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Mapping of the Tryptophan Genes of *Acinetobacter calcoaceticus* by Transformation

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Auxotrophs of *Acinetobacter calcoaceticus* blocked in each reaction of the synthetic pathway from chorismic acid to tryptophan were obtained after *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine mutagenesis. One novel class was found to be blocked in both anthranilate and *p*-aminobenzoate synthesis; these mutants (*trpG*) require *p*-aminobenzoate or folate as well as tryptophan (or anthranilate) for growth. The loci of six other auxotrophic classes requiring only tryptophan were defined by growth, accumulation, and enzymatic analysis where appropriate. The *trp* mutations map in three chromosomal locations. One group contains *trpC* and *trpD* (indoleglycerol phosphate synthetase and phosphoribosyl transferase) in addition to *trpG* mutations; this group is closely linked to a locus conferring a glutamate requirement. Another cluster contains *trpA* and *trpB*, coding for the two tryptophan synthetase (EC 4.2.1.20) subunits, along with *trpF* (phosphoribosylanthranilate isomerase); this group is weakly linked to a *his* marker. The *trpE* gene, coding for the large subunit of anthranilate synthetase, is unlinked to any of the above. This chromosomal distribution of the *trp* genes has not been observed in other organisms.

There is increasing interest in a group of gram-negative organisms (now placed in the genera *Moraxella* and *Acinetobacter*) which has been recovered from a wide variety of sources and has been unsatisfactorily classified with several other groups of bacteria (2-4). *Acinetobacter* is the genus consisting of the oxidase-negative representatives of this biochemically versatile group of organisms (4). *Acinetobacter calcoaceticus* was first isolated and described by Beijerinck (6) and later characterized by Taylor and Juni (31) and Baumann (2), among others. The organism is a nonmotile, oxidase-negative rod. Cells appear either singly, in pairs, or in short chains; they are spherical when growing in semisolid media but become short rods in liquid during exponential growth. After entry into the stationary phase they become coccoid. They frequently produce a capsular poysaccharide. This organism is often confused morphologically with various species of *Neisseria*, and biochemically, because of the great variety of carbon sources it can utilize, with *Pseudomonas*. The recent demonstration of transformation in *A. calcoaceticus* has made genetic analysis possible within the species (19).

The structural genes governing the synthesis of enzymes of the tryptophan biosynthetic pathway have been studied and mapped in many organisms (7, 8, 13, 16, 17, 22, 26, 36). Significant differences exist in the gene-enzyme relationships and the organization of the gene products into macromolecular aggregates (11, see also comparative nomenclature in Materials and Methods).

In the enteric bacteria (7, 36) the *trp* genes are clustered in a single typical operon. Until recently all the *trp* genes of *Bacillus subtilis* were thought to be linked in a typical operon (8, 14). However, a new unlinked *trp* locus, *trpX*, directing the synthesis of one component of anthranilate synthetase has recently been demonstrated (20, 21) in this organism. The order of genes in these operons, beginning at the operator, is *trpE-trpD-trpC-(trpF)-trpB-trpA*. The order of loci mapped in the tryptophan gene cluster of *Staphylococcus aureus* (26; Proctor and Kloos, *manuscript in preparation*) is similar to that of *B. subtilis*. Nothing is known about the *trpG* function in *Staphylococcus*, however. Another exception to the single operon pattern of *trp* loci in the eubacteria has been shown by Kloos and Rose (22) in *Micrococcus luteus* where four *trp* loci are linked in the sequence *trpE-trpC-trpB-*

trpA, but the *trpD* and *trpF* functions lie apart from this cluster.

The *trp* genes in *Pseudomonas putida* (13) are dispersed into three distinct linkage groups, *trpE-trpD-trpC*, *trpF*, and *trpB-trpA*. A *trpG* locus has been inferred in this organism from biochemical evidence (27) but has not yet been mapped. The trytophan loci in the fungi are even more dispersed (17).

In this paper we will show that the tryptophan enzymes in *A. calcoaceticus* are governed by seven genetic loci which are dispersed in a pattern unlike that observed in any other organism.

