In Lactobacillus plantarum, Carbamoyl Phosphate Is Synthesized by Two Carbamoyl-Phosphate Synthetases (CPS): Carbon Dioxide Differentiates the Arginine-Repressed from the Pyrimidine-Regulated CPS

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Received 19 January 2000/Accepted 22 February 2000

Carbamoyl phosphate (CP) is an intermediate in pyrimidine and arginine biosynthesis. Carbamoyl-phosphate synthetase (CPS) contains a small amidotransferase subunit (GLN) that hydrolyzes glutamine and transfers ammonia to the large synthetase subunit (SYN), where CP biosynthesis occurs in the presence of ATP and CO2. Lactobacillus plantarum, a lactic acid bacterium, harbors a pyrimidine-inhibited CPS (CPS-P; Elagöz et al., Gene 182:37–43, 1996) and an arginine-repressed CPS (CPS-A). Sequencing has shown that CPS-A is encoded by carA (GLN) and carB (SYN). Transcriptional studies have demonstrated that carB is transcribed both monocistronically and in the carAB arginine-repressed operon. CP biosynthesis in L. plantarum was studied with three mutants (ΔCPS-P, ΔCPS-A, and double deletion). In the absence of both CPSs, auxotrophy for pyrimidines and arginine was observed. CPS-P produced enough CP for both pathways. In CO2-enriched air but not in ordinary air, CPS-A provided CP only for arginine biosynthesis. Therefore, the uracil sensitivity observed in prototrophic wild-type L. plantarum without CO2 enrichment may be due to the low affinity of CPS-A for its substrate CO2 or to regulation of the CP pool by the cellular CO2/bicarbonate level.

Lactobacilli are fastidious gram-positive bacteria with complex nutritional requirements resulting from numerous genetic lesions in metabolic pathways which often revert to prototrophy (24). Natural auxotrophies involved in carbamoyl phosphate (CP) biosynthesis were shown to be reversed in most cases by incubating lactobacilli in CO2-enriched air (2; F. Bringel, unpublished data). Metabolically, lactobacilli are at the threshold of the transition from anaerobic to aerobic life (16): Lactobacillus plantarum grows in aerobiosis but prefers microaerobiosis or increased CO2 concentration.

The arginine and pyrimidine biosynthetic pathways share CP as a common precursor. CP synthetase (CPS) catalyzes the synthesis of CP from bicarbonate, glutamine, and two molecules of ATP via a complex reaction mechanism that leads to several unstable intermediates. The X-ray crystal structure of CPS in Escherichia coli has revealed the location of three separate active sites connected by two molecular tunnels that run through the interior of the protein (33) and that would allow substrate channeling and subsequent protection of the reactive intermediates (reviewed in reference 22). Furthermore, CP biosynthesis in Pyrococcus abyssi also includes metabolic channeling, a process whereby the product of one enzyme is directly transferred to the next enzyme in the pathway without being released in the bulk solvent; CPS may interact with two CP-utilizing enzymes in the pyrimidine (aspartate carbamoyltransferase) and the arginine (anabolic ornithine carbamoyltransferase) biosynthetic pathways (29). The heterodimeric CPS enzyme is composed of a small subunit (GLN) which functions as a glutamine amidotransferase and a large synthetase subunit (SYN) that fulfills the other catalytic properties (for a review, see reference 4). Allosteric regulation via the carboxy-terminal part of SYN subunit by effectors which are intermediates in the pyrimidine, arginine, and purine pathways has been identified for some prokaryotic CPSs (4) but not for others (27, 38).

Prokaryotic CPSs are encoded by two genes, commonly named carA and carB, which are organized as an operon with the gene order carAB. Prokaryotes harbor a single CPS regulated by both the pyrimidines and arginine levels in the cell (E. coli); alternatively, a single CPS regulated by the pyrimidines and CP is also produced by arginine degradation (Lactobacillus leichmannii and possibly Lactococcus lactis and Enterococcus faecalis), or a set of two CPSs specifically pyrimidine or arginine regulated is produced (Bacillaceae). In L. plantarum, earlier studies suggested the presence of two CPSs, CPS-P, and CPS-A. CPS-P is part of the pyr operon regulated by transcriptional attenuation under the control of the regulator PyrR (9, 10). The existence of L. plantarum CPS-A was suspected but not proven. Partial cloning of a carA-like sequence contiguous to the arginine-repressed biosynthetic genes (3) suggested the existence of CPS-A in L. plantarum. Furthermore, Lyman et al. observed in 1947 that L. plantarum (formerly named L. arabinosus) grew in the presence of uracil if incubated in air enriched with CO2. This CO2-conditional uracil sensitivity was confirmed in our laboratory. These data argue for the presence of a CPS-A in L. plantarum which is not inhibited by uracil and not functional in cells grown with ordinary air unless supplemented with CO2. In this study, we demonstrate that L. plantarum indeed harbors two functional CPSs. In contrast to other microorganisms, wild-type L. plantarum CP synthesis is dependent on higher CO2 requirements.

