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Why Is Carbonic Anhydrase Essential to Escherichia coli?

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A large proportion of the open reading frames (ORFs) located on fully sequenced chromosomes are of unknown or uncharacterized Escherichia coli K-12 ORFs, we have been replacing selected ORFs with reporter cassettes (23). One of our targets, yadF, has been demonstrated to be a carbonic anhydrase (CA) of the β-class. Its X-ray crystal structure has been solved, and its biochemical properties have been characterized (10). Our initial attempts to delete yadF were unsuccessful, suggesting that it might encode an essential gene product. Although CA has been reported to be involved in cell processes such as photosynthesis, respiration, and CO₂ transport in other organisms and in cyanate metabolism in Escherichia coli (see reference 33 for a recent review), none of these processes is expected to be necessary for the viability of E. coli.

CAs are zinc metalloenzymes that catalyze the interconversion of carbon dioxide (CO₂) and bicarbonate anion (HCO₃⁻). Why should this activity be needed? The well-understood function of CynT, the only E. coli paralog of YadF, suggests a reason. CynT is a normally repressed CA that is induced during cyanate metabolism. Its role is to prevent the depletion of intracellular bicarbonate which accompanies the cyanase-catalyzed bicarbonate-dependent hydrolysis of cyanate (16). CynT rehydrates the CO₂ which is produced and thus prevents its loss by rapid diffusion from the cell. Bicarbonate can be regarded as a coenzyme of cyanase (CynS), and CynT provides for its regeneration.

E. coli requires a supply of bicarbonate/CO₂ as a metabolic substrate during normal growth. It is needed not only for biosynthesis of various small molecules but also for fatty acid biosynthesis and in central metabolism. The small molecules, which include arginine, pyrimidines, and purines, can be provided as supplements and, interestingly, certain mutants limited in the production of these nutrients can be suppressed with increased levels of CO₂ (8). However, the need for CO₂ in central metabolism cannot be replaced with supplements. The only known supply pathway for bicarbonate is via the hydration of CO₂. Although it has long been known that E. coli requires CO₂ to grow (7), under most conditions an adequate supply is generated endogenously. CO₂ reacts spontaneously with water to produce bicarbonate, but we calculate here (see Discussion) that, during growth in minimal glucose medium in air, the demand for bicarbonate is 10³- to 10⁴-fold greater than would be provided by uncatalyzed hydration at the prevailing steady-state concentration of CO₂. We therefore propose that the function of yadF, which we show here to be expressed at all times, is to meet this shortfall.

Three classes of CA (α, β, and γ) have been reported to occur in bacteria. They are unrelated in sequence and widespread among organisms of all three kingdoms of life. Indeed, E. coli YadF can correct the oxygen-sensitive phenotype of a β-CA deletion mutant of Saccharomyces cerevisiae (10, 15). In addition to the β-CAs, YadF and CynT, E. coli also encodes several gene products (CaiE, PaaY, and YrdA) which are clearly homologous to γ-group CAs, with fully conserved active-site residues. CaiE and PaaY are, like CynT, included in inducible operons that are each concerned with a particular process: CaiE in carnitine metabolism (11) and PaaY in phenylacetic acid degradation (13). Eichler et al. (11) proposed that CaiE is involved in the generation or regeneration of a coenzyme for CaiB/CaiD. The function of these two proteins has been clarified recently (12) and CaiB shown to be an acyl coenzyme A transferase. We suggest that the biochemistry of this pathway may be analogous to that of fatty acid biosynthesis.

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and that bicarbonate may be a coenzyme for this transferase activity.

The *Ralstonia eutropha* homolog of YadF, Can, has recently been characterized (20) and found to be essential. Unpublished results of these authors (quoted in reference 20) show that Can is able to suppress an *E. coli* yadF mutant and that YadF can suppress a *can* mutant in *R. eutropha*. In view of the close homology, demonstrated activities, and likely interchangeability of the two proteins, we suggest that YadF also be assigned the name Can (and its encoding gene, *can*).

We both demonstrate and calculate here that CA is required for the growth of *E. coli* at the CO₂ concentration characteristic of air. CA activity is normally supplied by the product of *can*, which is always expressed. Can, however, can be replaced by CynT, if induced, by the archael α-Ca, Can, or by cultivation under sufficiently elevated levels of CO₂.

