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Carbonic Anhydrase Is Essential for Growth of *Ralstonia eutropha* at Ambient CO_2 Concentrations

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Mutant strain 25-1 of the facultative chemoautotroph *Ralstonia eutropha* H16 had previously been shown to exhibit an obligately high-CO_2-requiring (HCR) phenotype. Although the requirement varied with the carbon and energy sources utilized, none of these conditions allowed growth at the air concentration of CO_2. In the present study, a gene designated *can* and encoding a β-carbonic anhydrase (CA) was identified as the site altered in strain 25-1. The mutation caused a replacement of the highly conserved glycine residue 98 by aspartate in Can. A *can* deletion introduced into wild-type strain H16 generated mutant HB1, which showed the same HCR phenotype as mutant 25-1. Overexpression of *can* in *Escherichia coli* and mass spectrometric determination of CA activity demonstrated that *can* encodes a functional CA. The enzyme is inhibited by ethoxyzolamide and requires 40 mM $MgSO_4$ for maximal activity. Low but significant CA activities were detected in wild-type H16 but not in mutant HB1, strongly suggesting that the CA activity of Can is essential for growth of the wild type in the presence of low CO_2 concentrations. The HCR phenotype of HB1 was overcome by complementation with heterologous CA genes, indicating that growth of the organism at low CO_2 concentrations requires sufficient CA activity rather than the specific function of Can. The metabolic function(s) depending on CA activity remains to be identified.

Carbon dioxide and bicarbonate (dissolved inorganic carbon [DIC]) are essential growth factors for bacteria. The metabolic need for DIC is evident in autotrophs utilizing CO_2 as the sole carbon source, but heterotrophs also fix significant amounts of both carbon species. Although sufficient CO_2 is produced during catabolism, deprivation of atmospheric CO_2 leads to growth inhibition or even death of heterotrophs (7, 14, 22). Pathogenic bacteria seem to be adapted to high DIC concentrations in their host environment, as they usually require 5 to 10% (vol/vol) CO_2 for growth (9, 45, 60). Furthermore, elevated DIC was found to shorten the lag phase and accelerate growth of bacteria even though the organisms were not generally dependent on high DIC concentrations (46, 47, 60). This "sparking effect" is most pronounced when cultures are inoculated at low cell densities. The need for DIC is generally attributed to CO_2 fixation in anaplerotic or other biosynthetic reactions. Consequently, the requirement is often satisfied by supplementation of the growth media with metabolites, particularly intermediates of the tricarboxylic acid cycle such as oxaloacetate and 2-oxoglutarate (28). Most high-CO_2-requiring (HCR) mutants of *Escherichia coli* and other microorganisms regained the ability to grow at air concentrations of CO_2 (0.035% [vol/vol]) upon provision with appropriate metabolites, but some depended strictly on high CO_2 concentrations (5 to 10% [vol/vol]) (1, 10, 64). In contrast to their general DIC requirement, many microorganisms are inhibited by very high CO_2 concentrations (ca. 20% [vol/vol] and above), an effect used in food preservation (15). However, the sensitivity towards CO_2 varies widely among organisms and also depends on the nutritional and cultural conditions.

Although the uncatalyzed hydration-dehydration of CO_2-HCO_3^- proceeds at significant rates, the metabolic reaction is catalyzed by carbonic anhydrase (CA) (EC 4.2.1.1) to support various physiological functions involving DIC. CAs are known to participate in transport and autotrophic fixation of CO_2 in plants, algae, and cyanobacteria as well as in HCO_3^-- or H^+-coupled ion transport, pH regulation, or carboxylation reactions in higher eukaryotes (8, 11, 17, 20, 59). Four phylogenetically unrelated families of CA (α, β, γ, and δ) are currently differentiated (26, 51, 63). However, while CAs are common in bacteria, with β-CA apparently as the dominant type, there is little information about the physiological significance of the enzyme in these organisms (36, 51).

Ralstonia eutropha (formerly *Alcaligenes eutrophus*) is a respiratory, facultatively chemoautotrophic bacterium. Organic acids such as pyruvate, lactate, or succinate are preferred organic substrates. Assimilation of CO_2 during autotrophic growth with either hydrogen or formate as an energy source proceeds via the Calvin-Benson-Bassham cycle (5). An HCR mutant of *R. eutropha* H16, strain 25-1, that depended on increased CO_2 concentrations for growth on all substrates tested was isolated previously, although the CO_2 concentrations necessary for phenotypic restoration varied with the carbon source (1). Approximately 2.5% (vol/vol) CO_2 was required for the mutant to regain wild-type growth rates on succinate, about 5% (vol/vol) was required on fructose, and even 10% (vol/vol) was not sufficient on lactate. Growth of the mutant on complex media also needed elevated CO_2, as did lithoautotrophic growth. Supplementation with vitamins or various biosynthetic precursors did not alleviate the CO_2 requirement of the mutant. Accumulation of the storage polyes-

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics[a]	Reference or source
R. eutropha		
H16	Cfx Hox Fox; wild type	ATCC 17699, DSM 428
25-1	Cfx Hox Fox HCR; *can*; mutant of H16	1
HB1	Cfx Hox Fox HCR; Δ*can*; mutant of H16	This study
E. coli		
S17-1	Sm^r Mod^+ Res^-; *thi pro recA*; integrated RP4 (Tc::Mu-Km::Tn*7*)	48
JW1	*ara* Δ*(lac-proAB) rpsL thi* ϕ80(*lacZ*Δ*M15*) F′(*lacI*^{q} *lacZ*Δ*M15 proA*$^+$*B*$^+$)	34
BH1214	Hfr; *pyrE41 metB1 tonA22 relA1 spoT* PA02	24
BUM012	HfrR5; Δ*cynT lacY*$^+$	24
Plasmids[b]		
pUC19	Ap^r; *lacPOZ′*	66
pBluescript KS	Ap^r; *lacPOZ′*	Stratagene
pLAFR1	Tc^r Mob^+ Tra^-; λ-*cos*; broad-host-range cosmid	19
pMP92	Tc^r Mob^+ Tra^-; broad host range	54
pNHG1	Km^r Tc^r Mob^+; *sacB* RP4-*oriT* ColE1-*ori*	29
pBH2240	pUC19::224-bp *Bgl*II/*Hin*fI	39
pMP2240	pMP92(*Pst*I-*Xba*I)::224-bp *Bgl*II/*Hin*fI, recloned from pBH2240 as *Pst*I-*Xba*I fragment	This study
pKR1	pLAFR 1::20+3.1+1.7-kb *Eco*RI	This study
pKR100	pMP92::3,067-bp *Eco*RI from pKR1	This study
pKR200	pMP92::821-bp *Kpn*I-*Eco*RI from pKR100	This study
pCAN3000/3001	pBluescript KS::3067-bp *Eco*RI; different orientations of insert	This study
pCAN1701	pBluescript KS::1736-bp *Bgl*II-*Eco*RI from pCAN3001	This study
pCAN1701Δ	pCAN1701 carrying a 423-bp *Hin*cII in-frame deletion in *can*	This study
pNHG1701Δ	pNHG1(*Xba*I-*Pme*I)::1,313-bp *Bgl*II-*Eco*RI from pCAN1701Δ, recloned as *Xba*I-*Eco*RV	This study
pCAN8210	pUC19::821-bp *Kpn*I-*Eco*RI from pCAN3000	This study
pAL12	pUC118::1.3-kb *Eco*RI-*Hin*dIII carrying *cynT* from *E. coli*	24
pHCAII	pICB1::0.8-kb *Xho*I-*Bgl*II carrying the human HCAII gene	16
pMP-*cynT*	pMP2240(*Bam*HI-*Bgl*II)::0.7-kb *Hin*cII-*Bgl*II from pAL12	This study
pMP-HCAII	pMP2240(*Eco*RI-*Bgl*II)::0.8-kb *Xho*I-*Bgl*II from pHCAII	This study

