Identification and Characterization of a Cyanate Permease in *Escherichia coli* K-12

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***Escherichia coli* contains an inducible enzyme, cyanase, that catalyzes the decomposition of cyanate into ammonia and bicarbonate. The gene encoding cyanase, *cynS*, was cloned and found to be on a DNA fragment that contained the *lac* operon. Characterization of a plasmid encoding cyanase indicated that a 26-kilodalton (kDa) protein of unknown function was also induced by cyanate (Y.-C. Sung, D. Parsell, P. M. Anderson, and J. A. Fuchs, J. Bacteriol. 169:2639-2642, 1987). The gene encoding the 26-kDa protein was located between *cynS* and its promoter, indicating the existence of a *cyn* operon. The 26-kDa protein was identified as a cyanate permease that transports exogenous cyanate by active transport. *E. coli* was shown to contain a cyanate transport system that is energy dependent and saturable by cyanate.**

Escherichia coli K-12 contains an enzyme, cyanase, that catalyzes the bicarbonate-dependent decomposition of cyanate into ammonia and bicarbonate (2-4, 13, 25, 26). Cyanase is induced by addition of cyanate to the medium (25). Cyanate is formed in nature by the spontaneous dissociation of urea in aqueous solution (11) and by the spontaneous decomposition of carbamoylphosphate (1). Cyanate can react with a number of functional groups in proteins. The carbamylation of amino groups by cyanate is irreversible, while reactions of cyanate with thiols, imidazoles, phenolic groups, and carboxylates are reversible (20-22). A mutant strain deficient in cyanase activity is more sensitive to the toxic effects of cyanate than its parent strain (9, 10, 24). Cyanase from *E. coli* has been purified (2), sequenced (5), and characterized (3-5, 15). The gene encoding cyanase, *cynS*, was cloned, sequenced, and expressed at a high level (23, 24). The characterization of plasmids encoding cyanase indicated that a plasmid which encoded a protein with an M_r of 26,000 was also induced by cyanate in parallel to the induction of cyanase (24).

In this paper, we report that the 26-kilodalton (kDa) protein is encoded by the DNA located between the *cynS* gene and its promoter. The 26-kDa protein was found to be a cyanate permease. *E. coli* was shown to have an active transport system for the uptake of cyanate, and this system was partially characterized. Strains without *cynT* were found to be as sensitive to cyanate as strains deleted for both *cynT* and *cynS*, while strains containing *cynT* but not *cynS* were found to be hypersensitive to cyanate.

MATERIALS AND METHODS

Chemicals and enzymes. Scintillation counting fluid was obtained from Amersham Corp. KN^{14}CO (54.5 mCi/mmol) was obtained from New England Nuclear Corp. Membrane filters were obtained from Nuclepore Corp. All other chemicals were obtained from Sigma Chemical Co. or Bio-Rad Laboratories, Inc. All enzymes were obtained from Boehringer Mannheim Biochemicals and were used as specified by the manufacturer.

Bacterial strains, plasmids, and growth conditions. *E. coli* MM294A (*endA1 thi-1 hsdR17 gyrA96 supE44 recA*) was obtained from D. D. LaPorte (University of Minnesota).

JM101 [*thi supE* $\Delta(lac-proAB)$ (*F'* *traD36 proAB lacI^qZ* $\Delta M15$)] was obtained from J. Messing, and SJ100 [*thi supE* $\Delta(lac-proAB)$] was constructed by acridine orange treatment to eliminate the *F'* *lac*⁺ plasmid of JM101 (17, 24). Since the *lac* deletion of SJ101 was found to delete the *cynS* gene (24), this strain was used as a *cyn* mutant strain. The *F'* *lac* in JM101 contains the gene encoding CynS (24). Plasmid pMS421 was obtained from A. Das (University of Minnesota). Plasmid isolation, mapping of restriction sites, ligation reactions, and transformation were performed essentially as previously described by Maniatis et al. (16). The growth medium used was L broth (17) supplemented with 20 μg of thymine per ml or minimal medium (7) appropriately supplemented. When indicated, ampicillin, kanamycin, and chloramphenicol were added at concentrations of 50, 40, and 100 $\mu\text{g}/\text{ml}$, respectively. An assay of cyanase activity was performed as previously described (2).

