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# Cyanase-Mediated Utilization of Cyanate in *Pseudomonas fluorescens* NCIB 11764

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***Pseudomonas fluorescens* NCIB 11764 was capable of utilizing cyanate ($OCN^-$) as a sole nitrogen source for growth. Crude cell extracts from cells grown on cyanate, but not on ammonium sulfate, were induced for an enzyme catalyzing cyanate conversion to ammonia. Enzymatic activity was shown to be bicarbonate dependent and specific for cyanate as a substrate, suggesting that cyanate utilization in this organism is facilitated by an enzyme resembling cyanase (cyanate amidohydrolase; EC 3.5.5.3), as described previously in *Escherichia coli* and *Flavobacterium* sp.**

Cyanase (cyanate amidohydrolase; EC 3.5.5.3) is an enzyme that catalyzes the hydrolysis of cyanate to ammonia and bicarbonate: $OCN^- + H^+ + 2H_2O \rightleftharpoons NH_4^+ + HCO_3^-$. It has been shown to occur in *Escherichia coli* (3, 24, 25), *Flavobacterium* sp. (5), and plants (15, 16). In *E. coli* it is induced by cyanate and consists of 8 to 10 identical subunits with a molecular weight of 16,350 (3). Recently, the *cynS* gene encoding cyanase in *E. coli* K-12 was identified and the sequence was determined (22, 23). A characteristic feature of the enzyme is its unusual requirement for bicarbonate for catalytic activity (3–5). However, despite a variety of studies describing the physical and kinetic properties of this enzyme, its physiological role remains uncertain.

The toxicity of cyanate has long been recognized (20), and it has been suggested that one role of cyanase in *E. coli* may be to prevent the toxic effects of cyanate (3, 7). This hypothesis is supported by reports that cyanase-deficient mutants are more sensitive to cyanate, which among other effects (21) has been shown to specifically inhibit carbamoyl phosphate synthase and, thus, arginine biosynthesis (7, 8). In addition, it is known that cyanase confers on *E. coli* the ability to utilize cyanate as a sole nitrogen source (7, 23). The importance of these capabilities, however, remains to be resolved since the chemical origin of cyanate in nature is unclear. As far as is known, cyanate is not a product of any metabolic reaction, although it is known to arise from the spontaneous decomposition of carbamoyl compounds such as urea and carbamoylphosphate (1, 9). Hence, it has been proposed that one role for cyanase may be to maintain tolerable levels of toxic cyanate in the cell (3).

Systematic studies on the metabolism of cyanate by bacteria have not been conducted; however, Guilloton and Karst (7) did report that 60% of *E. coli*, 92% of *Pseudomonas aeruginosa*, and 100% of *Serratia marcescens* strains tested were resistant to this compound. In contrast, no strains of *Proteus*, *Streptococcus faecalis*, or *Staphylococcus aureus* were resistant, which they suggested was due to the lack of cyanase in these organisms, although this remains to be verified. To learn more about cyanate metabolism in bacteria, we examined whether this compound could serve as a source of nitrogen for the growth of *Pseudomonas fluorescens* NCIB 11764. This organism was chosen for study since it has the unusual capability of assimilating cyanide as a sole nitrogen source for growth (10–12, 18), and cyanate has been proposed as a possible intermediate in this process (12). We reasoned, therefore, that cyanate might also support the growth of this organism and report here that cyanase is responsible for this ability.

All experiments were conducted with *P. fluorescens* NCIB 11764, which was obtained from the National Collection of Marine and Industrial Bacteria, Torrey, Scotland. Cells were grown in minimal medium containing 67 mM $KH_2PO_4$ (pH 7.0) and inorganic salts consisting of 0.04% $MgSO_4 \cdot 7H_2O$ and 0.001% $FeSO_4 \cdot 7H_2O$, as described previously (13), with glucose serving as a carbon source (20 mM). For the growth curves described in Fig. 1, cells were initially cultured on 10 mM ammonium sulfate and washed 3 times in 50 mM sodium-potassium phosphate buffer (pH 7.0) before a 4% inoculum was provided to flasks containing either cyanate (KOCN) or $(NH_4)_2SO_4$ as the sole nitrogen source. KOCN (99%) was purchased from Aldrich Chemical Co., Inc. (Milwaukee, Wis.), and was recrystallized before use from a water-ethanol mixture as described by Scattergood (19). The recrystallized compound was then stored under $P_2O_5$ in a vacuum desiccator before use. Since cyanate undergoes slow spontaneous hydrolysis (2, 7), stock solutions for growth tests and enzyme assays were prepared just prior to use. All other compounds were of the highest purity commercially available.

