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# Why Is Carbonic Anhydrase Essential to *Escherichia coli*?

Christophe Merlin,† Millicent Masters,* Sean McAteer, and Andrew Coulson

*University of Edinburgh, Institute of Cell and Molecular Biology, Edinburgh EH9 3JR, Scotland*

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**The *can* (previously *yadF*) gene of *Escherichia coli* encodes a β-class carbonic anhydrase (CA), an enzyme which interconverts $CO_2$ and bicarbonate.Various essential metabolic processes require either $CO_2$ or bicarbonate and, although carbon dioxide and bicarbonate spontaneously equilibrate in solution, the low concentration of $CO_2$ in air and its rapid diffusion from the cell mean that insufficient bicarbonate is spontaneously made in vivo to meet metabolic and biosynthetic needs. We calculate that demand for bicarbonate is $10^3$- to $10^4$-fold greater than would be provided by uncatalyzed intracellular hydration and that enzymatic conversion of $CO_2$ to bicarbonate is therefore necessary for growth. We find that *can* expression is ordinarily required for growth in air. It is dispensable if the atmospheric partial pressure of $CO_2$ is high or during anaerobic growth in a closed vessel at low pH, where copious $CO_2$ is generated endogenously. CynT, the single *E. coli* Can paralog, can, when induced with azide, replace Can; also, the γ-CA from *Methanosarcina thermophila* can at least partially replace it. Expression studies showed that *can* transcription does not appear to respond to carbon dioxide concentration or to be autoregulated. However, *can* expression is influenced by growth rate and the growth cycle; it is expressed best in slow-growing cultures and at higher culture densities. Expression can vary over a 10-fold range during the growth cycle and is also elevated during starvation or heat stress.**

A large proportion of the open reading frames (ORFs) located on fully sequenced chromosomes are of unknown or unverified function. To gain functional information about 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 $CO_2$ 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 ($CO_2$) and bicarbonate anion ($HCO_3^-$). 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 $CO_2$ 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/$CO_2$ 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 $CO_2$ (8). However, the need for $CO_2$ in central metabolism cannot be replaced with supplements. The only known supply pathway for bicarbonate is via the hydration of $CO_2$. Although it has long been known that *E. coli* requires $CO_2$ to grow (7), under most conditions an adequate supply is generated endogenously. $CO_2$ 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 $10^3$- to $10^4$-fold greater than would be provided by uncatalyzed hydration at the prevailing steady-state concentration of $CO_2$. 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

* Corresponding author. Mailling address: University of Edinburgh, Institute of Cell and Molecular Biology, Darwin Building, King's Building, Mayfield Rd., Edinburgh EH9 3JR, Scotland. Phone: 44(0)131-650-5355. Fax: 44(0)131-650-8650. E-mail: M.Masters@ed.ac.uk.

† Present address: LEMiR/DEVM-DSV, CEA Cadarache, F-13108 Saint-Paul-Lez-Durance, France.

TABLE 1. Strains used in this study

| Name | Genotype[a] | Source or reference |
|---|---|---|
| BUM012 | *cynT1 glnV44(AS) gal-3 malT1 xylA7 mtlA2 thi-1* | CGSC[b] |
| EDCM367 | MG1655 Δ*lacZY* | 24 |
| EDCM421 | MG1655 Δ*lacZY araBAD*< >*can* | This study |
| EDCM464 | MG1655 Δ*lacZY can*< >FLK2 *araBAD*< >*can* | This study |
| EDCM636 | MG1655 Δ*lacZY can*< >FLK2 | This study |
| EDCM637 | MG1655 Δ*lacZY* Δ*can* | This study |
| EDCM638 | MG1655 Δ*lacZY can*< >FLK2 *rpoS*::Tn*10* (by transduction from RH90) | This study |
| MG1655 | Sequenced $\lambda^-$ and $F^-$ derivative of K-12 | CGSC |
| RH90 | MC4100 *rpoS359*::Tn*10* | 21 |
| SM1 | BL21(λDE3) *can*<>FLK2/pcam-AC (by transduction from EDCM464) | This study |

[a] The FLK2 cassette contains two FRT sites flanking a *lacZ* reporter gene, followed by the *aph* gene for kanamycin resistance. "<>" indicates replacement.
[b] CGSC, Coli Genetic Stock Center at Yale University (http://cgsc.biology.yale.edu/).

