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A Physiological Role for Cyanate-Induced Carbonic Anhydrase in *Escherichia coli*

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Cyanate induces expression of the *cyn* operon in *Escherichia coli*. The *cyn* operon includes the gene *cynS*, encoding cyanase, which catalyzes the reaction of cyanate with bicarbonate to give ammonia and carbon dioxide. A carbonic anhydrase activity was recently found to be encoded by the *cynT* gene, the first gene of the *cyn* operon; it was proposed that carbonic anhydrase prevents depletion of bicarbonate during cyanate decomposition due to loss of CO_2 by diffusion out of the cell (M. B. Guilloton, J. J. Korte, A. F. Lamblin, J. A. Fuchs, and P. M. Anderson, J. Biol. Chem. 267:3731–3734, 1992). The function of the product of the third gene of this operon, *cynX*, is unknown. In the study reported here, the physiological roles of *cynT* and *cynX* were investigated by construction of chromosomal mutants in which each of the three genes was rendered inactive. The Δ*cynT* chromosomal mutant expressed an active cyanase but no active carbonic anhydrase. In contrast to the wild-type strain, the growth of the Δ*cynT* strain was inhibited by cyanate, and the mutant strain was unable to degrade cyanate and therefore could not use cyanate as the sole nitrogen source when grown at a partial CO_2 pressures (pCO_2) of 0.03% (air). At a high pCO_2 (3%), however, the Δ*cynT* strain behaved like the wild-type strain; it was significantly less sensitive to the toxic effects of cyanate and could degrade cyanate and use cyanate as the sole nitrogen source for growth. These results are consistent with the proposed function for carbonic anhydrase. The chromosomal mutant carrying *cynS*::*kan* expressed induced carbonic anhydrase activity but no active cyanase. The *cynS*::*kan* mutant was found to be much less sensitive to cyanate than the Δ*cynT* mutant at a low pCO_2, indicating that bicarbonate depletion due to the reaction of bicarbonate with cyanate catalyzed by cyanase is more deleterious to growth than direct inhibition by cyanate. Mutants carrying a nonfunctional *cynX* gene (*cynX*::*kan* and Δ*cynT cynX*::*kan*) did not differ from the parental strains with respect to cyanate sensitivity, presence of carbonic anhydrase and cyanase, or degradation of cyanate by whole cells; the physiological role of the *cynX* product remains unknown.

Cyanase is an inducible enzyme in *Escherichia coli* that catalyzes the reaction of cyanate with bicarbonate, resulting in decomposition of cyanate according to the following reaction (12): $NCO^- + 3H^+ + HCO_3^- \rightarrow 2CO_2 + NH_4^+$. The gene encoding cyanase is part of the *cyn* operon, which includes three genes in the order *cynT* (encoding a carbonic anhydrase), *cynS* (encoding cyanase), and *cynX* (encoding a hydrophobic protein of unknown function) (10, 19, 22). It has been proposed that the carbonic anhydrase functions by recycling the CO_2 produced in the cyanase-catalyzed reaction back to bicarbonate (10). Assuming that the cell membrane is very permeable to CO_2 (11), decomposition of cyanate in cells without carbonic anhydrase activity would result in the conversion of bicarbonate to CO_2, followed by diffusion of CO_2 out of the cell faster than it can be spontaneously hydrated to bicarbonate. Decomposition of cyanate would thus result in depletion of cellular bicarbonate needed for continued cyanase activity and for bicarbonate-dependent reactions required for cell growth (e.g., phosphoenolpyruvate carboxylase, acetyl coenzyme A carboxylase, and carbamoyl phosphate synthetase).

The studies reported here were conducted to investigate the physiological role of *cynT*. A Δ*cynT* chromosomal mutant was constructed that expresses cyanase activity but not carbonic anhydrase activity when cyanate is added to the growth medium. The properties of this mutant strain as well as those of two other chromosomal mutant strains in which *cynS* and *cynX* were rendered inactive by insertions of the gene for kanamycin resistance are reported along with other properties of this unique system of cyanate and bicarbonate metabolism. The results establish that a function of the carbonic anhydrase is to protect the cell from bicarbonate depletion during cyanate metabolism.

MATERIALS AND METHODS

Bacterial strains. All bacterial strains used were derivatives of *Escherichia coli* K-12 and are listed in Table 1.

Media and chemicals. Minimal A medium supplemented with 4 mg of thiamine hydrochloride per liter (13) was used for physiological studies. LB broth (13) was used as the enriched medium. Bacto-Agar (Difco) was added to a final concentration of 2% for solid medium. When used in culture medium, these compounds were present at the indicated concentrations (in milligrams per liter), unless otherwise stated: arginine, 100; uracil, 50; kanamycin, 25; ampicillin, 50; and chloramphenicol, 20. Commercial-grade potassium cyanate was recrystallized from a saturated solution in 50% ethanol at 50°C as described previously (1). All other chem-

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TABLE 1. *E. coli* strains, plasmids, and phages used in this study

Strain, plasmid, or phage	Relevant genotype	Source or reference
Strains		
HfrR5	Hfr *thi lacY1 supE44 gal-3 malT1 xyl-7 mtlA2* PO47	8
JC7623	*thr-1 leu-6 thi-1 lacY1 galK2 ara-24 xyl-5 mtl-1 pro-2 his-2 argE3 str-3*(dl) *tsx-33 sup-37 sbcC201 recB21 recC22*	24
SJ100	*supE thi* Δ(*lac-proAB*)	22
BH1214	Hfr *pyrE41 metB1 tonA22 relA1 spoT* PA02	Lab strain
JM101	*thi supE* Δ(*lac-proAB*) F' *traD36 proA*$^+$*B*$^+$ *lacI*^{q}*Z*Δ*M15*	22
BUM012	HfrR5 Δ*cynT lacY*$^+$	This study
BUM015[a]	HfrR5 *lacY*$^+$	This study
BUM019	HfrR5 *cynX*::*kan*	This study
BUM025	HfrR5 Δ*cynT cynX*::*kan lacY*$^+$	This study
BUM031	HfrR5 *cynS*::*kan*	This study
Plasmids		
pSJ104		22
pSJ130		21
pSJ128		19
pUC4K		23
pAL4T		This study
pAL12		This study
pAL15		This study
pAL19		This study
Phages		
M13mp11::CAT		6
M13mp11::CATΔ*cynT*		This study
P1 *vir*		Lab strain

[a] Wild-type strain. The presence of *lacY*$^+$ had no observable effect on phenotype with respect to expression and function of the *cyn* operon.

icals were purchased from Sigma Chemical Co. Restriction endonucleases and molecular biology supplies were obtained from Boehringer Mannheim Biochemicals. Reagents for Western immunoblotting were purchased from Bio-Rad and Amersham Corp.