Cells used for the preparation of cell extracts were grown overnight in 1 liter of the same minimal medium described above containing either KOCN or $(NH_4)_2SO_4$ as the sole nitrogen source. To test the effect of ammonia on cyanase synthesis, cells were grown in either minimal medium containing $(NH_4)_2SO_4$ (10 mM) or in broth described by Lennox (14), each of which was supplemented with KOCN (5 mM). Inocula for all cultures were pregrown on the same medium and provided at 0.5% (vol/vol). Crude cell extracts were prepared as described earlier (13), and their protein content was determined by the method of Lowry et al. (17). Cyanase was assayed by measuring ammonia formation at 30°C essentially as described by Anderson (3). The standard assay mixture contained the following (in 5 to 10 ml): 50 mM $KH_2PO_4$ (pH 7.5), 3 mM bicarbonate, 2 mM KOCN, and crude extract (0.1 to 2 mg of protein per ml). Following the addition of enzyme, 0.5-ml samples were removed at intervals and mixed with an equal volume of Nessler reagent (1:3; Sigma Chemical Co., St. Louis, Mo.), and the $A_{420}$ was read.

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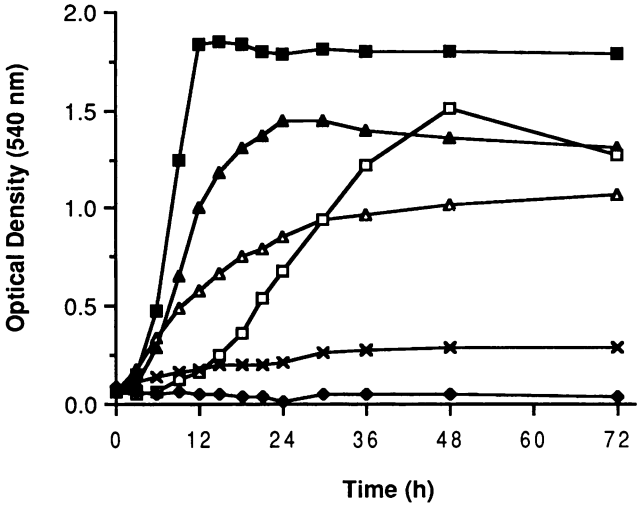


FIG. 1. Growth of *P. fluorescens* NCIB 11764 in glucose (20 mM) minimal medium supplied with the following sources of nitrogen: $(NH_4)_2SO_4$ (10 mM) (■); KOCN (0.5, 5, 10, and 20 mM; △, ▲, □, and ◆, respectively); and control (no fixed nitrogen) (×).

Cyanate was determined by using a modification of the procedure described by Guilloton and Karst (6) involving cyclization with anthranilic acid to give 2,4-(1*H*,3*H*)-quinazolinedione; the latter gives a characteristic maximum at $A_{310}$. For this purpose, equal volumes (0.5 ml) of sample and 10 mM anthranilic acid were mixed at pH 4 to 5 and heated at 40°C for 10 min. The final pH of this mixture was critical since coupling at higher pH values is unfavorable and lower values result in cyanate hydrolysis prior to derivatization (6). Following the 10-min incubation period, 1 ml of 6 N HCl was added and the samples were again heated at 100°C for 1 min before the $A_{310}$ was read with a spectrophotometer (Ultrospec II; LKB Instruments, Inc., Rockville, Md.).

Figure 1 shows the growth results obtained after *P. fluorescens* NCIB 11764 was cultivated in glucose minimal medium containing either ammonia or cyanate as the sole nitrogen source for growth. Although the growth rate was less than half that observed on ammonia (generation time [$t_{gen}$], 1.0 h), cells grew readily on cyanate (5 mM) ($t_{gen}$, 3.4 h) after an initial lag period of 6 h. A comparison of the growth results between separate cultures that were supplied various concentrations of cyanate revealed that optimal growth occurred at 5 mM. On 10 mM cyanate, a longer lag period (approximately 12 h) accompanied by a slower growth rate ($t_{gen}$, 5.6 h) was observed, which presumably could be attributed to cyanate toxicity. The complete absence of growth on 20 mM cyanate and the shorter lag period (approximately 3 h) observed at low concentrations (e.g., 0.5 mM) are consistent with this hypothesis.

