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Organization of Genes Necessary for Growth of the Hydrogen-Methanol Autotroph *Xanthobacter* sp. Strain H4-14 on Hydrogen and Carbon Dioxide

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Mutants unable to grow on H_2 and CO_2 were isolated in the hydrogen-methanol autotroph *Xanthobacter* sp. strain H4-14 and complemented with a clone bank constructed in a broad-host-range cosmid vector. The mutants fell into two classes. Class I mutants (Cfx^-) cannot grow on hydrogen or methanol and are deficient in one or more of the key enzymes of the Calvin Cycle. Class II mutants (Hox^-) can grow on methanol but not on hydrogen and lack hydrogenase activity. Restriction maps of the complementing clones show that each class is not linked to the other. Subcloning and Tn*5* mutagenesis have localized the regions of DNA complementing these mutants. The region complementing a class I mutant which is deficient in ribulosebisphosphate carboxylase activity is approximately 3.2 kilobase pairs in size. Expression of this enzyme activity from cloned DNA gave evidence for the orientation of an operon containing the structural genes for this enzyme. The region complementing most of the class II mutants is 3 to 4.5 kilobase pairs in size.

Xanthobacter spp. (35) are bacteria that can grow autotrophically with either hydrogen or methanol as an energy source (Fig. 1). CO_2 is assimilated through the Calvin Cycle, the carbon assimilation pathway used by all green plants and the majority of autotrophic procaryotes. This is carried out by the key enzymes ribulosebisphosphate carboxylase-oxygenase (Rubisco) and phosphoribulokinase (PRK). Methanol is oxidized via three consecutive two-electron steps to formaldehyde, formate, and finally CO_2 (2), while hydrogen is oxidized by a membrane-bound hydrogenase (29). Because of the significance of Rubisco, PRK, and hydrogenase to metabolism in autotrophic bacteria, it is important to study the genetic organization and transcriptional regulation of these enzymes. However, little is known about the genetics of these enzymes in autotrophic hydrogen bacteria.

The genes encoding the two subunits of Rubisco have been studied in both procaryotic and eucaryotic autotrophs. In higher plants, the large subunit of Rubisco is encoded by the chloroplast (19) and may or may not have introns (15), whereas the small subunit is encoded by the nucleus (19).

DNA encoding the large subunit of Rubisco has been cloned from cyanobacteria (5) and photosynthetic bacteria (30). It has recently been shown that the gene for the small subunit of Rubisco in *Anabaena* sp. is 0.5 kilobase pair (kbp) downstream from the gene for the large subunit (22), but nothing is known about the location of other genes involved in CO_2 fixation. In the autotrophic hydrogen bacterium *Alcaligenes eutrophus* CO_2 fixation genes were isolated by complementing CO_2 fixation-negative mutants with a genomic clone bank constructed in a broad-host-range vector. Further analysis suggested that CO_2 fixation genes may be present in *A. eutrophus* in duplicate copies, one on a plasmid and the second copy on the chromosome (1). The plasmid that encodes the CO_2 fixation genes also carries the gene involved in hydrogen oxidation (12). Hydrogen oxidation genes have been shown to be associated with plasmids in *Rhizobium leguminosarum* (4), *Pseudomonas facilis* (25), and *Nocardia opaca* (26). Recently, genes involved in hydrogen oxidation in *Rhizobium japonicum* were isolated by complementing hydrogen oxidation-negative mutants with a genomic clone bank constructed in a broad-host-range vector (11). In this study we report the use of a clone bank constructed in a broad-host-range cosmid vector to complement *Xanthobacter* strain H4-14 mutants deficient in enzymes for CO_2 fixation and hydrogen oxidation. We mapped the genes involved, and the evidence suggests that CO_2 fixation and hydrogen oxidation genes are not linked.

MATERIALS AND METHODS

Strains. Strains and plasmids used in this study are listed in Table 1.