MATERIALS AND METHODS

Gram-positive bacteria strains and culture conditions. L. plantarum CCM-1904 was grown on MRS medium (Difco Laboratories) supplemented with arginine (500 µg/ml), uracil (100 µg/ml), and erythromycin (2.5 µg/ml) when necessary. Nutritional requirements were tested at 30°C on agar plates of defined medium DLA (3) supplemented with 50 µg of arginine or uracil per ml in

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Plasmid pGID023Δcar was introduced by transformation in the L. plantarum wild-type strain, and erythromycin-resistant transformants were selected. After testing for plasmid marker loss, we obtained 13 erythromycin-sensitive exants that either harbored the CPS-A deletion or reverted to the wild-type allele. To discriminate between these two types of exants, Southern-type hybridization was done with a probe hybridizing with the remaining carB gene. The probe hybridized with genomic DNA digested with either NspI (5’2432) or HinfIIII, and a band of 4 kb or 3 kb, respectively, was detected with the wild-type CCM1904, whereas a band of 2.6 kb or 1.6 kb, respectively, was obtained with four exants (data not shown). CPS-A deletion was confirmed in exant FB335 using PCR amplifications (data not shown).

(iii) HN217. To construct an L. plantarum strain with no functional CPS, plasmid pGID023Δcar harboring the CPS-A deletion was introduced by electroporation in FB331, a strain having the CPS-P deleted. After the two steps of homologous recombination, pGID023Δcar integration at the car locus, and excision of the vector, erythromycin-sensitive exants were analyzed as described for FB335. Strain HN217 was an erythromycin-sensitive exant deleted of both CPS-A and CPS-P as demonstrated by Southern-type hybridization, PCR amplifications, and sequencing (data not shown).

Computer analysis. Database searches and sequence analyses were performed using the Genetics Computer Group package from the University of Wisconsin (6) and the advanced BLAST program (1). The ClustalX program (35) was used for protein alignments.

RESULTS AND DISCUSSION

Sequencing strategy without cloning. CPSs are generally encoded by two carA and carB clustered genes (see Introduction). We cloned part of carA while cloning the argF gene by complementation of a B. subtilis argF mutant since argF is 4.5 kb distant from carA (3). Our attempts to clone the L. plantarum car genes by functional complementation in a CPS-deficient E. coli strain (c600ΔcarB8) were unsuccessful (see Discussion of arginine repression studies for possible explanations). Thus, PCR without cloning was chosen to complete the carA sequence and find carB (Fig. 1). We added 3,954 bp to the 7,226-bp arg cluster sequence previously published.

Sequence analysis. (i) GLN subunit. Divergently oriented with respect to the arg cluster, an open reading frame (ORF) of 355 aa shares more than 40% identity with the GLN small subunit of different bacterial CPSs (B. subtilis, B. stea- thermophilus, B. subtilis, and E. coli; CPS-P of L. plantarum) and corresponds to the carA gene. Important residues were identified for E. coli GLN (Cys249, His335, Glu555, and His312) by biochemical studies, site-directed mutagenesis, and X-ray crystal structure analysis (33) and found in the L. plantarum GLN subunit (Cys240, His226, Glu278, and His344).

The pyrimidine-regulated GLN subunits have about 10 aa more than the gram-positive bacteria arginine-regulated GLN subunits. To investigate the putative localization of these missing amino acids, gram-positive bacteria GLN subunit sequences of CPS-A (B. stea- thermophilus, 354 aa; B. subtilis, 353 aa; L. plantarum, 355 aa) were aligned with the 364-aa-long sequences of CPS-P (B. caldolyticus, B. subtilis, and L. planta- rum). As a reference, the single E. coli CPS (382 aa) regulated by both the arginine and the pyrimidines was also aligned. As seen in Fig. 2, a stretch of 7 to 8 aa that is not conserved in CPS-A is absent only in CPS-A. When placed on the E. coli CPS quaternary structure (33) this segment, named CPS-AΔ (Fig. 2), was at the hinge of the NH2- and catalytic COOH-terminal subdomains of the bifold GLN subunit. When other prokaryotic CPS-A sequences are available, the significance of this proposed CPS-A specific deletion can be tested.

