Isolation and Characterization of *Escherichia coli* Mutants Lacking Inducible Cyanase

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(Received 14 July 1986; revised 17 September 1986)

To determine the physiological role of cyanate aminohydrolase (cyanase, EC 3.5.5.3) in bacteria, mutants of *Escherichia coli* K12 devoid of this inducible activity were isolated and their properties investigated. Five independent mutations were localized next to *lac*; three of them lay between *lacY* and *codA*. Thus cyanase activity could depend on the integrity of one gene or set of clustered genes; we propose for this locus the symbol *cnt*. Growth of the mutant strains was more sensitive to cyanate than growth of wild-type strains. This difference was noticeable in synthetic medium in the presence of low concentrations of cyanate (≤ 1 mM). Higher concentrations inhibited growth of both wild-type and mutant strains. Urea in aqueous solutions dissociates slowly into ammonium cyanate. Accordingly wild-type strains were able to grow on a synthetic medium containing 0.5 M-urea whereas mutants lacking cyanase were not. We conclude that cyanase could play a role in destroying exogenous cyanate originating from the dissociation of carbamoyl compounds such as urea; alternatively cyanate might constitute a convenient nitrogen source for bacteria able to synthesize cyanase in an inducible way.

INTRODUCTION

Cyanate aminohydrolase (EC 3.5.5.3), also termed cyanase, is an inducible enzyme in bacteria, which hydrolyses cyanate into ammonium and hydrogencarbonate:

This enzyme has been found in *Escherichia coli* B (Taussig, 1960) and in a *Flavobacterium* sp. (Guilloton & Hargreaves, 1972). Purification and kinetic studies of the enzyme from *E. coli* B have been achieved (Anderson, 1980). It has been demonstrated that carbon dioxide is an essential activator for the enzymes from both *Flavobacterium* sp. and *E. coli* (Guilloton & Hargreaves, 1972; Anderson, 1980). The covalent structure of the enzyme has been determined (Chin *et al.*, 1983).

The presence of this enzyme in bacteria raises the question of its physiological role, and also of the availability of its substrate, cyanate. Cyanate is not, to our knowledge, the product of any metabolic reaction; this molecule may be considered as a by-product of the metabolism of carbamoyl compounds, chiefly carbamoylphosphate and urea. Anderson (1980) proposed that cyanase might hydrolyse endogenous cyanate arising from the dissociation of carbamoylphosphate. On the other hand, urea dissociates slowly into ammonium cyanate (Marier & Rose, 1964); urea present in some biological fluids could constitute an exogenous source of cyanate. The experiments reported here were done in order to determine the possible roles of cyanase in bacteria.

METHODS

Bacterial stains and transducing phage. All strains were derivatives of *Escherichia coli* K12. They are listed in Table 1. The transducing phage used was P1 vir, kindly supplied by Dr Maxime Schwartz, (Institut Pasteur, Paris,

Table 1. *E. coli* strains

Strain	Genotype	Source or reference
H	Hfr (λ^+)	M. Schwartz
R5	Hfr <i>lacY mal mtl thi</i> (λ^+)	J. H. Miller
pop 1375	F ⁻ <i>trpA his supF recA srl::Tn10</i>	M. Schwartz
C600 F' <i>lac</i>	F ⁻ <i>cnt lac</i> /F' <i>cnt⁺ lac⁺</i>	F. Jacob
C600	F ⁻ <i>leu cnt lacY trp his</i> (λ^+)	
ED 8689	F ⁻ <i>cnt lac gal</i> (λ^-)	G. G. Wilson
PC 0698	F ⁻ <i>purA thr leu proA pyr codA lacY gal xyl mtl mal lam tsx phx rpsL</i>	P. G. de Haan
MC 4100	F ⁻ [<i>ara D139</i>] Δ (<i>argF-lac</i>)169 <i>flb-5301 ptsF25 relA1 rpsL150 deoC1</i> (λ^-)	B. J. Bachmann
X7026	F ⁻ Δ (<i>gpt-lac</i>)5 <i>supE44 relA1 spoT1 thi-1</i> (λ^-)	
JA 200	F ⁺ <i>thr leu lacY recA trpE5</i>	A. Piérard
BGM 017	F ⁻ <i>gal proC rpsL</i>	This work
BGM 022	as R5 but <i>cnt2</i>	
BGM 023	as R5 but <i>cnt3</i>	
BGM 024	as R5 but <i>cnt4</i>	
BGM 025	as R5 but <i>cnt5</i>	
BGM 035	as Hfr H but <i>cnt15</i>	
BGM 132	as PC 0698 but <i>gal⁺</i>	
BGM 133	as PC 0698 but <i>lac⁺ gal⁺</i>	
BGM 137	as PC 0698 but <i>cod⁺ cnt2 lacY gal⁺</i>	
BGM 160	F ⁻ <i>cnt lac srl::Tn10 recA</i>	
BGM 182	as PC 0698 but <i>gyrA</i>	