### MATERIALS AND METHODS

#### Strains and media.
Strains used in the present study are described in Table 1. Cultures were grown at 37°C in either LB broth (Lennox) (1% tryptone [Difco], 0.5% yeast extract [Difco], 1% NaCl) or VB minimal medium (37) supplemented with thiamine (10 μg/ml) and 0.2% glucose, unless otherwise stated. Kanamycin, ampicillin, and chloramphenicol were used at concentrations of 50, 100, and 20 μg/ml, respectively. Arabinose, when required for *pBAD* induction, was added at concentrations ranging from 0.2 to 1%. Azide (Sigma) was used at 0.1 mM for cynT induction. Cyanate (Aldrich) was added to VB medium-glucose agar at 0.5 mM to score CynT activity or functional suppression of cynT by other CAs. To obtain variations in growth time, supplements to minimal medium were used at the following concentrations: Casamino Acids, 0.5%; asparagine, 100 μg/ml; and tryptophan, adenine, uracil, or cytosine, 20 μg/ml. Anaerobic growth in jars with CO₂ was achieved with the Oxoid gas generating kit AN35, which produces an atmosphere with 4 to 10% CO₂ and 20% O₂ to water through a palladium-catalyzed reaction with H₂ supplied by the kit. BBL Dry Anaerobic Indicating Strips were used to confirm that anaerobiosis had been attained. For growth under H₂, air was exhausted and replaced with H₂ from a cylinder; a Pd catalyst ensured that any remaining O₂ was converted to H₂O. For liquid cultures bubbled with mixtures of CO₂, N₂, and O₂, gas composition and delivery were controlled from the Bio Console ADI1035 of an Applikon fermenter.

### RESULTS

Phylogenetic distribution of *can* and *cynT*. We analyzed the phylogenetic distribution and chromosomal neighborhoods of *can* and *cynT* bioinformatically by using the MicroBial Genome Database (http://mbgd.genome.ad.jp) and the NCBI database BLAST resources (http://www.ncbi.nlm.nih.gov:80/BLAST). The local genetic organization surrounding *can* is conserved only among the sequenced *Enterobacteriaceae*. Traces of this organization can still be observed in *Vibrio cholerae* but not in other sequenced bacteria in which *can* homologs are found. Although phylogenetic profiling analysis of sequenced genomes did not identify other genes sharing the *can* phylogenetic distribution, *can* is conserved, and homology searches suggest its vertical inheritance at least during the evolution of the γ-proteobacteria. *cynT*, in contrast, appears to be absent from *E. coli*'s closest relatives and is much more sporadic in its occurrence, suggesting recent horizontal transfer, perhaps initially from plants, where close homologs are common.

Deletion of *can*. The *can* deletion/replacement we designed spans coordinates 3334 to 3909 on section 12 of the *E. coli* K-12 chromosome (GenBank AN AE000122) and removes most of the coding sequence, including the region corresponding to the conserved Zn-binding residues of the postulated catalytic site.
EDCM421 (Table 1) has a copy of can growth medium contained sufficient deletant EDCM464 then became possible, provided that the araBAD copy should not be expressed from LB medium-glucose, a condition in which the complement-strain EDCM464 was able to grow for a complete growth cycle for growth in standard aerobic laboratory conditions. LB plates lacking arabinose. We conclude that Can is essential why growth stops; we believe that this ref

iments showed that EDCM464 can divide 7 to 8 times before the same medium and incubated further (Fig. 1). Repeated experiments showed that Can depletion does not cause any morphological abnormalities (data not shown); Can-depleted cells simply appear to stop growing.

CynT can replace Can. cynT transcription can be gratuitously induced with a sublethal concentration of azide (16). If the cynT CA can replace Can, it should be possible to maintain a can deleted strain by addition of azide. We found that the can<->FLK2 mutation could be P1 transduced from EDCM464 to EDCM637 (MG1655 ΔlacZ; Table 1) to create EDCM636, provided that transductants were selected and maintained on azide-containing plates. Thus, CynT synthesized from a single gene copy and from its own promoter can satisfy the cell’s need for CA activity, and Can is not specifically required for viability.