To test for a defect in cyanate metabolism, cell suspensions were spotted on a solid medium composed of nutrient broth (8 g/liter; Difco) containing 10 mM KNCO and 2 mM $CaCl_2$. Strains able to metabolize cyanate formed opaque, white patches, while strains unable to metabolize cyanate formed translucent patches, presumably resulting from localized alkalinization from excreted NH_4OH and subsequent precipitation of hydroxyapatite (8).

Strains were tested for their ability to synthesize inducible cyanase by growth in LB broth containing 0.1 mM sodium azide, a gratuitous inducer (8). This concentration of azide is low enough not to affect growth but is sufficient to induce synthesis of cyanase.

Growth conditions. For stable-isotope exchange studies with $^{13}C^{18}O_2$, a strongly buffered medium was required, and cells were grown in a 2× minimal medium (13). No change in generation time was observed in this 2× medium compared with that in 1× minimal medium.

For growth studies other than isotope studies, overnight cultures were used to inoculate 20 ml of medium in test tubes (25 by 200 mm) in a shaking water bath at 37°C. Mid-log-phase cultures were diluted in 20 ml of the same medium in tubes (25 by 200 mm) placed in a 37°C water bath, and flushed continuously either with air (0.03% CO_2) or with air containing 3% CO_2. The optical density at 600 nm (OD_{600}) of each tube was monitored with a Spectronic SP-20 solid-state spectrophotometer. A linear relationship between cell density and optical density was observed for OD_{600} values of up to 0.6. To monitor the effect of cyanate on cell growth, KNCO was added to cultures when the OD_{600} was 0.1, and the optical density was measured over time.

Construction of a chromosomal *cynT* deletion mutant strain. The 1.3-kb *Eco*RI-*Hin*dIII fragment of plasmid pSJ130 (21) containing the *cynT* sequence was cloned into the *Eco*RI and *Hin*dIII sites of plasmid pUC118. The resulting plasmid, pAL12, was cut with *Nde*I and *Acc*I to remove a 195-bp fragment of the *cynT* sequence. The sticky ends were filled with DNA polymerase (Klenow fragment) and ligated to obtain plasmid pAL15. Strain SJ100 containing pAL15 was negative for cyanate metabolism on plates containing the medium described above. The *Eco*RI-*Hin*dIII fragment from pAL15 was transferred into M13mp11::CAT, an M13mp11 derivative containing a chloramphenicol resistance gene (6). This M13mp11::CAT Δ*cynT* phage was used to infect strain BH1214, and chloramphenicol-resistant colonies were selected. These colonies had presumably integrated M13 into the chromosome by a single crossover in the region of homology shared by the phage and the chromosome. Cells cured of M13 by a single crossover were selected on LB plates containing 0.3% deoxycholate. Approximately 25% of the deoxycholate-resistant strains displayed a strong sensitivity to cyanate. The Δ*cynT* mutation was finally introduced into strain HfrR5 by transduction with phage P1 *vir*, with selection for Lac$^+$ colonies and screening for Cyn$^-$ done on nutrient broth agar plates containing 2 mM $CaCl_2$ and 10 mM KNCO. Approximately 93% of the Lac$^+$ transductants displayed a Cyn$^-$ phenotype resulting from the Δ*cynT* mutation; one of these clones, BUM012, was chosen for growth and physiological studies.

Construction of a chromosomal *cynS*::*kan* mutant strain. Plasmid pAL4T (10), a derivative of pSJ104 (22) with a 1,264-bp *Bam*HI fragment containing the kanamycin resistance gene from pUC4K (23) inserted into the *Bgl*II site within *cynS*, was linearized by digestion with *Eco*RI and used to transform strain JC7623, with selection for kanamycin resistance (24). All colonies selected were Cyn$^-$, as determined both by growth on nutrient broth with KNCO

and $CaCl_2$ and by cyanase assays of cells grown in the presence of 0.1 mM sodium azide. Phage P1 *vir* was grown on a selected derivative and used to transduce parental strain HfrR5 to kanamycin resistance, and all derivatives were found to be devoid of cyanase activity. A derivative, BUM031, was chosen for further studies.

Construction of a chromosomal Δ*cynT cynX*::*kan* mutant strain. Plasmid pSJ128, which contains the *Acc*I-*Nru*I (*cynS cynX*) fragment inserted into the *Sma*I site of pT7-5 (19), was digested with *Nco*I, and the protruding ends were filled in with DNA polymerase I (Klenow fragment). The *Hin*cII fragment of pUC4K (23) containing the gene for kanamycin resistance was ligated into the plasmid pSJ128 *Nco*I site to generate pAL19. Plasmid pAL19 was digested with *Eco*RI and *Bam*HI, and the insert was transferred into M13mp11::CAT. This phage was used to introduce the *cynX*::*kan* mutation into the chromosome of HfrR5 as described above for introduction of Δ*cynT* into the chromosome except that advantage could be taken of the *kan* marker. Since the insert does not contain homology upstream of *cynT*, Δ*cynT* remains in the chromosome and the resulting strain, BUM025, will be Δ*cynT cynX*::*kan*.

Construction of a chromosomal *cynX*::*kan* mutant strain. Plasmid pAL19 was linearized with *Eco*RI and used to transform strain JC7623 to kanamycin resistance by homologous recombination (24). Phage P1 *vir* was used to transduce strain BUM015 to kanamycin resistance to generate strain BUM019. Approximately 92% of the Kan^r derivatives were also Lac^-.