MATERIALS AND METHODS

Organism. The wild-type organism used in this study was the prototrophic strain BD413, a microencapsulated mutant of *A. calcoaceticus* BD4 (19) received from E. Juni. Stock cultures were maintained at 4 C on L-agar (23) slants transferred at 1- to 2-month intervals.

Selection of mutants. Tryptophan auxotrophs were obtained by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine treatment (1). Cells were grown in 10 ml of L-broth (23) to the exponential phase, centrifuged, and resuspended in 5 ml of sterile 0.1 M sodium citrate buffer, *p*H 5.0. The mutagen was added at 20 μg per ml of cell suspension, and the cells were incubated with shaking at 30 C for 60 min. Treated cells were centrifuged, washed once, resuspended in 5 ml of L-broth, incubated for 4 hr with shaking at 30 C, and then spread at appropriate dilutions on minimal agar plates supplemented with tryptophan. After colonies had developed they were replicated to similar plates lacking tryptophan. Colonies failing to grow were reisolated and characterized. Other auxotrophic markers were isolated from similarly treated cells plated on appropriate media.

Comparative nomenclature. Differences exist in the designations for genetic loci in the tryptophan pathway, some of which are clearly homologous (9). In this paper we shall adhere most closely to the nomenclature used by those working with *Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus*, and *Micrococcus luteus*. We shall use this nomenclature uniformly, even in referring to loci in *Salmonella typhimurium* or *Pseudomonas putida* where the original designations were different. Note that *trpF* refers to the gene for phosphoribosylanthranilate isomerase (PRAI) when this is separate from *trpC*, the gene for indoleglycerol phosphate synthetase (InGPS). At least some enteric bacteria (7, 28) have these two genetic functions fused into one cistron which is designated *trpC*. The gene for the small, glutamine-binding subunit of anthranilate synthetase (AS) will be designated *trpG* in this study. In *E. coli* (18) and *S. typhimurium* (5) this function appears to be fused to *trpD*, the gene for phosphoribosyl transferase (PRT). It may be evolutionarily related to the *trpX* (20, 21) of *B. subtilis*. The other designations used are *trpE*, the gene for the large component of AS, and *trpA* and *trpB*, coding for the α and β chains of tryptophan synthetase (TS).

Deoxyribonucleic acid (DNA) preparation. Cells grown to the early stationary phase in 10 ml of L-broth were centrifuged and resuspended in 2.5 ml of sterile saline-ethylenediaminetetraacetic acid (EDTA) (24). Sodium dodecyl sulfate was added to a final concentration of 0.25% and the cells were lysed by incubation for 10 min at 37 C. To clear the lysate, 0.05 ml of 5 M KCl was added, the solution was chilled in ice for 20 min, and then centrifuged at 39,000 × *g* for 20 min. The supernatant solution was then heated at 50 C for 5 min to kill surviving cells. DNA was tested for sterility by spreading 0.1 ml of DNA solution on an L-agar plate and incubating at 30 C for 24 hr.

Transformation. Cells were brought to competence by diluting an overnight L-broth culture 1:10 into fresh L-broth and incubating with shaking at 30 C for 2 hr. Cells were then centrifuged, washed once, and resuspended in minimal medium E (33). Transformations were routinely carried out by the "plate method" (19). Competent cells and appropriately diluted DNA (0.1 ml of each) were placed in the center of an agar plate and spread with a glass rod. Plates were incubated at 30 C for 24 to 30 hr or until colonies were of desired size.

Enzyme assays and accumulation tests. Enzymes were assayed as described by Hoch et al. (14). Chorismate was purified by the procedure of Gibson (11). For accumulation studies, mutants were grown in minimal medium E supplemented with 0.2% L-malate and 2.0 μg of tryptophan per ml. Anthranilate accumulation was determined by fluorescence after extraction from acidified supernatant fluids into ethyl acetate. The two fluorescent intermediates, anthranilate and 1-(*o*-carboxyphenylamino)-1-deoxyribulose (CDR) were distinguished by paper chromatography in methanol-*n*-butanol-benzene-water (2:1:1:1). All other accumulation tests were performed as described by Smith and Yanofsky (29, 30).