CA was depleted from EDCM636, previously grown overnight with 0.1 mM azide, by inoculating into LB medium with-out azide. As shown in Fig. 2, LB medium-grown cells of EDCM636 stop dividing 1.7 generations after azide removal, indicating rapid loss of CynT. Figure 2 also shows that EDCM636 cultivated in VB medium-glucose achieves only a 62% mass increase. Note that whereas EDCM464 pregrown in arabinose grows to stationary phase, growth stops earlier if EDCM464 is pregrown with azide rather than arabinose. This suggests, since cultures grown overnight with arabinose can complete a full growth cycle, that Can, expressed from PBAD, reaches a stationary-phase level manifold higher than is required for growth. Also, since EDCM464 inoculated from azide outgrows EDCM636, it appears that the PBAD expression system is leaky, at least during stationary-phase incubation. To avoid complications caused by this, EDCM636 was used, where possible, for all subsequent experiments.

Growth without oxygen. When either EDCM464 or EDCM636 is grown on LB plates in anaerobic jars under H2 and CO2 (see Materials and Methods), colonies indistinguishable from wild-type are formed; thus, Can appears to be dispensable for growth under these conditions. Is this because of the increased CO2 partial pressure (which also increases the rate of supply of HCO3−) or because of the lack of O2? Oxygen sensitivity has been reported as a phenotype associated with deletion of the can homolog NCE103 from Saccharomyces cerevisiae (15), and can mutants of R. eutropha can grow in an atmosphere enriched with CO2 (20). To distinguish between the need to exclude O2 and a requirement for an increased partial pressure of CO2, we tested the ability of EDCM636 to grow anaerobically under H2 alone and found that it cannot, indicating that it must be the increased partial pressure of CO2 in the earlier experiment that allowed growth.

Growth in air with added CO2. To determine whether increased CO2 partial pressure would restore growth in air, EDCM636, pregrown with azide, was grown in liquid with or without added CO2 (Fig. 3A). When a mixture of 21% O2 and 79% N2 was continuously bubbled through EDCM636 in LB medium, the usual CA depletion growth pattern was seen, whereas the parental strain EDCM367 grew with a total yield about twofold greater than usual. This demonstrates that atmospheric CO2 is not needed to support growth of the parental

![FIG. 1. Can is required for continued growth in air. EDCM464 and its parent EDCM421, grown overnight in LB medium-arabinose, were inoculated into LB medium-glucose at 37°C. Cultures were allowed either to grow for one complete cycle (※) or were kept growing by dilution into the same medium (□). ■. Total growth (OD × dilution factor) of the diluted cultures maintained at low OD.](http://jb.asm.org/)

![FIG. 2. Depletion of CA from can deleted strains. (Left panel) Growth curves of EDCM636 (▲) and its parent EDCM367 (●) in LB medium at 37°C. Strains were grown overnight in LB medium (with 0.1 mM azide for EDCM636) and inoculated into LB medium. (Right panel) Comparison of CA depletion in EDCM464 (triangles) and EDCM636 (▲) in VB minimal medium plus 0.2% glucose. EDCM636 was pregrown overnight in VB medium-glucose-azide and EDCM464 in VB medium-glycerol-arabinose (▲) or VB medium-glycerol-azide (△).](http://jb.asm.org/)
strain. If a 2% CO$_2$ plus 21% O$_2$ plus 77% N$_2$ gas mixture was bubbled through the culture medium instead, the parental strain EDCM367 grew as before, and growth of EDCM636 continued into stationary phase, albeit at a slower rate. Thus, increased CO$_2$ in the growth environment at least partially ameliorates the growth defect resulting from lack of Can, a finding consistent with the idea that Can is required to maintain the CO$_2$/bicarbonate concentration in the cell. Note the inflected shape of the growth curve of EDCM636 with CO$_2$. Initial rapid growth (to 100 min: dilution of preexisting CA) is followed by growth at a reduced rate (to 250 min); growth rate then increases again. can expression was measured during growth of the CA depleted cultures and will be described below.