Media and growth conditions. *Xanthobacter* strain H4-14 was grown at 30°C in minimal medium (10) with biotin (0.2 μg/ml) in flasks as described previously (16) or in nutrient broth. For autotrophic growth, 8 μM $NiCl_2$ and 10 mM $NaHCO_3$ were added separately as sterile supplements. For autotrophic growth with hydrogen, flasks were incubated under an atmosphere of 70% H_2–10% CO_2–20% air. For growth on all other substrates the atmosphere was 80% He–20% air. Sterile supplements were added separately at the following concentrations: methanol, gluconate, succinate, and citrate, 0.2% each (wt/vol); tetracycline (Tc), 10 μg/ml; kanamycin (Km), 50 μg/ml; nalidixic acid (Nx), 20 μg/ml; ampicillin (Ap), 50 μg/ml; streptomycin (Sm), 20 μg/ml. *Escherichia coli* strains were grown at 37°C in L broth (20). Agar was added to 1.5% (wt/vol) for plates.

Mutagenesis. *Xanthobacter* strain H4-14 was mutagenized with ethyl methanesulfonate for 1 h as described by Miller (21) followed by two rounds of glycine enrichment (36). Cells were plated on nutrient agar and transferred to minimal medium under H_2 and CO_2 to score for autotrophic growth. The streptomycin-resistant strain was isolated spontaneously by plating on nutrient agar containing streptomycin (20 μg/ml). Lambda::Tn*5* mutagenesis was carried out in *E. coli* as previously described (34).

Enzyme assays and mutant characterization. *Xanthobacter* strain H4-14 was grown on 0.2% gluconate under an atmosphere of 80% H_2–20% air. Under these conditions Rubisco,

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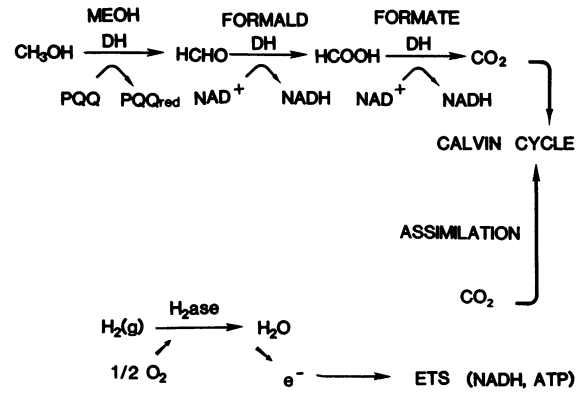

FIG. 1. Diagram of the two modes of energy generation that *Xanthobacter* strain H4-14 can couple to CO_2 fixation via the Calvin Cycle. Abbreviations: MEOH, methanol; g, gaseous; H_2ase, hydrogenase; DH, dehydrogenase; FORMALD, formaldehyde; e^-, electrons; ETS, electron transport system; PQQ, pyrrolo quinoline quinone.

PRK, and the membrane-bound hydrogenase are expressed although they are not necessary for growth (13). Cell extracts were prepared by harvesting cells during late-log phase and resuspending them in the appropriate buffer. They were then broken by two passes through a French pressure cell at 10,000 lb/in^2. Cell debris was removed by centrifugation at 10,000 × *g* for 5 min, and the supernatant fluid was used for enzyme assays. For the whole-cell Rubisco assay, cells were suspended in assay buffer (150 μl/5 ml of cell culture) and treated with 50 μl of toluene per 150 μl of cells for 20 min.

Enzymes were assayed by published procedures as follows: ribulosebisphosphate carboxylase (EC 4.1.1.39) (31), PRK (EC 2.7.1.19) (31), or hydrogenase (28).

DNA isolation. Plasmid DNA from *E. coli* was isolated by the rapid-screening method or the large-scale preparation described by Maniatis et al. (18).

Genomic DNA isolation was carried out as described previously (9) with the following modifications. Pronase (0.5 mg/ml) and proteinase K (0.1 mg/ml) were added to the lysis mixture. Lysis was carried out at 55°C for 30 min followed by 10 min at 65°C to destroy nucleases. Sodium perchlorate was added to 1 M before phenol extraction, and the solution was treated with RNase (RNase A [100 μg/ml] and RNase T [30 U/ml]) for 10 min at 65°C before a second phenol extraction.

