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Role of Bicarbonate/CO_2 in the Inhibition of *Escherichia coli* Growth by Cyanate

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Cyanase is an inducible enzyme in *Escherichia coli* that catalyzes the reaction of cyanate with bicarbonate to give two CO_2 molecules. The gene for cyanase is part of the *cyn* operon, which includes *cynT* and *cynS*, encoding carbonic anhydrase and cyanase, respectively. Carbonic anhydrase functions to prevent depletion of cellular bicarbonate during cyanate decomposition (the product CO_2 can diffuse out of the cell faster than noncatalyzed hydration back to bicarbonate). Addition of cyanate to the culture medium of a Δ*cynT* mutant strain of *E. coli* (having a nonfunctional carbonic anhydrase) results in depletion of cellular bicarbonate, which leads to inhibition of growth and an inability to catalyze cyanate degradation. These effects can be overcome by aeration with a higher partial CO_2 pressure (M. B. Guilloton, A. F. Lamblin, E. I. Kozliak, M. Gerami-Nejad, C. Tu, D. Silverman, P. M. Anderson, and J. A. Fuchs, J. Bacteriol. 175:1443–1451, 1993). The question considered here is why depletion of bicarbonate/CO_2 due to the action of cyanase on cyanate in a Δ*cynT* strain has such an inhibitory effect. Growth of wild-type *E. coli* in minimal medium under conditions of limited CO_2 was severely inhibited, and this inhibition could be overcome by adding certain Krebs cycle intermediates, indicating that one consequence of limiting CO_2 is inhibition of carboxylation reactions. However, supplementation of the growth medium with metabolites whose syntheses are known to depend on a carboxylation reaction was not effective in overcoming inhibition related to the bicarbonate deficiency induced in the Δ*cynT* strain by addition of cyanate. Similar results were obtained with a Δ*cyn* strain (since cyanase is absent, this strain does not develop a bicarbonate deficiency when cyanate is added); however, as with the Δ*cynT* strain, a higher partial CO_2 pressure in the aerating gas or expression of carbonic anhydrase activity (which contributes to a higher intracellular concentration of bicarbonate/CO_2) significantly reduced inhibition of growth. There appears to be competition between cyanate and bicarbonate/CO_2 at some unknown but very important site such that cyanate binding inhibits growth. These results suggest that bicarbonate/CO_2 plays a significant role in the growth of *E. coli* other than simply as a substrate for carboxylation reactions and that strains with mutations in the *cyn* operon provide a unique model system for studying aspects of the metabolism of bicarbonate/CO_2 and its regulation in bacteria.

Cyanase (EC 4.3.99.1) catalyzes the reaction of cyanate with bicarbonate to give two molecules CO_2 (15): $NCO^- + 3H^+ + HCO_3^- \rightarrow 2CO_2 + NH_4^+$. The synthesis of cyanase in *Escherichia coli* is induced by addition of cyanate to the growth medium (2). The gene for cyanase is part of the *cyn* operon, which includes three genes in the order *cynT*, *cynS*, and *cynX*, encoding carbonic anhydrase, cyanase, and a hydrophobic protein of unknown function, respectively (11, 30–33). The function of carbonic anhydrase appears to be to prevent depletion of cellular bicarbonate (which diffuses out of the cell as CO_2 faster than noncatalyzed hydration back to bicarbonate) during cyanate decomposition. A Δ*cynT* mutant strain (having a nonfunctional carbonic anhydrase but a functional cyanase) was found to have the following phenotypes: (i) extreme sensitivity to inhibition of growth by cyanate, (ii) inability to degrade cyanate, and (iii) inability to use cyanate as the sole source of nitrogen when grown under aeration with air (0.03% CO_2). However, these phenotypes were eliminated when aeration was with air supplemented with 3% CO_2. Thus, addition of cyanate to the culture medium of the Δ*cynT* mutant strain results in depletion of cellular bicarbonate. This leads to inhibition of growth and an inability to catalyze cyanate degradation, and this effect can be overcome by addition of higher concentrations of CO_2 (12).

The question considered in this report is why depletion of bicarbonate/CO_2 in the Δ*cynT* mutant strain as a result of the addition of cyanate has such an inhibitory effect. Inhibition could simply be due to a lack of bicarbonate/CO_2 required for metabolic carboxylation reactions, including synthesis of oxalacetate (required for the Krebs cycle and/or biosynthetic reactions) and/or carbamoyl phosphate, fatty acids, adenosine, and/or histidine biosynthesis. Alternatively, it may be that cyanate interacts at some other site that normally interacts with bicarbonate/CO_2 and the resulting inhibition is therefore enhanced by a lower bicarbonate/CO_2 concentration. These alternative possibilities have been investigated, and the results reported here indicate that the major effect is due to the latter possibility. In addition, our results indicate that the inhibitory effect of the absence of bicarbonate/CO_2 on growth of *E. coli* is more complex than merely the requirement for metabolic carboxylation reactions.