**Growth with no gas phase.** In vessels filled with liquid, once the dissolved O$_2$ is consumed during the early growth of the culture, further growth is necessarily anaerobic and fermentative, and CO$_2$ equilibrates between medium and cytoplasm rather than being lost by diffusion. During fermentative growth under acid conditions, *E. coli* produces CO$_2$ abundantly (in alkali, formic acid is excreted instead, see Discussion). To test whether endogenously produced CO$_2$ is sufficient to support growth of the mutant, cultures were inoculated at pH values ranging from 5 to 9 and then incubated with (flasks) or without (in filled containers) a gas phase. Figure 4A shows that, under aerobic conditions (with gas exchange), growth of the mutant quickly stops at any pH in the range. Figure 4B shows, however, that EDCM636 can grow quite well anaerobically at an acidic pH (pH 5 or 6), although only poorly (pH 7 or 8) or hardly at all (pH 9) under more alkaline conditions. Figure 4C confirms that the parental strain can grow without air at all pH values between 5 and 9, although the growth yield is slightly reduced at lower pH values. We attribute the ability of EDCM636 to grow in bottles without air at acid pH to the fact that endogenously produced CO$_2$ necessary for growth of the mutant, cannot escape. To further confirm this interpretation, we attempted to grow EDCM636 anaerobically in an open vessel to confirm that it was not the absence of O$_2$ that permitted growth. Flask cultures, in LB medium at pH 6, were continuously flushed with argon to both exclude O$_2$ and to facilitate the escape of endogenously generated CO$_2$. We found (data not shown) that EDCM636 was unable to grow under these conditions, a finding consistent with the idea that CO$_2$ is needed for anaerobic growth.

**Can expression responds to environmental changes.** In EDCM636 can is replaced by the reporter cassette FLK2, which permits the monitoring of gene expression by measurement of β-galactosidase activity. Because CA activity can be supplied by CynT, we can monitor can expression in the absence of Can protein.

(i) Expression is inversely proportional to growth rate. Figure 5A shows the expression of can during a single growth cycle (growth without dilution) in LB medium. Initially, as exponential growth starts in the presence of azide, the specific activity (SA) drops from the higher level characteristic of overnight cultures. There is then a short period during which SA remains low and constant. After this, as the growth rate decreases (MG1655 growing in LB medium always slows when it reaches an OD$_{600}$ of 0.4) the rate of expression of can increases and continues to do so as growth rate decreases further. When azide is absent, the initial growth rate is slow and specific expression of can is relatively high; expression continues even after growth has apparently ceased. To examine growth rate dependence in more detail, we grew EDCM636 with azide in several different media to vary the generation time from ~20 to ~150 min. Figure 5B shows that expression decreases with growth rate over a threefold range (from ~120 to ~40 Miller units [MU]). Although we did not achieve slower exponential growth rates, we were able to measure expression in cultures that were growing extremely slowly because azide, air, or CO$_2$
can expression, an rpoS derivative of EDCM636, EDCM638 was constructed. Figure 6A shows that can is expressed at the same levels in both strains at OD_{600} up to 2.6. At higher ODs the expression is greater, by ~1.5-fold, in the RpoS^+ strain. Since this difference is small and since RpoS controls the expression of a variety of proteins that regulate expression during stationary phase, we do not know whether the effect is direct or indirect.

(iv) can is not autoregulated. In EDCM464 can is under the control of P_{BAD}. Figure 6B shows that induction of Can synthesis from P_{BAD} does not affect the activity of the native can promoter; thus, can expression is not repressed by Can. Depletion of Can obviously does not cause significant derepression, as exponentially growing azide cultures of can deletion strains show low levels of expression. We conclude that can expression is not autoregulated.

(v) can expression is increased at high temperature. Annotation of can in the database suggests that it may have a heat shock promoter, although evidence of heat shock induction was not noted in a microarray experiment (31). We find (Fig. 6C) that there is a twofold increase in can expression after transfer from 30 to 42°C, in contrast to the characteristic decrease in expression which accompanies an increase in growth rate due to improved nutrient supply. We have not tested whether this response is dependent on the heat shock induction system since mutating dnaK or rpoH reduces the growth rate, which would itself cause increased can expression. Site-directed mutagenesis of the putative rpoH binding region has not been attempted.