Transport assay. Overnight minimal medium cultures were diluted 1:100, grown to an optical density at 660 nm (OD_{660}) of 0.35, and divided into two flasks. One flask was induced for 20 min with either 0.5 mM isopropyl- β -D-thiogalactopyranoside (IPTG) or 0.5 mM KNCO (depending on the promoter controlling *cyn* expression), and the parallel flask was not induced. The cultures were chilled on ice, and the cells were collected by centrifugation and washed twice with minimal medium at 4°C. The cells were suspended in fresh minimal medium appropriately supplemented to a final OD_{660} of 0.3 and grown to an OD_{660} of 0.45 to allow cells time to eliminate residual cyanate used for induction. The cells were harvested by centrifugation at 4°C and suspended in fresh minimal medium to a concentration of 10^{10} cells per ml (an OD_{660} of 1 corresponds to 8×10^8 cells per ml). These cells were used in the transport assay at a final concentration of 10^9 cells per ml. Transport assays were conducted in minimal medium containing KN^{14}CO , with aeration by shaking at 37°C. Transport rates were determined by taking a 0.4-ml sample every minute for up to 8 min after radiolabeled cyanate was added. When indicated, carbonyl cyanide *m*-chlorophenylhydrazone and dinitrophenol were added to an assay culture at final concentrations of 0.1 and 1 mM, respectively, just before labeled cyanate was added. The samples were filtered through filters (pore size, 0.45 μm), the filters were rinsed twice with 10 ml of minimal medium and

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FIG. 1. Physical and genetic maps of various plasmids. The restriction enzyme sites used in these constructions are not shown but are located in the polycloning site of the pUC13 vector. The sizes of all fragments were determined by restriction enzyme mapping or by DNA sequencing. The coding regions of *cynT* (cyanate permease) and *cynS* (cyanase) are indicated by arrows pointing in the direction of transcription. Parts of the *lac* operon are indicated by *lacY* and *lacA*. Various restriction fragments were tested for cyanase activity. Symbols: +, positive for cyanase and permease activity controlled by cyanate; +^a, positive for cyanase and permease activity controlled by IPTG; -, negative for cyanase and permease activity; ∇ , GATCCGTCGACG; Kb, kilobase; P1, *Pst*I; C, *Cl*aI; P, *Pvu*II; H, *Hin*II; A, *Acc*I; B, *Bgl*II; E, *Eco*RI.

dried, and activity was measured in a liquid scintillation spectrometer.

Toxicity assay. Overnight cultures were diluted 1:100 in fresh minimal medium and incubated at 37°C with aeration by shaking to an OD₆₆₀ of 0.25. Cells requiring IPTG induction were grown to an OD₆₆₀ of 0.15 and induced with 0.5 mM IPTG for 20 min. All cultures (OD₆₆₀ = 0.25) were diluted to an OD₆₆₀ of 0.15 with fresh minimal medium and divided into 10-ml cultures in 50-ml flasks containing various concentrations of cyanate. The samples were taken every 30 min, and the OD₆₆₀ was determined and plotted as a function of the cyanate concentration. Length of the lag phase was extrapolated to 0 to determine a MIC.

RESULTS

Location of gene encoding 26-kDa protein. It was previously observed that the plasmids pDP3 and pSJ104 synthesized a 26-kDa protein of unknown function in addition to cyanase following induction by cyanate (24). Subclones of the insert in plasmid pSJ104 were used to generate plasmids pSJ105 and pSJ147 and to further localize the region of DNA encoding the 26-kDa protein (Fig. 1). The cyanase activities of pSJ105 and pSJ147 were not induced by cyanate but were induced by IPTG, indicating that the *cyn* regulatory region was deleted and that the *cynS* gene encoding cyanase was expressed by readthrough transcription from the *lac* promoter on the pUC13 plasmid (23). Since plasmid pSJ105, but not pSJ147, contains approximately 700 base pairs upstream of *cynS* (Fig. 1), the possibility that the DNA region upstream of *cynS* encodes the 26-kDa protein was investigated. Strain JM101 containing pSJ105 or pSJ147 was grown to early log phase (OD₆₆₀ = 0.2) and induced with IPTG for 2 h. Samples were harvested and subjected to sodium dodecyl sulfate-15% polyacrylamide gel electrophoresis (14, 18).