[a] Ap^r, ampicillin resistant; Km^r, kanamycin resistant; Tc^r, tetracycline resistant; Cfx, ability for autotrophic CO_2 fixation; Hox, ability for H_2 oxidation; Fox, ability for formate oxidation; HCR, high CO_2 requirement.

[b] The last digit of the pCAN plasmid designations indicates the relative orientation of the cloned genes relative to *lacZ′* of the vector: 0, *can* colinear to *lacZ′*; 1, *can* divergent to *lacZ′*.

ter poly-β-hydroxybutyrate was not affected, indicating that the main pathways involved in heterotrophic catabolism, autotrophic CO_2 fixation, and synthesis of the storage compound were still functional.

In the present study we identified the *can* gene as the site of mutation in mutant 25-1 of *R. eutropha* H16. The gene encodes a β-CA whose function is essential for growth at air concentrations of CO_2. The HCR phenotype of mutant 25-1 was confirmed by the generation of a *can* deletion strain. High CA activities detected in *E. coli* after heterologous expression of *can* provided evidence that the gene encodes a functional CA. Phenotypic complementation of the mutants with several heterologous CA genes showed that there is a requirement for sufficient CA activity in *R. eutropha* but not for the specific function of the *can*-encoded CA.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. Strains of *R. eutropha* were propagated in nutrient broth or mineral salts medium at 30°C as described previously (65). The mineral medium was supplemented with organic substrates at a final concentration of 0.2% (wt/vol). Lithoautotrophic cultures were incubated under an atmosphere consisting of H_2, CO_2, and O_2 (8:1:1, vol/vol/vol). For growth of *R. eutropha* mutants 25-1 and HB1, small liquid cultures (10 or 50 ml) or agar plates were incubated in desiccators under air plus 10% (vol/vol) CO_2. Larger cultures (1 liter) used in growth experiments were continuously supplied with this gas mixture at a rate of 2.5 liters/min and stirred magnetically at 600 rpm. CO_2-limited cultures of HB1 were first gassed with air plus 10% (vol/vol) CO_2 until an optical density at 436 nm (OD_{436}) of 0.3 to 0.4 was reached. The cultures were then bubbled with ambient air for several hours.

Air-grown cells were obtained by initially growing the culture under air plus 10% (vol/vol) CO_2 to an OD_{436} of 0.3 to 0.4 followed by gassing with ambient air. To achieve better control of DIC (bicarbonate) concentration during growth experiments, the pH of the mineral medium was kept at 6.5. Anaerobic, denitrifying growth was tested under an atmosphere of N_2 or N_2 plus CO_2 (9:1, vol/vol) on fructose mineral agar containing 0.2% (wt/vol) KNO_3. The utilization of cyanate as a nitrogen source was checked on fructose mineral agar, in which the NH_4Cl of the standard medium was replaced by 0.05% (wt/vol) KCNO. Succinate mineral medium supplemented with various metabolites at a final concentration of 1 mM was used to test if the metabolites supported growth of *R. eutropha* HB1 at ambient CO_2.

E. coli was routinely grown in Luria-Bertani medium at 37°C. Growth in cyanate-containing Luria-Bertani medium was done as detailed earlier (24). Required antibiotics were added to media at the following concentrations: ampicillin, 50 μg/ml; kanamycin, 50 μg/ml for *E. coli*, 450 μg/ml for *R. eutropha* in mineral medium, and 120 μg/ml for *R. eutropha* in nutrient broth; and tetracycline, 15 μg/ml for *E. coli* and 20 μg/ml for *R. eutropha*.

Manipulation of DNA. Standard protocols were employed for DNA isolation and cloning (3). Restriction and DNA-modifying enzymes were used as recommended by the manufacturers. Oligodeoxynucleotide primers were purchased from MWG-Biotech (Ebersberg, Germany). PCRs were performed with *Taq* (Qiagen, Hilden, Germany) or *Pfu* (Promega, Mannheim, Germany) DNA polymerase. For Southern hybridizations, digested DNA was separated by agarose gel electrophoresis and vacuum blotted onto a Biodyne B nylon membrane (Pall, Dreieich, Germany). The DNA probes were labeled nonradioactively with digoxigenin, and DNA-DNA hybrids on the blots were detected in a staining reaction involving nitroblue tetrazolium, 5-bromo-4-chloro-3-indolyl phosphate, and alkaline phosphatase conjugated to antidigoxigenin Fab fragments (Roche, Mannheim, Germany).