Restriction endonuclease mapping, gel electrophoresis, ligation, and transformation. These procedures were all carried out according to the methods described by Maniatis et al. (18).

Clone bank construction. The clone bank was constructed in pVK100 by using a *Hin*dIII partial digest of genomic DNA as described previously (9) except that *Xanthobacter* strain H4-14 genomic DNA was used instead of *Pseudomonas* strain AM1 DNA.

Matings and complementation analysis. Plasmids were mobilized into *Xanthobacter* strain H4-14 in three-way crosses between an *E. coli* donor (HB101 or JM83), an *E. coli* mobilizer (MM294 carrying pRK2013 or pRK2073), and a streptomycin-resistant (Sm^r) *Xanthobacter* strain H4-14 recipient. Mid-log-phase cultures of donor, mobilizer, and recipient were mixed in a 1:1:4 ratio and filtered onto a membrane filter (pore size, 0.45 μm) which was incubated for 12 to 18 h on a nutrient agar plate at 30°C. Cells were washed from the filter, plated onto minimal medium containing tetracycline, and incubated under the autotrophic growth conditions described above. For complementation analysis of clones, subclones, and Tn*5* insertions, spot matings with the same ratio of donor, mobilizer, and recipient were carried out on plates containing 0.2% citrate and tetracycline. Individual colonies were then tested for growth on H_2 and CO_2. Mobilization of plasmids from *Xanthobacter* strain H4-14 to *E. coli* SF800 was carried out with filter matings as described above except the selective medium was L agar plus tetracycline. All putative transconjugants were purified by single-colony isolation to assure the absence of donor cells before successive steps were carried out.

RESULTS

Mutant characterization. The eight independent autotrophy mutants isolated fell into two phenotypic classes. Class I mutants (Cfx^-, carbon dioxide-fixation negative) could not grow on hydrogen or methanol (Table 2). At least one mutant in this class (Q13) lacked Rubisco activity, the key enzyme of the Calvin Cycle. All other mutants in this class contained Rubisco activity at 30 to 100% that of the wild-type strain. Class II mutants (Hox^-, hydrogen oxidation negative) could grow on methanol but not on hydrogen and completely lacked hydrogenase activity (Table 2).

Complementation analysis. The broad-host-range vector pVK100 is mobilized into *Xanthobacter* strain H4-14 by pRK2013 or pRK2073 at frequencies of 10^{-2}, which is sufficiently high to allow the cloning of specific genes by direct mutant complementation. Mobilization of a genomic clone bank constructed in pVK100 into a class I mutant (Q13) and a class II mutant (w42) resulted in distinct colonies that had regained the ability to grow on H_2 and CO_2. This occurred at a frequency of 10^{-4} per Tc^r recipient. These prospective recombinant clones were mated from these

TABLE 1. Characteristics of the strains, plasmids, and phage used in this study

Strain or plasmid	Characteristics[a]	Source or reference
Xanthobacter sp.		
Wild type	Facultative autotroph	16
Sm^r	Streptomycin resistant	This study
E. coli		
HB101	*recA*	18
MM294	$recA^+$	3
SF800	*thy gyrA polA*	6
JM83	Derivative of W3110 *ara* Δ(*lac-pro*) *rpsL thi* Φ80d Δ*lacZM15*	20
Plasmid or phage		
pVK100	Tc^r Km^r IncP1 *rlx*	14
pRK404	Tc^r IncP1 *rlx*, pUC9 linker	G. Ditta
pRK2013	Km^r ColE1 with RK2 *tra*	7
pRK2073	Sm^r ColE1 with RK2 *tra*	7
pUC8, pUC9	Ap^r ColE1	32
Lambda::Tn*5*	Defective lambda *rex*::Tn*5*	27