France). Strain BGM 017 is a Cnt⁺ Lac⁺ transductant of a *proC rpsL* derivative of strain ED 8689. Strain BGM 132 is a Gal⁺ transductant of strain PC 0698. BGM 133 is a Lac⁺ revertant of BGM 132. BGM 137 was obtained by infecting BGM 133 with phage P1 grown on BGM 022 and selecting for the Cod⁺ character; resulting transductants were scored for Lac⁻ Cnt⁻. In order to obtain strain BGM 160 (*recA*), strain C600 F'*lac⁺* was cured of the episome; the resulting F⁻ *cnt lac* strain was transduced to tetracycline resistance using a phage P1 grown on strain pop 1375 (*recA srl::Tn10*). This strain was scored as a Rec⁻ strain among the resulting Tet^R Srl⁻ transductants. Strain BGM 182 was obtained by plating PC 0698 on L broth supplemented with nalidixic acid (40 μ g ml⁻¹) (Hane & Wood, 1969).

Genetic techniques. Conjugation and P1 mediated transduction were done as described by Miller (1972).

Culture media. L broth and synthetic medium (minimal A medium) were prepared as described by Miller (1972). Glucose was added to this synthetic medium at a concentration of 0.4%. Indicator plates used in the detection of the Cnt⁺ phenotype were composed of Difco Nutrient Broth (0.8%) supplemented with Difco Bacto agar (2%, w/v, Batch no. 418470) and NaCl (0.5%); recrystallized KNCO was added to a final concentration of 10 mM, after sterilization and cooling to 60 °C.

Growth conditions. Bacteria were cultivated at 37 °C with shaking in 25 × 250 mm test tubes; the optical density (OD) of the tubes was measured at 575 nm with a Coleman Junior AII spectrophotometer. OD₅₇₅ was proportional to cell density up to 0.6. To study the effects of cyanate on the physiology of bacteria, overnight cultures in standard synthetic medium were diluted into the same medium to an OD₅₇₅ of approximately 0.05. Potassium cyanate was added when the OD₅₇₅ reached 0.2 and growth was monitored for a further 150 min.

Assay of cyanate. Spectrophotometric determination of cyanate was done according to Guilloton & Karst (1985).

Assay of cyanase. Cell-free extracts or toluene-treated cells were incubated at 37 °C in the presence of 20 mM-veronal buffer, 5 mM-KNCO and 5 mM-KHCO₃, pH 8. Ammonium produced by the enzymic hydrolysis of KNCO was estimated by using the Berthelot reaction (Lubochinsky & Zalta, 1955). One unit of cyanase is defined as the amount of enzyme which catalyses the production of 1 μ mol NH₄⁺ min⁻¹ under these conditions.

Test for the presence of cyanase in toluene-treated cells. Cells to be tested for the presence of cyanase were treated with toluene at 37 °C for 30 min and mixed with 1 ml of a medium containing 5 mM-veronal, 5 mM-KNCO, 1 mM-KHCO₃ and 0.1% bromothymol blue, pH 7. Cyanate hydrolysis resulted in an increase of the pH and the colour turned deep blue.

RESULTS AND DISCUSSION

Identification of strains with inducible cyanase activity

Description of the Cnt⁺ phenotype. Cyanase activity could be induced in wild-type *E. coli* cells growing exponentially in L broth when cyanate was present at concentrations ranging from 1 to 10 mM. Specific activities of cyanase ranging from 0.25 to 0.5 units (mg protein)⁻¹ were

Fig. 1. Induction of cyanase by azide. *E. coli* Hfr H was grown at 37 °C in standard synthetic medium. Sodium azide was added to exponentially growing cultures when the OD₅₇₅ reached 0.2. After a further incubation of 60 min, growth was stopped by addition of chloramphenicol (40 µg ml⁻¹), and the cells were harvested by centrifugation and washed with buffered saline. Cyanase activity was measured in toluene-treated cells (see Methods).

obtained. Higher concentrations of cyanate were toxic and led to growth inhibition and low enzyme levels.