(ii) SYN subunit. The second ORF encodes 1,020 aa sharing significant identity with the SYN large subunit of several bacterial CPSs (L. plantarum carA carP, 62%; B. caldolyticus, 59%; L. lactis, 59%; E. coli, 48%). Therefore, this ORF was named carB. The 7-nr overlap of the GLN and SYN subunits (Fig. 3)
suggests stoichiometric translational coupling. *E. coli* CPS crystal structure studies revealed an internal tunnel mediating substrate channeling; 21 conserved residues lining this tunnel (34) were also found in *L. plantarum* CPS-A (data not shown). The *L. plantarum* SYN subunit is the smallest of the arginine-specific SYN subunits described so far. Like CPS-A in *B. subtilis* (1,027 aa [27]) and *B. stearothermophilus* (1,064 aa) (protein alignments not shown). Yang et al. (38) correlated the difference in size (average of 34 aa) between CPS-A and CPS-P with the presence of the pyrimidine effector binding site in the carboxy terminus of CPS-P. So, is *L. plantarum* CPS-A, like other gram-positive bacteria CPS-A, unable to bind allosteric ligands?

(iii) Third ORF. Another gene was found with the opposite orientation of carB and located 106 bp after the carB stop codon. This ORF encodes a 124-aa protein truncated at its N terminus that shared no similarity with known proteins in the databank. Therefore, we designated this gene usg (upstream gene of the citrulline biosynthetic cluster).

Transcription studies and arginine repression. (i) *carB* is cotranscribed with *carA* and transcribed monocistrionically, mRNAs extracted from *L. plantarum* grown on DLA without arginine were probed with a *carA* fragment. A unique 4.2-kb transcript was detected (Fig. 4A), demonstrating cotranscription of the carAB genes. The *carA* transcription initiation site was localized using primer extension (Fig. 5), and the corresponding promoter was found (Fig. 3) by similarity with the *Lactobacillus* consensus sequence (−35 box [TTGACA] and −10 box [TATAAT] 17 nt apart) (28). Since the *carB* gene is transcribed monocistrionically in *P. aeruginosa* and in *L. lactis* (18, 20), independent *carB* transcription was assessed in *L. plantarum* using a specific *carB* probe in Northern hybridizations. Indeed, we detected a 4.2-kb transcript (Fig. 4B) but not the 3.1-kb band corresponding to the predicted *carB* transcript. Nonetheless, *carB* may be weakly transcribed. Therefore, primer extension experiments were done on mRNAs extracted from cells grown in the absence of arginine, and a transcription start site was identified (Fig. 5) with two different primers (data not shown). We concluded that *carB* is independently transcribed. However, in the vicinity of the transcription start site, no promoter-like sequence could be found. Moreover, as no *carB* transcript was revealed by Northern blots, the *carB* mRNA is probably not abundant. This could result from weak transcription in the tested conditions or *carB* mRNA instability.

(ii) Arginine repression studies. In *L. plantarum*, arginine inhibition of the biosynthetic ornithine transcarbamoylase activity was correlated to the presence of 11 *E. coli*-like ARG boxes in the intergenic *carA-argC* promoter region (3). Northern hybridizations with either a *carA* or a *carB* probe were assessed on mRNA extracted from *L. plantarum* grown in DLA with or without arginine. As a positive control, transcription of the constitutive gene *ldhL* (11) was visualized with a 1-kb band in all cases (data not shown). No transcripts were detected with the *carB* probes in presence of arginine (Fig. 4), demonstrating arginine repression of the *car* operon. Two regions were similar to the *B. subtilis* ARG box consensus sequence (23). The first region (5'-CATIAccAAAATGaaAT-3'; mismatches are in lowercase) was located on the *carA* initiation codon (Fig. 3); the second region is also similar to the *E. coli* ARG box consensus and was previously designated R9 (3). The lack of success in cloning *L. plantarum* carAB in *E. coli* may be explained by the arginine repressor binding to the ARG boxes present on the insert and not to its *E. coli* target. Moreover,
plasmid instability may occur since the arginine repressor is an accessory protein involved in ColE1 multicopy plasmid stability (reviewed in reference 32). We conclude that L. plantarum may harbor an arginine repressor binding in the presence of its corepressor to a specific ARG box, as found for E. coli or other gram-positive bacteria, B. subtilis (23), and B. stearothermophilus (7).