Crude extracts. For enzyme studies, tryptophan auxotrophs were grown in minimal medium E with 0.2% L-malate as carbon source. A growth-limiting concentration of L-tryptophan (4 μg/ml) was the only supplement used, except in the case of *trpG* mutants, where 1 μg of *p*-aminobenzoate (PABA) per ml was also added. Cells were grown with vigorous aeration at 30 C. When no further increase in turbidity was observed, the cells were harvested by centrifugation at 16,000 × *g*, washed once with 0.1 M potassium phosphate (*p*H 7.5), containing 10 mM L-glutamine, 2 mM $MgCl_2$, 0.1 mM EDTA, and 1.0 mM β-mercaptoethanol, and then resuspended in 2 ml of this buffer per gram wet cells. Cells were disrupted by sonic oscillation, and cell debris was removed by centrifugation at 39,000 × *g* for 20 min.

RESULTS

Tryptophan pathway. All of the enzymes which comprise the known tryptophan syn-

thetic pathway in other microbes and plants (Fig. 1) are present in extracts of wild-type *A. calcoaceticus* BD 413. This organism cannot grow with tryptophan as sole carbon source; in fact, we have found no evidence that either a tryptophanase or tryptophan pyrrolase activity can be formed by our wild-type strain.

Mutant isolation and characterization. The properties of 23 *A. calcoaceticus* tryptophan auxotrophs are summarized in Table 1. Enzyme assays, growth responses, and accumulation tests divide these mutants into seven classes. Mutants responding to anthranilate or anthranilate plus PABA fail to accumulate pathway intermediates. *trpB* mutants grow only on tryptophan and accumulate indole and indoleglycerol. Those mutants responding only to indole or tryptophan are further subdivided according to accumulation products and enzyme activities. *trpA* mutants accumulate indoleglycerol. *trpD* and *trpF* mutants, lacking PRT and PRAI activities, respectively, accumulate anthranilate and grow on indole. *trpC* mutants lacking InGPS activity accumulate either anthranilate or both anthranilate and CDR in culture fluids.

Pseudomonads blocked early in the tryptophan synthetic pathway grow much more slowly on 5 μg of indole per ml than on an equivalent amount of tryptophan (13). Recently it has been learned that nearly normal growth rates can be obtained at higher indole concentrations (Proctor and Crawford, *manuscript in preparation*). *A. calcoaceticus* mutants blocked after anthranilate synthetase also grow slowly on 5 μg of indole per ml yet utilize tryptophan readily. At indole concentrations of 40 μg or more per ml these strains grow at a rate equal to that in the presence of 10 μg of L-tryptophan per ml.

One unusual class of mutants, which we have designated *trpG*, was found to require both PABA (or folate) and tryptophan (or anthranilate or indole) for growth. Although blocked in two pathways, these mutants revert at a frequency of about 10^{-8} and are transformed to prototrophy by wild-type DNA at frequencies comparable to the other tryptophan auxotrophs. They appear to be defective in the synthesis of a subunit common to anthranilate and PABA synthetases.

Linkage of trpB to other trp loci. Cotransformation was sought when *trpB18* was transformed with DNA from representative strains of each mutant class. The presence of donor type transformants, able to grow on indole but not on minimal medium, was taken as evidence for linkage. Cells and DNA were spread on minimal agar plates supplemented with 5 μg of indole per ml (plus 1 μg of PABA per ml for *trpG* donors). Recombinants receiving the *trpD*, *trpC*, *trpF*, or *trpA* marker of the donor