Genomic DNA hybridization. Genomic DNA was isolated from saturated liquid cultures of *E. coli* HfrR5, BUM012, BUM019, and BUM025 as described in the miniprep CTAB (hexadecyltrimethyl ammonium bromide) protocol (5). DNA samples (4 μg) from each strain were digested with 40 U of *Pst*I and 34 U of *Nru*I. DNA digests were resolved by electrophoresis through 1% agarose gels. DNA was subjected to alkaline denaturation and transferred to Zeta probe-blotting membranes (Bio-Rad) by the procedure of Southern (17). Prehybridization and hybridization were conducted at high stringency in 50% (vol/vol) formamide at 65°C (5). Plasmids pSJ130 and p228AL (equivalent to plasmid pSJ128) (19) were digested with *Eco*RI and *Pst*I restriction enzymes. The 2.7-kb pSJ130 fragment carrying *cynR* (regulatory gene for the cyanase operon), *cynT*, *cynS*, and part of *cynX* and the 2-kb p228AL fragment with *cynS* and *cynX* gene sequences were isolated. These fragments were labeled with [α-^{32}P]dATP by random oligonucleotide-primed synthesis (5) and subsequently used in hybridization.

Cyanate determination. The cyanate concentration was determined by reaction with anthranilic acid and cyclization of the reaction product in 6 N HCl (7). Since this method was used to quantitate cyanate in minimal medium cultures that contained 0.1 M phosphate, pH 7.0, a 0.04 M solution of anthranilic acid in 0.3 M sodium acetate, pH 4.34, was used to maintain a pH of 4.7 in the 1:1 mixture of culture medium and anthranilic acid solution. The amount of quinazoline dione obtained after cyclization was estimated from a second-derivative spectrum obtained with a Beckman DU-70 spectrophotometer from 348 to 320 nm (scanning speed, 2,400 nm/s). The cyanate concentration in the culture medium was determined by comparison with a calibration curve. Second-derivative peak height at 334 nm was found to be proportional to cyanate concentration in standard culture medium up to 2 mM. It was found that a cell density of up to an OD_{600} of 0.6 and chemicals such as arginine (100 μg/ml) and uracil (50 μg/ml) did not interfere with this cyanate assay method.

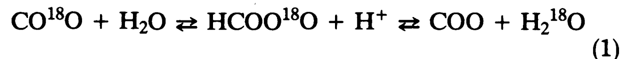
Purification of carbonic anhydrase and cyanase. Carbonic anhydrase was purified from strain SJ100/pAL4T as described previously (10). Cyanase was purified as described previously (18).

Detection of carbonic anhydrase and cyanase by Western blotting. Antibodies to highly purified cyanase and carbonic anhydrase were prepared by standard procedures, using two-step injection of rabbits with 1 ml of a 1-mg/ml enzyme solution dialyzed against water. Antisera from immunized rabbits were stored at −20°C and diluted for use without purification. Immunoblotting was done by the recommended protocols of and with reagents from Bio-Rad for electrophoresis (15% sodium dodecyl sulfate), blotting transfer, and reaction with primary and secondary antibodies and Amersham Corp. for detection based on a luminol oxidation chemiluminescent reaction. Some changes were introduced into the protocols to minimize nonspecific binding of both primary and secondary antibodies: the time of washing after each step was increased from 15 min to 2 h, and antiserum was highly diluted (1:10^6). No bands were detected with preimmune serum.

Samples for blotting were prepared as follows. Liquid cultures (20 ml) were grown in minimal medium at a high partial CO_2 pressure (pCO_2) as described above. KNCO (0.5 mM) was added to cultures when the OD_{600} was 0.1. After 30 min, cells were harvested, immediately suspended in 0.1 ml of denaturing buffer (0.05 M Tris [pH 6.8], 2.5% sodium dodecyl sulfate, 20% glycerol, 10% mercaptoethanol), and boiled for 1 min. The volume of sample added to the gel was 5 μl. Samples (5 to 10 μl) containing carbonic anhydrase (10 ng), cyanase (10 ng), or protein standards (5 μg) were also treated with denaturing buffer and boiled before application to gels. For carbonic anhydrase in whole-cell extracts, some unexplained nonspecific binding of lower intensity was observed, but this did not interfere with interpretation of the results.

Assay of cyanase activity. Cyanase activity was determined as described previously (3, 4). Growing liquid cultures were harvested and resuspended in 0.05 M phosphate buffer, sonicated twice during 40 s on ice, and centrifuged for 10 min at 15,000 × *g*. A small volume (50 μl) of supernatant was then assayed.

Detection of carbonic anhydrase activity in whole cells by ^{18}O exchange. The exchange of ^{18}O between CO_2 and water occurs because of the hydration-dehydration cycle, in which $H_2{}^{18}O$ is very greatly diluted by $H_2{}^{16}O$ (equation 1). Since carbonic anhydrase catalyzes this cycle, it also catalyzes the loss of ^{18}O from CO_2.



$$CO^{18}O + H_2O \rightleftharpoons HCOO^{18}O + H^+ \rightleftharpoons COO + H_2{}^{18}O \quad (1)$$

Carbonic anhydrase activity in whole cells of *E. coli* was tested by measuring the rate of depletion of ^{18}O from CO_2 in a membrane inlet to a mass spectrometer (16). In this method, whole cells are added to a solution containing ^{18}O-labeled CO_2 and HCO_3^- in chemical equilibrium. The presence of carbonic anhydrase activity is indicated by enhanced depletion of ^{18}O from CO_2; in the case of carbonic anhydrase compartmentalized in cells, this loss is frequently found to be described by the sum of two first-order exponential processes (16).

Overnight cultures were diluted and grown aerobically in 400-ml batches of the 2× minimal medium. Sodium azide (0.1 mM) was added to the cultures at an OD_{600} of 0.1, and

