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Genetic Control of Glutamine Synthetase in *Klebsiella aerogenes*

STANLEY L. STREICHER, ROBERT A. BENDER, AND BORIS MAGASANIK*
Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

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Mutations at two sites, *glnA* and *glnB*, of the *Klebsiella aerogenes* chromosome result in the loss of glutamine synthetase. The locations of these sites on the chromosome were established by complementation by episomes of *Escherichia coli* and by determination of their linkage to other genetic sites by transduction with phage P1. The *glnB* gene is located at a position corresponding to 48 min on the Taylor map of the *E. coli* chromosome; it is linked to *tyrA*, *nadB*, and *gua*. The *glnA* gene is at a position corresponding to 77 min on the Taylor map and is linked to *rha* and *metB*; it is also closely linked to *rbs*, located in *E. coli* at 74 min, indicating a difference in this chromosomal region between *E. coli* and *K. aerogenes*. Mutations in the *glnA* site can also lead to nonrepressible synthesis of active glutamine synthetase. The examination of the fine genetic structure of *glnA* revealed that one such mutation is located between two mutations leading to the loss of enzymatic activity. This result, together with evidence that the structural gene for glutamine synthetase is at *glnA*, suggests that glutamine synthetase controls expression of its own structural gene by repression.

We have previously shown that mutations resulting in the inability of *Klebsiella aerogenes* to produce glutamine synthetase can occur at two chromsomal sites, *glnA* and *glnB*, unlinked by transduction with phage PW52 (8). In the preceding paper, we have presented the evidence for considering *glnA* the structural gene for glutamine synthetase (5). The present paper describes the mapping of *glnA* and *glnB* on the chromosome of *K. aerogenes*. This was accomplished through the use of the transducing phage P1. The method used to isolate strains of *K. aerogenes* sensitive to this phage has already been published (6).

We also determined the relative positions of mutations within the *glnA* site. This fine structure mapping was of particular interest because these closely linked mutations result in different phenotypes; some mutants fail to produce any detectable product, others produce enzymically inactive antigen, and still others produce an active enzyme whose synthesis is not subject to regulation by ammonia. The genetic evidence suggests that all these mutations are allelic.

MATERIALS AND METHODS

Bacterial strains. *K. aerogenes* strains used were P1-sensitive derivatives (6) of *K. aerogenes* W-70. Strains used in this study are listed in Table 1. *Escherichia coli* strains were derivatives of *E. coli* K-12 and are listed in Table 2.

Media. The rich medium was LB (8). LBgln medium was LB supplemented with 0.2% L-glutamine (filter sterilized). LBKM medium was LB supplemented with 25 μg of kanamycin sulfate per ml. The minimal medium used for *K. aerogenes* strains was W salts (12) supplemented with carbon source at 0.4% and nitrogen source at 0.2%. The minimal medium used for *E. coli* strains was 0.M salts (11) supplemented with 0.4% carbon source. Amino acids were added when appropriate at a final concentration of 0.01% with the exception of glutamine (0.2%) and glutamate (0.1%). Nicotinic acid was added at a final concentration of 0.001%. Solid medium contained 1.5% agar. For the isolation of rhamnose- and ribose-negative mutants, MacConkey indicator plates were used with the appropriate carbon source added at a final concentration of 1.0%. Plates used for the test for the GlnC$^-$ phenotype (8) contained W salts supplemented with citrate (0.4%), ammonium sulfate (0.2%), and tryptophan (0.2%) and were supplemented with amino acids when required.

Cultivation of bacteria and preparation of phage lysates. *K. aerogenes* strains were grown in LB or LBgln medium to saturation aerobically at 30 C. *E. coli* strains were similarly grown at 37 C. *E. coli* strains used as episome donors were grown in LB medium without shaking at 37 C to a density of 2×10^8 to 4×10^8 cells/ml. The isolation of strains lysogenic for phage P1c1r100KM (hereafter referred to as P1) and the preparation of P1 lysates by thermal induction has been previously described (6).