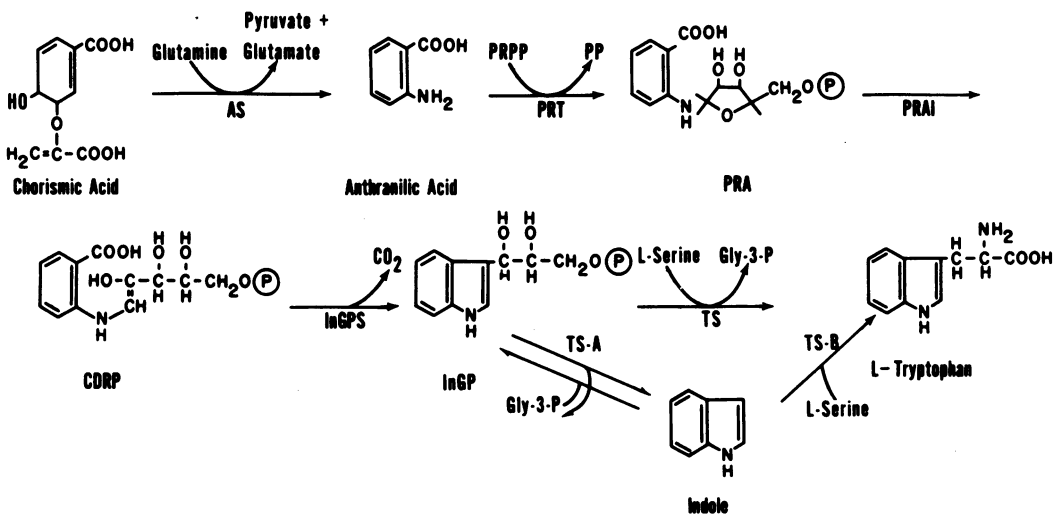

FIG. 1. *Pathway of tryptophan biosynthesis. Abbreviations: AS, anthranilate synthetase; PRT, phosphoribosyl transferase; PRPP, phosphoribosyl-5-pyrophosphate; PP, inorganic pyrophosphate; PRA, phosphoribosylanthranilate; PRAI, phosphoribosylanthranilate isomerase; CDRP, 1-(o-carboxyphenylamino)-1-deoxyribulose-5-phosphate; InGP, indoleglycerol phosphate; InGPS, indoleglycerol phosphate synthetase; Gly-3-P, glyceraldehyde-3-phosphate; TS, tryptophan synthetase; TS-A and TS-B, A and B reactions of tryptophan synthetase. The synthesis of p-aminobenzoate (PABA) is believed to occur from chorismate and glutamine in a reaction analogous to AS.*

TABLE 1. *Classification of Acinetobacter calcoaceticus tryptophan autotrophs*

Strain	Growth response[a]			Accumulation[b]	Enzyme assay[c]			Class[d]
	Anth	Ind	Trp		PRT	PRAI	InGPS	
trpB18	–	–	+	Ind + InG				TS-B
trpB26	–	–	+	InG				TS-AB
trpB28	–	–	+	InG				TS-AB
trpB50	–	–	+	Ind + InG				TS-B
trpA22	–	s	+	InG	+	+	+	TS-A
trpA23	–	s	+	InG	+	+	+	TS-A
trpA24	–	s	+	InG	+	+	+	TS-A
trpA34	–	s	+	InG				TS-A
trpC32	–	s	+	Anth	+	+	–	InGPS
trpC32 bdl-35^{+e}	–	s	+	Anth	+	+	–	InGPS
trpC41	–	s	+	Anth	+	+	–	InGPS
trpC43	–	s	+	Anth + CDR	+	+	–	InGPS
trpC44	–	s	+	Anth + CDR	+	+	–	InGPS
trpF20	–	s	+	Anth	+	–	+	PRAI
trpF29	–	s	+	Anth	+	–	+	PRAI
trpF29 bdl-35^{+e}	–	s	+	Anth	+	–	+	PRAI
trpF38	–	s	+	Anth	+	–	+	PRAI
trpD42	–	s	+	Anth	–	+	+	PRT
trpD42 bdl-35^{+e}	–	s	+	Anth	–	+	+	PRT
trpE27	+	+	+	0	+	+	+	AS
trpE33	+	+	+	0				AS
trpG35	p	p	p	0	+	+		AS-PABS
trpG53	p	p	p	0				AS-PABS

[a] None of the mutants showed growth on minimal agar plates. Growth response was determined on minimal plates plus: Anth, 5 μg of anthranilate per ml; Ind, 5 μg of indole per ml; Trp, 10 μg of L-tryptophan per ml. The letter "s" means slow growth, "p" means practically no growth, becoming normal when PABA (1 μg per ml) is present.