[a] *recA*, recombination deficient; *polA*, DNA polymerase I defective; ColE1, replication deficient; *gyrA*, gyrase defective, resistant to nalidixic acid; *rlx*, mobilization positive.

complemented mutants into *E. coli* strain SF800 and remated into the mutant they came from with selection on citrate-plus-tetracycline plates. Twenty putative transconjugants were toothpicked onto minimal medium and tested for growth on H_2 and CO_2. In both cases, 100% of the colonies tested (20 of 20) were able to grow on H_2 and CO_2, indicating that this ability was conferred by the recombinant plasmid involved and not reversion of the mutation. Control matings with the vector pVK100 instead of the clone bank did not give any colonies that could grow on H_2 and CO_2. Plasmid DNA was then isolated from the appropriate *E. coli* SF800 strains and analyzed by restriction endonuclease analysis (Fig. 2). Each clone was mapped with the restriction endonucleases that could be used in subcloning in the vector pRK404. This vector is a 10.6-kbp plasmid RK2 derivative (similar to pRK310 [9]) which has the linker of pUC9 and carries resistance to tetracycline. Recombinant plasmids recovered from the two mutant classes were distinct from one another by restriction sites and did not complement mutants from the other class. All mutants in class I were complemented by the recombinant plasmid recovered from Q13 (pCD102) except E25. The insert in this plasmid was approximately 24 kbp in size and had only one *Hin*dIII end. E25, which was not complemented by pCD102, was also not complemented by the clone bank. All mutants in class II were complemented by the recombinant plasmid recovered from w42 (pCD123). The insert in this plasmid was approximately 26 kbp in size, consisting of a *Hin*dIII fragment of 20 kbp and an additional 6 kbp of DNA which had only one *Hin*dIII end. Subcloning and Tn*5* mutagenesis were used to localize and define the regions of DNA defective in the mutants.

Cfx⁻ genes. pCD102 was mutagenized in *E. coli* with phage lambda::Tn*5* and conjugated into strain Q13 with selection on citrate plus tetracycline and kanamycin. The resulting colonies were transferred to minimal tetracycline-plus-kanamycin plates and checked for growth on H_2 plus CO_2. Two colonies (of 100) failed to grow on H_2 plus CO_2. Tc^r Km^r plasmids were mated from these mutants into *E. coli*, plasmid DNA was isolated, and the inserts were mapped by restriction endonuclease analysis. Both Tn*5* inserts (102:14, 102:28) mapped to the same 6.3-kbp *Bam*HI fragment, 0.5-kbp apart from one another (Fig. 2). This 6.3-kbp *Bam*HI fragment was subcloned into pRK404 in both orientations (pLL463 and pLL463R in Fig. 3, where R refers to the same DNA fragment in the reverse orientation). Both of these plasmids were able to complement strain Q13 and the other class I mutants that were complemented by pCD102. Cells carrying pLL463R grew on H_2 and CO_2 at approximately the same rate as the wild-type strain and about twice as fast as cells carrying pLL463. Tn*5* mutagenesis of these plasmids and construction of the plasmid pLL447 have narrowed the region of interest to approximately 3.2 kbp, the region between Tn*5* inserts 11 and 12 (Fig. 3). Tn*5* inserts within this region removed the ability of either pLL463 or pLL463R to complement Q13. However, only Tn*5* inserts between 28 and 102:28 prevented complementation of A3. This region has been marked in Fig. 4 with a bar designated A3. Tn*5* inserts outside this region were positive for complementation of this mutant. The plasmid pLL417R containing the 1.7-kbp *Sal*I fragment surrounding this region was able to complement A3 but not Q13. The plasmid pLL419R containing the adjacent 1.9-kbp *Sal*I fragment (Fig. 3) was able to complement Q13, but the cells with this plasmid grew slower than when they contained pLL463R. Additional restriction sites (*Sma*I, *Bgl*II, *Pvu*I, and *Sst*I) were mapped in the 6.3-kbp *Bam*HI fragment to determine the orientation of these *Sal*I fragments. Neither the 1.7-kbp *Sal*I fragment (pLL417) nor the 1.9-kbp *Sal*I fragment (pLL419) could complement A3 or Q13 when inserted in pRK404 in the opposite orientation.

