-^{14}C]glycine, 42.5 mCi/mmol; and [^{14}C]toluene, 4.5×10^5 dpm/ml, were obtained from New England Nuclear Corp., Boston, Mass.

Industrial wastewater studies. Effluent from an industrial wastewater lagoon was collected in sterile containers and transported and stored at 4°C. Batch cultures consisting of 250 ml of raw wastewater in 500-ml Erlenmeyer flasks were incubated at 100 rpm with and without illumination (4,304 lx from daylight fluorescent tubes on a 12 h of light, 12 h of dark cycle) at 15 and 30°C. Filter-sterilized and autoclaved controls were used; algal numbers were determined by the Whipple disk method (2).

Industrial wastewater was sterilized by filtration through a type HAWG 0.45-μm membrane filter (Millipore Corp., Bedford, Mass.) or by autoclaving. Filter-sterilized wastewater was inoculated with *P. fluorescens* S, 66, MS1, and MS4 and *P. aeruginosa* 100 and incubated at 15 and 30°C. The inoculum was prepared from washed cells which were suspended in physiological saline for 24 h at 4°C, centrifuged, and suspended in sterile wastewater. An initial cell density of approximately 10^6 viable cells per ml was obtained. Uninoculated sterile wastewater was used as a control.

Components of the defined growth medium were added to industrial wastewater. Stock solutions were prepared at concentrations 20 times greater than in the defined growth medium, adjusted to pH 7.0, and sterilized by autoclaving. A 5-ml amount of stock solution was added to 95 ml of wastewater. Unamended wastewater was used as a control, and cultures were incubated for 7 days at 100 rpm in 250-ml Erlenmeyer flasks.

Wastewater amended with all components of the defined growth medium was incubated in 250-ml volumes in 500-ml Erlenmeyer flasks with cyanide-free, filter-sterilized air passed over the surface. HCN was collected in 10 ml of 0.1 M NaOH. Cultures were incubated at 30°C and 100 rpm for 7 days. All experiments involving industrial wastewater were carried out in triplicate.

RESULTS

Distribution of cyanogenic bacteria. Of 200 water isolates screened, five strains of *P. fluorescens* and one strain of *P. aeruginosa* were cyanogenic. Four of the *P. fluorescens* strains (Q, S, MS1, and MS4) were isolated from rivers, whereas *P. fluorescens* 66 and *P. aeruginosa* 100 were isolated from an industrial wastewater lagoon.

Cyanogenesis in defined growth medium. The effects of temperature, pH, and phosphate concentration on cyanogenesis by *P. fluorescens* S and 66 were examined. Table 1 lists the conditions under which maximum cyanide production occurred. A linear relationship was observed between cyanide production and the log of iron concentration for both strains over a range of 3 to 300 μM, whereas growth was relatively unaffected.

At an added iron concentration of 300 μM, the concentration of cyanide in the growth medium was 21 and 14 μM for strains S and 66, respectively. Figure 1 shows total cyanide production, which is the sum of cyanide in the growth medium and HCN collected in NaOH, as a function of incubation time. The highest concen-

TABLE 1. Optimum temperature, pH, and phosphate concentration for cyanide production by *P. fluorescens* S and 66 in defined growth medium

Strain	Optimum temp (°C)	Cyanide concn	Optimum pH	Cyanide concn	Optimum phosphate (mM)	Cyanide concn
S						
Cyanide/ml[a]	30	3.1	7.7–8.1	2.9–3.5	0.3–1	7.2–7.3
Cyanide/cell[b]	25–30	5.8–6.3	7.7–8.5	6.3–7.0	300	88
66						
Cyanide/ml	30	9.2	6.6	14	100	18
Cyanide/cell	25–30	13	8.9	24	300	35

[a] Nanomoles of cyanide per milliliter.
[b] Nanomoles of cyanide per 10^8 viable cells.

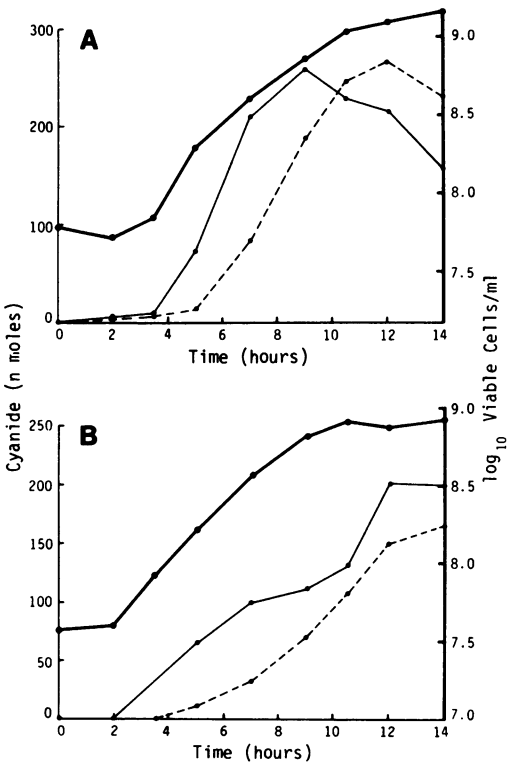

FIG. 1. Cyanide production as a function of incubation time by *P. fluorescens* strain S (A) and strain 66 (B). Symbols: ——, viable cells; ——, cyanide per 10^9 viable cells; ------, cyanide per milliliter.