MATERIALS AND METHODS

Bacterial strains. All bacterial strains used were derivatives of *E. coli* K-12 and were previously described (12). The genotypes and phenotypes of the strains are listed in Table 1. Construction of the plasmid pCAH, encoding a human carbonic anhydrase II, was described by Kozliak et al. (16). Strain BUM100 (Δ*cyn*) was constructed by P1 transduction of HfrR5 (26). P1 grown on SH210 was used to

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TABLE 1. *E. coli* strains used in this study

Strain	Relevant genotype	Expression of:		Reference
		Cyanase	Carbonic anhydrase	
BUM015	Wild type[a]	+	+	12
BUM012	Δ*cynT*	+	−	12
BUM031	*cynS*::*kan*	−	+	12
BUM100	Δ*cyn*[b]	−	−	This study

[a] HfrR5 *lac*$^+$ (HfrR5: Hfr *thi lacY1 supE44 gal-3 malT1 xyl-7 mtlλ2* PO47 [12]).
[b] HfrR5 Δ(*argF-lac*). The deletion is transduced from the strain SH210 (26).

transfer *zai-736*::Tn*10* by selection for tetracycline resistance, and the linked Δ(*argF-lac*)U169, which deletes the *cyn* operon, was identified by assaying for cyanase activity as previously described (4, 5). The phenotype of this strain was confirmed by Western blotting (immunoblotting) (12); neither cyanase nor carbonic anhydrase was detected after 2 h of induction with 0.5 mM cyanate (data not shown).

Media and chemicals. Glucose minimal A medium supplemented with 4 μg of thiamine hydrochloride per ml (19) and deprived of citrate was used for physiological studies. Unless stated otherwise, the minimal A medium was supplemented with arginine (100 mg/liter) and uracil (50 mg/liter). Succinate, α-ketoglutarate, citrate, isocitrate, malate, fumarate, aspartate, histidine, adenosine, and adenine were added in some experiments to this medium at a final concentration of 1 mM. All other media, additions to culture media, biochemicals, and other supplies were prepared or purchased as described previously (12, 16). The surfactant Brij 58 (polyoxyethylene 20 cetyl ether) was added (0.1%) to the culture media supplemented with palmitate and oleate (2 mM each) in order to solubilize the fatty acids (7).

Growth conditions. For growth 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), with air depleted of 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 OD_{600} was observed up to an OD_{600} of 0.6. Potassium cyanate was added to exponentially growing cultures at an OD_{600} of 0.1, and further growth was monitored by the increase in OD_{600}. To ensure exponential growth without lag periods in experiments with air depleted of CO_2, a high initial inoculum resulting in an OD_{600} of >0.08 was required; subsequent growth was monitored at 5-min intervals. Cultures which did not exhibit a normal growth rate were discarded, and only those with a generation time of about 50 min, which corresponds to the noninhibited growth, were used. Cyanate decomposition in vivo was measured as described previously (8).

RESULTS AND DISCUSSION

Inhibition of growth of the wild-type strain by the absence of atmospheric CO_2 can be alleviated by succinate and certain other Krebs cycle intermediates. If the inhibition of growth of BUM012 (Δ*cynT*) by cyanate is due to depletion of bicarbonate needed for normal metabolic functions, then the growth of the BUM012 (Δ*cynT*) mutant strain with cyanate added should be analogous to the growth of the wild-type strain in the absence of bicarbonate/CO_2. Neidhardt et al. (20) observed that the lack of growth in minimal medium under conditions of low bicarbonate concentration can be overcome by using a larger inoculum or by adding succinate. Their implied explanation for these effects was that (i) a larger inoculum provides more metabolic CO_2, thus increasing the concentration of bicarbonate/CO_2 in the cell (needed for oxalacetate formation from pyruvate), and (ii) adding succinate bypasses the need for oxalacetate formation to replenish Krebs cycle intermediates. Those studies provided an explanation for the long lag times often observed when the volume of inoculum was low; i.e., metabolically produced CO_2 diffused out of the cell at a rate that was faster than hydration to bicarbonate, thus keeping the cellular bicarbonate concentration low (20). Similar observations have been made by Repaske et al. (23, 24), who pointed out that in several bacterial species, aspartate (as a precursor of oxalacetate) can overcome the lag due to the absence of bicarbonate in the culture medium or the absence of CO_2 in the aerating atmosphere. We have extended these observations, showing that when CO_2 is absent from the aerating atmosphere, the growth of strains BUM012 (Δ*cynT*), BUM031 (*cynS*::*kan*), BUM100 (Δ*cyn*), and BUM015 (wild type) is insignificant (or the lag period is longer than can be reasonably determined) (Fig. 1 and data not shown). This result is obtained even with an inoculum giving an initial OD_{600} as high as 0.02 to 0.03. The lag time is greatly reduced under these conditions if succinate is present, but the subsequent generation time is somewhat higher than that observed under normal growth conditions (Fig. 1). This result is in some contrast to the finding by Neidhart et al. (20) that the addition of succinate restored normal growth; however, the composition of the growth medium was different from that used here, and CO_2 was not excluded from the aerating gases. We have also found that (i) aspartate, fumarate, and malate have effects similar to those of succinate; (ii) the presence of α-ketoglutarate restores growth to normal after a short lag period; and (iii) isocitrate, pyruvate, acetate, and citrate have no effect (Fig. 1 and data not shown). In Luria-Bertani (LB) medium, growth of these strains is normal when CO_2 is absent from the aerating atmosphere (data not shown); this is presumably due to a higher rate of metabolic CO_2 formation and/or the presence of metabolites that overcome the impact of the absence of CO_2.