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 $CO_2$ 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, Cam, or by cultivation under sufficiently elevated levels of $CO_2$.

## 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 [Beta Lab], 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 $P_{BAD}$ 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 generation 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 $CO_2$ was achieved with the Oxoid gas generating kit AN35, which produces an atmosphere with 4 to 10% $CO_2$ and converts $O_2$ to water through a palladium-catalyzed reaction with $H_2$ supplied by the kit. BBL Dry Anaerobic Indicator Strips were used to confirm that anaerobiosis had been attained. For growth under $H_2$, air was exhausted and replaced with $H_2$ from a cylinder; a Pd catalyst ensured that any remaining $O_2$ was converted to $H_2O$. For liquid cultures bubbled with mixtures of $CO_2$, $N_2$, and $O_2$, gas composition and delivery were controlled from the Bio Console ADI1035 of an Applikon fermentor. Anaerobic liquid cultures were grown without a gas-phase in syringes to allow maintenance of anaerobiosis during sample collection; glass beads and gentle shaking were used to ensure a uniform distribution of cells.

**Growth and expression experiments.** Overnight cultures were grown at 37°C in 5 ml of medium in 10-ml bottles or in shaking flasks filled to 10% of their volume, with appropriate selection, inducer, and supplementation. Cultures were diluted 100-fold, with centrifugation and washing when a change of medium was to follow, into the desired growth medium, and the optical density at 600 nm ($OD_{600}$) was measured at intervals. To obtain continuous exponential growth, cultures were diluted fourfold into prewarmed medium when $OD_{600}$ reached 0.2. Samples for β-galactosidase assay were taken at intervals into Z-buffer with $CHCl_3$ and assayed as described by Miller (25).

**Plasmid construction.** To clone *paaY*, *can*, *cynT*, *caiE*, and *yrdA* into pUC18 (GenBank AN L08752), primers containing *Nhe*I and *Xho*I sites upstream and downstream, respectively, and 24 bases homologous to the DNA flanking the gene to be cloned were synthesized and used in PCRs with *Pfu* DNA polymerase to amplify each of the genes from chromosomal DNA. The resulting PCR products were digested and ligated into pUC18 which had been digested with *Xba*I and *Sal*I. Constructs were verified by restriction analysis and, for *caiE* and *yrdA*, by sequencing. To clone *can*, *cynT*, *caiE*, and *yrdA* into pTRC99A (GenBank AN U13872), the same downstream primers used for pUC18 cloning and an upstream primer with an engineered *Nco*I site overlapping the predicted start codon of the gene, followed by 24 bases of sequence from within the gene, were used in PCRs with *Pfu* as described above. Products were digested and cloned into pTRC99A DNA which had been digested with *Nco*I and *Sal*I. Constructs were verified by restriction digestion analysis.

**DNA manipulation.** Plasmid DNA purification, genomic DNA preparation, DNA cleaning, and DNA gel extraction were performed by using the Wizard Plus SV Minipreps kit (Promega), the Quantum Prep AquaPure genomic DNA isolation kit (Bio-Rad), QIAquick PCR purification kit (Qiagen), and the QIAquick gel extraction kit (Qiagen), respectively.

Crossover PCR and analytical PCR were carried out on a PCR Sprint cycler (Hybaid) with *Pfu* DNA polymerase and *Taq* DNA polymerase, respectively, according to the manufacturer's recommendations. All primers used were purchased from MWG-Biotech.

Ligations were carried out as described in (32). Prior to ligation with T4 DNA ligase (Roche), vectors were dephosphorylated with calf alkaline phosphatase (Roche), and both vector and insert DNA were purified by using a QIAQuick PCR purification kit (Qiagen).