[b] Determined in liquid cultures grown in minimal medium plus 2 mg of L-malate and 4 μg of L-tryptophan per ml. Ind, indole; InG, indoleglycerol; Anth, anthranilate; CDR, 1-(*o*-carboxyphenylamino)-1-deoxyribulose; 0, none of the foregoing.

[c] Assayed in extracts of cells grown to a tryptophan limit as for accumulation. Absence of a symbol means the assays were not done.

[d] Missing activity or activities abbreviated as for Fig. 1, plus PABS, *p*-aminobenzoate synthetase.

[e] *bdl-35*$^+$ is a standard genetic background into which E. Juni transformed three of our mutants. The recipient contained a mutation (*bdl-35*) conferring inability to use butanediol as carbon source. The *trpC*, *F*, and *D* markers were introduced by congression (joint transfer of completely unlinked genes by DNA transformation). We found no significant genetic or biochemical changes following this procedure.

along with *trpB*$^+$ would be expected to grow slowly on the plates, whereas recombinants receiving *trpB*$^+$alone or in combination with *trpE* or *trpG* markers would grow at a normal rate. Where possible, the simple ratio of small to total colonies was taken as the cotransfer frequency. All colonies including those with *trpE* or *trpG* donors were tested by replication to minimal medium (or minimal plus PABA for the *trpG* donor). The results of these experiments are shown in Table 2. Apparently only *trpA* and *trpF* markers are cotransformed with the *trpB* locus. When tested, all the small colonies in these crosses failed to grow on minimal agar, while large colonies, including all those from *trpE* and *trpG* donors, could do so. These results indicate that only the loci for PRAI and the α component of TS are linked to the *trpB* gene.

Linkage of trpE to other trp loci. Transformation experiments designed to test for linkage between *trpE* and all the other *trp* loci failed to indicate any linkage. In these experiments, DNA of strain *trpE27* was mixed with competent cells of representative *trpA*, *B*, *C*, *D*, and *F* mutants on minimal agar supple-

TABLE 2. *Linkage of trpB18 to other trp loci*

Donor	Total colonies observed[a]	Small colonies (%)	Prototrophs		Cotransfer frequency[b]
			Small colonies (%)	Large colonies (%)	
trpE27	500	0		100	0.0
trpG35	100	0		100	0.0
trpD42 bdl-35$^+$	500	0		100	0.0
trpF29 bdl-35$^+$	500	89	0	100	0.89
trpC32 bdl-35$^+$	500	0		100	0.0
trpA23	500	66	0	100	0.66

[a] Transformants tested appeared as isolated colonies on minimal plates containing 5 μg of indole per ml (plus 1 μg of PABA for *trpG* donor) with *trpB18* cells as recipients.

[b] Cotransfer frequency is the uncorrected frequency with which the donor marker appears in the selected recombinants.

mented with 5 μg of anthranilate per ml. Colonies were then replicated to minimal agar to detect donor type transformants. In no case were donor types observed. Linkage of *trpE* to *trpG* could not have been detected by this method, of course, and was examined in a later experiment.

Linkage of trp loci to histidine markers. Several histidine-requiring auxotrophs were isolated and tested for linkage to the *trp* loci. The three *his* mutants shown in Table 3 are not identical, as shown by their ability to transform one another to prototrophy. As shown in the table, however, two are weakly linked to the *trpA*, *B*, and *F* loci, with cotransformation frequencies ranging from 0.06 to 0.27. We could not detect linkage of any *trp* markers to *his-22*. We think this suggests that not all of the *his* genes in this organism are present in a single cluster.