(vi) Expression with added CO_2. Expression from the can promoter in EDCM636 was monitored during growth under a CO_2-enriched atmosphere (Fig. 3). Growth is initially at a normal rate; it then ceases in the absence of CO_2 or proceeds more slowly in the presence of CO_2. During the initial growth phase, can expression decreases from its stationary phase level and then increases as growth slows in both the CO_2-supplemented and the nonsupplemented cultures. SAs (Fig. 3B) are comparable for the two cultures, despite the great difference in total enzyme synthesized (Fig. 3A). When the supplemented culture reached an OD_{600} of 0.8, the growth rate again increased and can expression decreased. It is clear that can expression in these cultures is responding to growth rate changes rather than to the presence or absence of CO_2.

Suppressors of Δcan. In order to see whether mutations which obviate the need for Can occur, suppressor mutants of EDCM636 were sought. Of 50 streaked colonies, 6 yielded progeny able to grow without azide. All of these mutations were at least 50% cotransductationally linked to lac, which is adjacent to the cyn operon; these mutants most likely produce CynT constitutively. We constructed a cynT can double mutant by selecting for transductants under CO_2 in anaerobic jars, where no CA is needed. A total of 10^{10} cells of double mutants was constructed. Figure 6A shows that there is a twofold increase in can expression.

Figure 4. Effect of pH on growth of EDCM636. Strains were inoculated into LB medium-glucose at pHs from 5 to 9 and grown either aerobically in flasks or anaerobically in filled syringes (see Materials and Methods). (A) EDCM636 grown in air; (B) EDCM636 grown anaerobically; (C) EDCM637 grown anaerobically.

FIG. 4. Effect of pH on growth of EDCM636. Strains were inoculated into LB medium-glucose at pHs from 5 to 9 and grown either aerobically in flasks or anaerobically in filled syringes (see Materials and Methods). (A) EDCM636 grown in air; (B) EDCM636 grown anaerobically; (C) EDCM637 grown anaerobically.

were limiting. These cultures, with an OD_{600} of between 0.1 and 0.4, all expressed can at high levels (~200 to ~700 MU).

(ii) Expression can also vary with cell density. In Fig. 5A, the SA does not remain constant even during the period of exponential growth. A constant SA could only be achieved during exponential growth by frequently diluting to maintain cell density within a narrow range. In the experiment shown in Fig. 5C, a culture of EDCM636 was divided into two; the OD_{600} of one culture was maintained below 0.07 and of the other between 0.1 and 0.2 by dilution as necessary. The SA of the denser is 1.5-fold greater than that of the more dilute.

(iii) RpoS has a small effect on can expression. Increased expression at high OD or during slowed growth is often attributable to the participation of the stationary-phase transcriptional sigma factor RpoS. To see whether RpoS is involved in...
tested. We therefore obtained a strain, BL21(DE3)/pcam-AC, with a copy of the Methanosarcina thermophila cam gene (1) cloned under the control of a T7 promoter from the laboratory of J. G. Ferry, where it had been demonstrated to produce large quantities of active protein. We introduced the can deletion into this strain (in the presence of azide) to create SM1. When IPTG (isopropyl-D-thiogalactopyranoside) was added to induce T7 RNA polymerase, SM1 was able to grow on LB plates in the absence of azide, although the colonies were small, indicating that this enzyme can at least partially substitute for a β-CA.

At least three native E. coli proteins—YrdA, CaiE, and PaaY—share significant similarity with Cam. The gene for each of these, along with cyanT and can, was amplified by using PCR and cloned into the high-copy-number vector pUC18. Of the three putative γ-CAs, only PaaY was visibly expressed, although CynT and Can supported growth in the absence of azide. Conjecturing that the expression signals of the others might be weak or that massive overexpression might be toxic, we cloned them instead into pTRC99A, which provides all expression signals up to and including an initiation codon and from which expression of the cloned genes is IPTG inducible. Because pTRC99A carries the lacIq gene, the cloned genes are not expected to be expressed until IPTG is added. The plasmids were then tested for their ability to suppress can mutations. A single colony of each type of EDCM636 transformant was streaked onto LB medium plus IPTG with or without azide and also used to inoculate a broth culture, supplemented with azide and IPTG, which was used, after overnight growth, to prepare material for the protein gel shown in Fig. 7. Each of the proteins, other than CynT, is clearly visible as an overexpressed protein band of about the expected size. The large amounts of PaaY obtained presumably reflect the high copy number of the pUC18 vector. Although the cyanT and can genes cloned in parallel efficiently complemented and/or suppressed can, plasmids containing the possible γ-CA genes did not suppress the need for Can. If the expressed proteins are enzymatically active, we conclude that they are not likely to have significant CA activity.