Transformation of bacteria with engineered plasmid DNA was done by using a classical $CaCl_2$ preparation, and recombinant DNA was recovered in DH5α (32). After the FLK2-cassette cloning step, recombinant DNA was recovered in TOP10 by using One Shot TOP10 competent cells (Invitrogen).

**Gene deletion and/or replacement.** Precise *yadF* deletion was performed as described in detail previously (23). The primer pairs NiYadF (CGCTCTTGCGGCCGCTTGGAACGGCACCAGCATTTTTGACCATAGTGC)-NoYadF (AAAAACTGCAGTCCCAACTGACGTTTAGCGTTCAC ) and CiYadF (CGTTCCAAGCGGCCGCAAGAGCGCTCAAGCTGAAACACGCCAACCAC)-CoYadF (AAAAAGTCGACTTTATGACCGCCTCCAGC) were used to amplify ~400-bp segments flanking the *can* region.

## 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 *Enterobacteriacae*. 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

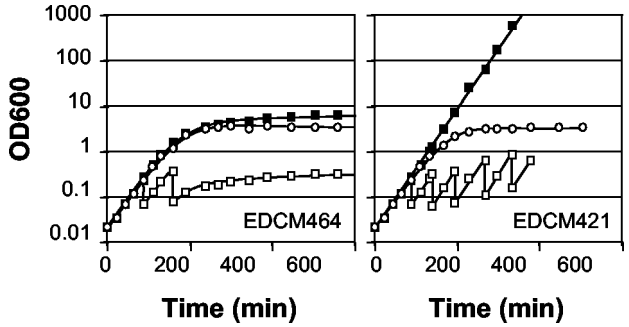


FIG. 1. Can is required for continued growth in air. EDCM464 and its parent EDCM421, grown overnight in LB medium-arabinose, were inoculated into LB medium-glucose at 37°C. Cultures were allowed either to grow for one complete cycle (○) or were kept growing by dilution into the same medium (□). ■, Total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

(10). Gene replacement with the removable reporter cassette (FLK2: FRT-*lacZ-aph*-FRT) failed several time when attempted in the uncomplemented strain EDCM367 (Table 1), and we speculated that the gene might be essential. We therefore followed the procedure described by Arigoni et al. (2) to insert a controllable second copy of *can* at the *ara* locus. Strain EDCM421 (Table 1) has a copy of *can*, under $P_{BAD}$ control, in place of *araBAD* on the chromosome. Construction of the deletant EDCM464 then became possible, provided that the growth medium contained sufficient arabinose to maintain the expression of *can*; colonies of EDCM464 are not formed on LB plates lacking arabinose. We conclude that Can is essential for growth in standard aerobic laboratory conditions.

**Depletion of Can from EDCM464.** In order to determine why *can* is essential, we attempted to deplete Can by removing arabinose from growing liquid cultures (Fig. 1). Surprisingly, strain EDCM464 was able to grow for a complete growth cycle in LB medium-glucose, a condition in which the complementing copy should not be expressed from $P_{BAD}$. Cessation of growth was observed only when cells were diluted into the same medium and incubated further (Fig. 1). Repeated experiments showed that EDCM464 can divide 7 to 8 times before growth stops; we believe that this reflects a high level of expression from the arabinose promoter in the overnight cultures used as inoculum (see below) and suggests that Can itself is probably not required in large quantities. Microscopic analysis 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 Δ*lacZ*; 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 without 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 $P_{BAD}$, reaches a stationary-phase level manyfold higher than is required for growth. Also, since EDCM464 inoculated from azide outgrows EDCM636, it appears that the $P_{BAD}$ 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 $H_2$ and $CO_2$ (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 $CO_2$ partial pressure (which also increases the rate of supply of $HCO_3^-$) or because of the lack of $O_2$? 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 $CO_2$ (20). To distinguish between the need to exclude $O_2$ and a requirement for an increased partial pressure of $CO_2$, we tested the ability of EDCM636 to grow anaerobically under $H_2$ alone and found that it cannot, indicating that it must be the increased partial pressure of $CO_2$ in the earlier experiment that allowed growth.