Hox⁻ genes. The 8.3- and 11.6-kbp *Hin*dIII-*Bam*HI fragments of pCD123 (Fig. 2) were subcloned into pRK404. The 8.3-kbp *Hin*dIII-*Bam*HI fragment (pLL483, Fig. 4) complemented w42 and B2 but not A1. Tn*5* mutagenesis of pLL483 and further subcloning narrowed this complementing region to less than 4.5 kbp. pLL451, containing the 5.1-kbp *Hin*dIII-*Pst*I fragment of pLL483, complemented both of the class II mutants complemented by pLL483; but pLM432, containing the 3.2-kbp *Hin*dIII-*Sal*I fragment, did not. When either w42 or B2 containing pLM432 was transferred to autotrophic medium, a few colonies appeared within the streak. This result suggests that only part of the gene or operon involved is included in this fragment and that complementation could have occurred via recombinational rescue. Tn*5* mutagenesis of plasmid pLL483 yielded results consistent with the analysis by subcloning (Fig. 4). A1 was not complemented by pLL483, although it was complemented by the original clone (pCD123). The 11.6-kbp *Hin*dIII-*Bam*HI fragment of pCD123 was cloned in a two-step process. The 19.9-kbp *Hin*dIII fragment of pCD123 was cloned into pUC9 with the 11.6-kbp *Hin*dIII-*Bam*HI fragment closer to the *Bam*HI site in the linker. This fragment was then cut out as a *Bam*HI fragment and inserted into pRK404 in both orientations (pLL411 and pLL411R). The fragment was not able to complement A1 in either orientation. There were no convenient restriction sites that could be used to subclone the region outside the *Hin*dIII fragment.

Expression of Rubisco from cloned DNA. When grown on succinate or nutrient broth, *Xanthobacter* strain H4-14 contains no detectable Rubisco. This is consistent with regulation patterns observed in other hydrogen autotrophs (8). When strain Q13 or the wild-type strain carrying either of the

TABLE 2. Properties of *Xanthobacter* strain H4-14 autotrophy mutants

Class	Strain	Growth on:			Activity of enzymes (% of wild type)		
		H_2	Methanol	Citrate	Rubisco[a]	PRK[b]	H_2ase[c]
	Wild type	+	+	+	100	100	100
I (Cfx^-)	Q13	−	−	+	<1	<1[d]	100
	A3	−	−	+	30	40	100
	N29	−	−	+	86	105	100
	Y19	−	−	+	107	40	100
	E25	−	−	+	+[f]	+	+
II (Hox^-)	w42	−	+	+	99[e]	96	<1
	B2	−	+	+	100	100	<1
	A1	−	+	+	78	55	<1

[a] Wild-type activity is 55 nmol/min per mg of protein.

[b] Because this activity is measured with the whole-cell assay, it is a function of the cellular level of Rubisco. Wild-type activity is 40 nmol/min per mg of protein.

[c] H_2ase, Hydrogenase. Wild-type activity is 270 to 330 nmol/min per mg of protein.

[d] In a cell-free assay which is independent of cellular Rubisco, this mutant is PRK positive.

[e] These mutants were characterized after growth on gluconate with formate instead of gluconate with H_2 since they are hydrogenase mutants. Under these conditions wild-type activities are approximately the same as described above.

[f] This mutant was not characterized quantitatively because it could not be complemented. It had <100% Rubisco and PRK activity.