Isolation of mutants. Mutagenesis by ethyl methane sulfonate has been previously described (9). Mutagenesis by ICR-191E was as described by Roth

TABLE 1. *List of K. aerogenes strains and their characteristics*

Strain	Relevant genotype	Comments
MK9000	Prototroph	Derivative of MK53 (9)
MK9001	*leu-1 met-1 glnA10*	This laboratory
MK9011	*ilvA1 glnA6*	Derivative of MK104 (6)
MK9013	*ilvA1 ppc-63*	This laboratory
MK9014	As MK9011 but *rha-1*	EMS[a] of MK9011
MK9019	*glnA29 glnB3*	Revertant of MK93 (8)
MK9020	As MK9014 but ∇ [*fad-1*]Chl^R	Chl^R of MK9014
MK9021	*ilvA1 glnA10 rha-1*	This laboratory
MK9040	*glnA4*[b] *glnB3*	Derivative of MK94 (8)
MK9052	*metB4 glnA5*	Derivative of MK103 (8)
MK9057	*ilvA3 met-7 glnB3*	This laboratory
MK9080	*ilvA3 thr-1 argH2 glnB3*	This laboratory
MK9083	*ilvA3 leu-5 tyrA1 glnB3*	This laboratory
MK9086	*ilvA3 leu-5 glnB3 rbs-2*	This laboratory
MK9096	*ilvA3 ppc-63 rha-1*	This laboratory
MK9110	*ilvA1 guaB1*	This laboratory
MK9111	*ilvA3 tyrA2 rha-1*	This laboratory
MK9113	*ilvA3 nadB1 rha-1*	This laboratory
MK9120	*glnA20 rha-2 rbs-4*	This laboratory
MK9127[c]	*metB9 glnA51 asm-200 rha-4 rbs-3*	This laboratory
MK9203[c]	*glnA51 asm-200 rha-4*	EMS of MK9267
MK9266[c]	*glnA50 asm-200*	Derivative of MK189 (3)
MK9267[c]	*glnA51 asm-200*	Derivative of MK189 (3)
MK9271	*ilvA1 metB6 rha-1*	This laboratory
MK9281[c]	*glnA20*	Spontaneous Gln$^-$
MK9282[c]	*glnA20 rha-2*	EMS of MK9281
MK9341	*metB6 ppc-63 rha-1*	This laboratory
KG2[c]	Prototroph	Derivative of MK1 (6)
KG27[c]	*metB6*	EMS of KG2

[a] EMS, Ethyl methane sulfonate.
[b] Previously designated as *glnC4* (8).
[c] These strains are *hutC*$^+$; all other strains listed also have the *hutC515* (9) mutation.

TABLE 2. *List of E. coli strains and their characteristics*

Strain	Relevant genotype	Source
RS127	*proC ilv*	R. Sanders
LS518	*trpA36 metB argH rif*	L. Soll
LS519	*metB rha*	L. Soll
FS321	*his-1 argG metB glnA200*	S. Kang
FS323	*his-1 glnA200*	Derivative of FS321
AB1206	F14	B. Bachmann (7)
KLF11/JC1553	F111	B. Bachmann (7)
KLF12/JC1553	F112	B. Bachmann (7)
KLF33/JC1553	F133	B. Bachmann (7)
KLF42/KL253	F142	B. Bachmann (7)
KLF43/KL259	F143	B. Bachmann (7)
KLF5/AB2463	F105	B. Bachmann (7)
JG85	F197	B. Bachmann (7)