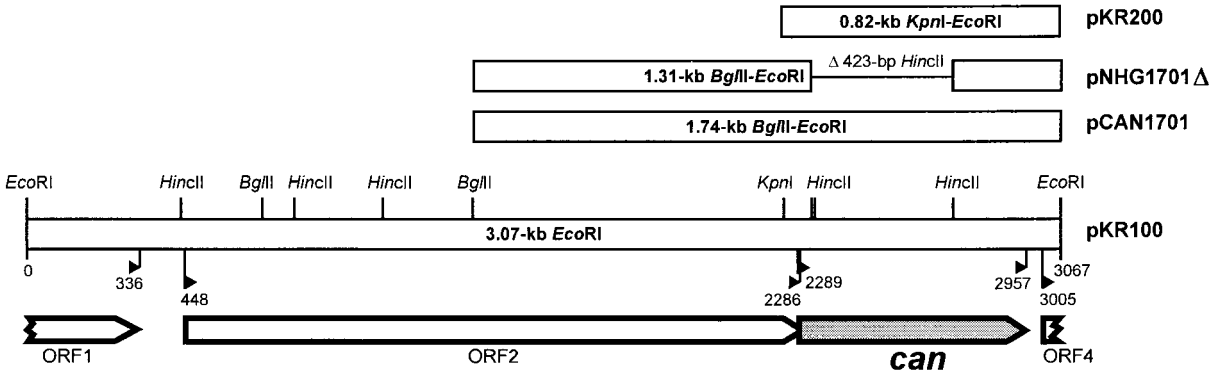

FIG. 1. Genetic organization within the 3,067-bp *Eco*RI fragment of *R. eutropha* H16. The orientation and sizes of the identified ORFs (ORF1, ORF2, *can*, and ORF4) are indicated by arrows. Fragments cloned in the respective plasmids are shown by bars together with the cleavage sites of relevant restriction endonucleases. Flagged marks indicate the positions (in base pairs) of the start and stop codons of the ORFs.

DNA sequencing and analysis. DNA sequences were determined by the dideoxy chain termination method, using cycle sequencing reactions (SequiTherm cycle sequencing kit; Biozym, Hessisch Oldendorf, Germany) with α-^{35}S-dATP (ICN Biomedicals, Eschwege, Germany) as the labeled nucleotide. The *can* gene of *R. eutropha* was amplified by PCR with primers CA-PCR1-2 (20-mer) and CA-PCR2 (24-mer), which annealed to positions 2244 to 2263 and 3032 to 3009, respectively, of the 3,067-bp *Eco*RI fragment. Amplified fragments from two independent PCRs were sequenced to localize the site of mutation in strain 25-1. Basic analyses of nucleotide and deduced protein sequences were performed with the Genetics Computer Group (Madison, Wis.) program package, version 10.0. Similarity searches within various sequence databases were done using the BLAST programs (2). Sequence alignments were generated with the ClustalX program, version 1.8 (61).

Phenotypic complementations. Broad-host-range cosmid (pLAFR1) clones of a genomic library of *R. eutropha* H16 (18) were conjugally transferred from *E. coli* S17-1 into HCR *can* mutant 25-1. Transconjugants were selected under air plus 10% (vol/vol) CO_2 on fructose mineral agar containing tetracycline and subsequently checked for growth on the same agar under low (air) CO_2 concentrations. For verification, the transferred plasmids were isolated from apparently complemented transconjugants, retransformed into *E. coli* S17-1, and finally retransferred into mutant 25-1. Cosmids complementing the mutant were subjected to restriction analysis. Subclones were generated and used for complementation of mutants 25-1 and HB1 to delimit the size of the complementing DNA fragment. Mutant HB1 was also complemented with heterologous CA genes cloned in pMP2240. Transconjugants were tested for growth under air or air plus 10% (vol/vol) CO_2 on mineral agar containing various carbon and energy sources.

Construction of plasmids. The 3.1-kb *Eco*RI fragment of pKR1 was recloned into pBluescript KS to yield pCAN3000 and pCAN3001. Cloning of the same fragment into broad-host-range vector pMP92 generated pKR100. Digestion of pCAN3001 with *Bam*HI and *Bgl*II removed a 0.7-kb *Bam*HI-*Bgl*II and a 0.6-kb *Bgl*II fragment prior to religation, producing pCAN1701. To delete a *can*-internal 423-bp *Hin*cII fragment (Fig. 1) from the 1.74-kb insert of pCAN1701, the plasmid was cleaved with *Hin*cII and the resulting large vector-insert and 341-bp *Hin*cII fragments were religated in their original orientations, yielding pCAN1701Δ. The 1.31-kb insert of pCAN1701Δ was recloned as an *Xba*I-*Eco*RV fragment into pNHG1, producing pNHG1701Δ. Plasmid pCAN8210 was constructed by cloning the 821-bp *Kpn*I-*Eco*RI fragment of pCAN3000 into pUC19. Recloning of this fragment in pMP92 gave pKR200. Expression vector pMP2240 was constructed by inserting the 0.23-kb *Xba*I-*Pst*I fragment of pBH2240, containing the chromosomal *cbb* operon promoter of *R. eutropha*, into pMP92. The human HCAII gene was recloned from pHCAII as a 0.8-kb *Xho*I-*Bgl*II fragment into pMP2240 to generate pMP-HCAII. For the construction of pMP-*cynT* the 0.7-kb *Hin*cII-*Bgl*II fragment of pAL12, containing the *E. coli cynT* gene, was ligated into pMP2240.

Construction of *can* deletion strain HB1. A *can* deletion strain of *R. eutropha* H16 was constructed by gene replacement mutagenesis. For this purpose plasmid pNHG1701Δ carrying a 423-bp in-frame deletion within *can* (Fig. 1) was conjugally transferred from *E. coli* S17-1 into *R. eutropha* H16. Subsequent recombinations and selections of hetero- and homogenotes were done as described previously (29). Since the *can* mutant was expected to exhibit an HCR phenotype, recombinants were grown under air plus 10% (vol/vol) CO_2. The mutant genotype was verified by Southern hybridization and PCR.

Overexpression of *can* and preparation of cell extracts. The *can* gene in pCAN8210 was expressed in *E. coli* JW1 after induction by isopropyl-β-D-thiogalactopyranoside as described before (38). Proteins in lysates obtained from the induced cells were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Crude cell extracts of *E. coli* and *R. eutropha* were prepared from cells suspended in CA buffer (50 mM Bicine-NaOH [pH 8.0] containing 40 mM $MgSO_4$, 5 mM dithioerythritol, and 1 mM phenylmethylsulfonyl fluoride) and disrupted by sonication. The extracts were obtained after centrifugation at 14,000 × *g* for 20 min to remove unbroken cells and cell debris. Up to 100 μl of the extracts was assayed for CA activity.