**Growth in air with added $CO_2$.** To determine whether increased $CO_2$ partial pressure would restore growth in air, EDCM636, pregrown with azide, was grown in liquid with or without added $CO_2$ (Fig. 3A). When a mixture of 21% $O_2$ and 79% $N_2$ 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 $CO_2$ is not needed to support growth of the parental

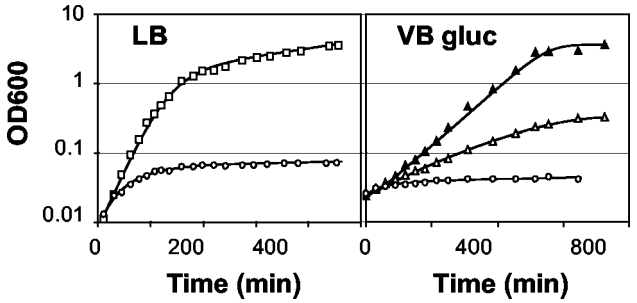


FIG. 2. Depletion of CA from *can* deleted strains. (Left panel) Growth curves of EDCM636 (○) and its parent EDCM367 (□) in LB medium at 37°C. Strains were grown overnight in LB medium (with 0.1 mM azide for EDCM636) and inoculated into LB medium. (Right panel) Comparison of CA depletion in EDCM464 (triangles) 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 (△).

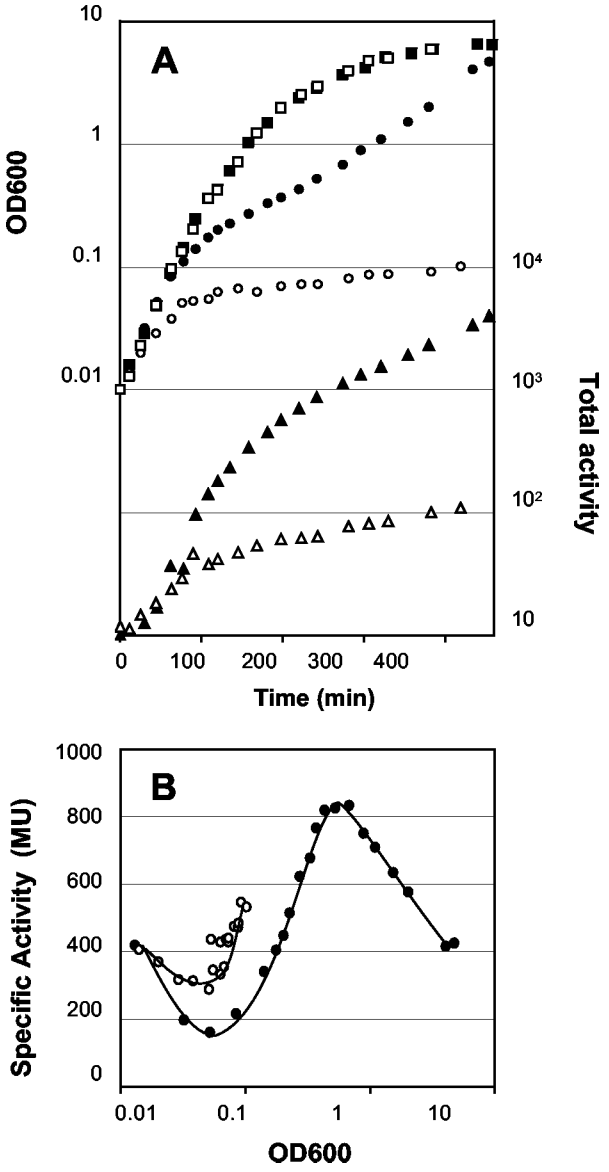


FIG. 3. Growth of EDCM636 in a $CO_2$-controlled atmosphere. (A) Growth of EDCM636 (circles) and its parent EDCM367 (squares) in LB medium at 37°C with or without added $CO_2$. The cultures were bubbled (~55 ml/min/flask) with a gas mixture of either 2% $CO_2$–21% $O_2$–77% $N_2$ (solid symbols) or 21% $O_2$–79% $N_2$ (open symbols). Total β-galactosidase synthesized by EDCM636 from the *can* promoter (SA × OD) is shown on the right-hand axis (△, no $CO_2$; ▲, 2% $CO_2$). (B) Variations in synthesis from the *can* promoter versus the culture OD in EDCM636 calculated from the data in panel A. Symbols: ○, 2% $CO_2$; ●, no $CO_2$. Specific activities are expressed in MU.