(10). Mutagenized bacteria were grown out in LB or LBgln medium overnight at 30 C. Desired auxotrophs were enriched for by penicillin treatment. LB medium cultures of mutagenized bacteria were centrifuged and resuspended in saline (0.85% NaCl) and diluted 1:100 into minimal medium containing glucose and ammonium sulfate (GN medium) and appropriate supplements (complete medium). The bacteria were grown in complete medium to a density of 1 $\times 10^8$ to 2 $\times 10^8$ cells/ml, then centrifuged and washed two times with saline and suspended in GN medium deficient in the particular nutrient of interest (starvation medium). Incubation was continued for 2 to 3 h to allow the desired mutants to stop growing. The starved culture was diluted 1:10 into fresh starvation medium, and penicillin was added to a final concentration of 5,000 U/ml. Incubation was continued for 3 to 4 h. Surviving bacteria were harvested by centrifu-

gation and washed three times with saline and finally suspended in complete medium or LB or LBgln medium and grown up overnight at 30 C with shaking. Starvation medium for the enrichment of glutamine auxotrophs was supplemented with 0.1% glutamate to avoid enrichment of glutamate auxotrophs. Penicillin-enriched cultures were diluted and spread onto complete medium plates (100 to 200 colonies/plate) and incubated at 30 C for 18 to 24 h. Colonies were picked with sterile toothpicks and streaked onto appropriate plates. Such plates were incubated for 24 h at 30 C and presumptive mutants were picked and purified by single-colony isolation on LB or LBgln plates. To simplify mutant hunts, starvation medium plates were supplemented with 1% LB medium. Auxotrophs form tiny colonies after 24 h and could easily be distinguished from prototrophs. Glutamine auxotrophs were similarly detected by plating cells on unsupplemented LB plates. Glutamine-requiring mutants utilize the limiting amounts of glutamine present in the tryptone and yeast extract of the medium.

Several auxotrophic markers isolated with the above described procedures were further characterized by nutritional supplementation. Specific assignment of genetic defects such as *ilvA*, *metB*, and *argH* (13) was made according to the nutritional supplementation data (Table 3) and complementation with *E. coli* episomes.

Mutants defective in the utilization of ribose or rhamnose were isolated by plating mutagenized bacteria on MacConkey plates containing the appropriate sugar. After incubation for 24 h at 30 C, nonutilizing mutants appear white, while the wild-type colonies appear deep red with a zone of precipitated salts surrounding the colonies. Putative mutants were purified by single-colony isolation on MacConkey plates. All of the mutants isolated in this manner and used in the present study have proved to be leaky. They all grow to some extent in minimal medium supplemented with the specific sugar they appear not to utilize as judged by MacConkey plate indications. Each mutant was checked for utilization of other carbohydrates (MacConkey indication) and was positive. Several other carbohydrate mutants also isolated on MacConkey plates proved to be leaky. Because of these results, we were able to use the ribose and rhamnose markers only as unselected markers in mapping experiments.

P1-mediated transduction. Transductions were performed as previously described (6). Recipient bacteria were grown overnight in LB or LBgln medium and harvested by centrifugation. The bacteria were washed once with saline and resuspended in adsorbing medium (0.005 M $CaCl_2$ and 0.01 M $MgSO_4$) at a concentration of 2×10^9 cells/ml. Sterile phage were added at a multiplicity of infection of 0.1 to 0.5, and the infected bacteria were incubated at 30 C for 30 min and then spread (0.1 ml) on selective plates. The plates were incubated for 18 to 36 h at 30 C or until colonies appeared. For transductions that selected for recombinations events within the *glnA* gene, the multiplicity of infection was increased to 1.0. Transductant colonies were purified by single-colony isolation on selective plates prior to scoring for transfer of negative markers from the donor strain. Transfer of positive markers from the donor strain was tested with the transductant colonies without further purification.

Episome transfer. *E. coli.* episomes were transferred into *K. aerogenes* strains by plate mating. Overnight cultures of recipient bacteria were harvested by centrifugation and were washed with saline. One drop of the suspension of the recipient culture was placed on a selective plate and allowed to dry. A drop of donor *E. coli* was placed over the dry area of the recipient bacteria and allowed to dry. Separate areas of recipient and donor bacteria served as controls on the same plate. Plates were incubated 30 C for 24 to 48 h. Growth within the mixed areas and no growth in the control areas indicated episome transfer and complementation. Donor bacteria were counterselected by the addition of chloramphenicol (100 μg/ml) to the plates or by the use of citrate as the sole source of carbon. *K. aerogenes* strains used are resistant to this antibiotic (2), and *E. coli* is unable to utilize citrate. The presence of the episome in *K. aerogenes* strains was demonstrated by allowing segregation to occur and recovering the original markers