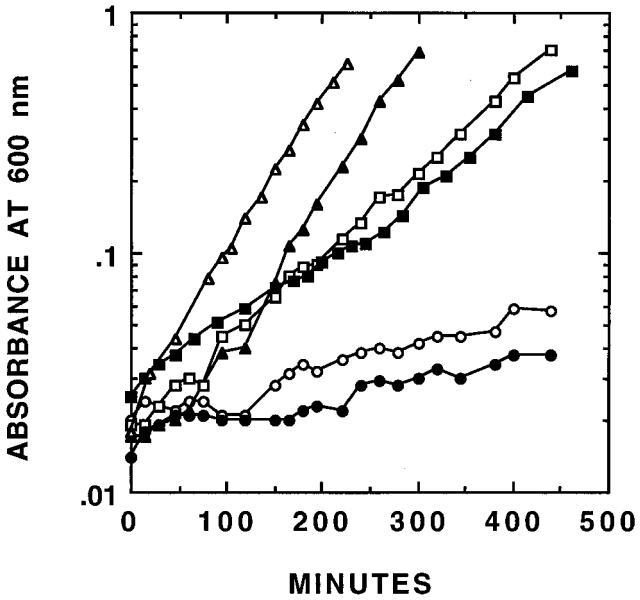

FIG. 1. Growth of the wild-type strain (BUM015) while aerated by air with no CO_2. Glucose minimal medium was inoculated at zero time with a low cell count to obtain an OD_{600} of between 0.01 and 0.02. The medium contained no additives (●) or was supplemented with citrate (○), malate (■), succinate (□), or α-ketoglutarate (▲) (1 mM each). The growth curve of the wild-type strain (BUM015) aerated by air with a normal pCO_2 (0.03%) is shown for comparison (△).

Succinate and other Krebs cycle intermediates do not alleviate the inhibition of BUM012 (Δ*cynT*) growth by cyanate. If the inhibition of growth of BUM012 (Δ*cynT*) by cyanate is due only to depletion of cellular bicarbonate required for replenishing Krebs cycle intermediates, then according to the results described in the previous paragraph, succinate, fumarate, aspartate, malate, and, especially, α-ketoglutarate should alleviate inhibition by cyanate. However, as shown in Fig. 2 (and data not shown), these compounds do not alleviate the inhibi-

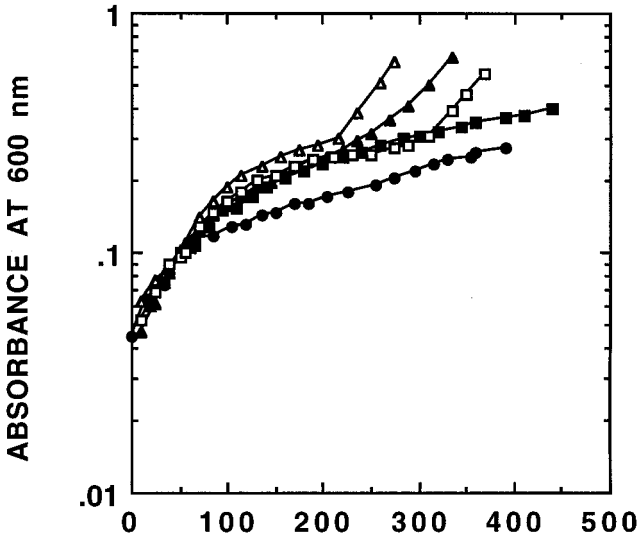

FIG. 2. Effect of several Krebs cycle intermediates on the inhibition of growth of the Δ*cynT* mutant strain (BUM012) by cyanate. Growth in glucose minimal medium (supplemented with arginine and uracil) was as described in Materials and Methods. Cyanate (1 mM) was added when the OD_{600} was 0.1. The growth medium contained no additives (●) or was supplemented with malate (■), aspartate (□), succinate (▲), or α-ketoglutarate (△) (1 mM each).

tion of growth of BUM012 (Δ*cynT*) by 1 mM cyanate. Some temporary initial protection is afforded, resulting in a short delay in the decline in growth, but this is followed by a long period of severely reduced growth until all cyanate is degraded (Table 2), at which time normal growth resumes.

The presence of aspartate, succinate, and, particularly, α-ketoglutarate, does, however, reduce the time required for cyanate degradation (Table 2). The metabolism of these three compounds apparently provides for a somewhat higher steady-state bicarbonate concentration in the cell that is sufficient to support a slow rate of cyanate degradation. However, bicarbonate formed is continually removed as the result of the activity of cyanase so that growth is not supported until all cyanate has been degraded. The relative effects of α-ketoglutarate, succinate, and aspartate on accelerating the rate of cyanate degradation correlate with their effects on reducing the lag time of cell growth in the absence of CO_2 in the aerating gas (see previous section) (Table 2). However, malate and fumarate, which are just as effective as succinate in alleviating inhibition of growth by the absence of CO_2 in the aerating gas, do not affect the rate of cyanate degradation (Table 2). Apparently, these two compounds (in contrast to α-ketoglutarate, succinate, and aspartate) are not catabolized to give CO_2 at a rate sufficient to significantly affect cyanate degradation catalyzed by cyanase.