Such induction was also observed in wild-type cells cultivated on solid nutrient media. Colonies of wild-type cells growing on cyanase indicator plates (see Methods) showed a white precipitate in their centre (constituting the Cnt⁺ phenotype) while colonies of cyanase-lacking strains remained translucent. This colony appearance of wild-type cells was strongly dependent upon the presence of both phosphate and calcium ions in the medium. Thus the Cnt⁺ phenotype was believed to result from the precipitation of a calcium phosphate salt. The formation of this insoluble salt is probably driven by the alkalization of the medium following cyanate hydrolysis. This precipitate also formed in replica-plated colonies and in spot tests of wild-type cells grown on the same indicator medium. This identification of the Cnt⁺ phenotype was particularly useful in screening the clones obtained after conjugation or transduction experiments.

Induction of cyanase by azide. Azide (N=N=N)⁻, which is isosteric and isoelectronic to cyanate (N=C=O)⁻ (Wagner, 1965), induces cyanase in a *Flavobacterium* sp. (Guilloton & Hargreaves, 1972). It also induced this activity in *E. coli*, a maximum specific activity of 2.3 units (mg protein)⁻¹ being obtained with strain Hfr H grown in standard synthetic medium in the presence of 1 mM-NaN₃ (Fig. 1). Since this concentration (and higher ones) substantially inhibited cell growth, induction of cyanase was routinely obtained with an azide concentration of 0.3 mM both in liquid and solid media. Cyanase could be detected by the cyanase test on toluene-treated cells (see Methods).

Routinely, all the clones obtained after conjugation or transduction experiments were grown on the cyanase indicator plates and on L broth/azide plates. All the strains exhibiting the Cnt⁺ phenotype were shown to possess a cyanase activity inducible by either cyanate or azide. Conversely none of the Cnt⁻ strains synthesized cyanase in the presence of either cyanate or azide.

Genetic characterization of cnt mutants

Mutagenesis and selection of mutants. Wild-type cells (Cnt⁺ phenotype) were plated on solid L broth medium and mutagenized by UV until 1% survival was obtained. After an incubation of 24 h, the cells were scraped, suspended in buffered saline and plated on cyanase indicator medium in order to obtain 300 colonies per plate. Translucent colonies were subcloned on the

Table 2. *Analysis of recombinants obtained from conjugation between Hfr H and C600 F⁻ (leu cnt lac trp his)*

Selected phenotype	No. of recombinants	Non-selected phenotypes (linkage, %)				
		Leu ⁺	Lac ⁺	Cnt ⁺	Trp ⁺	Lac ⁺ Cnt ⁺
Leu ⁺	88	—	16 (18%)	16 (18%)	0 (0%)	16 (18%)
Lac ⁺	98	73 (74%)	—	98 (100%)	0 (0%)	98 (100%)
Cnt ⁺	88	64 (73%)	88 (100%)	—	1 (1%)	88 (100%)
Trp ⁺	81	16 (20%)	7 (9%)	7 (9%)	—	7 (9%)

Table 3. *Cotransduction of lac with cnt*

Donor strain (relevant genotype)	Recipient strain (relevant genotype)	Selected phenotype (no.)	Unselected phenotype		Linkage (%)
			Cnt ⁺	Cnt ⁻	
Hfr H	ED 8689 (<i>cnt lac</i>)	Lac ⁺ (665)	665	0	100
Hfr H	BGM 022 (<i>cnt2 lacY</i>)	Lac ⁺ (211)	184	27	87
BGM 133	BGM 137 (<i>cnt2 lacY</i>)	Lac ⁺ (324)	284	40	88
BGM 017	BGM 023 (<i>cnt3 lacY</i>)	Lac ⁺ (358)	358	0	100
Hfr H	BGM 024 (<i>cnt4 lacY</i>)	Lac ⁺ (190)	153	37	80
Hfr H	BGM 025 (<i>cnt5 lacY</i>)	Lac ⁺ (99)	98	1	99
BGM 035 (<i>cnt15</i>)	R5 (<i>lacY</i>)	Lac ⁺ (772)	174	598	77

same medium and their phenotype was checked. One Cnt⁻ strain could be isolated for every 2000 Cnt⁺ colonies.