Physiological analysis of the L. plantarum deletion mutants. Since CPS produce CP for arginine and pyrimidine biosynthetic pathways, CPS-deficient derivatives were tested for arginine and uracil auxotrophy. Moreover, different growth conditions were tested such as aerobiosis and anaerobiosis because oxygen limitations induce arginine catabolism in some microorganisms (12), and CP produced from arginine catabolism provides pyrimidine biosynthesis in some lactobacilli (15). Our anaerobiosis conditions implied both oxygen depletion (less than 2%) and CO2 enrichment from 4 to 20%. Thus, to evaluate the effect of CO2 alone, we tested growth conditions in ordinary air enriched with either 4 or 20% CO2 and no change of oxygen concentration. We found no difference of growth between anaerobiosis and CO2-enriched air at either 4 or 20% CO2 (Table 1). On the contrary, incubation with less than 0.01% of CO2 (ordinary air) had a dramatic effect on L. plantarum growth in the presence of uracil alone. Thus in our tests, the important factor to alleviate uracil sensitivity in L. plantarum was CO2 and not oxygen.

(i) In L. plantarum, no CP-producing system other than CPS-A and CPS-P is functionally significant. To test if L. plantarum can produce CP for arginine and pyrimidine biosynthetic pathways using a system different from the two CPSs, strain HN217 (double CPS deletant) was tested for its ability to grow on defined media. Such an alternative CP-producing system may result from carbamate kinase activity. A carbamate kinase-like CPS has been identified in the prokaryote Pyrococcus furiosus (8) and shown to be enzymatically and structurally a carbamate kinase that produced rather than degraded CP in vivo (37). To assess if an anabolic carbamate kinase may be present in L. plantarum, the growth requirement of HN217 was tested. The absence of both CPSs in HN217 resulted in arginine and pyrimidine auxotrophy (Table 1). This result was confirmed in the single CPS deletants under conditions repressing the remaining CPS (Table 1; FB335 was uracil sensitive and FB331 was unable to grow in the presence of arginine alone in CO2-enriched air). Thus, in the tested conditions, no CP-producing system other than CPS-A and CPS-P is present or functionally significant in L. plantarum.

(ii) CPS-P is the major source of CP in L. plantarum. To assess the CPS-P relative role in L. plantarum and whether CPS-P can supply CP necessary for arginine and pyrimidine biosynthesis, we tested the growth requirements of strain FB335 with only a functional CPS-P. FB335 was prototrophic for arginine and uracil (Table 1, without supplement). Thus, CPS-P alone can provide sufficient CP for the pyrimidine and arginine biosynthetic pathways. Despite its natural lability, CP can be transferred from the L. plantarum pyrimidine-specific CPS-P to the arginine biosynthetic CP-consuming enzyme, ornithine carbamyltransferase. This is reminiscent of the absence of CP pool compartmentalization found in B. subtilis, which also harbors two CPSs, and is consistent with the case of prokaryotes having a unique CP such as E. coli but is different from the eukaryote Neurospora (27). Since unlike CPS-P deletion (see below), CPS-A deletion did not lead to auxotrophy, we concluded that CPS-P is the major source of CP in L. plantarum.

(iii) CPS-A is functional in L. plantarum grown in CO2-enriched air and provides CP only for arginine biosynthesis.
With only a functional CPS-A, FB331 was unable to grow on DLA (Table 1, without supplement). Thus, CPS-A is unable to provide CP for both the arginine and pyrimidine biosynthetic pathways. Therefore, unlike CPS-P, CPS-A is not essential for \textit{L. plantarum} growth in minimal medium. FB331 grew in the presence of both arginine and uracil, demonstrating that only these corresponding biosynthetic pathways were impaired in FB331. FB331 grew in the presence of uracil only when CO₂ was added to air (similar to the wild-type strain, where CPS-P was repressed and only CPS-A was theoretically available for CP biosynthesis [Table 1, with uracil in ordinary air]). The conditional pyrimidine auxotrophy suggests that CPS-A provides enough CP for arginine biosynthesis and is active only in CO₂-enriched air. Since unlike CPS-P, CPS-A is not able to provide CP for pyrimidine biosynthesis, CPS-P may be more abundant than CPS-A, or possibly CPS-A activity remains lower than CPS-P activity even in presence of increased CO₂ concentrations.