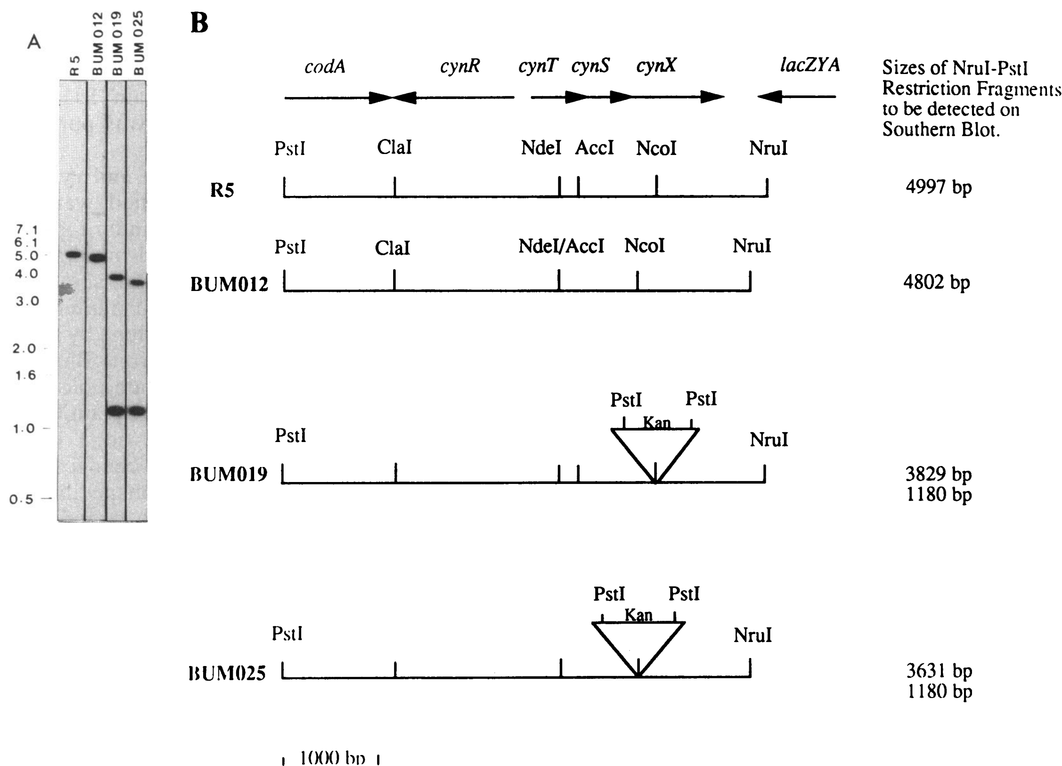


FIG. 1. (A) Autoradiogram of Southern blot of chromosomal DNA from *E. coli* HfrR5 (R5) and mutants. Chromosomal DNA digested with *Pst*I and *Nru*I was separated on a 1% agarose gel, blotted, and probed with labeled fragments carrying the *cyn* operon genes and regulatory elements. Positions of molecular size standards are indicated on the left (in kilobases). (B) Scheme showing the *Pst*I-*Nru*I chromosomal fragment carrying the *cyn* operon. The sizes of the expected restriction fragments are indicated to the right of each strain.

growth was continued to an OD_{600} of 0.4. The cultures were harvested by centrifugation at room temperature, washed with an equal volume of 2× minimal medium, and resuspended in 6 ml of this medium. Samples of this suspension (2.0 ml) were mixed in the membrane inlet vessel with 6.0 ml of 2× minimal medium containing a total 5.0 mM concentration of all species of CO_2 at chemical equilibrium; these species of CO_2 were enriched (99 atom%) in ^{13}C (that is, $[^{13}CO_2] + [H^{13}CO_3^-] = 5.0$ mM). The temperature was maintained at 37°C, and the pH after mixing was 7.2. This final suspension contained between 3.6×10^9 and 4.8×10^9 bacterial cells per ml, as determined from the OD_{600}, assuming that 1 OD unit = 8.1×10^8 cells per ml. Mass spectrometric measurements of the atom fraction of ^{18}O in ^{13}C-containing CO_2 ensured that this procedure was not significantly affected by endogenous CO_2 initially present in the *E. coli* cells. For each sample, two rate constants were determined: Θ_1 is the first-order rate constant, describing the decrease in the atom fraction of ^{18}O in $^{13}CO_2$ in the rapid initial exchange, and Θ_2 is the slower subsequent exchange, as described by Silverman et al. (16).

RESULTS

Construction of chromosomal mutant strains carrying Δ*cynT*, *cynS*::*kan* *cynX*::*kan*, and Δ*cynT* *cynX*::*kan*. Strains BUM012 (Δ*cynT*), BUM031 (*cynS*::*kan*), BUM019 (*cynX*::*kan*), and BUM025 (Δ*cynT cynX*::*kan*) were constructed as described in Materials and Methods. When the chromosomal DNA of BUM031 was subjected to Southern analysis, the fragments generated were larger than expected, indicating that an extra fragment may have been inserted (data not shown). The results presented in this article indicate that BUM031 has carbonic anhydrase activity but not cyanase activity and that it does contain the insert giving kanamycin resistance, indicating that if an additional fragment was added, it did not affect the phenotype. Confirmation of the construction of the other mutants by Southern analysis is shown in Fig. 1. Fragments differing from the parental fragments by the expected number of bases were obtained. The *cynS*::*kan* mutant strain (BUM031), however, appears to have an insertion that is somewhat larger than expected but nevertheless has the expected phenotype with respect to absence of cyanase activity. The *cynT* and *cynS* mutants showed the expected phenotypes with respect to expression and/or activity of carbonic anhydrase and cyanase, respectively; this was demonstrated by an enzyme assay (Tables 2 and 3) and by immunoblotting (Fig. 2). As noted in Fig. 2, expression of either carbonic anhydrase or cyanase occurs only after induction by cyanate (or by azide, a gratuitous inducer [8] [data not shown]).

Assay of carbonic anhydrase activity. Induced carbonic anhydrase activity in the wild-type strain BUM015 is normally very low and could not be measured by standard methods involving pH changes, as used when carbonic anhydrase is overexpressed (10). Carbonic anhydrase activity was therefore measured in the wild type and in the chromosomal mutants by the ^{18}O exchange method (16). In this method, CO_2 hydration activity is determined by the rate of depletion of ^{18}O from CO_2 as this label is exchanged from CO_2 to H_2O. This rate of depletion is typically biphasic

TABLE 2. First-order rate constants Θ_1 and Θ_2 for the two segments of the depletion of ^{18}O from CO_2 in suspensions of *E. coli*[a]

Strain	Induced with 0.1 mM azide	Θ_1 (10^{-3} s^{-1})	Θ_2 (10^{-3} s^{-1})
BUM015 (wild type)	−	5.8	6.3
	+	13	7.3
BUM012 (Δ*cynT*)	−	7.1	4.8
	+	7.6	4.6
BUM019 (*cynX*::*kan*)	−	8.8	5.1
	+	14	6.8

[a] All experiments were carried out at 37°C at a pH after mixing of 7.2. Measurements for the initial segment of ^{18}O exchange (used to obtain Θ_1) were collected in the first 30 s after adding cells; data for the second segment were collected in the following 6 min. Under these conditions, the first-order rate constant for the uncatalyzed depletion of ^{18}O from CO_2 is $\Theta_{uncat} = 4.7 \times 10^{-3}$ s^{-1}. The standard errors for all values of Θ were less than 20%; the standard errors were less than 10% for $\Theta > 1 \times 10^{-2}$ s^{-1}.