Strains containing plasmid pSJ105 but not pSJ147 produced the 26-kDa protein in response to IPTG induction (Fig. 2). This observation indicates that the 26-kDa protein must be encoded by the 700-base-pair DNA region between the

FIG. 2. Coomassie brilliant blue R-stained sodium dodecyl sulfate-15% polyacrylamide gel of proteins synthesized from JM101 (pSJ105) and JM101(pSJ147). Cultures were grown to an OD₆₆₀ of 0.3 and induced with 0.5 mM IPTG. Samples were harvested at 0, 1, and 2 h after induction, processed, and run on this gel. The plasmid contained in JM101 and the time of induction are indicated above each lane of the gel. Lane M contained marker proteins (phosphorylase b, 94 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; soybean trypsin inhibitor, 20.1 kDa; and α -lactalbumin, 14.4 kDa). *cynT* and *cynS* indicate the positions of cyanate permease (26 kDa) and cyanase (17 kDa), respectively.

FIG. 3. (A) Kinetics of KN^{14}CO uptake by *E. coli* cells in minimal medium. The experimental procedures were as described in Materials and Methods. Symbols: $\circ$, JM101 plus KNCO ; $\bullet$, SJ100 plus KNCO . (B) Kinetics of KN^{14}CO uptake by *E. coli* cells in minimal medium. The experimental procedures were as described in the Materials and Methods. The insert is a Lineweaver-Burk plot of the KN^{14}CO uptake by JM101 induced with cyanate. Symbols: $\circ$, JM101 plus KNCO ; $\square$, JM101 plus KNCO in the presence of 100 μg of chloramphenicol per ml 5 min before KNCO induction; Δ , SJ100 plus KNCO . (C) Kinetics of KN^{14}CO -uptake by SJ100 containing various plasmids. The experimental procedures were as described in the Materials and Methods. Symbols: $\bullet$, SJ100(pSJ104) plus KNCO ; $\circ$, SJ100(pDP3) plus KNCO ; $\square$, SJ100 (pUC13) plus KNCO . (D) Kinetics of KN^{14}CO uptake by SJ100(pMS421) containing pSJ105 and pSJ147 in minimal medium. The experimental procedures were as described in the Materials and Methods. Symbols: $\circ$, SJ100(pSJ105, pMS421); $\bullet$, SJ100(pSJ105, pMS421); Δ , SJ100(pSJ147, pMS421); $\blacksquare$, SJ100(pSJ105, pMS421); $\square$, SJ100(pSJ105, pMS421); $\triangle$, SJ100 (pSJ105, pMS421).

HincII site and the *cynS* gene on plasmid pSJ105 (Fig. 1). This result and the fact that both the 26-kDa protein and cyanase are induced by cyanate indicate that the genes encoding these proteins are part of a *cyn* operon.

Since cyanate (NCO^-) exists as an anion under physiological conditions (pK 3.7), the possibility that the 26-kDa protein is a permease was investigated.

Uptake of cyanate in *cyn* and *cyn* $^+$ *E. coli* strains. To test the possibility that the 26-kDa protein may be involved in the transport of cyanate, an assay for specific transport of cyanate was developed. *cyn* strain SJ100 [the $\Delta(\text{lac-proAB})$ includes the region encoding *CynS*] and its *cyn* $^+$ parent, JM101 (*F'* *lac* also encodes *Cyn*), were induced by 0.5 mM cyanate for 20 min, washed twice with cyanate-free medium, incubated for 0.5 generation to allow degradation or diffusion of cyanate from cells, and then assayed for uptake of