Phenylacetic acid (PAA) is an inducer of the enzymes of the Paa pathway, which allows it to be used as a sole carbon source; EDCM 637 is able to form colonies on plates containing no
other source of carbon. EDCM636, however, does not grow on PAA plates or on glucose or LB plates containing PAA, indicating that induction of PaaY from its chromosomal gene does not provide a CA activity that can support growth.

**DISCUSSION**

The experiments described here show that *E. coli* cannot grow in air in the complete absence of a functional CA (either Can or CynT when induced) and that this inhibition is relieved by added or endogenous high concentrations of carbon dioxide. The reactions catalyzed by CA are the forward and reverse processes in the formation of bicarbonate, which is at rapidly established equilibrium with carbonic acid:

$$\text{CO}_2 + \text{OH}^- \rightleftharpoons \text{H}^+ + \text{HCO}_3^-$$

$k_a = 6.35$

The uncatalyzed rates of the hydration and dehydration of CO$_2$ were first accurately measured by Mills and Urey (26). These authors showed that the forward pseudo-first-order rate constant $k_1$ is 0.0275 s$^{-1}$ at 25°C and estimated the backward rate constant $k_{-1}$ to be 80 s$^{-1}$.

CO$_2$/bicarbonate is both produced and consumed by *E. coli* metabolism. If hydration or dehydration were the only rapid process involved, the system would approach equilibrium with a half-time of ca. 10 ms, there would be no reason to distinguish between the metabolic roles of CO$_2$ and its hydration products, and CA would not be required. However, molecular CO$_2$ is rapidly lost from the cell by passive diffusion and, if this causes the steady-state to remain far from equilibrium, the rates of production and consumption of CO$_2$ and bicarbonate have to be kept individually in balance.

The main metabolic reactions that generate CO$_2$/bicarbonate in *E. coli* during aerobic growth are those catalyzed by the enzymes pyruvate dehydrogenase, 2-oxoglutarate dehydrogenase, isocitrate dehydrogenase, 6-phosphogluconate dehydrogenase, and β-ketoacyl[acyl carrier protein] synthase. The mechanisms of these and related enzymes have been extensively studied (29, 30, 34, 38, 39), and in all cases the decarboxylation mechanism generates molecular CO$_2$. During fermentation, the formate dehydrogenase component of formate-hydrogen lyase provides another potential source of CO$_2$/bicarbonate.
Although less conclusive in this case, mechanistic studies (3, 6) strongly suggest that molecular CO₂ is the true product of formate oxidation.

The principal reactions in which CO₂/bicarbonate is consumed are those catalyzed by the enzymes phosphoenolpyruvate carboxylase, carbamoyl phosphate synthetase, 5-aminimidazole ribotide carboxylase, and biotin carboxylase. The mechanisms of these enzymes have been extensively studied (9, 18, 27, 35, 36), and in every case it has been shown that the true substrate is bicarbonate.

In summary, endogenous CO₂/bicarbonate is generated in E. coli predominantly and perhaps wholly in the form of molecular CO₂, both during aerobic growth and in fermentation. A steady-state concentration of this species is established in the cell, such that the rate of loss by diffusion is equal to the net rate of production. The only significant source of the bicarbonate required as a substrate for metabolism is the hydration of this CO₂. In the absence of CA, the rate of generation of bicarbonate depends on the steady-state concentration of CO₂ and on the rate constant for the uncatalyzed hydration reaction. Under the conditions in which CA is an essential enzyme for growth of E. coli, the rate of the uncatalyzed reaction at the prevailing intracellular concentrations of CO₂ is too low to meet the demand for bicarbonate.