in suspensions of cells containing carbonic anhydrase; it is usually monophasic when carbonic anhydrase is homogeneous in solution (16). There was no evidence of carbonic anhydrase activity in any uninduced strain tested, as determined by the single uncatalyzed ^{18}O exchange rate, about 5 $\times 10^{-3}$ s^{-1}, measured over the entire ^{18}O exchange process (Table 2); there was also no significant indication for two phases of ^{18}O depletion of CO_2. For induced cells, there was clear evidence of carbonic anhydrase activity for wild-type (BUM015) and BUM019 (*cynX*::*kan*) cells, as determined from the ^{18}O exchange rate constants and the presence of two phases of depletion of ^{18}O from CO_2 (Table 2). These rate constants may underestimate the activity of carbonic anhydrase in the cells because of the presence in the washed cells of residual azide, a known inhibitor of carbonic anhydrases. The rate constant for the second phase was indistinguishable from the uncatalyzed rate. As expected, there was no evidence for carbonic anhydrase activity in the induced BUM012 (Δ*cynT*) cells (Table 2).

Properties of a Δ*cynT* mutant (BUM012). The 195-bp deletion resulting in the Δ*cynT* mutation (BUM012) eliminates 65 amino acids from the normal carbonic anhydrase

TABLE 3. Specific activity of cyanase in extracts of various strains grown in minimal medium and induced with 0.5 mM cyanate or 0.1 mM azide

Strain	Arginine + uracil added	pCO_2	Inducer	Cyanase sp act (μmol/min/mg)
BUM015 (wild type)	−	Low	None	<0.01
	−	Low	Cyanate	1.17
	+	Low	Cyanate	1.56
	−	High	Cyanate	0.66
BUM012 (Δ*cynT*)	−	Low	Cyanate	0.12
	+	Low	Cyanate	0.52
	−	High	Cyanate	0.50
BUM015 (wild type)	−	Low	Azide	0.83
	−	High	Azide	0.87
BUM012 (Δ*cynT*)	−	Low	Azide	0.43
	−	High	Azide	0.38
BUM019 (*cynX*::*kan*)	−	Low	Azide	0.45
BUM031 (*cynS*::*kan*)	−	Low	Azide	<0.01
BUM025 (Δ*cynT* *cynX*::*kan*)	−	Low	Azide	0.17

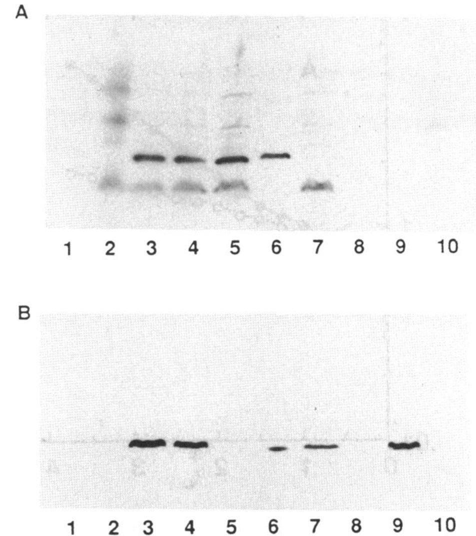


FIG. 2. Immunoblots of purified carbonic anhydrase and cyanase and of extracts of various strains. All extracts were grown in minimal medium at high pCO_2 and, where indicated, induced with 0.5 mM cyanate. Samples were prepared as described in Materials and Methods. Unless indicated otherwise (wild type), all extracts are from induced cells. (A) Carbonic anhydrase immunoblots. Lane 1, cyanase; lane 2, wild type (BUM015), noninduced; lane 3, wild type (BUM015); lane 4, BUM019 (*cynX*::*kan*); lane 5, BUM031 (*cynS*::*kan*); lane 6, carbonic anhydrase; lane 7, BUM025 (Δ*cynT* *cynX*::*kan*); lane 8, standard protein size markers (locations not indicated); lane 9, BUM012 (Δ*cynT*); lane 10, wild type (BUM015), preimmune sample. (B) Cyanase immunoblots. Lanes are the same as in panel A except for lane 1 (carbonic anhydrase) and lane 6 (cyanase).

protein yet maintains the correct reading frame and would thus not be expected to alter the products of the downstream *cynS* and *cynX* genes. The results given in Table 2 and Fig. 2 show that no detectable cyanate-induced carbonic anhydrase activity or protein, respectively, was made in BUM012 (Δ*cynT*). The 195-bp deletion in *cynT* does not prevent the expression of cyanase, as determined by the cyanase assay (Table 3) and by immunoblotting (Fig. 2).

The growth properties of the Δ*cynT* mutant (BUM012) compared with those of the wild-type strain (BUM015) are illustrated in Fig. 3. In glucose minimal medium, the growth of the Δ*cynT* mutant strain was significantly inhibited by the addition of 0.5 mM cyanate to the medium aerated with air i.e., 0.03% CO_2 (low pCO_2), but growth was the same as for the wild-type strain when aeration was done with air containing 3% CO_2 (high pCO_2). (The pH of the growth medium was not significantly altered by the higher pCO_2.) In contrast, the growth of the wild type (BUM015) was not significantly affected under either aeration condition by the addition of 0.5 mM cyanate. These results are consistent with the proposed role of carbonic anhydrase. In a Δ*cynT* mutant strain, in which cyanase but not carbonic anhydrase activity is present, conversion of added cyanate to CO_2 would deplete the cell of bicarbonate. This effect is alleviated by an increased CO_2 concentration in the medium. Depletion of bicarbonate could result in an inability to catalyze bicarbonate-dependent reactions essential for growth and an inability to catalyze rapid decomposition of cyanate, which inhibits growth. Results similar to those shown in Fig. 3 were obtained when the concentration of