Determination of CA activities. CA activities were determined by a mass spectrometric method based on the loss of ^{18}O from doubly labeled $^{13}C^{18}O_2$ to water (58). The ^{18}O decline was monitored with a quadrupole mass spectrometer (MSD 5970; Hewlett-Packard, Waldbronn, Germany) coupled to a 10-ml thermostatted (30°C) reaction cuvette via a membrane inlet system. Changes in mass signals $m/z = 45$ ($^{13}C^{16}O_2$), $m/z = 47$ ($^{13}C^{18}O^{16}O$), and $m/z = 49$ ($^{13}C^{18}O_2$) were recorded and used to calculate the ^{18}O fraction (as percent enrichment) in doubly labeled CO_2 as follows: log (% enrichment) = log ($^{13}C^{18}O_2/^{13}CO_2$) = log [100 − 49(45 + 47 + 49)].

$H^{13}C^{18}O_3^-$ was added (1 mM) to the assay buffer (50 mM Bicine-NaOH [pH 8.0] plus 40 mM $MgSO_4$), and the cuvette was closed. Isotopic changes in CO_2 were recorded until isotopic equilibrium (blank) was reached. The CA-catalyzed reaction was then initiated by injecting cell extract. CA activity was calculated from the linear decrease in log (% enrichment) before and after addition of the sample as [log (% enrichment)$_{sample}$ − log (% enrichment)$_{blank}$]/log (% enrichment)$_{blank}$ and normalized on a protein basis (58).

For in vivo CA assays, cells were harvested from freshly grown cultures (OD_{436} of 2 to 3) and resuspended in mineral salts medium at an OD_{436} of 50. Up to 400 μl of the cell suspension, corresponding to about 2.5 mg of total cell protein, was used per assay. Addition of cells caused a biphasic decline of log (% enrichment) resulting in an initial and a final slope. The apparent internal CA activity was expressed as the difference in loss of enrichment by extrapolating the final slope back to the time point of cell addition (43, 57). Control assays were run with mineral medium to evaluate the effects of pH change and dilution.

Nucleotide sequence accession number. The nucleotide sequence of the 3,067-bp *Eco*RI fragment was deposited in the EMBL/GenBank/DDBJ databases under accession number AJ310671.

RESULTS AND DISCUSSION

The defect in HCR mutant 25-1. Phenotypic complementation of mutant 25-1 by hybrid cosmids from a genomic library of wild-type strain H16 resulted in the isolation of a transconjugant harboring pKR1. Growth of the transconjugant at ambient CO_2 concentrations was restored. Subcloning identified a 3.07-kb *Eco*RI fragment (pKR100) that complemented the mutation. A 0.82-kb *Kpn*I-*Eco*RI subfragment (pKR200) (Fig.

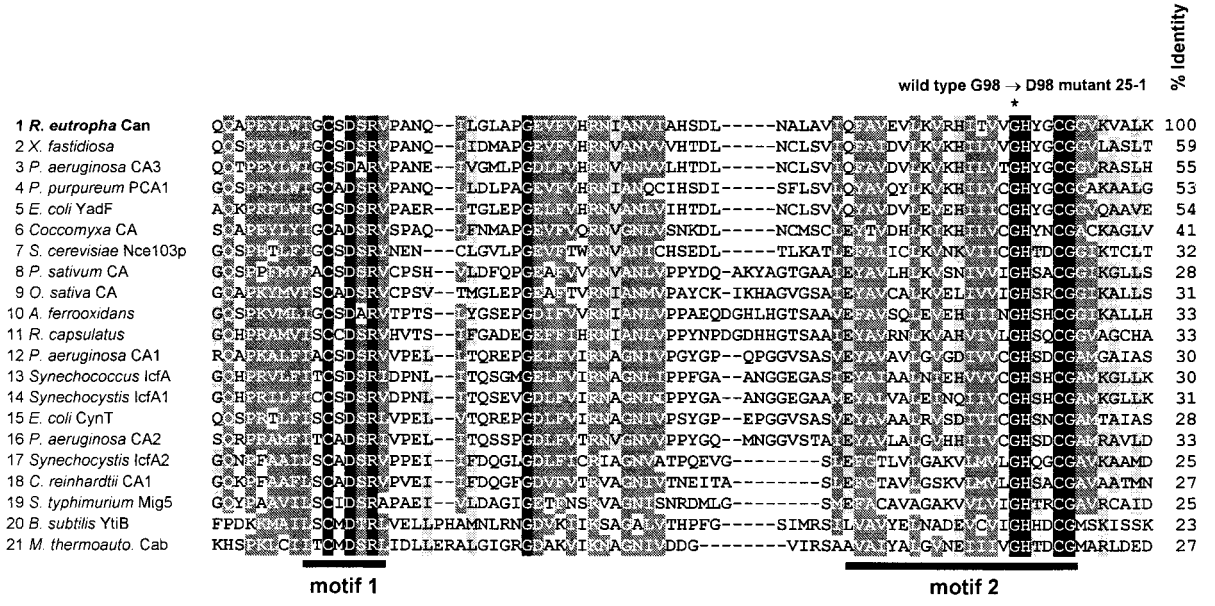

FIG. 2. Partial sequence alignment of various (putative) β-CAs. The selected segment corresponds to amino acid residues 33 through 110 of Can from *R. eutropha* and covers the two most conserved motifs (motifs 1 and 2, indicated by bars) involved in binding of Zn^{2+} within the active center of the enzyme. The alignment was performed by means of the program ClustalX (version 1.8) with a blosum62 matrix (gap-opening penalty, 11; gap extension penalty, 1). Different shadings indicate the relative similarity of amino acid residues (dark shading, 100% identity; medium shading, 100 to 75% conservation; light shading, 74 to 50% conservation) based on the following groupings: (D, E, H, K, R), (N, Q, S, T), and (L, I, V, M, F, Y, W, A, G). The asterisk marks the highly conserved glycine residue found to be the site of mutation in *R. eutropha* HCR mutant 25-1. Overall identities of the CA sequences with that of *R. eutropha* Can are given at the right. Origins of sequences (accession number or source): 1, *R. eutropha* (AJ310671); 2, *X. fastidiosa* (AAF83690); 2, *P. aeruginosa* (AAG08063); 4, *P. purpureum* PCA1 (D86050); 5, *E. coli* YadF (AE000122); 6, *Coccomyxa* sp. (U49976); 7, *S. cerevisiae* (U52369); 8, *Pisum sativum* (X52558); 9, *Oryza sativa* (AB016283); 10, *Acidithiobacillus ferrooxidans* (The Institute for Genomic Research); 11; *Rhodobacter capsulatus* (University of Chicago and Institute of Molecular Genetics, Prague); 12, *P. aeruginosa* (AAG03492); 13, *Synechococcus* sp. strain PCC 7942 IcfA (M77095); 14, *Synechocystis* sp. strain PCC 6803 IcfA1 (U45962); 15, *E. coli* CynT (AE000141); 16, *P. aeruginosa* (AAG05441); 17, *Synechocystis* sp. strain PCC 6803 IcfA2 (D64001); 18, *Chlamydomonas reinhardtii* CA1 (CRU41189); 19, *Salmonella enterica* serovar Typhimurium Mig5 (AF020806); 20, *Bacillus subtilis* YtiB (Z991119); and 21, *Methanobacterium thermoautotrophicum* Cab (AE000918). The sequences of the putative CAs from *A. ferrooxidans* and *R. capsulatus* were derived from unfinished genome sequences and may thus contain errors.