TABLE 2. Decomposition of 1 mM cyanate by BUM012 (Δ*cynT*) in glucose minimal medium supplemented with Krebs cycle intermediates or their precursors

Strain and additive	Time (min) required for cyanate degradation
BUM012	
No addition	>500
Pyruvate	>500
Citrate	>500
Isocitrate	300
α-Ketoglutarate	185
Succinate	260
Malate	500
Fumarate	490
Aspartate	275
Acetate	>500
Wild type	125

Similar results were obtained when these experiments were carried out with LB medium. As noted in the previous section, growth of BUM012 (Δ*cynT*) in LB medium is not affected by the absence of atmospheric CO_2. However, even when grown at atmospheric partial CO_2 pressure (pCO_2), cyanate significantly inhibits growth of BUM012 (Δ*cynT*) in LB medium (after a short period of continued normal growth following addition of cyanate, as noted in a later section). Addition of succinate or α-ketoglutarate does not protect against this inhibition (data not shown).

These observations indicate that the inhibition of growth of BUM012 (Δ*cynT*) by cyanate is not directly related to a deficiency of Krebs cycle intermediates resulting from the depletion of bicarbonate in the cell. Inhibition of growth resulting from a deficiency of bicarbonate due to the absence of CO_2 in the aerating gas can be overcome by the presence of certain Krebs cycle intermediates. Inhibition of growth resulting from the absence of bicarbonate induced by cyanase-catalyzed degradation of added cyanate cannot be overcome by the presence of these Krebs cycle intermediates. Of course, it is likely that the depletion of bicarbonate induced by the presence of cyanate does result in depletion of Krebs cycle intermediates. However, the fact that succinate and the other Krebs cycle intermediates do not prevent inhibition of growth indicates that there is an additional and very significant inhibitory effect by cyanate that is related to the concentration of bicarbonate/CO_2.

Inhibition of BUM012 (Δ*cynT*) growth by cyanate is not a result of depletion of bicarbonate required for other known carboxylation reactions. An alternative explanation for the inhibitory effect of cyanate could be that inhibition of BUM012 (Δ*cynT*) growth by cyanate is due to depletion of bicarbonate required for carboxylation reactions that are not related to the Krebs cycle. These reactions include histidine and purine syntheses (5-aminoimidazole-4-carboxylic acid ribonucleotide carboxylase), fatty acid synthesis (acetyl-coenzyme A carboxylase), and arginine and pyrimidine syntheses (carbamoyl phosphate synthetase [CPSase]).

Charles and Roberts (6) have reported CO_2-requiring mutants that are auxotrophic for either purines or histidine while growing at a low pCO_2. We found that addition of histidine and either adenine or adenosine has no effect on inhibition of growth of BUM012 (Δ*cynT*) by cyanate (data not shown). Concerning fatty acid synthesis, Repaske et al. (24) reported that oleate can eliminate the lag period in growth due to the absence of bicarbonate in the medium and CO_2 in the aerating gas in some species of bacteria. Harder et al. (13) have shown that palmitate and oleate provide for growth in mutants with impaired lipid synthesis. We found that addition of a mixture of these two lipids to either minimal or LB medium had no effect either on the inhibition of growth of BUM012 (Δ*cynT*) by cyanate or on cyanate decomposition by BUM012 (Δ*cynT*) (data not shown).

CO_2-requiring mutants that are auxotrophic for arginine and/or uracil have been reported by Charles and Roberts (6); these mutants are likely the result of alterations in the enzyme CPSase. An additional consideration with respect to CPSase is