N^{14}CO^- . N^{14}CO^- was added to cultures of JM101 and SJ100 to a final concentration of 50 μM . Samples of the cultures were collected at 1-min intervals and washed by filtration, and activity was determined in a scintillation counter. Cyanate uptake was linear in JM101 for up to 8 min, whereas cyanate uptake was negligible in SJ100 (Fig. 3A). These results indicate that JM101 contains a component of cyanate transport that is not present in SJ100. This component is presumably encoded by the DNA deleted in SJ100 but present on the *F'* *lac* of JM101. To determine the concentration dependence of cyanate uptake, various concentrations of labeled cyanate were added to a culture of JM101 for 5 min before cyanate uptake was quantitated. JM101 showed a concentration-dependent uptake of cyanate that was saturable and inhibited at cyanate concentrations greater than 0.7 mM cyanate (Fig. 3B). SJ100 showed only minimum

uptake of cyanate. A K_m of 400 μM and a $V_{\max}$ of 4.44 nmol/min per 10^9 cells for cyanate uptake by JM101 were calculated from the Lineweaver-Burk plot (8) (Fig. 3B, insert).

Since the *cyn*-deleted strain SJ100 does not exhibit cyanate uptake activity, various plasmids containing different segments of *cyn* DNA were tested for their abilities to confer cyanate uptake ability on SJ100. SJ100(pSJ104) and SJ100 (pDP3) had high levels of cyanate uptake (Fig. 3C), indicating that plasmids pDP3 and pSJ104 contain a gene which is involved in the uptake of cyanate. Since both plasmids pDP3 and pSJ104 contain a gene encoding cyanate permease as well as additional genetic information in the insert, subclones of the insert in plasmid pSJ104 were used to generate plasmids pSJ105 and pSJ147 (Fig. 1), and these were used further to show that the 26-kDa protein encoded by plasmid pSJ105 is required for cyanate transport. Since strain SJ100 containing pSJ105 and pSJ147 produces cyanase constitutively, plasmid pMS421, which encodes *lacI^q* and contains the p15A origin that is compatible with pUC derivatives (6), was transformed into SJ100. The resulting strain was used as a recipient for transformation of plasmids pSJ105 and pSJ147. The cyanate uptake of SJ100(pSJ105, pMS421) was dependent on IPTG induction, and SJ100(pSJ147, pMS421) did not exhibit significant uptake of labeled cyanate under any condition (Fig. 3D). These results and the observation that plasmid pSJ105 but not pSJ147 encodes a 26-kDa protein strongly indicate that the 26-kDa protein is a cyanate permease protein. The gene encoding this protein was designated *cynT*.

Cyanate transport is energy dependent. To further characterize cyanate transport, strain SJ100(pSJ105, pMS421) was used, since the synthesis of cyanate permease in this strain is induced by IPTG. IPTG induction eliminates any possible interference in the transport assay of residual cyanate used for induction. Cyanate uptake was strongly inhibited by uncouplers of oxidative phosphorylation, suggesting that uptake of labeled cyanate is energy dependent (Fig. 3D). *m*-Chlorophenylhydrazine carbonyl cyanide at 10^{-4} M and dinitrophenol at 10^{-3} M completely inhibit uptake of labeled cyanate. The complete loss of cyanate uptake was also observed at 0°C without addition of inhibitor.

Effect of cyanate permease on cyanate toxicity. A *cyn*-deleted strain, SJ100, is more sensitive to cyanate than is its parent, JM101 (24). To determine the role of cyanate permease and cyanase in the inhibition of *E. coli* growth by cyanate, various combinations of *cynT* and *cynS* alleles were used. An experiment was conducted in which various concentrations of cyanate were added to exponentially growing cultures and the OD_{660} was monitored every 30 min for 3.5 h. An examination of the growth curve indicated that cyanate caused growth inhibition followed by a return to uninhibited growth. The length of the lag time in growth was plotted as a function of cyanate concentration for various strains. The MIC was determined. Extrapolation of the length of lag time to 0 lag was used to determine the MIC. Strain SJ100 (Δcyn) had an increased lag time compared with that of JM101 (*cyn*⁺) (MICs, 0.5 versus 1.3 mM, respectively). SJ100 (pDP3) and SJ100(pSJ104), which contain *cynT*⁺ and *cynS*⁺ on multicopy plasmids, were less sensitive to cyanate than JM101 (1.7 versus 1.3 mM, respectively), indicating that additional copies of the *cyn* operon further decrease cyanate toxicity. SJ100(pSJ147, pMS421) was only slightly less sensitive to cyanate than SJ100 (MICs, 0.7 versus 0.5 mM, respectively), although it contains very high cyanase activity when induced by IPTG, but it does not contain permease,