Mapping of cnt mutations. We have studied five selected mutants (BGM 022, 023, 024 and 025, derivatives of Hfr R5, and BGM 035, a derivative of Hfr H) along with two 'naturally' Cnt⁻ strains held by our laboratory (ED 8689 and C600 F⁻).

An interrupted mating experiment using Hfr H and C600 F⁻ (*leu cnt lac trp his*) showed that the time of entry was identical for the Cnt⁺ and Lac⁺ characters. Phenotypic analysis of the selected recombinants showed a strong linkage between the *cnt* and *lac* markers (Table 2). Thus, the *cnt* mutation studied lies close to *lac* at 8 min on the linkage map (Bachmann, 1983).

In order to determine the location of the *cnt* mutations more precisely, transductional crosses were made between Cnt⁺ and Cnt⁻ strains. Since the cyanase indicator medium cannot be used as a selection medium for the Cnt⁺ clones, mapping experiments were done by transduction of characters linked to *cnt* and subsequent analysis of the transductants. Transduction experiments using Lac⁺ donor strains and Lac⁻ recipient strains showed that the Lac and Cnt characters were closely linked. [Strain C600 F⁻ (*leu cnt lac trp his*) could not be transduced for Lac⁺ by P1 generalized transduction; generalized transduction by T4GT (Wilson *et al.*, 1979) also failed to transduce this strain for the Lac⁺ phenotype.] The frequencies of cotransduction were between 77 and 100% (Table 3). Localization of three *cnt* mutations (*cnt2*, *cnt4* and *cnt15*) was achieved

Table 4. Mapping of *cnt* mutations using three-point analysis

Cross (relevant phenotype)	Selected phenotype (no. of clones tested)	Unselected phenotypes (no. of clones)	Frequency (%)
ϕ P1 [BGM 035] (<i>cnt15</i>)	Cod ⁺ (306)	Cnt ⁻ Lac ⁺ (218)	71
×		Cnt ⁻ Lac ⁻ (43)	14
PC 0698 (<i>codA lacY</i>)		Cnt ⁺ Lac ⁻ (30)	10
ϕ P1 [BGM 022] (<i>cnt2 lacY</i>)	Cod ⁺ (92)	Cnt ⁺ Lac ⁺ (15)	5
×		Cnt ⁻ Lac ⁺ (20)	22
BGM 133 (<i>codA</i>)		Cnt ⁻ Lac ⁻ (54)	59
ϕ P1 [BGM 024] (<i>cnt4 lacY</i>)	Cod ⁺ (52)	Cnt ⁺ Lac ⁻ (1)	1
×		Cnt ⁺ Lac ⁺ (17)	18
BGM 133 (<i>codA</i>)		Cnt ⁻ Lac ⁺ (22)	42
ϕ P1 [BGM 133] (<i>codA</i>)	Cod ⁺ (324)	Cnt ⁻ Lac ⁻ (22)	42
×		Cnt ⁺ Lac ⁻ (0)	0
BGM 133 (<i>codA</i>)		Cnt ⁺ Lac ⁺ (8)	15
ϕ P1 [BGM 133] (<i>codA</i>)	Lac ⁺ (324)	Cod ⁻ Cnt ⁺ (220)	68
×		Cod ⁺ Cnt ⁺ (64)	20
BGM 137 (<i>cnt2 lacY</i>)		Cod ⁺ Cnt ⁻ (38)	12
		Cod ⁻ Cnt ⁻ (2)	1

Table 5. Phenotypes of sexductants obtained by crossing *F*⁻ strains with C600 *F'**lac*

Strain [relevant genotype]	Phenotypic trait		
	Cod	Cnt	Lac
MC 4100 [Δ (<i>argF-lac</i>)]	wt	—	—
MC 4100 [Δ (<i>argF-lac</i>)] <i>F'</i> <i>lac</i>	wt	+	+
X 7026 [Δ (<i>gpt-lac</i>)]	wt	—	—
X 7026 [Δ (<i>gpt-lac</i>)] <i>F'</i> <i>lac</i>	wt	+	+
C600 [<i>cnt lac</i>]*	wt	—	—
C600 [<i>cnt lac</i>] <i>F'</i> <i>lac</i> *	wt	+	+
BGM 132 [<i>codA lacY</i>]	—	wt	—
BGM 132 [<i>codA lacY</i>] <i>F'</i> <i>lac</i>	+	wt	+