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 anerobically 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 continually 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 $CO_2$

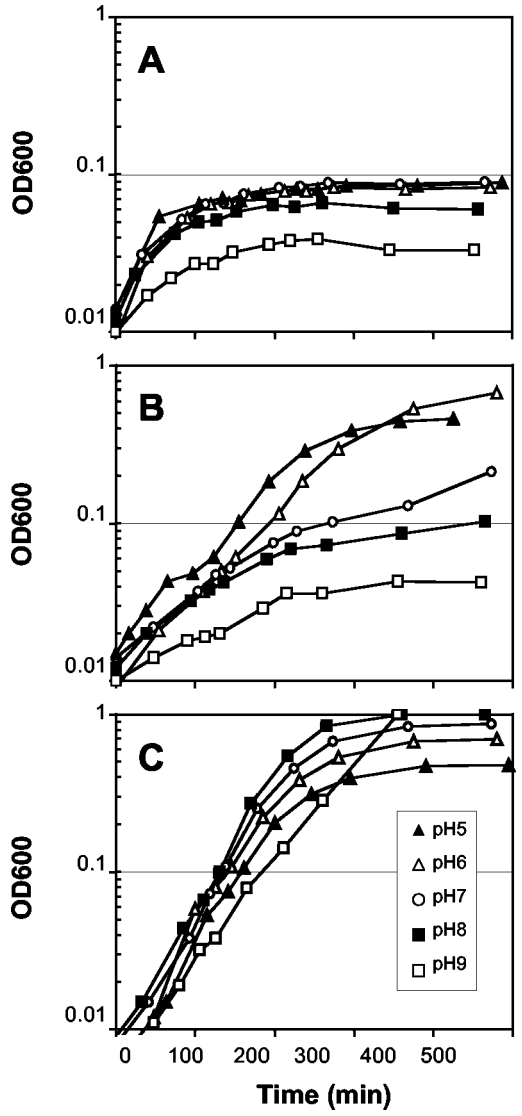


FIG. 4. Effect of pH on growth of EDCM636. Strains were inoculated into LB medium-glucose at pHs from 5 to 9 and grown either aerobically in flasks or anaerobically in filled syringes (see Materials and Methods). (A) EDCM636 grown in air; (B) EDCM636 grown anaerobically; (C) EDCM367 grown anaerobically.

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 *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 Δ*can*.** 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% cotransductionally 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 were screened for the ability to grow in air. About 40 slow-growing colonies which have not yet been further characterized were found and should prove interesting, since they indicate that *E. coli* can become, by mutation, independent of β-CA activity.

**A γ-CA can substitute for Can.** It has been shown that a human α-CA can substitute for the *R. eutropha* Can (20). The ability of γ-CAs to substitute for bacterial β-CAs has not been