TABLE 3. *Patterns of nutritional supplementation of some mutants in K. aerogenes*[a]

Mutation	Stimulates growth	Does not stimulate growth
argH2	Arginine	Citrulline, ornithine, *N*-acetyl ornithine, argininosuccinate
guaB2	Guanine	Hypoxanthine, xanthine, adenine
ilvA1, *ilvA3*	Isoleucine	Leucine, valine
metB4, *metB6*	Methionine, cystathionine, homocysteine	Succinyl homoserine, B_{12}
ppc-63	Citrate,[b] succinate, glutamate, aspartate	
tyrA2	Tyrosine	Phenylalanine, tryptophan

[a] Mutants were radially streaked on plates containing glucose, ammonia, W salts, and all other required supplements except for the one in question. A few crystals of the relevant compound were placed in the center of the plate. Plates were incubated at 30 C for 18 to 36 h and growth stimulated by the added compound was noted.

[b] Glucose was omitted from the medium.

of the recipient strain. Nonselective growth (LB) resulted in the loss of episome in 5 to 25% of the isolated colonies. The presence of the episome could be directly checked by the loss of sensitivity of such *K. aerogenes* strains to the phage T7. Upon loss of the episomes, the strains regain sensitivity towards phage T7.

RESULTS

Complementation of K. aerogenes glnA and glnB with E. coli episomes. Because of the similarity of *K. aerogenes* and *E. coli*, the map position of a gene in *E. coli* might reflect a similar position for the analogous gene in *K. aerogenes*. The complementation of a mutation in *K. aerogenes* by an episome derived from the *E. coli* chromsome ought therefore to be a useful method in localizing the mutation on the *K. aerogenes* chromosome. *K. aerogenes* strains carrying various mutations in *glnA* were used as recipients for a variety of *E. coli* episomes representing almost the entire *E. coli* chromsome. Complementation of *glnA* was determined first by direct selection of Gln$^+$ exconjugants. The results of these experiments suggested that the *glnA* gene may be located near *metB* and *ilv*. We subsequently isolated derivatives of *glnA* mutants carrying additional mutations in *metB* or *ilv*. We then selected episomes capable of complementing *metB* or *ilv* and tested the exconjugants for the Gln$^+$ phenotype, the ability to grow in the absence of glutamine. Table 4 shows the complementation pattern for *E. coli* episomes carrying the chromosomal regions corresponding to 74 to 80 min of the Taylor map. Four of the episomes complement *glnA*. The chromosomal regions carried by these episomes are shown in Fig. 1. The pattern of complementation suggests that *glnA* is located in *E. coli* between *metB* and *ilv*.

TABLE 4. *Complementation of K. aerogenes mutations with E. coli episomes*[a]

Episome	*Klebsiella* markers[b]			
	ilv	*glnA*	*metB*	*rha*
F14	+	+	+	+
F105	−	+	+	−
F111	+	+	+	+
F112	−	−	+	−
F133	+	+	+	+
F197	+	ND	−	ND

[a] Exconjugants were purified and scored for complementation of the indicated markers. The episomes complemented the indicated markers in several different recipient strains with allelic mutations.

[b] (+) Episome complements the indicated marker; (−) no complementation; ND, not done.

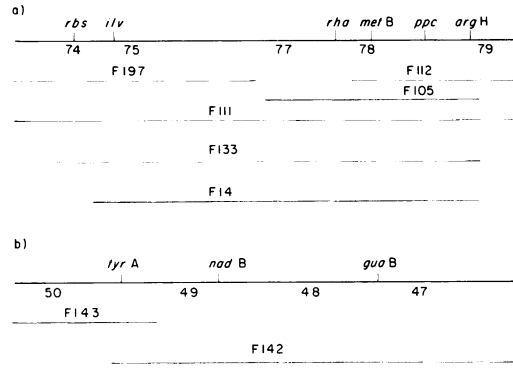

FIG. 1. *(a) Map of the rbs-argH region of E. coli. The lines below represent episomes from E. coli (7). (b) Map of the tyrA-guaB region of E. coli. The lines below represent episomes from E. coli (7).*