Linkage of trpC, D, and G loci. The aforementioned results imply that there are three or more unlinked clusters for the *trp* genes of *A. calcoaceticus*: *trpABF* (order unknown), *trpE* (with or without *trpG*), and *trpD* and *C* (linked or unlinked). We next tried to find a non-*trp* marker linked to *trpC32* by mutagenizing this strain with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and seeking colonies which would not grow on tryptophan-supplemented plates. Of many prospects, only one double auxotroph, which required high concentrations of glutamate in addition to tryptophan, could be readily transformed to complete prototrophy with wild-type DNA. The genotype of this double mutant is *trpC32 glt-43*, the *glt-43* mutation conferring a growth requirement for high concentrations of glutamate, which can be partially satisfied by aspartate, proline, or arginine, though glutamate is definitely a better supplement than any of these. We found this linked glutamate marker easiest to work with when 0.025% acid-hydrolyzed casein was used instead of glutamate as the supplement. Thus, when the double mutant *trpC32 glt-43* was transformed with wild-type DNA on either tryptophan-supplemented or acid-hydrolyzed casein-supplemented plates, 75% of the colonies were able to grow on minimal agar. From among the 25% still retaining the *glt-43* auxotrophic marker, we selected one of the *trp*$^+$ *glt-43* transformants for the experiments shown in Table 4. As this strain grows more slowly than wild-type even on acid-hydrolyzed casein- supplemented plates, DNA was prepared from it and used to transform representatives of all the *trp* loci to tryptophan independence in the presence of acid-hydrolyzed casein. Cotransfer of the donor marker along with the *trp*$^+$ character was manifested by small, glutamate-requiring colonies, and again

TABLE 3. *Linkage of trp markers to his loci*

Donor	No. of colonies observed[a]	Cotransfer frequency		
		his-135	*his-17*	*his-22*
trpE27	100	0	0	0
trpG35	100	0	0	0
trpD42 bdl-35$^+$	100	0	0	0
trpF29 bdl-35$^+$	100	0.10	0.06	0
trpC32	100	0	0	0
trpC32 bdl-35$^+$	100	0	0	0
trpA23	100	0.18	0.27	0
trpB18	100	0.08	0.12	0

[a] Number of colonies tested with each recipient for cotransfer of the tryptophan or tryptophan + PABA requirement.

TABLE 4. *Linkage of glt-43 to various trp markers*

Recipient	No. of colonies observed[a]	Small colonies (%)	Large colonies (%)	Cotransformation frequency
trpE27	500	0	100	0
trpG35	500	77	23	0.77
trpD42 bdl-35$^+$	500	66	34	0.66
trpF29 bdl-35$^+$	500	0	100	0
trpC32	500	66	34	0.66
trpC32 bdl-35$^+$	500	61	39	0.61
trpB18	500	0	100	0
trpA23	500	0	100	0

[a] Transformants tested appeared as isolated colonies on minimal agar plates plus 0.025% acid-hydrolyzed casein. On this medium the *glt-43* donor makes colonies much smaller than wild type.

cotransfer frequency was ascertained by the ratio of small to total colonies. *trpC*, *D*, and *G* mutants were all shown to be linked to the *glt-43* marker to about the same extent, with an average cotransfer frequency of 66%. Reciprocal experiments with the *glt-43* strain as recipient and DNA from the various *trp* mutants as donors gave comparable cotransfer frequencies for *trpG35*, *trpC32*, and *trpD42*, and again no cotransfer with *trpA*, *B*, *E*, and *F* markers (Table 5).

The results presented indicate a dispersal of the *trp* genes of *A. calcoaceticus* into three regions unlinked to each other by transformation. The disposition is *trpFBA* (order unknown), *trpGDC* (order unknown), and *trpE*. To confirm the linkage of *trpG* to *trpC* and *trpD* we made use of its unique requirement for PABA in addition to tryptophan. *trpG35* cells were mixed with DNA from *trpA* through *F* mutants and spread on tryptophan-supplemented plates. PABA-independent transformants appearing as colonies over the faint background growth were picked to liquid medium, and then streaked on minimal and tryptophan-supplemented plates. Only with the *trpC* and *trpD* donors was cotransfer observed (Table 6); the cotransfer frequency was about 0.9 in each case.

TABLE 5. *Linkage of trp markers to glt-43 recipient*

Donor	No. of colonies observed[a]	Auxotrophs found	Cotransfer frequency
trpE27	100	0	0
trpG35	100	73	0.73
trpD42 bdl-35$^+$	100	71	0.71
trpF29 bdl-35$^+$	100	0	0
trpC32	100	68	0.68
trpB18	100	0	0
trpA23	100	0	0

[a] Transformants were selected on minimal plates containing 10 μg of L-tryptophan per ml, and then replicated to minimal plates except in the case of *trpG35* as donor, where all plates also contained 1 μg of PABA per ml.