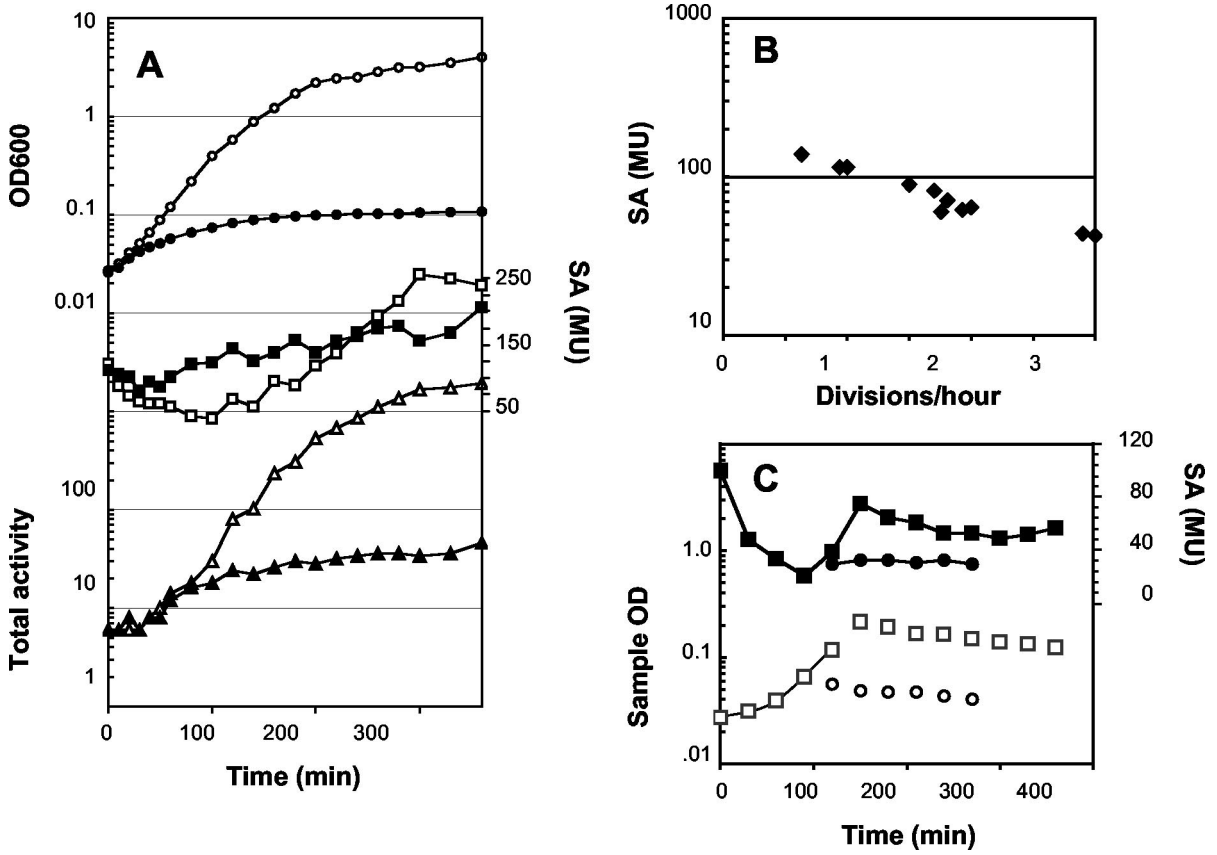


FIG. 5. Variation of *can* expression with growth rate and cell density. (A) An overnight LB medium-azide culture of EDCM636 was inoculated into LB medium with (open symbols) or without (solid symbols) azide and growth (circles) and β-galactosidase synthesis (squares for SA and triangles for total activity) were monitored. (B) EDCM636 was grown overnight with azide either in the medium to be used the following day or in a less-rich medium. The following media were used and corresponding approximate divisions/hour recorded: VB medium-glycerol, 0.63; VB-glucose, 1.0; VB medium-CAA-Asn-Trp, 1.5; VB medium-CAA-Asn-Trp-adenine-uracil-cytosine, 1.7 to 1.8; VB medium-glucose-CAA, 1.8 to 2.0; LB medium-glucose, 3.0. Culture generation time was individually calculated for each point plotted. (C) Steady-state growth of EDCM636 on LB medium at two optical densities. When an $OD_{600}$ of 0.1 was reached, the culture was divided in half. One half was diluted twofold (circles), and the other was allowed to reach an $OD_{600}$ of 0.2 (squares). Cultures were then diluted once/doubling time and always sampled at about the same OD. Samples taken during this dilution regimen are shown as unconnected points on the OD curve (open).

tested. We therefore obtained a strain, BL21(λDE3)/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-β-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 *cynT* 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 *lacI*$^q$ 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 *cynT* 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