Figure 2 shows the results obtained when crude extracts from cells of *P. fluorescens* NCIB 11764 grown on 2 mM cyanate were incubated with cyanate and assayed simultaneously for ammonia formation and cyanate disappearance. Cyanate was quickly consumed and converted quantitatively to ammonia (molar ratio, 1.13 ± 0.25 [$n$ = 3]), which is consistent with the expected stoichiometry for cyanase (3, 24). In all cases the rates of cyanate conversion in the presence of cell extract were significantly higher than those due to spontaneous hydrolysis (e.g., half-life = 27 h) (7), indicating that this reaction was truly enzyme dependent. Although the data are not shown, cyanase activity was found

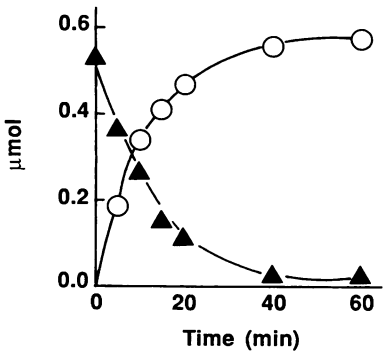


FIG. 2. Conversion of cyanate to ammonia by crude cell extracts containing cyanase from *P. fluorescens* NCIB 11764. Reaction mixtures contained 0.5 mM KOCN and crude extract (4 mg of protein) in the standard assay mixture described in the text. At the indicated times, 1.0-ml samples were withdrawn and analyzed for cyanate (▲) and ammonia (○).

to be bicarbonate dependent (e.g., the initial rates in the presence of $NaHCO_3$ were 2 times those observed in its absence), and neither urea nor thiocyanate (KSCN) served as a substrate. The specific activity of cyanase following growth on 5 mM cyanate was 168 nmol $min^{-1}$ $mg^{-1}$ (±67 [$n$ = 3]) but could not be detected in $(NH_4)_2SO_4$ (10 mM)-grown cells (<5 nmol $min^{-1}$ $mg^{-1}$). We therefore concluded that cyanase synthesis in this organism is inducible. These findings parallel those reported previously for inducible cyanase from *E. coli* (3) and *Flavobacterium* sp. (5) and strongly support the hypothesis that a similar enzyme is responsible for the growth of *P. fluorescens* NCIB 11764 on cyanate. Moreover, activity was still present when cells were grown in either ammonia-containing minimal medium or the broth described by Lennox (14) supplemented with cyanate (specific activity, 158 and 97 nmol $min^{-1}$ $mg^{-1}$, respectively), suggesting that no significant repression of cyanase synthesis by ammonia occurs.

Harris and Knowles (12) reported the detection of cyanase in *P. fluorescens* NCIB 11764 in a previous report, but no details were presented. Results of our study verify these earlier observations and provide the first evidence for the existence of this enzyme in a representative member of the genus *Pseudomonas*. The induction of this enzyme after growth on cyanate and the conversion thereof to ammonia by crude extracts provide strong evidence that this enzyme is responsible for cyanate utilization. However, a detoxification role cannot be excluded since induction by cyanate still occurred when cells were grown in excess ammonia. Nonetheless, results of this study support earlier hypotheses that cyanase may play a role in the breakdown of cyanate, which has been proposed as a hypothetical intermediate in cyanide assimilation by this organism (11, 12). Additional studies are needed, however, to establish unequivocally that cyanate is an intermediate in this pathway before firm conclusions regarding the origin of this compound and the potential physiological role of cyanase in *P. fluorescens* NCIB 11764 can be drawn.