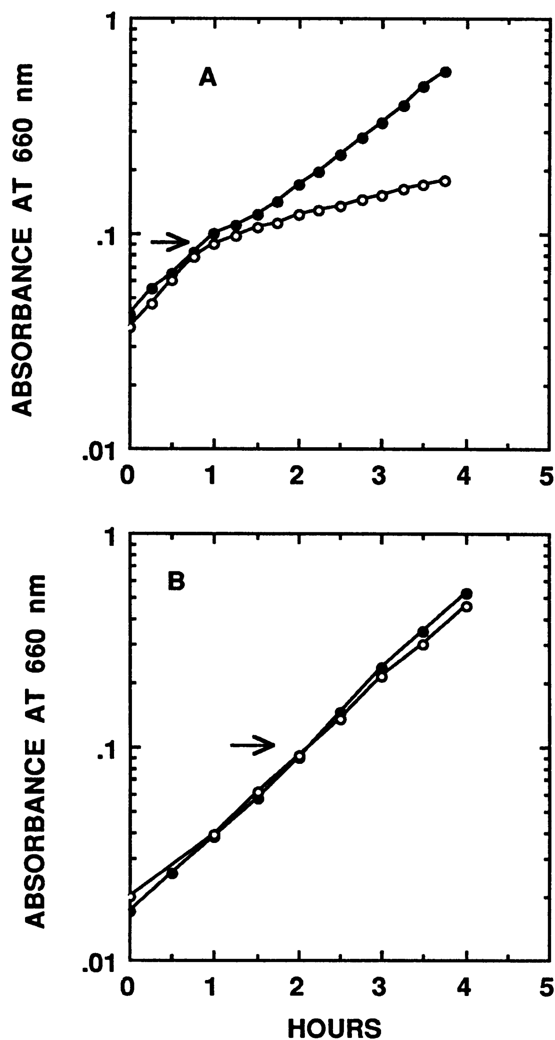


FIG. 3. Inhibition of growth of wild-type (●, BUM015) and Δ*cynT* mutant (○, BUM012) strains by cyanate. Strains were grown in glucose minimal medium as described in Materials and Methods; cyanate (0.5 mM) was added when the OD_{600} reached 0.1 (indicated by arrows). (A) Low pCO_2. (B) High pCO_2.

cyanate added was 0.75, 1.0, or 1.5 mM or when enriched medium (LB broth) was used instead of glucose minimal medium.

Properties of a *cynS*::*kan* mutant (BUM031). The *cynS*::*kan* mutant strain BUM031 was constructed by inserting a kanamycin resistance gene into the *cynS* gene. As a result of this insertion, no active cyanase is synthesized after induction, but the upstream *cynT* gene encoding carbonic anhydrase is expressed normally (Table 3 and Fig. 2). It is possible that construction of the *cynS*::*kan* mutation may also have affected the expression of *cynX*, but as shown below, even if this did occur, a defective *cynX* product does not appear to alter the functions of the other two gene products. The *cynS*::*kan* mutant strain was found to have a sensitivity to cyanate similar to that of other mutants devoid of inducible cyanase activity (8). In contrast, growth of the Δ*cynT* mutant strain (BUM012) was more sensitive to inhibition by cyanate (Table 4).

A major physiological effect of cyanate on growth is inhibition of carbamoyl phosphate synthetase, which cata-

TABLE 4. Generation times of various strains in the presence or absence of 0.5 mM cyanate in medium with and without arginine plus uracil (low pCO_2)

Strain	Arginine + uracil added	Generation time (min)	
		Without cyanate	With cyanate
BUM015 (wild type)	−	46	105 (57)[a]
	+	46	54
BUM012 (Δ*cynT*)	−	47	195
	+	41	176
BUM031 (*cynS*::*kan*)	−	50	79
	+	49	57

[a] The value in parentheses is the generation time after all cyanate has been degraded.

lyzes the first step in both arginine and pyrimidine biosynthesis (2, 9). Guilloton and Karst (9) have shown that inhibition of growth by cyanate in minimal medium in strains lacking an inducible cyanase activity can be partially alleviated by the presence of arginine and almost completely alleviated by the presence of both arginine and uracil, but that the presence of uracil alone increases sensitivity to cyanate. These observations were confirmed with the *cynS*::*kan* mutant strain; the presence of arginine (100 μg/ml) partially relieved the growth inhibition by cyanate, and uracil (50 μg/ml) increased the inhibitory effect of cyanate (data not shown). The effects of arginine plus uracil together on BUM015 (wild type), BUM012 (Δ*cynT*), and BUM031 (*cynS*::*kan*) are compared in Table 4. As noted above, the growth of the *cynS*::*kan* mutant was significantly less sensitive to inhibition by 0.5 mM cyanate than was that of the Δ*cynT* mutant at low pCO_2. Moreover, the addition of arginine plus uracil significantly relieved inhibition by cyanate for both the *cynS*::*kan* mutant and wild-type strains but not for the Δ*cynT* mutant. Thus, induced cyanase activity in the Δ*cynT* mutant strain at low pCO_2 appears to readily create a shortage of bicarbonate, and this depletion of bicarbonate is more serious than the direct inhibitory effect of cyanate on growth, as observed in the *cynS*::*kan* mutant.

Growth on cyanate as the sole nitrogen source. Previous studies (8, 22) have shown that induction of the *cyn* operon allows the wild-type strain to grow on medium containing cyanate as the sole source of nitrogen, i.e., induction of cyanase results in decomposition of cyanate to ammonia, which provides the nitrogen needed for growth. As shown in Fig. 4A, BUM012 (Δ*cynT*) did not grow at a significant rate with cyanate as the sole source of nitrogen at low pCO_2 but did grow at high pCO_2 (Fig. 4B). As noted in Fig. 4, BUM031 (*cynS*::*kan*) did not grow at a significant rate under either condition, as would be expected. The slow rate of growth that was observed for the Δ*cynT* and *cynS*::*kan* mutant strains can be accounted for by the spontaneous decomposition of cyanate (half-life of 6 h at 37°C in minimal medium). When ammonium chloride replaced cyanate at the same concentration (0.5 mM), the growth rate was the same as that observed for the parental strain with cyanate except that there was no lag period (the lag period in the parental strain reflects inhibition of growth by cyanate and ammonium shortage before cyanase is induced) (data not shown). These observations suggest that even though an active cyanase activity is induced by cyanate in the Δ*cynT* mutant (Table 3), the mutant strain cannot catalyze decomposition of cyanate to ammonia at a rate sufficient to sustain growth, presumably