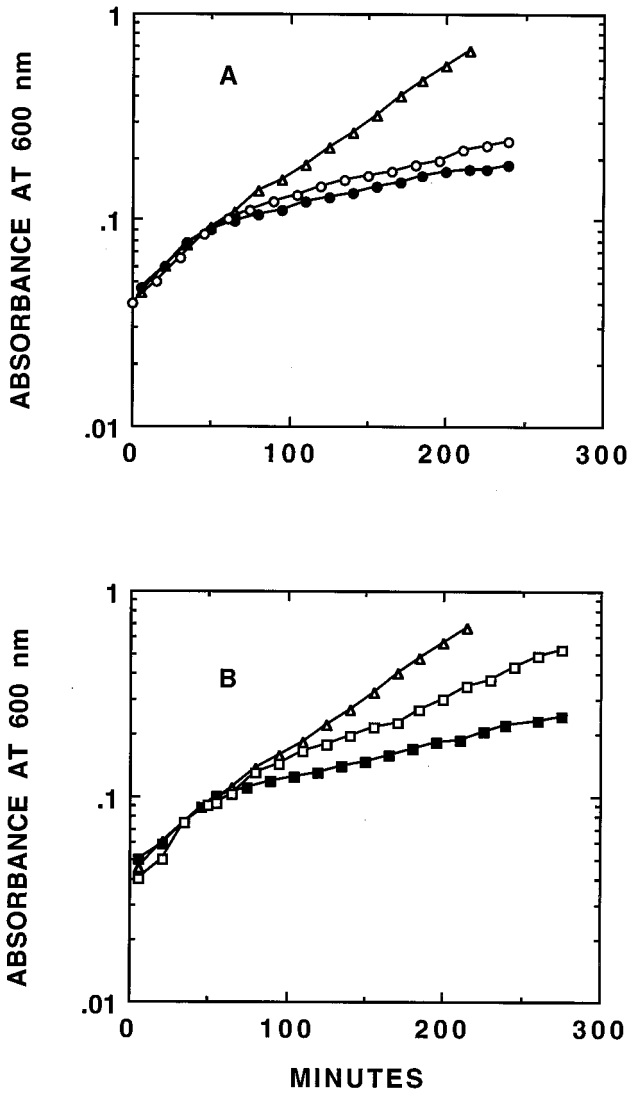

FIG. 3. Inhibition of growth of the wild-type strain (BUM015) and the Δ*cyn* (BUM100) and Δ*cynT* (BUM012) mutant strains by cyanate. The experiments were carried out without arginine or uracil added (closed symbols) and with arginine and uracil present (open symbols). Cells were grown in glucose minimal medium as described in Materials and Methods and were aerated with air (0.03% CO_2); cyanate (0.5 mM) was added when the OD_{600} was 0.1. △, wild type; ● and ○, Δ*cynT* mutant (BUM012); ■ and □, Δ*cyn* mutant (BUM100).

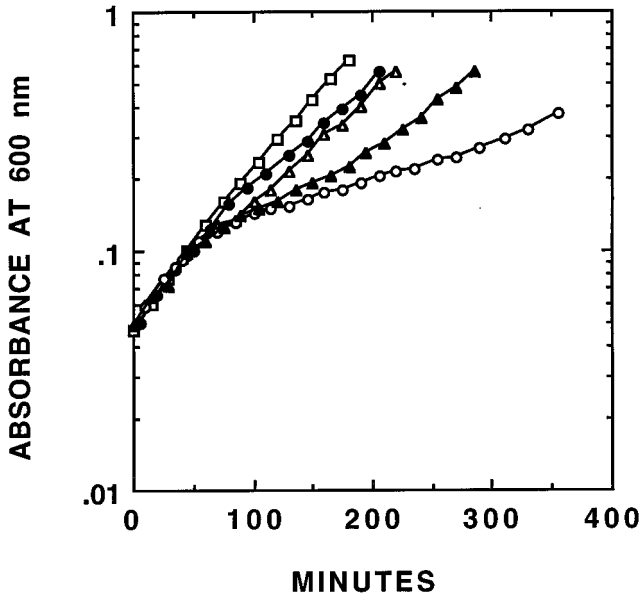

FIG. 4. Effect of addition of α-ketoglutarate, expression of intracellular carbonic anhydrase, and high pCO_2 on inhibition of growth of the Δ*cyn* mutant strain (BUM100) by cyanate. Growth in glucose minimal medium (supplemented with arginine and uracil and aerated with air containing 0.03% CO_2 unless indicated otherwise) was as described in Materials and Methods. Cyanate (1 mM) was added when the OD_{600} was 0.1. ○, BUM100 (Δ*cyn*); ●, BUM100 supplemented with 1 mM α-ketoglutarate; □, BUM100 (Δ*cyn*) aerated with air containing 3% CO_2; ▲, BUM100/pCAH; △, BUM031 (*cynS*::*kan*).

that the enzyme is inhibited by cyanate (3), and this inhibition can be observed physiologically (9, 10); the resulting inhibitory effect on growth can be overcome by addition of arginine and uracil (end products of the two pathways) to the growth medium. We found that addition of arginine and uracil to the minimal medium does afford some protection against the inhibition of growth of BUM012 (Δ*cynT*), as well as BUM100 (Δ*cyn*) (see following section), by cyanate (Fig. 3). However, the degree of this protection by arginine and uracil is quite small, indicating that the inhibition of CPSase (either by bicarbonate depletion or by reaction with cyanate) cannot account for the inhibitory effect of cyanate on growth of either of these two strains.

Finally, simultaneous addition of all metabolites related to carboxylation reactions (succinate, histidine, adenine, oleate and palmitate, arginine, and uracil) in either minimal medium or LB medium was also found to have little effect on cyanate inhibition of growth of BUM012 (Δ*cynT*) (data not shown). This indicates that the predominant inhibitory effect of cyanate on growth of BUM012 (Δ*cynT*), which can be overcome by a high pCO_2 (12), is apparently not due to the shortage of metabolites whose syntheses are dependent on carboxylation reactions.