TABLE 6. *Linkage of trpG to other trp loci*

Donor	No. of colonies observed[a]	Auxotrophs found	Cotransfer frequency
trpE27	100	0	0
trpD42 bdl-35$^+$	100	91	0.91
trpF29	100	0	0
trpC32	100	88	0.88
trpB18	100	0	0
trpA23	100	0	0

[a] Transformants were selected on minimal plates containing 10 μg of L-tryptophan per ml and then tested for growth on unsupplemented minimal medium.

DISCUSSION

We have isolated and mapped point mutants of *A. calcoaceticus* blocked in each of the five reactions of the tryptophan synthetic pathway. Two of these reactions, the first and last in the pathway, appear to be catalyzed by multi-subunit enzymes; each enzyme is under the influence of a pair of genetic loci. *Acinetobacter* mutants blocked in the last reaction are similar to those observed in other bacteria (34), that is, deficiencies in the first (TS-A) or second (TS-B) or both half-reactions are observed. In other bacteria these have been correlated with defects in the α or β subunits of an $\alpha_2\beta_2$ multimeric protein (34). The gene-enzyme relationships for *A. calcoaceticus* TS have not yet been extensively studied, however, and will not be discussed further here. The situation with respect to the first enzyme, AS, is presently unique, however. Two kinds of auxotrophs were isolated. Both are able to synthesize tryptophan from anthranilate; therefore, they possess the last four enzymes of the pathway and lack only the first. One type requires only tryptophan, indole, or anthranilate for growth on minimal medium. The other requires PABA or folate in addition to tryptophan, indole, or anthranilate. The present results show that the genes conferring these two phenotypes are unlinked. This suggests that AS may also be a multimeric enzyme having two dissimilar subunits.

The seven *trp* loci in *A. calcoaceticus* are arranged in three groups that are not cotransferable by transformation. *trpE*, one of the loci affecting AS, is unlinked to the other six. The other AS locus, *trpG*, is linked to the genes for PRT and InGPS, *trpD* and *trpC*. The remaining three loci, affecting PRAI and TS, *trpF*, *trpA* and *trpB*, are associated with each other in a third region. Gene order in the two three-gene clusters is not yet known, but these clusters will be discussed as though the order were the same as that of similar functions in the enteric bacteria, *trp(GDC)* and *trp(FBA)*, parentheses indicative of uncertainty of order.

The *his* marker that we used to confirm the clustering of the *trpF*, *B*, and *A* loci may not be close enough to permit us to order these three genes with respect to one another. In several experiments a *trpF* marker was cotransferred with *trpB18* at a higher frequency than *trpA23* or *trpA24*, however. It seems un-

likely that the *trpA* and *trpB* loci, affecting the α and β subunits of a single enzyme, are not adjacent in *Acinetobacter* as they are in all other bacteria so far studied, so the order *trpFBA* may be inferred but cannot be considered proved. In the *trp(GDC)* cluster, cotransformation frequencies with the marker *glt-43* suggest the order *glt-43-trpG35-trpD42-trpC32*, but the small number of transformants scored, the paucity of mutants studied, and the unreliability of two-point cotransformation results for purposes of ordering make these data indecisive. Three-point transformation tests using *glt-43* as a nonselective marker might be employed to establish order in the *trp(GDC)* cluster. Even though the gene order within the two three-gene clusters is not firmly established, the pattern of *trp* gene distribution on the chromosome is clearly unlike that of any other organism studied so far.