To make this description more quantitative, it is necessary to propose an explicit model for the steady-state concentration of CO₂. The diffusion coefficient for a dilute solution of CO₂ in water at 20°C is 1.78 × 10⁻⁹ m² s⁻¹ (22), and this provides the principal constraint on the overall diffusion rate, unless cell boundary structures provide a significant extra barrier. No direct measurements of the diffusivity of molecular CO₂ across the cell boundary of E. coli cells appear to have been made, but in the case of red blood cells the most recent and sensitive measurements (14) have shown that the cell membrane provides only a small additional diffusive barrier. Diffusion equations (4) can be combined with the aqueous diffusion coefficient and the shape and size of a typical E. coli cell (19) to estimate that if the net rate of production of CO₂ in a cell is F mol s⁻¹, the excess steady-state concentration (above the equilibrium concentration corresponding to the gas phase composition) will be about F × 10¹¹ mol liter⁻¹.

The results of careful measurements of the flux rates of individual processes in the central metabolic pathways of E. coli growing under a variety of precisely defined conditions have been presented (17). For E. coli ML308 growing at 0.94 h⁻¹ aerobically on glucose, the overall net production rate is 14.78 mol of CO₂·kg⁻¹ h⁻¹ (dry weight biomass)·h⁻¹. An average cell (28) has a dry weight of 2.8 × 10⁻¹² g and would therefore produce 1.15 × 10⁻¹⁷ mol of CO₂·s⁻¹ under these conditions. If the proposed model for diffusion provides a reasonable approximation, the expected excess intracellular concentration of CO₂ is ca. 10⁻⁷ mol liter⁻¹. If the gas phase concentration is zero, this will support a maximum uncatalyzed flux from CO₂ to bicarbonate of ca. 3 × 10⁻²⁸ mol·s⁻¹ in a single cell. Air normally contains 0.03% by volume of CO₂, and this will produce a concentration of 10 μM in the cell; in the absence of CA, this could support a flux of ca. 3 × 10⁻²² mol·s⁻¹.

The potential demand for bicarbonate can be estimated by considering the following three classes of pathway in which it is required.

(i) Incorporation into E. coli cell mass. During growth on simple substrates, one gram atom in each mole of arginine or nucleotide base is derived from bicarbonate ion, incorporated by the reactions catalyzed by carbamoyl phosphate synthetase and 5-aminimidazole ribotide carboxylase. In the cells described by Holms, generation of 14.78 mol of CO₂·kg⁻¹ h⁻¹ accompanied a growth rate of 0.94 h⁻¹. From the analysis of 1 g (dry weight) of E. coli (see reference 28, p. 135), it may be calculated that this corresponds to a requirement for 7.4 × 10⁻²⁰ mol·s⁻¹ of bicarbonate for a single cell. In growth on complex media, biosynthesis of amino acids and nucleic acid precursors is not necessary, and these pathways should contribute no demand for bicarbonate under these conditions.

(ii) Bicarbonate/CO₂ cycles. Bicarbonate is incorporated into malonyl coenzyme A, via biotin carboxylase, in the first committed step of fatty acid biosynthesis and is released as CO₂ in a subsequent condensation step. One molecule of bicarbonate is required for each two-carbon unit incorporated into lipid. Under the conditions outlined in the previous paragraphs, this corresponds to a flux of 1.79 × 10⁻¹⁹ mol·s⁻¹ in a single cell.

Bicarbonate/CO₂ is cycled in a similar way during the metabolism of cyanate, except that incorporation and release occur during the single step catalyzed by cyanase. Cyanate can be used by E. coli as its sole source of nitrogen and, under these conditions, there would be an additional demand for 7.46 × 10⁻¹⁹ mol of bicarbonate·s⁻¹ in a single cell (normally provided by CynT).

(iii) Carboxylation in central metabolism. Bicarbonate is the substrate for phosphoenolpyruvate carboxylase; the flux through this reaction varies considerably when E. coli is grown aerobically on a variety of simple substrates (17). For E. coli ML308 growing at 0.94 h⁻¹ aerobically on glucose, the measured flux corresponds to the use of 2.01 × 10⁻¹⁰ mol of bicarbonate·s⁻¹ in a single cell.