1) was eventually shown to be sufficient for complementation, indicating that the mutation resided within this region.

Sequencing of the *Eco*RI fragment revealed 3,067 bp comprising two incomplete and two complete open reading frames (ORFs) (Fig. 1). The deduced product of the first incomplete ORF (ORF1) shares highest sequence similarity (74% amino acid identity) with a putative isovaleryl coenzyme A dehydrogenase from *Pseudomonas aeruginosa* (55). ORF2, located 111 bp downstream of ORF1, is complete and resembles *aceK*, encoding the dual-function isocitrate dehydrogenase kinase/phosphatase in *E. coli* (33). The amino acid sequence of AceK is 42% identical to the deduced amino acid sequence of ORF2 (613 residues).

The second complete ORF, designated *can*, encodes a polypeptide of 223 amino acid residues that has a calculated molecular mass of 24,909 Da and represents a presumptive β-CA. Sequencing of *can* amplified by PCR from 25-1 DNA identified a G→A transition at position 293 of the gene that converted a highly conserved glycine residue of the Can protein into an aspartate (G98D) (Fig. 2). To verify the phenotypic effect of the mutation in strain 25-1, a 423-bp in-frame deletion was introduced into the *can* gene of wild-type H16, generating mutant HB1. Like mutant 25-1, HB1 was unable to grow at air concentrations of CO_2 regardless of the substrate provided, confirming an essential physiological role of Can. In addition, the HCR phenotype of the deletion mutant was relieved by providing the *can*-containing 0.82-kb *Kpn*I-*Eco*RI fragment in *trans* (pKR200). Growth of the wild-type strain at ambient CO_2 concentrations therefore requires a functional *can* gene.

The 5′-terminal region of the second incomplete ORF (ORF4) was identified 46 bp downstream of *can*. Its potential product comprises 21 amino acid residues and shows high similarity (68% identity within 19 residues) to the *phaA*-encoded β-ketothiolase (EC 2.3.1.16) of *P. aeruginosa* (55). ORF4 may represent the gene of a third β-ketothiolase in *R. eutropha*, which has been postulated before (50). Conspicuous promoter structures were not detected on the entire fragment. The putative operon-like organization did not provide a hint at a common metabolic function of all genes.

***can* encodes a β-CA.** The amino acid sequence of the Can protein is more than 50% identical to those of putative β-CAs identified in *Xylella fastidiosa* (49) and *P. aeruginosa* (55), the almost-identical PCA1 and PCA2 of the red alga *Porphyridium purpureum* (41), and the *yadF*-encoded CA of *E. coli* (13). However, β-CAs are highly diverse, with overall sequence identities ranging to below 25%, limiting the similarities almost to the Zn^{2+}-binding motifs within the catalytic centers of the enzymes (Fig 2). The mutated glycine 98 in Can of *R. eutropha* mutant 25-1 is located next to a histidine residue that is present

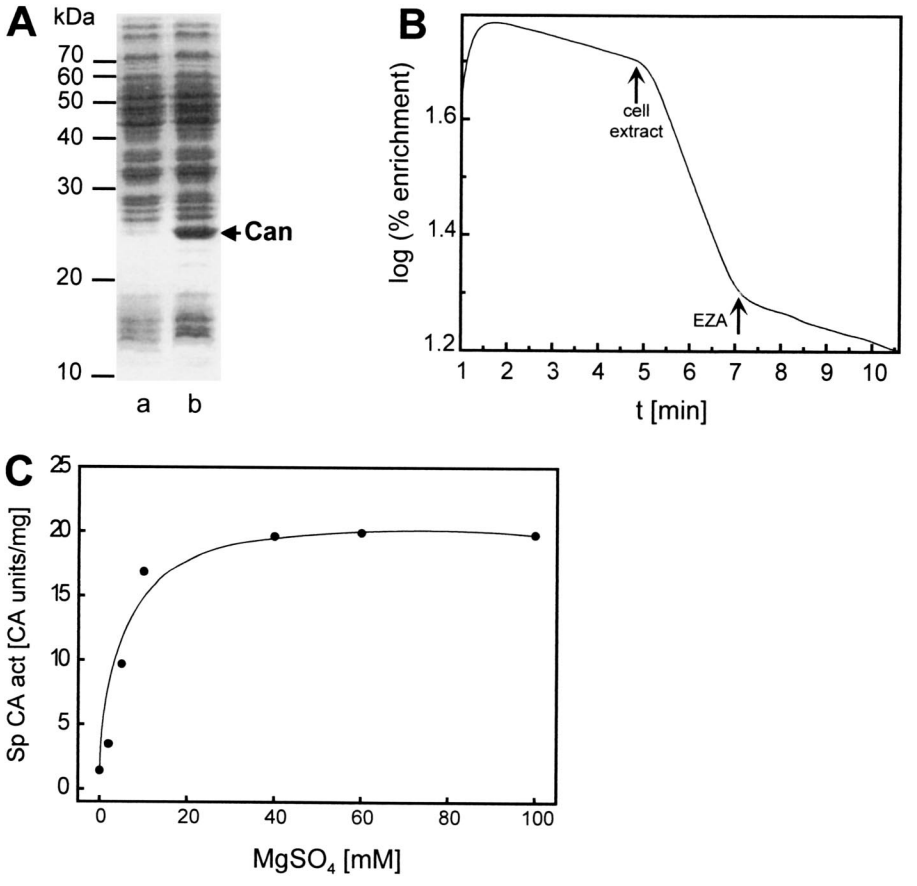

FIG. 3. Heterologous overexpression of the *can* gene from *R. eutropha* H16 in *E. coli* (A) and mass spectrometric assays of CA activities in a crude cell extract of transformant *E. coli* JW1(pCAN8210) (B and C). (A) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (14% [wt/vol] acrylamide) of cell lysates of transformants harboring pUC19 (lane a) or pCAN8210 (lane b). The arrow indicates the Can protein overproduced in *E. coli* JW1(pCAN8210). Molecular masses of reference proteins are indicated. (B) Representation of a CA activity assay in a cell extract of *E. coli* JW1(pCAN8210). The initial slope represents the uncatalyzed exchange reaction between $^{13}C^{18}O_2$ and unlabeled CO_2 after addition of $NaH^{13}C^{18}O_3$. Arrows mark the time points at which cell extract or EZA (0.5 mM) was added to the assay mixture. (C) Dependence of the CA activity on the presence of $MgSO_4$.

in all known β-CAs and that has been shown to participate in binding of Zn^{2+} (13, 32, 42, 56). Thus, the mutant Can in 25-1 is either nonfunctional or less active than the wild-type enzyme.