tration of cyanide in the growth medium was 250 μM for strain S after 12 h of incubation and 155 μM for strain 66 after 14 h. The ratio between cyanide concentration of the medium and the collection rate of HCN reached constant values after 9 h of incubation. The maximum rate of cyanide production by both *P. fluorescens* strains occurred during the transition from exponential to stationary growth phase.

TABLE 2. Conversion of radioactively labeled glycine to cyanide carbon by *P. fluorescens* and *P. aeruginosa*

Organism	Substrate	Sp act of cyanide (gbq/mol)	% Sp act of cyanide/ glycine
P. fluorescens S	[1-^{14}C]glycine	0.029	0.7
	[2-^{14}C]glycine	3.7	92
P. aeruginosa 1-73	[1-^{14}C]glycine	0.066	1.7
	[2-^{14}C]glycine	3.6	94

Cyanide biosynthesis from glycine. Radioactive tracer experiments indicate that the cyanide carbon originates from the number 2 carbon of glycine (Table 2). The specific activities of [^{14}C]cyanide produced from [2-^{14}C]glycine were 92 and 94% of the specific activity of the substrate for *P. fluorescens* S and *P. aeruginosa* 1-73, respectively.

Cyanide metabolism in industrial wastewater. Industrial wastewater collected over a 6-month period had initial cyanide concentrations of <0.1 to 0.5 μM. Net cyanide production did not occur in batch cultures of raw wastewater incubated at 15 to 30°C, with or without illumination. Filter-sterilized and autoclaved wastewater inoculated with pure cultures of cyanogenic *Pseudomonas* strains did not support detectable cyanogenesis.

Net production of cyanide was observed in industrial wastewater amended with the components of the defined growth medium (Fig. 2). Wastewater incubated at 30°C and amended with individual amino acids or with a portion of the components produced less cyanide than wastewater amended with all components. Cyanide production in wastewater amended with all components was greater at 30°C than at 15°C. Total HCN collected and concentration of cya-

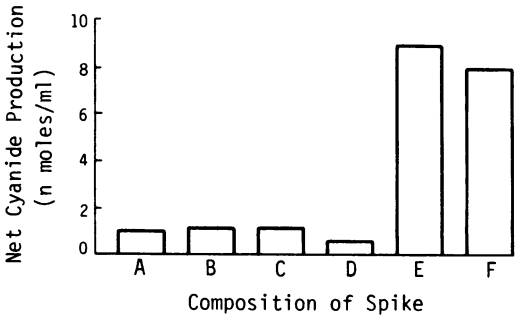

FIG. 2. Cyanide production in industrial wastewater amended with components of synthetic growth medium. A, Glutamate; B, glycine; C, glutamate, glycine, and methionine; D, Tris, sodium citrate, $MgSO_4$, K_2HPO_4, and $FeCl_3$; E, all components, 30°C; F, all components, 15°C.

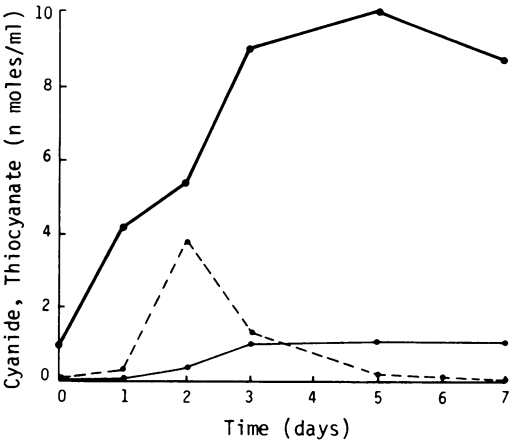

FIG. 3. Cyanide and thiocyanate concentration in industrial wastewater amended with all components of synthetic growth medium as a function of incubation time. Symbols: ——, thiocyanate; —, total cyanide collected; ------, cyanide.

nide and thiocyanate as a function of time in wastewater amended with all components are shown in Fig. 3. Cyanide concentration in the wastewater was highest after 2 days of incubation, whereas thiocyanate reached its highest level after 5 days.

DISCUSSION

The distribution of cyanogenic bacteria is a factor which must be considered to evaluate bacterial cyanide production in aquatic environments. The five bacterial species reported to produce cyanide are native to water and soil (5, 11). In this study, cyanide-producing bacteria were isolated from four of the five water sources sampled, indicating that cyanogenic bacteria are common inhabitants of aquatic habitats.