Cyanate inhibits growth of BUM100 (Δ*cyn*), and the inhibition is alleviated by high pCO_2. A difficulty in studying the influence of bicarbonate/CO_2 on the effect of cyanate on growth of BUM012 (Δ*cynT*) is that cleavage and removal of cyanate occur and complicate interpretations. This difficulty can be eliminated by using strains such as BUM100 (Δ*cyn*) in which cyanase is not expressed and no cyanate-induced shortage of bicarbonate occurs. We found that BUM100 (Δ*cyn*) is, in fact, less sensitive to inhibition by cyanate. As shown in Fig. 3, the inhibitory effect on growth is somewhat less than that observed for BUM012 (Δ*cynT*), especially if the medium is supplemented with arginine and uracil. However, BUM100 (Δ*cyn*) is still significantly inhibited by 0.5 and 1 mM cyanate at a low pCO_2 (Fig. 3B and 4, respectively), and, most importantly, this inhibition can be almost completely alleviated by a higher pCO_2 (Fig. 4). The inhibition can also be partly alleviated by expressing carbonic anhydrase, which likely results in a higher intracellular concentration of bicarbonate (for example, with BUM100/pCAH or BUM031 [*cynS*::*kan*], which expresses carbonic anhydrase but not cyanase) (Fig. 4). Although a high pCO_2 provides significant protection, it does not completely alleviate inhibition of growth of BUM100 (Δ*cyn*) by 1 mM cyanate, in contrast to the effect on inhibition of growth of BUM012 (Δ*cynT*) by cyanate (Table 3 and Fig. 4). The effect of a high pCO_2 is even less for 2 and 3 mM cyanate (Table 3 and data not shown). This probably reflects the fact that in BUM012 (Δ*cynT*) at a high pCO_2, the concentration of cyanate

TABLE 3. Ratios of the generation times of different mutant strains of *E. coli* grown in glucose minimal medium in the absence of cyanate to the generation times of those grown in the presence of cyanate at various concentrations[a]

CO_2 concn (%)	Cyanate concn (mM)	Generation time ratio for strain:			
		BUM012 (Δ*cynT*)	BUM100 (Δ*cyn*)	BUM031 (*cynS*::*kan*)	BUM100/pCAH
0	0	1.00	1.00	1.00	ND[b]
	0.1	0.19	0.70[c]	0.99[d]	ND
	0.3	0.18	0.62[c]	0.95[d]	ND
	0.5	0.15	0.12	0.82[d]	ND
	0.75	0.13	0.11	0.60[d]	ND
	1.0	0.12	0.09	0.20	ND
	2.0	0.11	0.06	0.18	ND
0.03	0	1.00	1.00	1.00	1.00
	0.1	0.86	0.99	1.00	1.00
	0.3	0.27	0.86	0.98	0.97
	0.5	0.25	0.56	0.85	0.92
	0.75	0.22	0.44	0.82	0.82
	1.0	0.20	0.33	0.78	0.56
	2.0	0.19	0.24	0.69	ND
3	0	1.00	1.00	1.00	ND
	0.1	1.00[e]	0.99	0.99	ND
	0.3	1.00[e]	0.96	0.94	ND
	0.5	0.98[e]	0.93	0.87	ND
	0.75	0.97[e]	0.90	0.86	ND
	1.0	0.92[e]	0.84	0.81	ND
	2.0	0.76[e]	0.50	0.69	ND

[a] The generation times (in minutes) of BUM012, BUM100, and BUM031, respectively, grown in the absence of cyanate were as follows: with no CO_2, 50.4, 50.4, and 50.3; with 0.03% CO_2, 48.1, 48.8, and 50.0; and with 3% CO_2, 48.6, 45.8, and 50.0. The generation time of BUM100/pCAH with 0.03% CO_2 was 50.0 min.
[b] ND, not done.
[c] The generation time was determined after a lag period of about 180 min. The nature of this lag period is unknown.
[d] The generation time was determined after the lag period necessary for carbonic anhydrase induction.
[e] Cyanate is decomposed rapidly.

decreases as the result of a significant rate of degradation, thus apparently overcoming inhibition completely; in BUM100 (Δ*cyn*), the concentration of cyanate remains high, since it is not degraded.

These results with BUM100 (Δ*cyn*) are consistent with the results obtained with BUM012 (Δ*cynT*) described in previous sections. Apparently, inhibition of growth by cyanate is not primarily due to depletion of bicarbonate required for biosynthetic carboxylation reactions. Instead, there appears to be some other process involving bicarbonate/CO_2 that is very important for growth and that is inhibited by cyanate when the concentration of bicarbonate/CO_2 is reduced.