In bacteria, β-CA seems to be the most frequent type (25, 51). These enzymes are also found in some archaea, lower eukaryotes such as yeast and fungi, and generally in algae and higher plants. However, the number and type of CAs in bacteria vary widely, and many organisms have multiple genes encoding CAs that belong to the same or a different family (25, 51). In *P. aeruginosa* three putative β-CA (Fig. 2) and γ-CA genes were recognized (55). Two β-CAs, CynT and YadF, are known to function in *E. coli* (Fig. 2), while a third protein, CaiE, represents a presumptive γ-CA (51). In *Neisseria gonorrhoeae* and *Helicobacter pylori*, putative β- and γ-CA genes were identified (51, 62). The α-CAs of these organisms have been characterized recently (12, 27). The cyanobacterium *Synechococcus* sp. strain PCC 7942 produces an α-CA (EcaA) (53) and a β-CA (IcfA [CcaA]) (21), whereas another cyanobacterium, *Synechocystis* sp. strain PCC 6803, has two β-CAs (CcaA and EcaB) (30, 52). Although there is preliminary evidence for additional CA genes in *R. eutropha* H16, the potential CA activities of their products are apparently not sufficient to support growth of the organism under air in the absence of Can.

Heterologous expression of *can*. The *can* gene cloned in pCAN8210 was expressed in *E. coli*, resulting in an overproduced protein with a molecular mass of about 25 kDa (Fig. 3A), which is in close agreement with the value calculated from the deduced amino acid sequence of Can. High specific CA activity (20.5 CA U/mg) was detected in cell extracts of transformant *E. coli*(pCAN8210), whereas the reference strain *E. coli*(pUC19) showed only very low activity (0.2 CA U/mg), strongly suggesting that *can* encodes a functional CA. The general CA inhibitor ethoxyzolamide (EZA) (40) almost completely inhibited this activity at a concentration of 0.5 mM (Fig. 3B). Maximal CA activity was observed in the presence of 40 mM $MgSO_4$ (Fig. 3C), similar to the case for IcfA of *Synechococcus* sp. strain PCC 7942, which requires 20 mM $MgSO_4$ (67). $MgCl_2$ only slightly stimulated Can. This effect was also seen with IcfA and might be attributed to inhibition exerted by the chloride anions counteracting the Mg^{2+} stimulation of Can. However, in contrast to the case for IcfA, the reducing

TABLE 2. CA activities in cell extracts of *R. eutropha* wild-type strain H16 and *can* mutant HB1 grown in pyruvate-mineral medium under different aerations

Strain	Aeration[a]	Sp act of CA (CA U/mg of protein)[b]	
		Without EZA	With EZA
Wild type H16	Air	0.06	0.03
	Air + CO_2	0.08	0.04
	Shift	0.07	0.05
Mutant HB1	Air + CO_2	<0.01	ND[c]
	Shift	<0.01	ND

[a] Aeration of the cultures with air or air plus 10% (vol/vol) CO_2. For shifting from high to low CO_2, cells were initially grown under air plus 10% (vol/vol) CO_2 before the CO_2 supply was turned off when the culture reached an OD_{436} of about 0.3. Cells were harvested at an OD_{436} of about 1 or 4 h after the shift.

[b] CA activities were determined by the mass spectrometric assay in the absence or presence (0.5 mM) of the CA inhibitor EZA.

[c] ND, not determined.

agent dithioerythritol showed no significant influence on the CA activity of Can (data not shown).

CA activities in *R. eutropha*. Very low but significant CA activities were found in cell extracts (Table 2) as well as whole cells (Fig. 4) of wild-type strain H16, as determined by the mass spectrometric assay. The conventional assay measuring the drop in pH as result of CO_2 hydration was not sufficiently sensitive to detect these activities. EZA partially inhibited the CA activity detected in the cell extract that was apparently absent in the *can* mutant HB1. These findings strongly correlate the activity with Can and suggest that Can is the most prominent CA present in the cells under the growth conditions tested. The biphasic reaction kinetics of the whole-cell assays (Fig. 4) were indicative of a nonhomogeneous distribution of Can in the assay mixture and suggested an intracellular localization of the enzyme (43). Although the measured CA activity was low, it is apparently essential for growth of strain H16 at air concentrations of CO_2. Similarly low activities were also found for the β-CAs CynT of *E. coli* (24) and IcfA of *Synechococcus* sp. strain PCC 7942 (4, 67). Moreover, inactivation of the corresponding genes led to HCR phenotypes of the resulting mutants (21, 24). Aeration of the *R. eutropha* cultures by low (air) or high (10% [vol/vol]) CO_2 concentrations as well as shifts from high to low CO_2 concentrations did not significantly alter the CA activities (Table 2). The *can* gene of *R. eutropha* might thus be expressed constitutively at low levels, a conclusion supported by preliminary transcriptional studies (data not shown).