indicating that cyanase itself is not sufficient to overcome cyanate toxicity. In contrast, SJ100(pSJ131), which contains permease but not cyanase, was found to be extremely sensitive to cyanate (MIC, less than 0.1 mM). Since plasmid pSJ131 contains the *cynT* gene but not the *cynS* gene, this observation indicates that uptake of cyanate by cyanate permease is deleterious in the absence of functional cyanase to degrade cyanate. SJ100(pSJ105, pMS421), containing *cynT* and *cynS* under control of the *lac* promoter, was slightly less sensitive to cyanate than SJ100 in the absence of IPTG (MICs, 0.7 versus 0.5 mM, respectively), indicating that at least a low level of *cynT* and *cynS* may be expressed without IPTG induction. However, when SJ100(pSJ105) was induced by IPTG for 20 min before various concentrations of cyanate were added, it was very resistant to growth inhibition by cyanate (MIC, 20 mM). The increased resistance of these cells compared with that of SJ100(pDP3) is presumably due to the induction of *cynT*⁺ and *cynS*⁺ by IPTG before exposure of the cells to cyanate as well as to the higher level expression of *cynT*⁺ and *cynS*⁺ under these conditions.

DISCUSSION

The results of this study indicate that the 26-kDa protein encoded by a previously cloned gene (23, 24) is a cyanate permease, and the gene was designated *cynT*. The *cynT* gene was localized to a region of DNA between *cynS* and its promoter, indicating that *cynT* and *cynS* are part of a polycistronic operon. The observations that the uptake of cyanate is sensitive to temperature and to an uncoupler of oxidative phosphorylation suggest that the uptake of cyanate is energy dependent. The uptake of cyanate appears to be saturable, but it exhibits strong inhibition at a high concentration of cyanate (0.7 mM). Since cyanate at 1 to 5 mM inhibits *E. coli* growth, the substrate inhibition may reflect inhibition of energy metabolism rather than a direct effect on cyanate uptake. If energy metabolism starts to be inhibited at around a 0.7 mM cyanate concentration, the uptake of cyanate, which is energy dependent, may be decreased at this concentration. This indicates that the calculated K_m and $V_{\max}$ values may be overestimated.

While numerous cation transport systems in *E. coli* have been well characterized, few anion transport systems are known. Like cyanate, phosphate, sulfate, and citrate are transported by specific inducible transport systems (12, 13, 19). However, unlike the other anion transport systems, cyanate permease transports a toxic molecule.

The observation that a strain with cyanase on a multicopy plasmid that does not encode a permease is as sensitive to cyanate as a strain devoid of both cyanase and permease suggests that cyanate can inhibit *E. coli* growth without entering the cell. This indicates that cyanate can inactivate protein or other components located outside the cytoplasmic membrane, which results in inhibition of cell growth. On the basis of this observation, we suggest that *E. coli* has evolved not only a cytoplasmic enzyme, cyanase (unpublished data), but also a permease that is required for detoxification of cyanate. In an alternate evolutionary scenario, cyanase would have evolved as a periplasmic enzyme without cyanate permease. However, since *E. coli* can use low concentrations of cyanate as its sole nitrogen source and since endogenous carbamoylphosphate can be decomposed into toxic cyanate, it may be advantageous for *E. coli* to have cyanase localized in the cytoplasmic space. The phenotypes of mutants containing various combinations of *cynT* and *cynS* are very similar to phenotypes of corresponding mu-

tants with mutations in the plasmid-encoded mercury resistance operon. In this system, Hg^{2+} is transported by the *merT* gene product and then detoxified by reduction to Hg^0 by mercury reductase. Since mercury reductase requires a source of NADPH, this enzyme may need to be in the cytoplasm, resulting in a requirement for a Hg^{2+} transport system. This system, like the *cyn* operon, uses a combination of a cytoplasmic detoxification enzyme and a permease to actively transport a toxic compound.

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