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## LITERATURE CITED

1. **Allen, C. M., and M. E. Jones.** 1964. Decomposition of carbamylphosphate in aqueous solutions. Biochemistry 3:1238–

1247.
2. **Amell, A. R.** 1956. Kinetics of the hydrolysis of cyanic acid. J. Am. Chem. Soc. **78**:6234–6238.
3. **Anderson, P. M.** 1980. Purification and properties of the inducible enzyme cyanase. Biochemistry **19**:2882–2888.
4. **Anderson, P. M., and R. M. Little.** 1986. Kinetic properties of cyanase. Biochemistry **25**:1621–1626.
5. **Guilloton, M., and A. B. Hargreaves.** 1972. Hydrolyse du cyanate par une bactérie du genre *Flavobacterium*. C. R. Acad. Sci. Ser. D **275**:1827–1830.
6. **Guilloton, M., and F. Karst.** 1985. A spectrophotometric determination of cyanate using reaction with 2-aminobenzoic acid. Anal. Biochem. **149**:291–295.
7. **Guilloton, M., and F. Karst.** 1987. Isolation and characterization of *Escherichia coli* mutants lacking inducible cyanase. J. Gen. Microbiol. **133**:645–653.
8. **Guilloton, M., and F. Karst.** 1987. Cyanate specifically inhibits arginine biosynthesis in *Escherichia coli* K12: a case of by-product inhibition. J. Gen. Microbiol. **133**:655–665.
9. **Hagel, P., J. J. T. Gerding, W. Fieggen, and H. Bloemendal.** 1971. Cyanate formation in solutions of urea. Calculation of cyanate concentrations at different temperature and pH. Biochim. Biophys. Acta. **243**:366–373.
10. **Harris, R., and C. J. Knowles.** 1983. Isolation and growth of a *Pseudomonas* species that utilizes cyanide as a source of nitrogen. J. Gen. Microbiol. **129**:1005–1011.
11. **Harris, R. E., A. W. Bunch, and C. J. Knowles.** 1987. Microbial cyanide and nitrile metabolism. Sci. Prog. Oxford **71**:293–304.
12. **Harris, R. E., and C. J. Knowles.** 1983. The conversion of cyanide to ammonia by extracts of a strain of *Pseudomonas fluorescens* that utilizes cyanide as a source of nitrogen for growth. FEMS Microbiol. Lett. **20**:337–341.
13. **Kunz, D. A., D. W. Ribbons, and P. J. Chapman.** 1981. Metabolism of allylglycine and *cis*-crotylglycine by *Pseudomonas putida* (*arvilla*) mt-2 harboring a TOL plasmid. J. Bacteriol. **148**:72–82.
14. **Lennox, E. S.** 1955. Transduction of linked genetic characters of the host bacteriophage P1. Virology **1**:190–206.
15. **Lisanti, L. E.** 1963. Distribution of cyanase in plants. I. Cyanatolytic activity in *Cicer arietinum*. Ann. Speri. Agrar. **17**:45–51.
16. **Lotti, G.** 1965. Cyanase of wheat. Chim. Ind. (Paris) **45**:561–563.
17. **Lowry, O. H., N. J. Rosenbrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
18. **Rollinson, G., R. Jones, M. P. Meadows, R. E. Harris, and C. J. Knowles.** 1987. The growth of a cyanide-utilising strain of *Pseudomonas fluorescens* in liquid culture on nickel cyanide as a source of nitrogen. FEMS Microbiol. Lett. **40**:199–205.
19. **Scattergood, A.** 1946. Alkali metal cyanates, p. 86–90. *In* W. Conard Fernelius (ed.), Inorganic syntheses, vol. II. McGraw-Hill Book Co., New York.
20. **Schütz, F.** 1949. Cyanate. Experientia **5**:133–172.
21. **Stark, G. R.** 1972. Modification of proteins with cyanate. Methods Enzymol. **25B**:579–584.
22. **Sung, Y.-C., P. M. Anderson, and J. A. Fuchs.** 1987. Characterization of high-level expression and sequencing of the *Escherichia coli* K-12 *cynS* gene encoding cyanse. J. Bacteriol. **169**:5224–5230.
23. **Sung, Y.-C., D. Parsell, P. M. Anderson, and J. A. Fuchs.** 1987. Identification, mapping, and cloning of the gene encoding cyanase in *Escherichia coli*. J. Bacteriol. **169**:2639–2642.
24. **Taussig, A.** 1960. The synthesis of the induced enzyme, "cyanase" in *E. coli*. Biochim. Biophys. Acta **44**:510–519.
25. **Taussig, A.** 1965. Some properties of the induced enzyme cyanase. Can. J. Biochem. **43**:1063–1069.