Thus, during growth at a moderate rate even on complex media, E. coli has a demand for ca. 2 × 10⁻¹⁰ mol of bicarbonate·s⁻¹ per cell. This demand will increase somewhat if biosynthesis of amino acids and nucleic acid bases is required and will increase by a factor of up to 3 to 4 if nitrogen is derived solely from cyanate. During aerobic growth, the diffusion calculation suggests that the intracellular steady-state concentration of CO₂ will be 10⁻² to 10⁻³-fold, which is too low to meet this demand in the absence of CA. These estimates provide a quantitatively convincing explanation of why CA is essential during aerobic growth of E. coli.

Anaerobic conditions provide a more complex situation because most of the growth substrate is converted into fermentation products rather than being incorporated into bacterial cell mass. Growth still requires bicarbonate—most unavailably to be cycled in fatty acid biosynthesis—but the generation of CO₂ by dehydrogenases is eliminated or strongly constrained by the need to recycle the NADH produced concomitantly by these enzymes. Formic acid is a major fermentation product, and this may be disproportionated to CO₂ and H₂ by the formate hydrogen lyase reaction. This activity is constrained by the need for pH homeostasis (5). Loss of CO₂ by diffusion raises the pH, so the enzyme is only active at low pH. In a fermenting culture that is effervescing CO₂, the intracellular concentration is ca. 20 mM. Under anaerobic conditions,
E. coli may generate enough bicarbonate for growth, even in the absence of CA, provided the pH does not rise unduly. The results of growth experiments with Can-depleted E. coli match expectations based on this analysis of can function. No growth occurs in aerobic cultures at any pH (pH 5 to 9), but slow growth is restored at pH 7 by bubbling 2% CO₂ through the culture medium. This concentration is 66-fold higher than the normal concentration in air and can provide a correspondingly increased flux of bicarbonate. Anaerobic growth is possible without added CO₂, but only in acid conditions, when there is abundant endogenous generation of CO₂ and when culture vials are closed (preventing CO₂ loss by diffusion out of the medium). This analysis also provides a somewhat different perspective on the role of CynT. Through the activity of the cyanase pathway, E. coli is able to grow with cyanate as its sole source of nitrogen. Since bicarbonate is dehydrated in the cyanase reaction, metabolism in this case requires the supply of one mole of bicarbonate for each gram atom of nitrogen incorporated into growing cell mass. This implies a 20- to 30-fold increase in the bicarbonate requirement, and it is this extra need that is met by the induction of CynT.

In the present study we have also monitored can expression under a variety of conditions in order to see how it might be controlled. We find that its level is not autoregulated, nor does it respond, as might be expected, to the partial pressure of carbon dioxide in the gas phase. On the other hand, it is responsive to increasing cell density, to temperature upshift and, during late stationary phase, to the availability of RpoS. In each case expression increases by ~2-fold. Transcription after temperature shift, with an overshoot followed by reduced transcription, is characteristic of σ32 responsive promoters, supporting the correctness of the sequence-based annotation, and may indicate an increased need for HCO₃⁻ during one or more of the stresses that increase σ32 availability.

However, the most notable conditions affecting expression are changes in growth rate and growth phase (although it is difficult to separate these two variables). What we observe is that the slower the growth, the greater the expression of can, with a 10- to 20-fold range of expression observed. We have not attempted to use genetics to further investigate the molecular mechanism of this variation (although RpoS may be involved), because mutations in genes such as relA, involved in many cases of growth rate regulation, are themselves characterized by slow growth, making it difficult to distinguish specific effects from indirect effects due to growth rate changes.

Why, however, should E. coli need more Can per mass when it is growing least quickly? One factor, of course, is that during growth on minimal medium, bicarbonate is needed to synthesize essential amino acids and nucleotides, which are supplied in broth. However, another possibility is that slow-growing cells, which are smaller, have a higher surface/volume ratio than faster-growing cells and therefore require proportionately more fatty acid biosynthesis. Cells entering stationary phase remain metabolically active, although cell mass no longer increases, and undergo major shifts in protein and lipid composition, as well as decreasing in size. Although we do not yet know why more Can should be required by slow-growing or starved cells than by rapidly growing ones, among the processes requiring bicarbonate, lipid synthesis appears to be the one most likely to make a relatively increased demand during slowed growth. It is also possible that an elevated level of Can is needed during slow growth within host animals for as-yet-unknown reasons.

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