A competitive relationship between bicarbonate/CO_2 and cyanate exists. The above-described considerations suggest that cyanate may bind at a specific CO_2/bicarbonate binding site(s) in competition with CO_2/bicarbonate and that this results in inhibition of growth. The effects on growth of four different strains by six different concentrations of cyanate at 0, 0.03, and 3% CO_2 in the aerating gas are shown in Table 3. For each concentration of cyanate, (i) increased resistance to inhibition with increased CO_2 concentration is observed, (ii) the resistance of the strains decreases in the order BUM031 (*cynS*::*kan*) ≅ BUM100/pCAH > BUM100 (Δ*cyn*) > BUM012 (Δ*cynT*), and (iii) for each strain and each concentration of CO_2, an increased cyanate concentration gives increased inhibition (Table 3). With respect to the phenotypes of the different strains, resistance to inhibition of growth by cyanate correlates with an expected increased intracellular concentration of bicarbonate/CO_2; i.e., the presence of carbonic anhydrase without cyanase correlates with greater resistance than the absence of both cyanase and carbonic anhydrase, which in turn correlates with greater resistance than the absence of carbonic anhydrase and presence of cyanase. One minor exception is that the generation time of BUM012 (Δ*cynT*) is lower than that of BUM100 (Δ*cyn*) with no CO_2 at high concentrations of cyanate. This may reflect an unusual situation in which the concentration of bicarbonate/CO_2 is so low that the bicarbonate-consuming action of cyanase in BUM012 (Δ*cynT*) makes little difference. However, the presence of cyanase in BUM012 (Δ*cynT*) results in the degradation of some cyanate entering the cells, thus decreasing the cellular concentration of cyanate, which might alleviate to some extent the inhibition of growth by cyanate.

These observations are consistent with a proposed competitive relationship between bicarbonate/CO_2 and cyanate. Such a competitive relationship could simply be a result of cyanate (or isocyanic acid) competing with bicarbonate/CO_2 for a regulatory or catalytic binding site; cyanate and isocyanic acid are structural analogs of CO_2 (18). Alternatively, cyanate/isocyanic acid may carbamoylate a nucleophilic site that normally undergoes nonenzymatic carboxylation as a result of reaction with bicarbonate/CO_2. Such reactions with CO_2 of physiological importance have been reported (17, 22, 25). Cyanate also reacts with the same functional nucleophilic groups (e.g., -SH, -NH_2, -OH, and -COOH) and may, therefore, compete with CO_2 (3, 28, 29).

The competitive relationship reported here is not unique to Δ*cynT* strains, but it is most conspicuous in the Δ*cynT* strain (BUM012) because an artificially low concentration of bicarbonate occurs when cyanate is added, for the reason cited above. An additional important point in the context of the subject of this study is that inhibition of growth of all strains reported here by cyanate can be alleviated by a higher pCO_2. The observation that full protection is not afforded by 3% CO_2

is not unexpected in such a competitive relationship. Complete protection against inhibition of growth by cyanate by bicarbonate/CO_2 would be observed only at extremely high concentrations of bicarbonate/CO_2, which are inhibitory to growth (16).

Succinate and certain other Krebs cycle intermediates partially alleviate the inhibition of growth of BUM100 (Δ*cyn*) and BUM031 (*cynS*::*kan*) by cyanate. In contrast to their lack of effect on inhibition of growth of BUM012 (Δ*cynT*) by cyanate, α-ketoglutarate, aspartate, and succinate do partially alleviate inhibition of growth of both BUM100 (Δ*cyn*) (Fig. 4) and BUM031 (*cynS*::*kan*) (data not shown) by cyanate. Normal growth proceeds for a short period of time after addition of cyanate; this is followed by a slower growth rate that is faster than the growth rate without these additions (Fig. 4). These effects are likely due to the fact that bicarbonate formed in these two strains as a result of the catabolism of these three compounds would not be immediately removed by the reaction of cyanate with bicarbonate catalyzed by cyanase, as would occur in BUM012 (Δ*cynT*).

As noted above, malate and fumarate cannot substitute for succinate, aspartate, and α-ketoglutarate in reducing the time required for cyanate degradation in BUM012 (Δ*cynT*). These compounds also have no effect on inhibition of growth of BUM100 (Δ*cyn*) or BUM031 (*cynS*::*kan*) by cyanate (data not shown). The reason why malate and fumarate can overcome the inhibition of growth resulting from depletion of bicarbonate due to the absence of CO_2 in the aerating gas but not that resulting from depletion of CO_2 due to the presence of cyanate is not known. It would seem to be due to differences in the rates at which these metabolites can be catabolized to CO_2. The explanation may be related to the observation that under aerobic conditions with glucose as the carbon source, the Krebs cycle apparently does not function at a rapid rate beyond the formation of succinyl coenzyme A in *E. coli* (21).

The delay in inhibition of growth of BUM100 (Δ*cyn*) and BUM031 (*cynS*::*kan*) in LB medium after addition of cyanate is dependent on pCO_2. When 0.5 to 1.0 mM cyanate is added to BUM100 (Δ*cyn*) or BUM012 (Δ*cynT*) in minimal medium, inhibition of growth is immediate. When succinate or other Krebs cycle intermediates are present, a short delay is observed before inhibition of growth occurs (Fig. 2 and 4). In LB medium, however, a delay in inhibition by cyanate is observed without the addition of any additional metabolites (Fig. 5). The length of this delay is dramatically affected by the pCO_2; the higher the pCO_2, the longer the delay of onset of inhibition. Apparently LB medium contains components that exert the same effect as Krebs cycle intermediates on growth of BUM100 (Δ*cyn*) or BUM012 (Δ*cynT*) in minimal medium, i.e., a delay in inhibition of growth by cyanate. These results suggest that the effect of these compounds is to provide a higher concentration of metabolic CO_2 such that inhibition by cyanate is delayed. The temporary nature of the protective effect of these compounds and the effect of external pCO_2 on the length of delay in inhibition emphasize the importance of the intracellular CO_2 concentration in alleviating inhibition by cyanate. A higher external CO_2 concentration contributes to the intracellular CO_2 concentration, thus increasing the effectiveness of these compounds in delaying inhibition by cyanate, but a high (3%) external CO_2 concentration itself is not sufficient for extended protection.