* The strain C600 employed was C600 (*leu cnt lac trp his*).

by three-point analysis using *codA* strains (de Haan *et al.*, 1972). These experiments are summarized in Table 4. The results of four analyses showed that the three mutations *cnt15*, *cnt2* and *cnt4* were located between *codA* and *lacY* (Table 4). Cotransduction frequencies of *cnt* with *codA* were between 80 and 88%. Three-point analysis was not done with strains BGM 023 (*cnt3*), BGM 025 (*cnt5*) and ED 8689 since the cotransduction frequencies of *lac* with the *cnt* mutations were too high.

From these experiments, it appeared that the *cnt* mutations studied were located at the same locus. The strong linkage between *cnt*, *codA* and *lac* was further demonstrated by two independent lines of evidence. Strain C600 *F'**lac*⁺ is phenotypically Lac⁺ Cnt⁺ and becomes Cnt⁻ Lac⁻ when cured of the episome. From the results presented in Table 5 it can be concluded that the *F'**lac* episome also carries *cnt* and *codA* wild-type genes since all sexductants obtained from *F*⁻ Lac⁻ Cnt⁻ became Lac⁺ Cnt⁺ whereas strains BGM 132 (*codA lacY*) acquired the Cod⁺ and Lac⁺ phenotypes after sexduction. Moreover we have detected in a copy of the Clarke and Carbon colony bank of *E. coli* (Clarke & Carbon, 1976) a clone, pLC19-3, which is able to transmit both the Cnt⁺ phenotype in a Cnt⁻ strain (as BGM 160) and the Cod⁺ phenotype in a Cod⁻ strain (as BGM 182). In addition cells harbouring pLC19-3 showed a sixfold amplification of azide-induced cyanase over JA 200 (*cnt*⁺) or cells harbouring other clones from the colony bank. pLC19-3 from our copy gave the same qualitative and quantitative results as pLC19-3 obtained from Dr B. Bachmann (Coli Genetic Stock Center).

Fig. 2. Growth of strains Hfr R5 (Cnt⁺) and BGM 023 (Cnt⁻) in standard synthetic medium with the addition of cyanate (1 mM or 2 mM) when the OD₅₇₅ reached 0.2 (arrows). Generation times relative to this experiment are listed in Table 6.

Table 6. Doubling times of strains Hfr R5 and BGM 023 in the presence of various concentrations of cyanate

KNCO concn (mM)	Doubling time (min)	
	Hfr R5	BGM 023
0	60	60
0.2	62	68
0.5	64	88
1.0	150*	130
	67*	
2.8	>400	>400
4.0	>600	>600

* Addition of 1 mM-cyanate to a culture of Hfr R5 was followed by two-phase growth (see text and Fig. 2).

Growth inhibition induced by cyanate

Effect of cyanate on wild-type and mutant strains. The physiological difference between wild-type strains (Cnt⁺) and Cnt⁻ mutants lies in the sensitivity towards cyanate added to cultures in synthetic medium (Table 6, Fig. 2). Addition of moderate concentrations of cyanate (< 1 mM) to cultures of wild-type strains (e.g. Hfr R5) did not produce perceptible inhibition and the added cyanate was progressively hydrolysed. On the other hand cultures of mutant strains (e.g. BGM 023) showed growth inhibition in the presence of cyanate concentrations as low as 0.2 mM (Table 6); generation times observed for this strain were always higher than those for Hfr R5 in the presence of the same cyanate concentrations, and no cyanate hydrolysis occurred. Addition of 1 mM-KNCO to a culture of Hfr R5 induced a two-phase growth pattern (Fig. 2): a first phase with a generation time of 150 min was followed by almost normal growth with a generation time of 67 min; added cyanate was completely hydrolysed at the end of the growth inhibition phase. Addition of 1 mM-KNCO to a culture of mutant strain BGM 023 resulted in a transient growth inhibition after which a stable generation time of 130 min was observed. No cyanate hydrolysis occurred. Growth of both strains was equally inhibited by cyanate concentrations of 2 mM and higher: generation times were always longer than 400 min (Table 6, Fig. 2); no cyanate hydrolysis could be detected in either case and assay of cyanase in strain Hfr R5 showed that very low enzyme levels were obtained [~ 0.01 units (mg protein)⁻¹].