The conditions used to isolate and screen for cyanide production select for bacteria which produce cyanide under the specific assay conditions employed. Organisms which are cyanogenic under different conditions or produce low levels of cyanide may appear to be cyanide negative.

Cyanide production per milliliter of culture was greatest at 30°C for both strains. Castric (7) reported that 34 to 37°C allowed maximum cyanide production by *P. aeruginosa*; cyanide production by *C. violaceum* was unaffected by growth temperature over a range of 25 to 35°C (18). Cyanide production per milliliter by *P. fluorescens* was greatest at pH 6.6 for strain 66 and at pH 7.7 to 8.1 for strain S. For an unidentified *Pseudomonas* species, the largest quantities of cyanide were produced over a pH range of 7.3 to 8.3 (23). A large variation was observed in the optimum phosphate concentration for cyanide production on a volume basis by *P. fluorescens* S and 66. Maximum cyanogenesis by *P. aeruginosa* has been shown to occur over a range of 1 to 10 mM phosphate (13), whereas increasing phosphate concentration resulted in increasing cyanide production by *C. violaceum* (18). A linear relationship was observed between the log of iron concentration and cyanide production by *P. fluorescens* over a range of 3 to 300 μM. Stimulation of cyanogenesis by iron has also been demonstrated for *P. aeruginosa* (7) and *C. violaceum* (18).

Three types of relationships between cell growth and cyanide production per cell by *P. fluorescens* were observed.

(i) Maximum cyanide production per cell under favorable growth conditions. Cyanide production on a per-cell basis by strains S and 66 was greatest at the optimum growth temperature for *P. fluorescens*. Maximum cyanogenesis by strain S occurred over a pH range that was favorable for growth.

(ii) Maximum cyanide production per cell under unfavorable growth conditions. Optimum cyanide production per cell by strain 66 occurred at pH 8.9, at which growth was inhibited. For both *P. fluorescens* strains, cyanide production per cell was greatest at 300 mM phosphate, whereas growth was severely inhibited.

(iii) Increasing iron concentration caused an increase in cyanogenesis, whereas cell growth was virtually unaffected. Trace iron present in the medium was sufficient for growth.

The maximum rate of cyanide production by *P. fluorescens* occurred during the transition from exponential to stationary growth phase. Similar results have been reported for *P. aeruginosa* (7), an unidentified *Pseudomonas* species

(23), and *C. violaceum* (14, 18).

Radioactive tracer studies revealed that the cyanide carbon originates from the number 2 carbon of glycine for *P. fluorescens* S and *P. aeruginosa* 1-73. Synthesis of cyanide from the number 2 carbon of glycine has also been reported for *C. violaceum* (3, 15) and a type B snow mold fungus (22). Castric (8) reported that [^{14}C]cyanide is produced from both [1-^{14}C]glycine and [2-^{14}C]glycine by *P. aeruginosa* 9-D2. Variations in glycine metabolism between *P. aeruginosa* 1-73 and 9-D2 may account for the different results.

Net cyanide production did not occur in batch cultures of raw industrial wastewater or in sterile wastewater inoculated with cyanogenic bacteria, but cyanide was produced in wastewater amended with components of the defined growth medium. Stimulation of cyanide production was due to three factors: (i) addition of nutrients which increased bacterial growth and resulted in higher cell densities; (ii) modification of wastewater pH, phosphate concentration, and iron concentration which enhanced cyanogenesis; and (iii) strain selection for cyanogenic bacteria originally present in low numbers.

Cyanide concentration in industrial wastewater amended with all components of the growth medium was highest after 2 days of incubation and decreased to barely detectable levels at the end of 7 days. HCN collected in NaOH accounted for approximately one-fourth of the cyanide present at day 2, whereas biological and/or chemical processes were responsible for the elimination of the remainder of the cyanide.

The purpose of bacterial cyanogenesis remains obscure. It is unlikely that cyanide production gives the producing organism a competitive advantage over cyanide-sensitive organisms because low levels of cyanide are removed by physical, chemical, and biological processes. It has been demonstrated that cyanide produced by *C. violaceum* can be assimilated in the synthesis of certain amino acid derivatives (4). Cyanide produced in low levels by the alga *Chlorella vulgaris* (21) and the cyanobacterium *Anacystis nidulans* (17) appears to function in regulatory mechanisms.

It has been proposed that bacterial cyanogenesis is a form of secondary metabolism which may serve to regulate glycine levels as growth rate slows (7). Bu'Lock (6) has hypothesized that secondary metabolism provides a selective advantage by maintaining essential cell multiplication mechanisms when growth is no longer possible. Cyanide production by certain *Pseudomonas* species may serve as a secondary metabolic process which enhances cell viability when transitions from active growth to senescence take place.

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