**How can CO₂ stimulate CP biosynthesis in \textit{L. plantarum}?**

CO₂ may regulate CPS-A expression as described for various microbial genes (31). To assess if CO₂ was absolutely required for \textit{carAB} transcription, Northern hybridization and primer extensions were done on mRNAs extracted from cells cultivated in air with agitation. The 4.2-kb \textit{carAB} transcript was detected, and primer extension of the 5' end of the \textit{carB} transcript was obtained (data not shown). These transcriptional results do not exclude CO₂ as a modulator of the \textit{car} genes but strongly suggest that the main effect of CO₂ stimulation on CPS-A is not at the transcriptional level.

The most obvious explanation for CO₂ stimulation is that CPS-A has a low affinity for CO₂/HCO₃⁻ as a substrate. Non-auxotrophic \textit{E. coli} mutants with sensitivity to uracil alleviated by raising CO₂ in the gas phase were isolated (21) and recently correlated to amino acid substitutions within the large CPS

**FIG. 3.** Transcriptional and translational elements of the \textit{L. plantarum carAB} operon. Numbers correspond to the citrulline biosynthetic cluster sequence (accession number X99978). The +1 labels indicate transcriptional start sites, which are marked with thick arrows. Residues involved in the \textit{carA} promoter are double underlined. \textit{A. subtilis} AR box-like sequence (23) around the +1 site is indicated with a blackened box. The proposed translational initiation and stop codons are indicated with boxes for the CPS-A GLN (\textit{carA} gene) and SYN (\textit{carB} gene) subunits and for the adjacent divergently oriented ORF. Good putative ribosome binding sites which exhibit complementarity to the \textit{L. plantarum} 3' terminal and 5' terminal 16S rRNA sequence (EMBL accession number M58827) are boldface and underlined, respectively. The free energy (Δ\textit{G}) characterizes the base pairing between the different RNAs. Two arrows indicate the inversely repeated (IR) sequences preceding a row of T residues, which suggests a transcriptional rho-independent terminator (estimated Δ\textit{G} of -12 kcal/mol for IR pairing).

**FIG. 4.** Transcription studies and arginine repression with Northern hybridizations using mRNAs extracted from \textit{L. plantarum} grown on defined medium without and with arginine (tracks 1 and 2, respectively) and probed with a \textit{carA} (A) or \textit{carB} (B) probe. The size of the transcript was determined using the Gibco BRL 0.24- to 9.5-kb RNA ladder (track M), which was revealed by hybridization with digoxigenin-labeled λ DNA.
subunit (5). These residues (Pro$^{165}$, Pro$^{170}$, Ala$^{192}$, and Pro$^{260}$, equivalent to L. plantarum Pro$^{258}$) were conserved between L. plantarum and E. coli wild-type SYN subunits (protein alignment not shown). The CO$_2$ bicarbonate level may be naturally low in L. plantarum grown under laboratory conditions since the two CO$_2$-producing steps of the Krebs cycle (isocitrate dehydrogenase and 2-oxoglutarate dehydrogenase) are inoperative, or other systems regulate CPS-A or the CP pool when a normal affinity for its substrate and CPS-P may have an isoenzyme specificity. The CO$_2$-enriched air, is not yet known.

The uracil sensitivity in aerobiosis but not in a CO$_2$-enriched air was found not only in strain CCM1904 but also in 90

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<td>CPS-A deficient (FB335)</td>
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<td>CPS-P and CPS-A deficient (HN217)</td>
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*Three days of growth on agar plates incubated at 30°C in different air conditions as described in Materials and Methods. Numbers in parentheses represent percent O$_2$, percent CO$_2$, +, +, +, and – refer to very good, good, and no growth, respectively.

ACKNOWLEDGMENTS

We thank J. Delcour for providing plasmid pGID023 and R. Cunin for strain C0000Mexar88.

REFERENCES


TABLE 1. Nutritional requirements of wild-type L. plantarum and CPS deletion mutants

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