Although *A. calcoaceticus* is also unique in having a locus, *trpG*, where single point mutations can give rise to an auxotrophic requirement for both tryptophan and PABA, this situation has some parallels to one described by Kane and Jensen (20, 21) in *B. subtilis*. In that organism, as in certain pseudomonads (27) and *Serratia marcescens* (16, 37) the enzyme AS freely dissociates into large and small subunits. The large subunit can catalyze the synthesis of anthranilate from chorismate and ammonium ions, but requires the presence of the small subunit to utilize the physiological amide group donor, L-glutamine, in the reaction. Although no mutations affecting the small component have been found in *Pseudomonas* or *Serratia*, Kane and Jensen have described one in *B. subtilis* (20). This mutant, *trpX7*, which is unlinked to the other *trp* genes, does not require tryptophan or PABA for growth. The participation of the small AS subunit in PABA synthesis is made evident when the mutant is grown on tryptophan, however, because exogenous tryptophan represses the synthesis of the small component and evokes a requirement for PABA or folate. Extracts of the *trpX7* mutant have undetectably low levels of the enzyme PABA synthetase, and in addition the mutant is abnormally sensitive to sulfathiazole (20). The authors conclude that the small AS subunit is in fact also a component of PABA synthetase, functioning as an L-glutamine amidotransferase in each case. Our *trpG* mutants of *A. calcoaceticus* have precisely the phenotype expected from mutants in a glutamine-binding subunit of AS and PABA synthetase, assuming that glutamine is an obligate substrate in both reactions. In a separate communication (Sawula and Crawford, *manuscript in preparation*) we will describe enzymatic experiments supporting this interpretation. Kane and Jensen (20) did not establish whether the prototrophic behavior of *trpX7* is due to a residual, unstable glutamine transferase activity in their mutant protein or the ability of *B. subtilis* to use ammonium ions in place of glutamine in vivo in both reactions. Our results indicate that *A. calcoaceticus* cannot use ammonium ions in place of glutamine as a nitrogen source in these reactions, for tryptophan does not alleviate the PABA requirement of *trpG* mutants or vice versa (Sawula and Crawford, *in preparation*).

The AS of *E. coli* and *S. typhimurium* differs from those discussed so far by being associated in a multienzyme complex with PRT (5, 17). Recently it has become clear that the glutamine-binding function of AS resides in a discrete portion of the PRT polypeptide chain coded by the operator-proximal portion of the *trpD* cistron (5, 12, 25, 35). As no mutations in the *trp* operon of these bacteria result in a PABA requirement, it is unlikely that the glutamine-binding function of the *trpD* cistron participates in PABA synthetase. Huang and Gibson (15) described two genetically distinct components of PABA synthetase in *E. coli*, a large and small component. It seems likely, therefore, that in the enteric organisms the glutamine-binding functions for the two pathways reside on separate polypeptide chains.

The enteric bacteria *E. coli* and *S. typhimurium* have many differences in their tryptophan genes and enzymes from bacteria such as *P. putida* and *A. calcoaceticus* where several *trp* gene clusters have been found. Amino acid sequence analysis of the α chains of TS from *E. coli* and *P. putida* clearly demonstrate a common genetic ancestry for this component of the pathway, however (9). It seems likely that translocation and gene fusion or splitting may have occurred during the evolution of bacteria, rather than extensive mutational substitutions resulting in functional alterations. It is interesting to note, therefore, that the *trp* operon of *E. coli* could be converted to the chromosomal arrangement of *A. calcoaceticus* by a modest number of operations. First *trpE* could be translocated away from the remaining *trp* genes. Then the glutamine-binding function of PABA synthetase could be deactivated and its function supplied by the small segment of the PRT chain serving that purpose for AS. Another translation stop and restart signal in the middle of the *E. coli trpC* gene would sep-

arate the InGPS (proximal) and PRAI (distal) portions of this polypeptide chain. Translocation to separate the remainder of the operon into two three-gene clusters, *trpGDC* (old *trpD* and the proximal half of old *trpC*) and *trpFBA* (the distal half of the old *trpC* and the TS genes) would complete the process, assuming that suitable control elements were made available. This hypothetical exercise is not meant to imply the direction of the evolutionary change, from one operon to several clusters, or that the pathway from *E. coli* to *A. calcoaceticus* was a direct one, or that *Acinetobacter* is more closely related to enteric species than to *Pseudomonas*.

Regulation of the *trp* genes at the several chromosomal locations in *A. calcoaceticus* was not investigated in this study. Published results of Twarog and Liggins (32) suggest that only AS is repressed by exogenous tryptophan in this organism. Additional studies using some of the mutant types reported in this investigation would appear to be desirable to strengthen their conclusions.

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