Conclusions. The function of the carbonic anhydrase among enzymes encoded in the *cyn* operon is to maintain bicarbonate/CO_2 in the cell as cyanate is degraded. The depletion of bicarbonate/CO_2 in the Δ*cynT* mutant strain when cyanate is added is the primary cause of the inhibition of cell growth by cyanate. The apparent major effect of a reduced amount of bicarbonate/CO_2 is that it increases the susceptibility to cyanate inhibition; i.e., there appears to be competition between bicarbonate/CO_2 and cyanate at an unknown site. This site may be quite important for growth; perhaps this explains why cyanate is more toxic for rapidly growing tumor cells (14, 27).

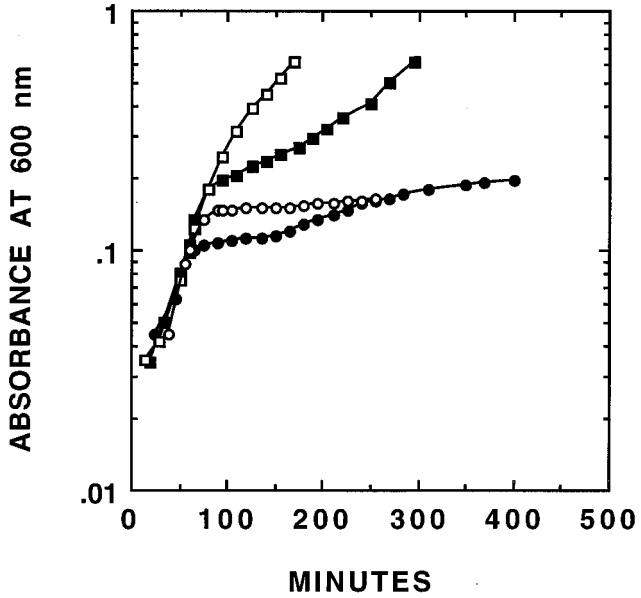

FIG. 5. Effect of pCO_2 on inhibition of growth of the BUM100 (Δ*cyn*) mutant strain by cyanate in LB medium. Cells were grown as described in Materials and Methods. Cyanate (3 mM) was added when the OD_{600} was 0.1. ○, no CO_2; ■, 0.03% CO_2; □, 3% CO_2. A growth curve for cells grown in glucose minimal medium aerated with 0.03% CO_2 is shown for comparison (●).

These results suggest that CO_2 may play a previously unrecognized role(s) other than providing bicarbonate needed for replenishing Krebs cycle intermediates or for other carboxylation reactions. Under normal circumstances this function of CO_2 would not be easily observed except at a very low pCO_2, but under such conditions the carboxylation reactions become growth limiting. The Δ*cynT* mutant strain grown in the presence of cyanate, as well as the other strains with mutations in the *cyn* operon, represents a model system for depleting the cell of bicarbonate at normal atmospheric pCO_2 and may afford a unique opportunity to study some aspects of the effects of bicarbonate/CO_2 on metabolism in bacteria.

Carbonic anhydrase appears to be more important than cyanase for alleviating inhibition of growth by cyanate. If only cyanase is present (BUM012 [Δ*cynT*]), the cells are very susceptible to inhibition of growth by cyanate. However, if only carbonic anhydrase is present (BUM031 [*cynS*::*kan*]), cell growth is not particularly sensitive to cyanate at physiological concentrations of bicarbonate/CO_2. The importance of the presence of carbonic anhydrase is emphasized by the observation that the growth of BUM031 (*cynS*::*kan*) is much less sensitive to cyanate inhibition than is the growth of BUM100 (Δ*cyn*) at a low pCO_2. Thus, the presence of carbonic anhydrase alone significantly reduces cyanate toxicity (presumably by increasing the intracellular concentration of bicarbonate/CO_2). This suggests that the role of the entire *cyn* operon (encoding cyanase plus carbonic anhydrase) is to provide ammonia from cyanate rather than to detoxify cyanate. This may be reflected in the fact that many organisms do not possess a *cyn* operon and apparently do not need to utilize cyanate as a source of nitrogen. Protection against inhibition of growth by

cyanate could be significant for some organisms, such as thermophiles, in which carbamoyl phosphate would be rapidly degraded to cyanate at high temperatures (1). It is possible that such protection may be accomplished by maintaining a high intracellular bicarbonate/CO_2 concentration by simply expressing a carbonic anhydrase rather than the entire *cyn* operon.

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