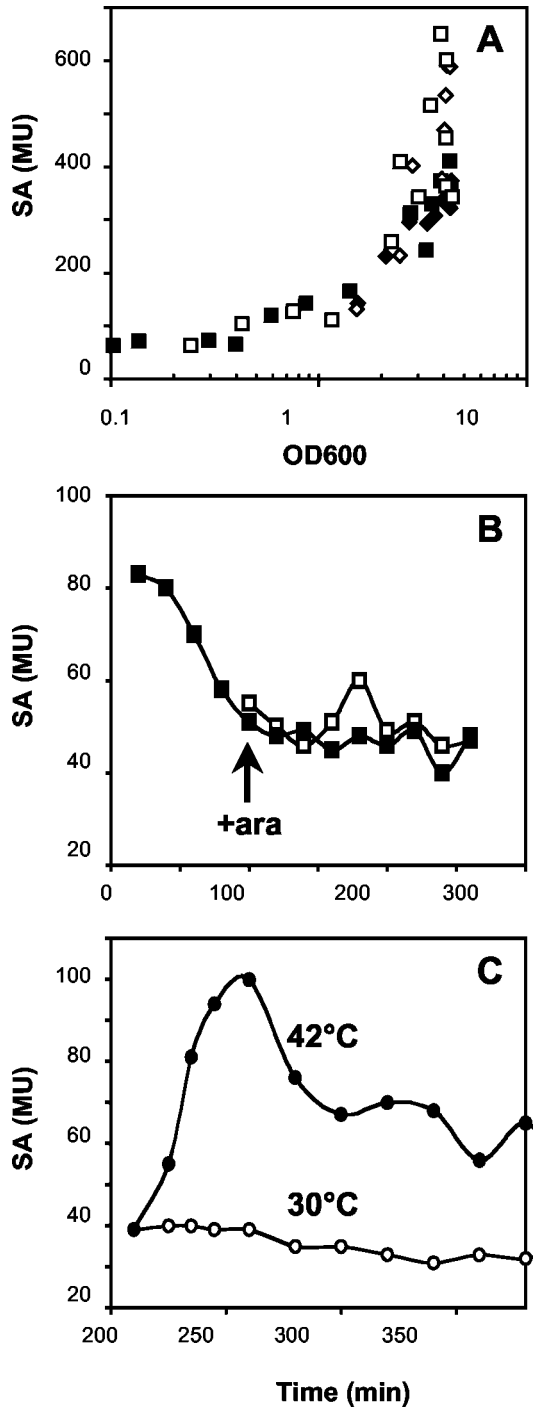


FIG. 6. Testing for other regulators of *can* expression. The growth medium was LB medium-azide. (A) RpoS. EDCM636 and EDCM638 (RpoS$^-$) were sampled and assayed for β-galactosidase. SA is plotted in MU as a function of OD. The data from two separate experiments are shown. Solid symbols, EDCM636 *rpoS*; open symbols, EDCM636. (B) Autoregulation. EDCM464 was grown in LB medium-azide. At 100 min, arabinose was added to a culture of EDCM464 to induce *can* expression. □, Induced culture samples (C) Heat shock. At 210 min, a 30°C culture of EDCM636 was divided, and fractions were incubated at either 30°C or transferred to 42°C. Both cultures were sampled, and β-galactosidase was assayed.

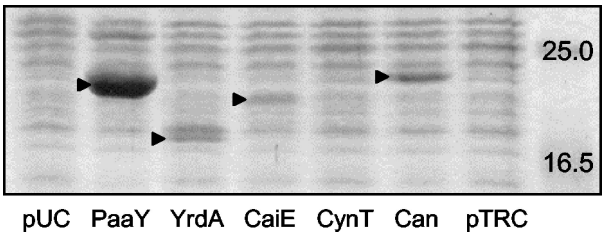


FIG. 7. Overproduction of β-CAs and possible γ-CAs. Overnight cultures of EDCM636 containing plasmids expected to overexpress the indicated proteins were grown overnight on LB medium plus azide plus IPTG, and 25 μl/lane was prepared and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis according to standard procedures (32). Extracts of cells transformed with the vector plasmids, pUC18 and pTRC99A, are in the left and right lanes, respectively.

