We have previously isolated ineffective (Fix-) mutants of *Rhizobium meliloti* 104A14 requiring both arginine and uracil, and thus probably defective in carbamoylphosphate synthetase. We describe here the molecular and genetic analysis of the *R. meliloti* genes coding for carbamoylphosphate synthetase. Plasmids that complement the mutations were isolated from a *R. meliloti* gene bank. Restriction analysis of these plasmids indicated that complementation involved two unlinked regions of the *R. meliloti* chromosome, carA and carB. Genetic complementation between the plasmids and mutants demonstrated a single complementation group for carA, but two overlapping complementation groups for carB. The cloned *R. meliloti* genes hybridize to the corresponding *E. coli* carA and carB genes which encode the two subunits of carbamoylphosphate synthetase. Transposon Tn5 mutagenesis was used to localize the carA and carB genes on the cloned *R. meliloti* DNA. The cloned *R. meliloti* carA and carB genes were unable to complement *E. coli* carA or carB mutants alone or in combination. We speculate on the mechanism of the unusual pattern of genetic complementation at the *R. meliloti* carB locus.

## INTRODUCTION

Carbamoylphosphate (CP), an intermediate required in the synthesis of both arginine and pyrimidines, is made by the enzyme carbamoylphosphate synthetase (EC 6.3.5.5). In prokaryotes and lower eukaryotes, the enzyme consists of two subunits (Pikrard *et al.*, 1973). The small subunit hydrolyses glutamine and transfers the ammonia that is released to the large subunit. The large subunit phosphorylates bicarbonate using ATP to generate carbonylphosphate, which then reacts with the ammonia to form carbamate (Fig. 1). A second molecule of ATP is used to phosphorylate carbamate to give CP (Trotta *et al.*, 1974).

In *Escherichia coli*, CP synthetase is subject to both transcriptional and allosteric regulation. The enzyme is encoded by the carAB operon. The operon is transcribed from tandem promoters: the upstream promoter is regulated by pyrimidines, and the downstream promoter by arginine (Bouvier *et al.*, 1984; Piette *et al.*, 1984). The activity of CP synthetase is modulated post-translationally by the allosteric activators inosine 5'-monophosphate and ornithine, and the inhibitor uridine 5'-monophosphate (Piérard *et al.*, 1976). Thus, CP is made only when end-products of either pathway are absent and other intermediates required to complete the synthesis are present at sufficiently high concentration.

As a general goal, we wish to understand the organization of intermediary metabolism in *Rhizobium* and its relationship to symbiotic nitrogen fixation. From the results presented in the

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**Abbreviations**: CP, carbamoylphosphate; SSC, saline sodium citrate (150 mM-NaCl, 15 mM-sodium citrate).

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preceeding paper (Kerppola & Kahn, 1988) we concluded that CP synthetase is required for successful symbiosis between R. meliloti and alfalfa (Medicago sativa). CP synthetase uses glutamine as a nitrogen donor and is regulated both by nucleotides and by amino acids. We therefore felt that studying the regulation of this key enzyme might be useful in understanding rhizobial nitrogen metabolism, and we describe here the preliminary characterization of the R. meliloti genes for CP synthetase.

METHODS

Bacterial strains and plasmids. R. meliloti 104A14, its derivatives that require arginine and uracil, and the general procedure of complementing auxotrophs using a P2 cosmid gene bank have been described (Kerppola & Kahn, 1988). E. coli MI178 carA and JEF8 carB (Mergeay et al., 1974) were obtained from Barbara Bachmann, E. coli Genetic Stock Center, Yale University. P2vir1, an immunity-sensitive derivative of bacteriophage P2, and P2vir22, a virulent mutant of P2 (Bertani & Bertani, 1971) were from R. Calendar, University of California, Berkeley. E. coli C-la was used to propagate P2vir1 and P2vir22. The P2 lysogen C-2324 was used as an intermediate host for the isolation of transposon insertion mutants. pMC40 (Nyunoya & Lusty, 1983) was provided by Marjolaine Crabeel and André Piérard, Université Libre de Bruxelles. Plasmids pPH1JI and R751 (Hirsch & Beringer, 1984) were obtained from Fred Ausubel, Massachusetts General Hospital, Boston, Mass., USA.