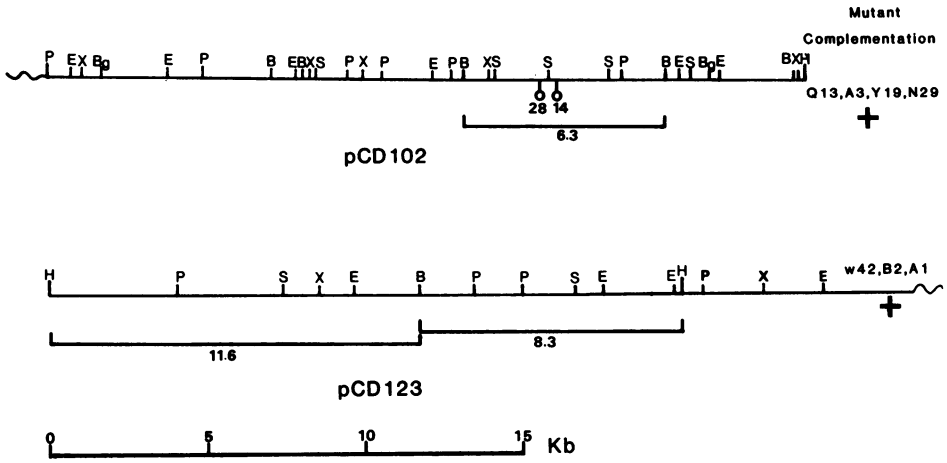

FIG. 2. Restriction endonuclease maps of *Xanthobacter* strain H4-14 DNA inserts in pCD102 and pCD123. Restriction sites are shown for *Eco*RI (E), *Bam*HI (B), *Pst*I (P), *Sal*I (S), *Bgl*II (Bg), *Xho*I (X), and *Hin*dIII (H). Subcloned fragments and their sizes (in kilobase pairs) are indicated by bars underneath each clone. The two open circles marked 28 and 14 show the position of the Tn*5* inserts in pCD102 that were negative for complementation of Q13. Mutants complemented by each clone are listed next to the clone.

vectors pVK100 or pRK404 was grown under the same conditions no Rubisco activity was present. However, when Q13 carrying any of the recombinant plasmids pCD102, pLL463, or pLL463R was grown on succinate, substantial Rubisco activity was present (Table 3), with pLL463R showing about five times as much activity as pLL463. This observation is in agreement with the fact that strain Q13 grows faster on H_2 and CO_2 when it contains pLL463R instead of pLL463. Rubisco activity is not present when strain Q13 carrying the identical plasmids is grown on nutrient broth.

DISCUSSION

The complementation analysis described in this study shows that both classes of autotrophy mutants (Cfx^- and Hox^-) are complemented by distinct fragments of DNA. Within the two fragments described in this paper, the region necessary for complementation maps at least 5 kbp from the end of the insert. This suggests that these regions involved in hydrogen oxidation and CO_2 fixation are not within one operon and are at least 10 kbp apart. Mutants within a class appear to map within one defined region, suggesting that they may reside within operons. However, the complementation analysis shows at least one mutant in each class (E25 in class I, A1 in class II) to be outside the potential operon.

We do not yet know the nature of the cloned *cfx* genes, but they may be (i) structural genes for Rubisco and PRK, (ii) regulatory genes, or (iii) unknown *cfx*-specific genes.

The region complementing the class I mutants is approximately 3.2 kbp in size. From protein gels it appears that in *Xanthobacter* strain H4-14 PRK and the large and small subunits of Rubisco are about the same size as these proteins in other autotrophs: 35,000, 54,000, and 15,000 daltons, respectively (unpublished data). To encode the structural genes for these three proteins, 3.2 kbp is sufficient. It is not clear whether these two enzymes are coordinately regulated in *Xanthobacter* strain H4-14 (unpublished data), and therefore the existence of a Calvin Cycle or Aut operon is still uncertain. Regulatory patterns in *Xanthobacter* strain H4-14 show these two enzymes to be repressed in the presence of organic carbon sources. The expression of Rubisco activity