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:

$$CO_2 + OH_2 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \left[ H^+ + HCO_3^- \overset{pK_a = 6.35}{\rightleftharpoons} H_2CO_3 \right]$$

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 $CO_2$ is the true product of formate oxidation.

The principal reactions in which $CO_2$/bicarbonate is consumed are those catalyzed by the enzymes phosphoenolpyruvate carboxylase carbamoyl phosphate synthetase, 5-aminoimidazole 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 $CO_2$/bicarbonate is generated in *E. coli* predominantly and perhaps wholly in the form of molecular $CO_2$, 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 of metabolism is the hydration of this $CO_2$. In the absence of CA, the rate of generation of bicarbonate depends on the steady-state concentration of $CO_2$ 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 $CO_2$ 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 $CO_2$. The diffusion coefficient for a dilute solution of $CO_2$ in water at 20°C is $1.78 \times 10^{-9} \cdot m^2 \cdot s^{-1}$ (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 $CO_2$ 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 $CO_2$ in a cell is $F$ $mol \cdot s^{-1}$, the excess steady-state concentration (above the equilibrium concentration corresponding to the gas phase composition) will be about $F \times 10^{11}$ $mol \cdot liter^{-1}$.

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 $CO_2 \cdot kg^{-1}$(dry weight biomass) $\cdot h^{-1}$. An average cell (28) has a dry weight of $2.8 \times 10^{-13}$ g and would therefore produce $1.15 \times 10^{-18}$ mol of $CO_2 \cdot s^{-1}$ under these conditions. If the proposed model for diffusion provides a reasonable approximation, the expected excess intracellular concentration of $CO_2$ is ca. $10^{-7}$ $mol \cdot liter^{-1}$. If the gas phase concentration is zero, this will support a maximum uncatalyzed flux from $CO_2$ to bicarbonate of ca. $3 \times 10^{-24}$ $mol \cdot s^{-1}$ in a single cell. Air normally contains 0.03% by volume of $CO_2$, and this will produce a concentration of 10 μM in the cell; in the absence of CA, this could support a flux of ca. $3 \times 10^{-22}$ $mol \cdot s^{-1}$.

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-aminoimidazole ribotide carboxylase. In the cells described by Holms, generation of 14.78 mol of $CO_2 \cdot kg^{-1} \cdot h^{-1}$ 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 $7.4 \times 10^{-20}$ $mol \cdot s^{-1}$ 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/$CO_2$ cycles.** Bicarbonate is incorporated into malonyl coenzyme A, via biotin carboxylase, in the first committed step of fatty acid biosynthesis and is released as $CO_2$ 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 $1.79 \times 10^{-19}$ $mol \cdot s^{-1}$ in a single cell.

Bicarbonate/$CO_2$ 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 $7.46 \times 10^{-19}$ mol of bicarbonate $\cdot s^{-1}$ 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 $2.01 \times 10^{-19}$ mol of bicarbonate $\cdot s^{-1}$ in a single cell.

Thus, during growth at a moderate rate even on complex media, *E. coli* has a demand for ca. $2 \times 10^{-19}$ mol of bicarbonate $\cdot s^{-1}$ 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 $CO_2$ will be $10^3$- to $10^4$-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 unavoidably to be cycled in fatty acid biosynthesis—but the generation of $CO_2$ 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 $CO_2$ and $H_2$ by the formate hydrogen lyase reaction. This activity is constrained by the need for pH homeostasis (5). Loss of $CO_2$ by diffusion raises the pH, so the enzyme is only active at low pH. In a fermenting culture that is effervescing $CO_2$, 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% $CO_2$ 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 $CO_2$ but only in acid conditions, when there is abundant endogenous generation of $CO_2$ and when culture vials are closed (preventing $CO_2$ 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 $HCO_3^-$ 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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