Media and chemicals. LB broth, yeast mannitol broth (YMB), yeast sucrose broth (YSB), M9 minimal medium and minimal mannitol medium have been described (Somerville & Kahn, 1983). When specified, E. coli media were supplemented with kanamycin sulphate at 75 µg ml⁻¹ or tetracycline at 25 µg ml⁻¹ and R. meliloti media were supplemented with tetracycline at 5 µg ml⁻¹ and kanamycin sulphate at 200 µg ml⁻¹ or as indicated. Tryptone, yeast extract and agar were from Difco. Salts and glucose were from J. T. Baker Chemical Co. All other chemicals and antibiotics were from Sigma.

Transduction into R. meliloti. This was done as described in the accompanying paper (Kerppola & Kahn, 1988).

DNA manipulations. Plasmid isolation and characterization followed described procedures (Kahn et al., 1979; Kerppola & Kahn, 1988). The DNA fragments to be used as probes in Southern hybridization experiments were isolated from preparative agarose gels and radioactively labelled by nick-translation (Thomasow et al., 1980). Restriction fragments were separated by agarose gel electrophoresis and transferred to nitrocellulose filters (Southern, 1975). The filter membranes were incubated with labelled probe, washed under the conditions specified, and analysed by autoradiography.

Complementation tests. Plasmids to be tested for their ability to complement R. meliloti mutants were transformed into E. coli C-la and transducing lysates were prepared. These lysates were infected into R. meliloti mutants and tetracycline-resistant colonies were isolated on YMB medium. The colonies were then transferred to minimal medium to determine their ability to grow without supplements. For complementation of E. coli, plasmids were transformed into the E. coli mutants and colonies were selected on LB medium. These were transferred to M9 minimal medium to assess the nutritional requirements.

Transposon Tn5 insertion mutagenesis. The cloned R. meliloti genes were mutagenized with transposon Tn5 by infecting E. coli JEF8 carrying each plasmid with the suicide Tn5 vehicle, A467, and selecting for kanamycin-resistant colonies (de Bruijn & Lupski, 1984). Mutagenized plasmids were identified by two methods. In the first method (de Bruijn & Lupski, 1984), plasmid DNA was isolated from the mutagenized culture and transformed into E. coli C-la, and kanamycin-resistant colonies were selected. Plasmid DNA was isolated from individual colonies and analysed by agarose gel electrophoresis. The plasmids with Tn5 insertions in the cloned DNA were tested for their ability to complement CP synthetase mutants of R. meliloti as described above.

Isolation of marker-exchange mutants. Marker exchange mutants were isolated by conjugating either of the IncP1 plasmids pPH1JI (Ruvkun & Ausubel, 1981) or R751 (Hirsch & Beringer, 1984) into R. meliloti 104A14-derived strains that contained a recombinant plasmid with a Tn5 insert. Transconjugants were isolated on medium that contained gentamicin (pPH1JI) or trimethoprim (R751) together with kanamycin and streptomycin (Tn5). The IncP1 plasmid destabilized the recombinant plasmid, which was readily lost if kanamycin was not present. Clones that had lost the tetracycline-resistance marker associated with the recombinant plasmid replicon were isolated after several passages on selective medium. The resistance to kanamycin and streptomycin in marker-exchange
Rhizobium carboxamidophosphate synthetase genes

Fig. 1. Biochemical reactions of CP synthetase and related pathways. The reaction pathway spanned by the brackets was suggested by Trotta et al. (1974): the hydrolysis of glutamine is catalysed by the small subunit, the rest of the reaction sequence by the large subunit.

Fig. 1.

RESULTS AND DISCUSSION

Characterization of R. meliloti CP synthetase mutants

We have described R. meliloti mutants that require a pyrimidine and arginine for growth (Kerppola & Kahn, 1988). Since CP is required in the synthesis of both of these compounds (Fig. 1), mutants with this phenotype are probably defective in CP synthetase (Mergeay et al., 1974). All of the mutants formed ineffective nodules when inoculated onto sterile alfalfa seedlings. Prototrophic revertants had normal symbiotic phenotypes, and complementation of the defect by the cloned CP synthetase genes also restored normal symbiosis (Kerppola & Kahn, 1988).