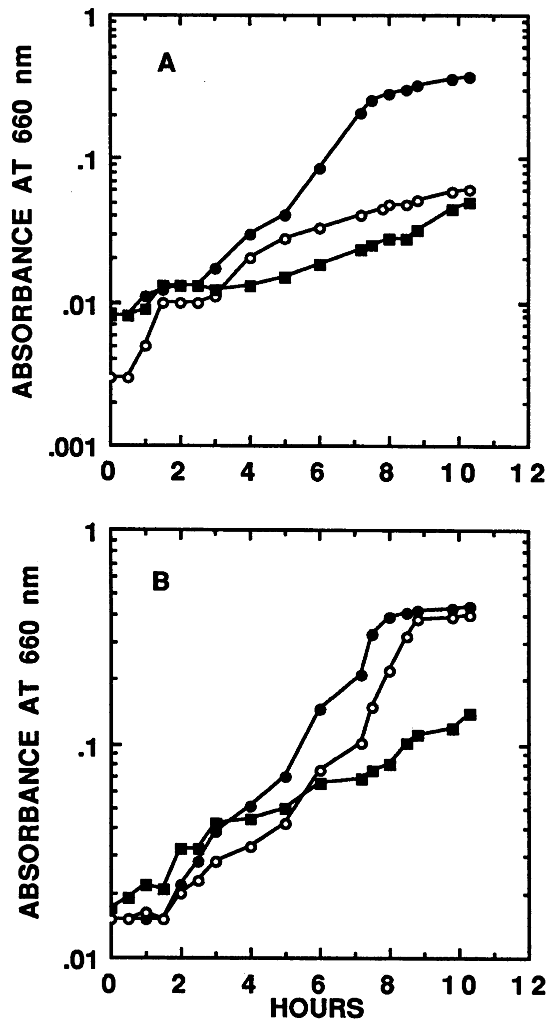


FIG. 4. Growth of wild-type and mutant strains on cyanate as a sole nitrogen source. Strains were grown in glucose minimal medium as described in Materials and Methods except that the medium did not contain any ammonium salt; cyanate (0.5 mM) was added before inoculation. (A) Low pCO_2. (B) High pCO_2. ●, BUM015 (wild type); ○, BUM012 (Δ*cynT*); ■, BUM031 (*cynS*::*kan*).

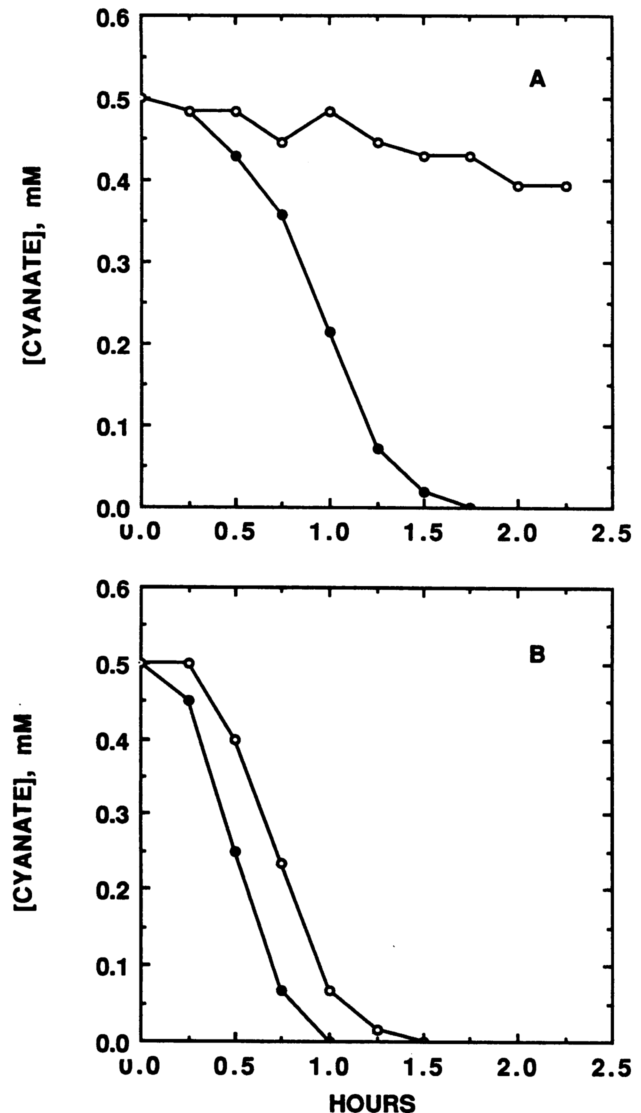


FIG. 5. Cyanate degradation by wild-type (●, BUM015) and Δ*cynT* mutant (○, BUM012) strains. Cultures were grown in glucose minimal medium as indicated in Materials and Methods, and 0.5 mM cyanate was added when the OD_{600} reached 0.1. Cyanate concentration was determined as described in Materials and Methods. (A) Cells aerated with flushed air (low pCO_2). (B) Cells aerated with air containing 3% CO_2 (high pCO_2).

because of the absence of carbonic anhydrase activity and the resulting rapid depletion of bicarbonate due to loss of CO_2.

Cyanate degradation in a Δ*cynT* mutant (BUM012). The results in Fig. 5 confirm that, at low pCO_2, BUM012 (Δ*cynT*) did not degrade cyanate at a significant rate relative to degradation in the wild type (BUM015) but did degrade cyanate at high pCO_2. The specific activity of induced cyanase in BUM012 (Δ*cynT*) grown at low pCO_2 (Fig. 5A) was only about 15% of that of the cyanase induced in the wild type (Table 3). Since growth inhibition by cyanate would reflect decreased protein synthesis, the greater sensitivity of the Δ*cynT* mutant than of the wild type to cyanate could account for the low cyanase activity. It is possible that the lower level of cyanase activity could explain the lack of cyanate degradation by BUM012 (Δ*cynT*) shown in Fig. 5. Since inhibition of growth by cyanate can be significantly reduced by addition of arginine and uracil, the experiment (Fig. 5) was repeated with minimal medium supplemented with arginine (100 μg/ml) and uracil (50 μg/ml). Under these conditions, the levels of induced cyanase were found to be much higher than observed under the original conditions (Fig. 5 and Table 3); however, cyanate decomposition by the wild type and the Δ*cynT* mutant were essentially the same as shown in Fig. 5, i.e., at low pCO_2, despite the presence of cyanase activity, the mutant strain did not catalyze decomposition of cyanate at a significant rate, indicating that carbonic anhydrase is needed for cyanate degradation.