Growth characteristics of HCR mutant HB1. Like the original HCR mutant 25-1, *can* deletion mutant HB1 failed to grow

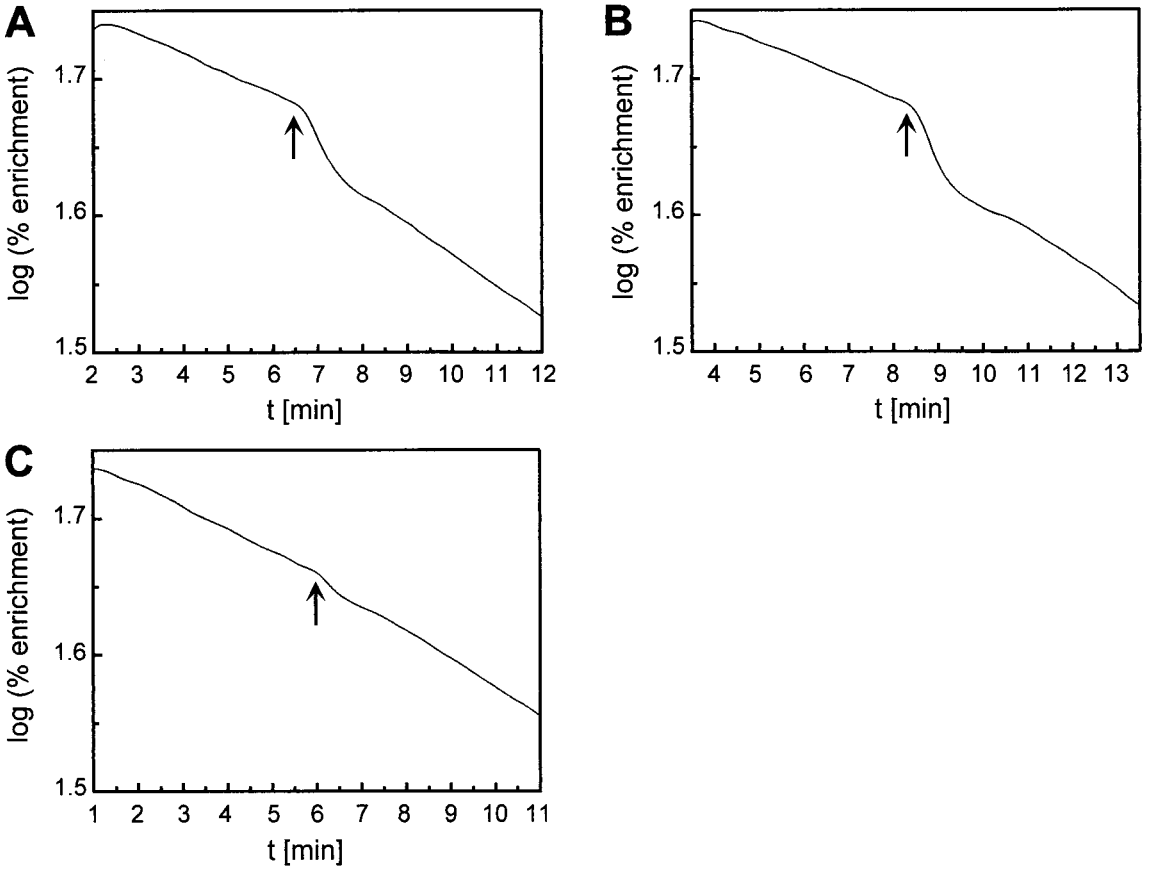

FIG. 4. Mass spectrometric assay of CA activity in whole cells of *R. eutropha* wild-type strain H16 and *can* mutant HB1. Cells were grown in pyruvate-mineral medium under different aerations. (A) H16 grown under air. (B) H16 grown under air plus 10% (vol/vol) CO_2. (C) HB1 grown under air plus 10% (vol/vol) CO_2. Arrows mark the time points at which cells were added to the assay mixture.

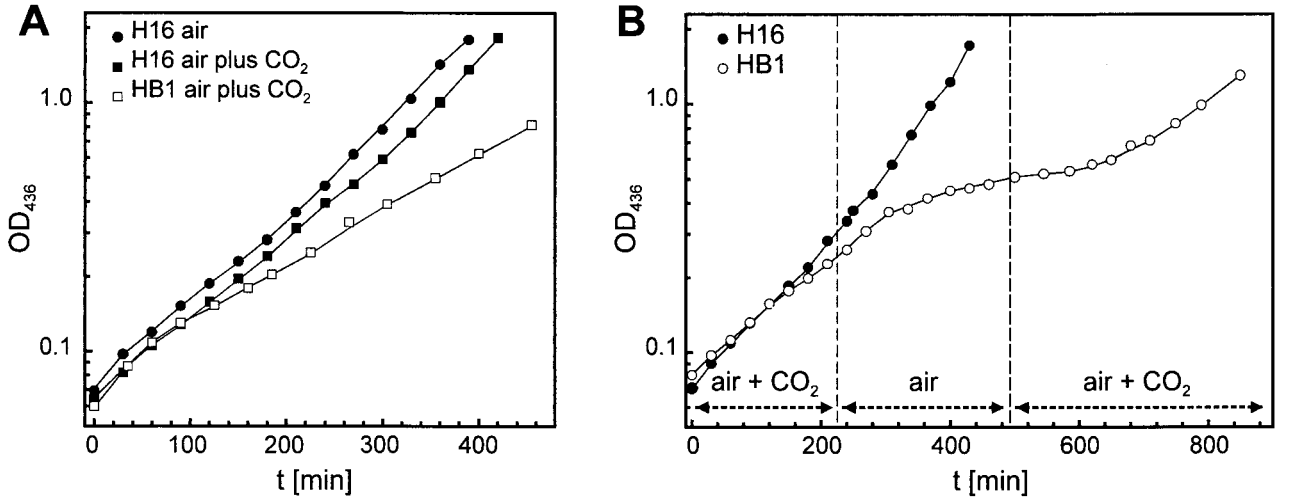

FIG. 5. Growth of *R. eutropha* wild-type strain H16 and *can* mutant HB1 in pyruvate-mineral medium under different aeration. (A) Constant aeration with air or air plus 10% (vol/vol) CO_2. (B) Shift from air plus 10% (vol/vol) CO_2 to air and back to air plus 10% (vol/vol) CO_2.

both heterotrophically and autotrophically regardless of the carbon and energy sources supplied when incubated on agar plates under air or air containing ≤0.1% (vol/vol) CO_2. Supplementation of the media with metabolites (malonate, 2-oxoglutarate, proline, histidine, arginine, hypoxanthine, adenine, thymine, uracil, or oleate) known to replace high CO_2 requirements of mutants of other microorganisms (6, 10, 28, 64) was not effective for HB1. Even growth on complex nutrient broth medium was dependent on high CO_2 concentrations. Wild-type growth of HB1 was restored in the presence of highly elevated CO_2 (3 to 10% [vol/vol]) in air, depending on the substrate utilized. Similar obligate HCR phenotypes have been described previously for mutants of *E. coli* (10) and *Streptomyces coelicolor* (64) without assigning them to a specific metabolic/genetic defect.