To study the R. meliloti CP synthetase genes in more detail, we have analysed a large number of plasmids that complemented the various R. meliloti mutants. [Plasmids are designated with a number that describes the mutant isolate that they rescued, i.e. plasmid pTK11A rescued mutant 11A. The number in the mutant designation describes the phenotypic class of the mutant (see Kerppola & Kahn, 1988): for example, all mutants that we would classify as carA belong to class 11.] Composite restriction maps for representative plasmids are shown in Figs 2 and 3. Plasmids pTK11A, pTK11M and pTK11Y share three HindIII fragments with a total size of 9-7 kb (Fig. 2). The additional HindIII fragments in pTK11M and pTK11Y do not appear to be essential for complementation. Plasmids pTK12K, pTK12N and pTK12X share five HindIII fragments with a total size of 12-2 kb (Fig. 3). Plasmids pTK12L and pTK12S contain three of these fragments. Plasmids pTK13C and pTK13F contain two fragments and do not overlap with plasmids pTK12L and pTK12S. Plasmids pTK13C and pTK13F also have restriction fragments from a region adjacent to the pTK12-series plasmids. Since the plasmids in the pTK11 group have no region in common with the pTK12 and pTK13 groups, the original mutations must affect at least two different and unlinked genes required for CP synthesis in R. meliloti.

Determination of complementation groups

We reintroduced each plasmid into the mutant it had originally rescued by selecting for tetracycline resistance. The resistant colonies were prototrophic, which confirmed that the plasmid carried DNA that could complement the original mutation. To determine the cross-complementation behaviour of the different groups of plasmids, the plasmids pTK11A,
Fig. 2. Restriction map of the carA plasmids. A restriction map for the plasmids that complement the group 11 mutants was determined. Enzyme abbreviations are X, XhoI; P, PstI; H, HindIII; B, BglII. The HindIII fragments are labelled H1–H5, according to size. The region that hybridizes to the E. coli carA probe is shown above the line: strong hybridization is shown by the thick line, weaker hybridization by the thinner line. The arrows indicate the sites of the Tn5 insertion mutations in pTK11A. Insertions labelled (−) (white arrowheads) abolish complementation; those labelled (+) (black arrowheads) do not change the phenotype.

pTK11M, pTK11Y, pTK12K, pTK12L, pTK12X, pTK12S, pTK13C and pTK13F were introduced into several auxotrophic mutants (Table 1). Three complementation groups (11, 12 and 13) were defined. The 11 group was complemented by all of the pTK11 group of plasmids but not by the pTK12 or pTK13 groups of plasmids. Thus, the genetic data agree with the restriction analysis in separating the pTK11 group of plasmids from the pTK12 and pTK13 groups. However, the complementation results with the 12 and 13 groups are unusual. The pTK12 plasmids complemented all of the mutants in both groups 12 and 13 but the pTK13 plasmids complemented only mutants in the 13 group. Since some of the pTK12-group plasmids do not overlap with the pTK13-group plasmids, this indicates that mutants in group 13 were complemented by two different DNA sequences within this region.

Rescue could be explained either by complementation or by recombination. In control experiments where samples of the infected cells were plated on YMB medium with tetracycline as well as on minimal medium, the number of prototrophs was comparable to the number of drug-resistant transductants. This result is consistent with rescue by complementation and could be obtained by recombination only if recombination were both fast and efficient. Other experiments with this strain indicate to us that recombination is neither (J. Somerville, personal communication). Complementation tests using mutant plasmids containing Tn5 (below) also suggest that recombination occurs slowly and at low efficiency. These data indicate that recombination cannot account for the rescue and that rescue occurs by the production of complementing proteins.

Homology with E. coli carA and carB genes

Two E. coli genes, carA and carB, code for the small and large subunits of E. coli CP synthetase. The genes are organized in an operon that is transcribed from a common promoter (Bouvier et al., 1984; Piette et al., 1984). DNA fragments carrying the E. coli carA and carB genes were isolated by preparative agarose electrophoresis of HindIII + BstEII double digests of pMC40 DNA (Nyunoya & Lusty, 1983). The carA fragment carries all of the carA gene except for 15 bp at the 3' end of the carA coding region and also contains 900 bp of upstream sequence.
carBI

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+++++
+

++
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carBII

+++ ++++
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Fig. 3. Restriction map for the carB plasmids. A restriction map was determined for the plasmids that complement mutants of groups 12 and 13. Enzyme abbreviations are defined in the legend to Fig. 2. The HindIII fragments are labelled H1–H8, according to size. The region that hybridizes to the E. coli carB probe is shown above the line (notation as for Fig. 2). The arrows indicate the sites of the Tn5 insertion mutations. Insertions shown to the right of the map occur in the plasmid cloning vehicle. Insertions into plasmid pTK13C begin with C, those into plasmid pTK12X begin with X. Insertions labelled (−) (white arrowheads) abolish complementation; those labelled (+) (black arrowheads) do not change the phenotype.
Table 1. Complementation groups

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* Plasmid designations were derived by adding pTK in front of the name of the mutant that was originally complemented by them.