A second approach to increasing the level of induced cyanase activity in the Δ*cynT* mutant strain involved induction of cyanase by the gratuitous inhibitor azide prior to addition of cyanate. Under these conditions, the level of induced cyanase was also significantly increased (Table 3). The results were also essentially the same as those shown in

Fig. 5 except that there was no lag period in cyanate degradation for the wild type at low or high pCO_2 or for the Δ*cynT* mutant at high pCO_2; the lack of a lag period under these conditions is expected, since cyanase activity is already present and a period of time to synthesize the enzyme is not required. The level of induced cyanase in the Δ*cynT* mutant under these two different conditions (presence of arginine plus uracil and induction with azide) is comparable to that observed for the *cynX*::*kan* mutant strain BUM019 (Table 3), which degrades cyanate at a rate similar to that observed for the wild type (BUM015) at low pCO_2. Thus, the lack of cyanate decomposition observed in the Δ*cynT* mutant strain appears to be due to the absence of carbonic anhydrase activity rather than a consequence of reduced levels of cyanase activity.

Properties of the *cynX*::*kan* (BUM019) and Δ*cynT* *cynX*::*kan* (BUM025) mutant. A chromosomal mutation of *cynX* (*cynX*::*kan*) was constructed by incorporating a gene for resistance to kanamycin into the *cynX* gene. No change in the phenotype of the mutant strain compared with the parental strain was observed. This strain behaved identically to the parental strain with respect to growth in the presence or absence of cyanate (low or high pCO_2, enriched or minimal medium; data not shown) and to induction of carbonic anhydrase or cyanase, as detected by activity or immunoblotting (Tables 2 and 3 and Fig. 2). The observed phenotype of the double mutant strain BUM025 (Δ*cynT* *cynX*::*kan*) is the same as that of BUM012 (Δ*cynT*) with respect to growth under different conditions (data not shown) and to induction of carbonic anhydrase and cyanase (Tables 2 and 3 and Fig. 2). As in the Δ*cynT* mutant strain, the level of cyanase activity in the *cynX*::*kan* mutant strain after induction with azide was about 40% of that obtained with the wild-type strain, and the level of induced cyanase activity in the Δ*cynT* *cynX*::*kan* double mutant strain (BUM025) after induction with azide was even lower, about 20% of the wild-type level (Table 3). The reason for these reduced levels of expression of cyanase by the mutant strains is not known but may be related to effects on the rates of transcription or translation or on the stability of the transcript.

DISCUSSION

The discovery that the product of the *cynT* gene of the *cyn* operon was a unique cyanate-induced carbonic anhydrase, together with the established fact that the cyanate-induced cyanase catalyzes the reaction of bicarbonate with cyanate to give ammonia and two molecules of CO_2, led to the proposal that a function of the product of the *cynT* gene is to prevent cellular depletion of bicarbonate (10). The reasoning was that CO_2 would diffuse out of the cell faster than it would be hydrated nonenzymatically to bicarbonate, thus depleting the cell of the bicarbonate required for bicarbonate-dependent metabolic reactions and/or decomposition of cyanate. The results presented here provide clear evidence that the action of induced cyanase on added cyanate in the absence of carbonic anhydrase does have a significant inhibitory effect on both cell growth and cyanate degradation at low pCO_2. These effects are likely the result of bicarbonate depletion in the cell as a result of the reaction of bicarbonate with cyanate catalyzed by cyanase to give two molecules of CO_2, which diffuse out of the cell faster than they can be hydrated to bicarbonate; the requirement for carbonic anhydrase can be eliminated by aeration at higher pCO_2. Under these conditions of low pCO_2 and absence of carbonic anhydrase, added cyanate is not degraded. However, the inhibitory effect of cyanate on cell growth due to the absence of carbonic anhydrase does not appear to be primarily related to a direct inhibitory effect of cyanate, since the *cynS*::*kan* mutant (no cyanase activity) is less sensitive to cyanate than is the Δ*cynT* mutant (no carbonic anhydrase activity) (Table 4). The role of the induced carbonic anhydrase in protection against bicarbonate depletion and subsequent inhibition of growth appears to be related to some essential bicarbonate-dependent function in the cell. The dependence of enterobacteria on the presence of CO_2 for growth has been reported previously (14, 15). The rather nonphysiological situation presented by the Δ*cynT* mutant strain has highlighted a significant dependence of growth on the availability of bicarbonate, which provides an opportunity to investigate the basis for this requirement in more detail.

The product of the *cynT* gene was originally proposed to be a cyanate permease (20). This conclusion was based on the observation that [^{14}C]cyanate was rapidly incorporated into *E. coli* cells harboring plasmids expressing the *cynT* gene ($CynT^+$ cells) but was not incorporated into $CynT^-$ cells. Since all uptake assays on $CynT^+$ cells were conducted on cells that were $CynS^+$, these results could also be consistent with the observation that *cynT* encodes a carbonic anhydrase activity. In a *cynT*-deficient strain, [^{14}C]cyanate would be converted to $^{14}CO_2$, which would diffuse out of the cell, while in a $CynT^+$ strain, the $^{14}CO_2$ would be rapidly converted to [^{14}C]bicarbonate in equilibrium with $^{14}CO_2$; much of the [^{14}C]bicarbonate would remain in the cell and be incorporated into stable metabolites and macromolecules. This system was strongly inhibited by concentrations of cyanate above 0.7 mM. This may be interpreted to indicate that carbonic anhydrase (and perhaps also cyanase) is sensitive to cyanate inhibition (10). Figure 5 shows that even the parental strain decomposes 0.5 mM cyanate more rapidly when the pCO_2 is increased, suggesting that carbonic anhydrase activity is limiting, presumably by cyanate inhibition (10). This suggests that the induced carbonic anhydrase activity may not be sufficient to meet the bicarbonate requirement of the cell during the decomposition of high concentrations of cyanate (at least under our culture conditions in minimal medium) and that the operon may be designed to decompose lower concentrations of cyanate. Although the physiological function of the *cyn* operon has not been clearly established, it has been suggested that its function is likely related to utilization of the low levels of cyanate resulting from the breakdown of urea or carbamoyl phosphate (1, 9).

Under the culture conditions and the growth parameters used, no differences were detected in a *cynX*::*kan* mutant strain compared with its parental strain. The function of the *cynX* gene remains unknown. Because this protein is hydrophobic in nature, its function, if any, would appear to involve interaction with a membrane. It is possible that this gene has no function related to cyanate metabolism.

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