Influence of pH on inhibition by cyanate. This was studied with the Cnt⁻ mutant strain BGM 023 (Table 7). Growth inhibition by KNCO was enhanced at higher H⁺ concentrations.

Table 7. *Influence of pH on growth inhibition mediated by cyanate in synthetic medium*

Standard synthetic medium was supplemented with 0.1 M-MES and the pH of the resulting solution was adjusted to the indicated values with concentrated H_3PO_4 or 60% (w/v) KOH. The inoculum consisted of a mid-exponential-phase culture of BGM 023. The control doubling time for each pH value is the mean value of four determinations, with the standard deviation in parentheses. Potassium cyanate was added to test cultures when the OD_{575} reached 0.2.

pH	Doubling time (min)		
	Control	0.1 mM-KNCO	1 mM-KNCO
7.5	61 (6)	68	80
7.0	58 (4)	68	127
6.5	58 (6)	75	> 600
6.0	61 (6)	103	> 600
5.5	57 (5)	> 600	> 600

Within experimental error, lowering the pH by 1 unit had the same effect on generation time as raising cyanate concentration by a factor of 10. Since pK_a of isocyanic acid is 3.7 (Boughton & Keller, 1966), isocyanic acid concentration is proportional to $[\text{H}^+]$ in the pH range 7.5–5.5. Thus, growth inhibition induced by cyanate is likely to be mediated by isocyanic acid.

We have observed that growth inhibition is essentially the consequence of a specific effect of cyanate on arginine biosynthesis (see the accompanying paper: Guilloton & Karst, 1987). Since the metabolic target is likely to be intracellular, the influence of pH on cyanate-mediated growth inhibition might indicate that isocyanic acid (and not cyanate) is the molecular species that crosses the cell membrane.

Physiological role of cyanase

Cyanase and endogenous cyanate. Anderson (1980) postulated that cyanase could play a role in the detoxification of endogenous cyanate arising from the spontaneous dissociation of carbamoylphosphate. Carbamoylphosphate dissociates into cyanate and phosphate and its half-life is 38 min at 37 °C (Allen & Jones, 1964). Assuming that endogenous cyanate is toxic, inducible cyanase would represent an advantage for all bacteria synthesizing carbamoylphosphate.

We have screened our collection of *E. coli* strains and we found that only half of them are Cnt^+ . However, one should take this result with caution since laboratory strains of *E. coli* are propagated on media on which cyanase – according to the above hypothesis – would not be very useful; numerous transfers may lead to the loss of the Cnt^+ character. Moreover, some $\text{Lac}^- \text{Cnt}^-$ strains harboured deletions in the *lac* locus and these deletions might extend into the *cnt* locus.

We screened a number of strains purified from clinical swabs for the Cnt^+ character and found that only 60% of the *E. coli* strains (82/137) tested possessed the Cnt^+ phenotype. Inducible cyanase was present in *Serratia marcescens* (16/16) and *Pseudomonas aeruginosa* (61/66). Cyanase activity could not be demonstrated among a variety of other bacteria including *Proteus* species (0/53), *Streptococcus faecalis* (0/43) and *Staphylococcus aureus* (0/73). It was of particular interest that the five *Salmonella typhimurium* strains tested were all Cnt^- . These observations suggest that if cyanase plays a role in destroying endogenous cyanate, this role may not be absolutely essential for bacterial survival.