The carB fragment is missing 120 bp from the 5' end of the carB coding region and contains 1100 bp of downstream sequence. These fragments were labelled by nick-translation and were used in Southern hybridization experiments to probe plasmids from each of the three complementation groups (Fig. 4).

The pTK11 group of plasmids hybridized to E. coli carA (Fig. 2, Fig. 4a), and thus presumably code for the small subunit of R. meliloti CP synthetase. We propose to call this locus carA. The hybrids were stable at 55 °C in 0.3 x SSC but could be dissociated by washing at 55 °C in 0.1 x SSC. The maximum similarity between the two DNA sequences is thus about 80% (Howley et al. 1979).

The pTK12 and pTK13 groups of plasmids hybridized to the E. coli carB probe (Fig. 3, Fig. 4b). This region probably codes for the large subunit of R. meliloti CP synthetase and we propose to call this locus carB. The region homologous to E. coli carB was not completely contained in two of the pTK12-group plasmids nor was it completely contained within either of the pTK13-group plasmids. These two complementation groups separate the carB region into two subregions, carBI and carBII (Fig. 3). The hybrids were stable at 55 °C in 0.3 x SSC but not at 68 °C in the same buffer. This stability corresponds to DNA sequences with between 80% and 85% similarity (Howley et al., 1979).

Rhizobium genes will sometimes function in E. coli and complement E. coli mutants (Somerville & Kahn, 1983). To determine if the cloned R. meliloti car genes could complement E. coli CP synthetase mutants, the genes were introduced singly or together into E. coli strains MI178, a carA mutant, and JEF8, a carB mutant (Mergeay et al., 1974). No complementation of the E. coli mutants was observed in any combination (data not shown).
Rhizobium carbamoylphosphate synthetase genes

Fig. 4. Homology between \textit{R. meliloti} \textit{car} plasmids and \textit{E. coli} \textit{car} genes. Lane A contains 1 ng of a \textit{HindIII} + \textit{BstEII} digest of pMC40 (Nyunoya & Lusty, 1983). The other lanes contain \textit{HindIII} digests of 1 \textit{ig} DNA from plasmids pTK11A (B), pTK11M (C), pTK11Y (D), pTK13C (E), pTK12N (F), pTK12X (G), pTK12K (H), pTK12S (I) and pTK12L (J). The fragments were separated on a 1\% (w/v) agarose gel.

(a) The \textit{carA} probe was a 1.6 kb fragment from a \textit{HindIII} + \textit{BstEII} digest of pMC40 that had been labelled by nick translation with [\textit{32P}]\textit{ATP}. Hybridization was at 55 °C for 12 h in 3 \times \textit{SSC}. The filters were washed several times in 3 \times \textit{SSC} at room temperature and twice in 0.3 \times \textit{SSC} at 55 °C. The bound radioactivity was determined by autoradiography. The positions of \textit{carA} \textit{HindIII} fragments H1–H5 (Fig. 2) are indicated to the right of the autoradiogram. The probe fragment is indicated by an asterisk. The degree of contamination of this probe by the \textit{carB} fragment can be estimated by the degree of hybridization to the band marked +. We attribute the hybridization in lanes E–J to the contaminating \textit{carB} fragment.

(b) The \textit{carB} probe was a 4.2 kb fragment from a \textit{HindIII} + \textit{BstEII} digest of pMC40. The positions of \textit{carB} \textit{HindIII} fragments H1–H8 (Fig. 3) are indicated to the right of the autoradiogram. The position of the probe fragment is indicated by an asterisk. The degree of contamination of this probe by the \textit{carA} fragment can be estimated by the degree of hybridization to the band marked +. We attribute the hybridization in lanes B–D to the contaminating \textit{carA} fragment.