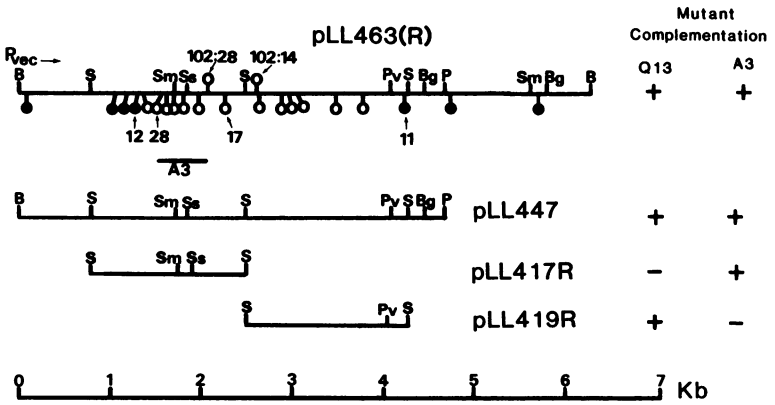

FIG. 3. Physical maps of *Xanthobacter* strain H4-14 inserts in pLL463(R), pLL447, pLL417R, and pLL419R. Additional restriction sites mapped: *Sma*I (Sm), *Pvu*I (Pv), and *SSt*I (Ss). Open circles are Tn*5* inserts in pLL463 or pLL463R which are negative for complentation of Q13; closed circles are positive for complementation. The two original Tn*5* inserts in pCD102 are indicated above the map. The solid bar marked A3 shows the region containing Tn*5* inserts that are negative for complementation of the mutant A3. The arrow marked Pvec indicates the direction of vector promoters relative to the inserts in pLL463R, pLL417R, and pLL419R.

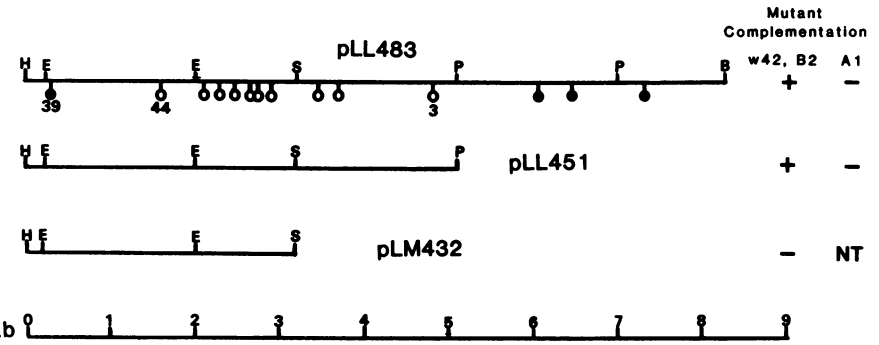

FIG. 4. Physical maps of *Xanthobacter* strain H4-14 DNA inserts in pLL483, pLL451, and pLM432. Open circles are Tn*5* inserts that are negative for complementation of w42 and B2; closed circles are positive for complementation. NT, Not tested.

from cloned DNA under conditions that normally repress this activity (growth on succinate) is consistent with the following hypothesis. Growth on heterotrophic carbon sources such as succinate may result in the synthesis of a repressor that binds to an Aut operon. When this operon is present in 6 to 10 copies per cell as a cloned piece of DNA, insufficient repressor may be produced for normal regulation of all copies, and a partial level of expression would be observed. When cells are grown on an even richer medium such as nutrient broth, sufficient repressor may be present to regulate all copies of the operon. Orientation of this operon in the same direction as a strong vector promoter (pLL463R as compared with pLL463) would enhance this expression. It is possible that the original mutant (Q13) was deficient in some positive regulatory gene that turns on autotrophy genes and that when this cloned gene is present in 6 to 10 copies per cell, autotrophy genes that are normally repressed are activated. This possibility, however, is not consistent with the regulatory pattern that is observed in *Xanthobacter* strain H4-14 or other autotrophs. It is clear that more than one gene is present in this region since A3 maps to a region distinct from that of Q13. These observations are consistent with the existence of an operon with transcription reading left to right as shown by the arrow in Fig. 3. The operon would start near Tn*5* insertion 12. The A3 gene would be upstream from the Q13 gene since Tn*5* insertions that remove complementation of A3 also remove complementation of Q13, even though the Q13 gene appears to be contained within the 1.9-kbp *Sal*I fragment (Fig. 3). The gene within this fragment is only functional when oriented in the same direction as the vector promoters because it has been separated from its presumptive natural promoter. One would expect that the 1.7-kbp *Sal*I fragment would complement A3 in either orientation, since it appears that this fragment carries this presumptive natural promoter and the whole A3 gene. It is possible that transcription from the natural promoter is not strong enough to oppose two strong vector promoters over this short distance (33) or that anti-mRNA from the vector promoter could inhibit normal mRNA (24). This would agree with the observed result that the 1.7-kbp *Sal*I fragment only complements A3 when oriented with the vector promoters.