The obligate HCR phenotype of mutant HB1 excludes a specific involvement of Can in autotrophic metabolism of *R. eutropha*. In contrast, IcfA appears to play a specific role in the autotrophic CO_2 assimilation of *Synechococcus* sp. strain PCC 7942. IcfA is a β-CA located in the carboxysomes of the organism that functions as a component of the CO_2-concentrating mechanism required for photosynthetic growth at low CO_2 concentrations (31). Inactivation of the *icfA* gene caused an HCR phenotype of *Synechococcus* (21, 44, 67). Because the cyanobacterium is obligately autotrophic, it is not easily possible to differentiate between the specific CA function of IcfA in CO_2 assimilation and a potential additional role in the general CO_2 metabolism of the organism. An increased sensitivity towards oxygen as reported for the *nce103* mutant strain of the yeast *Saccharomyces cerevisiae*, which is defective in a putative β-CA (23), was not observed with *R. eutropha* mutant HB1. The HCR phenotype of HB1 was also evident when it was grown anaerobically under denitrifying conditions (data not shown). In contrast, yeast mutant *nce103* was found to grow well aerobically at elevated CO_2 concentrations (D. Sültemeyer, unpublished data).

Growth of wild-type H16 and mutant HB1 was studied in more detail in liquid culture with mineral medium containing fructose or pyruvate as a substrate. As anticipated, the mutant failed to grow when gassed with air, whereas air plus 10% (vol/vol) CO_2 restored wild-type growth rates on fructose (data not shown). In contrast, growth of the mutant on pyruvate remained significantly slower than that of H16 even under air plus 10% (vol/vol) CO_2 (doubling time of 2.5 versus 1.5 h) (Fig. 5A). Mutant 25-1 has previously been found to exhibit a similar behavior during growth on lactate (1). Growth of mutant HB1 on pyruvate under air plus 10% (vol/vol) CO_2 ceased after a shift to air but returned to the initial rate upon resupply of CO_2 (Fig. 5B). This effect was most evident when the cultures were shifted at low cell densities (OD_{436} of up to about 0.4). Shifts to air at higher densities simply led to decreased growth or had no detectable effect (data not shown). In these cases the metabolically generated CO_2 presumably was sufficient to compensate for the high CO_2 requirement of the mutant. High concentrations of metabolic CO_2, occurring at high cell densities, seem to mask the need for CA activity in the HCR mutant. Changes in the CO_2 content of the atmosphere did not affect the growth of wild-type H16 (Fig. 5B), although *R. eutropha* has been shown to require elevated initial CO_2 concentrations to shorten the lag phase of low-density cultures (47). It is conceivable that low CO_2 concentrations present in such cultures due to limiting metabolic CO_2 do not allow the cells to convert sufficient CO_2 into HCO_3^- before CO_2 diffuses out (35). Bicarbonate is essential for some carboxylation reactions. Carbonic anhydrase would support the provision of bicarbonate to these reactions. However, the CO_2 demand of the wild type during the lag phase is different from the general high CO_2 requirement of the *can* mutants, since the latter could not be suppressed by added metabolites. The lag-phase CO_2 demand might contribute to the substrate-dependent variations in the CO_2 requirement of the *can* mutants.

Phenotypic complementation of HCR mutant HB1 by heterologous CA genes. Mutant HB1 was subjected to complementation analyses to check whether expression of heterologous CA genes encoding different types of CA could alleviate the HCR phenotype. For this purpose, the β-CA gene *cynT* from *E. coli* (pMP-*cynT*) or the human α-CA gene CAII (pMP-HCAII) was transferred into HB1. Both resulting transconju-

gants regained the ability to grow under air, indicating that sufficient CA activity is apparently required for *R. eutropha* to grow at ambient CO_2 concentrations. Can is not specifically needed, as its function can be replaced by other CAs which may belong to different families.

The *cynT* gene is part of the *cyn* operon of *E. coli*, which enables the organism to utilize cyanate as the sole nitrogen source. Inactivation of *cynT* caused an HCR phenotype of the mutant when it was growing in the presence of cyanate (24). Similar to the case for the *R. eutropha can* mutants, the *E. coli cynT* mutant was unable to grow under air unless the cyanate inhibition was overcome by elevated CO_2 concentrations or complementation by the human CAII (37). The phenotype of the *cynT* mutant has been attributed to inhibition by cyanate of a metabolic function involving DIC, rather than to a specific involvement of CynT in cyanate degradation (36, 37) as had been proposed previously (24). Since *R. eutropha* is also able to utilize cyanate as the sole nitrogen source (data not shown), Can might allow growth of the organism in presence of cyanate in much the same way that CynT does in *E. coli.* However, in contrast to the case for the *E. coli cynT* mutant, the HCR phenotype of mutant HB1 is not correlated to the presence of cyanate. This notion gained support by the phenotypic complementation of the *E. coli cynT* mutant expressing the *can* gene of *R. eutropha* (data not shown). It is likely that YadF, a second β-CA in *E. coli* (13) sharing high similarity with Can, might be the target of the CO_2-suppressible cyanate inhibition in vivo. The following observations support this conclusion: (i) the *cynT* mutant did not exhibit the HCR phenotype in the absence of cyanate (35), (ii) the CA activity of YadF but not that of CynT is strongly inhibited by cyanate in vitro (36; F. von Götz, B. Kusian, and B. Bowien, unpublished data), (iii) a *yadF*-deficient mutant showed an HCR phenotype except in the presence of cyanate (von Götz et al., unpublished data), and (iv) *can* and *yadF* complemented the *yadF* mutant and mutant HB1 of *R. eutropha*, respectively (von Götz et al., unpublished data). Therefore, the physiological role of YadF in *E. coli* seems to correspond to that of Can in *R. eutropha*. CynT would replace the function of YadF in the presence of cyanate.

Concluding remarks. The wide distribution and multiple occurrence of CAs in bacteria suggest a fundamental physiological significance of these enzymes in DIC metabolism by cells. Our results indicate that growth of *R. eutrophus* at air levels of CO_2 is principally dependent on sufficient CA activity, which is provided by the β-CA Can. In *E. coli* the β-CA YadF seems to serve this function. Further evidence suggests that growth of other organisms in air also requires CA activity. Although the involvement of CA in autotrophic growth of cyanobacteria at low CO_2 concentrations has been known for a long time, this is the first report relating obligate high-CO_2 requirements of heterotrophs directly to the lack of CA. In view of these findings, CAs must be assigned an essential role in DIC metabolism, at least at low CO_2 levels in the environment. However, the metabolic functions depending on CA activity under these conditions still remain to be identified. In this context, the availability of CO_2-HCO_3^- for carboxylation reactions, pH homeostasis, and possibly DIC-directed gene regulation are potential areas of interest.

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