\textit{Transposon Tn5} insertion mutagenesis of cloned \textit{R. meliloti} \textit{car} genes

Several Tn5 insertion mutations were isolated in each of the cloned \textit{R. meliloti} \textit{car} genes. The complementation phenotypes of the mutant plasmids were determined by transducing the mutated plasmids into the \textit{R. meliloti} auxotroph that was originally complemented by the parent plasmid (Figs 2 and 3). For plasmids without Tn5 inserts, there was always a clear and stable distinction between plasmids that could rescue a particular mutant and those that could not. Some of the plasmids with Tn5 inserts produced colonies with a delayed rescue phenotype. While complementing plasmids restored normal growth, these mutant plasmids gave slow growth which improved over the course of a week. Our interpretation of this class of plasmids is that the Tn5 insert had inactivated the complementing activity, but that recombination between the plasmid and the original chromosomal mutation was able to regenerate a functional gene.

The locations of the Tn5 inserts in a number of mutagenized plasmids (Figs 2 and 3) were determined by restriction mapping. Insertions that inactivated the complementation of \textit{R. meliloti carA} and \textit{carBI} mutants were found only in the regions homologous to the \textit{E. coli carA} and \textit{carB} genes. Insertions that inactivated \textit{carBII} complementation by the pTK13 group of plasmids were located in the regions of homology, in the flanking DNA, and also in the plasmid cloning vehicle. It is possible that these insertions affect the activation of the cloned DNA via their effect on a promoter on the cloning vehicle, or that the insertions lower plasmid copy number and thereby decrease the amount of protein produced from the cloned DNA.
The Tn5-mutated carA and carB genes were recombined into the chromosome by marker-exchange mutagenesis (Ruvkun & Ausubel, 1981). When the Tn5 insertion was in the region of homology between E. coli and R. meliloti, these marker-exchange mutants were auxotrophic and required arginine and uracil. Strains derived by recombination with plasmids that contained Tn5 inserts outside the regions of homology were prototrophic. In particular, prototrophic recombinants were obtained by recombination with the transposon insertion mutations C-284 and C-287, which abolish carBII complementation. The Tn5 insertions in these plasmids therefore do not eliminate complementation by disrupting a gene involved in CP synthesis, but rather interfere with some function specific for expression of carBII-rescuing activity from these plasmids.

Could CarB biochemistry account for carB genetics?

We have isolated R. meliloti genes involved in the production of CP synthetase genes by complementation. Based on the ability of these genes to hybridize to E. coli carA and carB DNA, we have named the R. meliloti genes carA and carB and suggest that they may correspond to the genes encoding the large and small subunit of CP synthetase. Complementation of mutant strains by various plasmids clearly separates carA from carB but has yielded the unusual result that two groups of plasmids with no sequences in common complement a set of the carB mutants. In addition, plasmids pTK12L and pTK12S are able to complement all of the carB mutants even though they contain only a part of the region that hybridizes to the E. coli carB gene.

The ability of part of the carB region to rescue all of the mutants may be explained by the unusual structure of the CarB protein. In E. coli the carB gene encodes one protein that contains a very large internal duplication: there is strong conservation between amino acids 1–400 and amino acids 553–933, and weaker conservation between amino acids 401–552 and 884–1072 (Nyunoya & Lusty, 1983). The large subunit of CP synthetase catalyses three distinct reactions (Fig. 1): the phosphorylation of bicarbonate to give carbonylphosphate, the transfer of ammonia to carbonylphosphate to give carbamate, and the phosphorylation of carbamate to give CP. The similarity between the first and third reactions suggests that each of the two similar domains may carry out one reaction. The E. coli CP synthetase can form oligomeric associations but, because the monomeric form is active and responds to all of the allosteric effectors that regulate CP synthetase expression (Anderson, 1977), it seems very likely that the two domains of the protein have extensive interactions.

Although our genetic data are not strong enough to rule out many other possibilities, the results we have obtained in Rhizobium could incorporate this biochemistry in a number of ways. Biosynthetic intermediates could be released by one defective protein and further processed by a protein with a complementary defect. Alternatively, a composite protein could form through the interaction of the functional domain from a mutated protein and the partial protein produced from the cloned fragment of carB. We would predict from this that carB1 mutants would complement carBII mutants, although we have not yet tested interactions between these mutants. These explanations would not account for the totipotency of the cloned carB1 region and for this reason we suggest that the protein produced by plasmids like pTK12S might be able to carry out all of the reactions of the intact protein. All of these hypotheses predict that the CP synthetase in a complemented mutant will have properties that are different from those of the wild-type enzyme. Further work will focus on clarifying the biochemistry of this unusual pattern of genetic complementation.
Rhizobium carbamoylphosphate synthetase genes

REFERENCES