TABLE 3. Rubisco activity in Q13 and wild-type strains containing vectors or cloned DNA and grown on succinate

Strain(clone)	Rubisco activity (relative to pCD102)[a]
Q13(pCD102)	100
Q13(pLL463R)	80–90
Q13(pLL463)	15–20
Q13(pRK404)	0
WT(pCD102)	100
WT(pVK100)	0

[a]Activity in Q13 or wild type (pCD102) was 21 nmol/min per mg of protein.

The Tn*5* insertions show the A3 gene to be between 0.5 and 0.9 kbp in size. This is large enough to be the small subunit of Rubisco but not large enough to be the large subunit. It is probably not the small subunit either, however, since the mutant A3 is not totally lacking in carboxylase activity. It is known that functional small subunit is required for activity in all Rubisco enzymes that have a small subunit. This gene also appears to be too small to encode a PRK enzyme of 35,000 daltons, and the mutant A3 does not totally lack this activity either.

We do not yet know the nature of the cloned *hox* genes, but they may be (i) structural genes for hydrogenase and its cytochrome, (ii) regulatory genes, or (iii) unknown *hox*-specific genes.

The class II mutants all show the same phenotype and are all deficient in hydrogenase activity. The membrane-bound hydrogenase of *Xanthobacter autotrophicus* is approximately 72,000 daltons in size and is tightly associated with a *b*-type cytochrome of 37,000 daltons (29). It is not known whether both of these components or any additional electron transport components are necessary for a positive reaction in the methylene blue assay used to test for hydrogenase activity; therefore, the exact nature of the Hox^- mutations is not known. However, the membrane-bound hydrogenase of *R. japonicum* is able to donate electrons directly to methylene blue (17). The difference between a mutation in hydrogenase itself can then be distinguished from a mutation in one of the electron transport components that is known to be involved in but not necessarily specific to hydrogen oxidation (23). The number of Hup^- (hydrogen uptake) mutants in *R. japonicum* that appeared to be in electron transport components compared with the number of mutants in hydrogenase itself was 3 versus 50 (17). If *Xanthobacter* spp. are similar to *R. japonicum* in this respect, then the mutations are most likely in the hydrogenase structural gene. The region in pLL483 defined by Tn*5* mutagenesis and subcloning is at least 3.2 kbp in length but no greater than 4.5 kbp. Approximately 3.4 kbp of DNA would be necessary to encode a hydrogenase of 72,000 daltons and a cytochrome of 37,000 daltons. If *Xanthobacter* strain H4-14 has hydrogenase protein similar to *X. autotrophicus*, the region defined

here is sufficiently large to encode both of these genes. Mutation A1 has not been precisely mapped but could lie in the region outside the *Hin*dIII fragment of pCD123 or across the single *Bam*HI site.

Our attempts to study the expression of cloned DNA in *E. coli* maxicells have been unsuccessful, possibly due to the high G+C ratio of *Xanthobacter* spp. DNA (69%). Therefore, we are currently attempting to determine the identity of these cloned autotrophy genes by studying the expression of cloned DNA in *Xanthobacter* strain H4-14. Further biochemical characterization of the mutants which we already have and construction of new mutants using marker exchange are also being attempted. These experiments should help define the exact nature and organization of these autotrophy genes.

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