Cyanase and exogenous cyanate. From the preceding results it may be seen that low concentrations of extracellular cyanate (<0.2 mM) could inhibit growth of Cnt^- cells provided that H^+ concentration is sufficiently high. Looking for such environmental conditions we found that solutions of urea (0.1–0.5 M) could be considered as potential sources of cyanate. Calculations after Hagel *et al.* (1971) indicate that 0.3–0.5 M solutions of urea at pH 6–7 are able to generate concentrations of cyanate in the 0.1 mM range. Assay of cyanate in such urea solutions demonstrated that these concentrations were actually obtained after an incubation of

Fig. 3. Assimilation of ammonium from cyanate by Cnt^+ cells. *E. coli* strains BGM 023 (Cnt^-) (a) and C600 $F' \text{lac}$ (Cnt^+) (b) were cultivated in minimal medium from overnight cultures in the same medium. When the cultures reached mid-exponential phase they were diluted 1/20 in minimal medium modified with respect to the nitrogen source ($\circ$, 1 mM- NH_4^+ ; $\bullet$, 1 mM- NCO^- ; $\triangle$, no added nitrogen source); the OD_{575} of the diluted cultures was then monitored. Residual growth in the medium without nitrogen source could be accounted for by the presence of approximately 0.4 mM- NH_4^+ carried over in the inoculum. Growth of strain BGM 023 in the presence of cyanate could be accounted for by a slow assimilation of ammonium from the inoculum and spontaneous hydrolysis of cyanate (which has a half-life of 27 h at 37 °C in the culture medium).

Table 8. Cyanate formation in dilute solutions of urea

Freshly made solutions of urea were sterilized by filtration and incubated at 37 °C. Cyanate concentration was estimated according to Guilloton & Karst (1985).

Time (h)	Cyanate concn after incubation (mM)		
	Initial urea concn:		
	0.3 M	0.4 M	0.5 M
22	0.14	0.20	0.23
49	0.37	0.46	0.72
76	0.54	0.59	0.92

Table 9. Influence of urea on growth of Cnt^+ and Cnt^- strains

Suspensions of Cnt^+ and Cnt^- strains were deposited on a solid synthetic medium composed of 0.1 M-MES, 5 mM- KH_2PO_4 , 8 mM- $(\text{NH}_4)_2\text{SO}_4$, 1.7 mM-trisodium citrate (pH adjusted to 6.5 with concentrated KOH), plus glucose (0.4%), thiamin hydrochloride (1 $\mu\text{g ml}^{-1}$) and 2% agar. The salts solution and additives were mixed with molten sterile agar at 60 °C. Urea from a 10 M filter-sterilized solution was added to the plates. Plates were incubated for 24 h at 37 °C in order to ensure a preliminary dissociation of urea. Cell suspensions were deposited on the agar surface and plates were incubated for a further 48 h. Growth was estimated semi-quantitatively from + + + + (excellent growth, as in the controls) to 0 (no growth).

Strain (genotype)	Urea concn . . .			
	0.3 M	0.4 M	0.5 M	
Hfr R5	+ + + +	+ + + +	+ + +	
BGM 022 (<i>cnt2</i>)	+	0	0	
BGM 023 (<i>cnt3</i>)	+ + +	0	0	
BGM 024 (<i>cnt4</i>)	+ + +	+ +	0	
BGM 025 (<i>cnt5</i>)	+ + +	+	0	
Hfr H	+ + + +	+ + + +	+ +	
BGM 035 (<i>cnt15</i>)	+ + +	0	0	

22 h at 37 °C (Table 8). We tested the growth of wild-type (Cnt⁺) and mutant (Cnt⁻) strains on a solid synthetic medium containing various amounts of urea (Table 9). Urea at concentrations of 0.4–0.5 M inhibited growth of Cnt⁻ strains while growth of Cnt⁺ strains was little affected. Natural fluids, such as urine from ureotelic animals, can contain 0.3–0.4 M-urea. Therefore in these media cyanase-bearing strains could probably be at an advantage over other bacteria. We have also observed that *E. coli* strains with inducible cyanase (Cnt⁺) could grow in a synthetic medium with cyanate as the only nitrogen source whereas Cnt⁻ mutants could not (Fig. 3). Therefore cyanate might constitute a convenient source of nitrogen for Cnt⁺ bacteria.

Thus, cyanase could play a role in relation to exogenous cyanate; taxonomic studies of the Cnt⁺ phenotype should help to identify the environmental conditions capable of maintaining cyanase in bacterial cells.

We wish to acknowledge Dr P. G. de Haan and Dr B. J. Bachmann for supplying *E. coli* strains, Dr M. Schwartz for supplying the P1 phage, Dr A. Piérard for a gift of a copy of the Clarke–Carbon colony bank and Dr Y. Rautlin de la Roy who supplied numerous purified bacterial strains from swabs. We thank Dr F. Nau for valuable collaboration in preparing the manuscript.

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