The localization of *glnB* was accomplished by corresponding procedures with different episomes. One episome, F'142, complements *glnB3* and presumably indicates the location of the *E. coli glnB* gene. No *glnB* mutants of *E. coli* have been isolated, and thus this assignment cannot be directly tested. Since strains carrying the *glnB3* mutation readily revert to glutamine independence, unequivocal results could only be obtained after selection for complementation of a putatively linked mutation, e.g., *tyrA*. Strain MK9083 was initially used as the recipient for *glnB* complementation experiments and exconjugants carrying F'142 and F'143 were obtained by selection of tyrosine independence. Only strains carrying F'142 were found to be glutamine independent.

The presence of episomes in exconjugants was determined primarily by observing the segregation of the episomes when the strains were grown in nonselective media such as LB medium supplemented with glutamine. Segregation was observed for all exconjugants and ranged from 5 to 25% varying with episome and the *K. aerogenes* background. On rare occasions after many passages on selective media, it was found that the chromosomal markers were not all recovered after loss of the episome. This could be the result of rare homogenotization between the *K. aerogenes* chromosome and the *E. coli* episome. Such strains were not examined in detail. Another convenient way of determining the presence or absence of episomes was the use of phage T7. Phage T7 is female specific, and its growth is interfered with by genes of the F factor (1). P1-sensitive mutants of *K. aerogenes* are also sensitive to T7. Such strains are resistant to phage T7 when *E. coli* episomes are present. Segregants of these strains regain

phage sensitivity. We have failed to observe the appearance of sensitivity towards male specific phages such as f_2 or Q_β. This may be due to the failure of such male *K. aerogenes* strains to produce F-pili and is also reflected in the inability of episomes to transfer out of *K. aerogenes* strains. R factors are known to repress F factor expression, and since K. aerogenes contains at least one R factor and a *lac* plasmid (2), its inability to transfer the episomes is understandable.

Mapping of glnA in K. aerogenes by transduction with phage P1. We assumed the map position of *glnA* in *K. aerogenes* to be similar to its map position in *E. coli*. We therefore used phage P1 to determine the linkage of *glnA* to genes located in the chromosomal region corresponding to 74 to 79 min on the Taylor map. Appropriate mutants were isolated by mutagenesis of *glnA* mutants. The genotypes of these mutants can be inferred from their phenotypes (Table 3) by analogy with corresponding mutants of *E. coli*.

The results of a series of transduction experiments are summarized in Table 5. Crosses 1 to 6 show that *glnA* is linked approximately 90% with *rbs*, approximately 30% with *rha*, and approximately 12% with *metB*. In addition, cross 5 indicates that *metB* is more closely linked to *glnA* than to *rbs*, and crosses 6 and 7 indicate that *metB* is more closely linked to *rha* than to *glnA*. The order suggested by these findings is *rbs-glnA-rha-metB*.

Crosses 4 and 8 show that *glnA* is not linked to *ilvA*, and cross 9 shows that *rbs* is not linked *ilvA*. On the other hand, crosses 4, 7, and 8 show

TABLE 5. *Mapping of the glnA region of the K. aerogenes chromosome by P1-mediated transduction*

Cross	Donor		Recipient		Selected phenotype	No. transductants examined	Donor character (%)						
	Strain	Relevant genotype	Strain	Relevant genotype			Rbs	Gln	Rha	Met	Ppc	Arg	Ilv
1	MK9086	*rbs-2*	MK9011	*glnA6*	Gln$^+$	80	90						
2	MK9086	*rbs-2*	MK9282	*glnA20 rha-2*	Gln$^+$	161	88		25				
3	MK9000	+	MK9120	*glnA20 rbs-4 rha-2*	Gln$^+$	208	95		29				
4	KG27	*metB6*	MK9014	*glnA6 ilvA1 rha-1*	Gln$^+$ Ilv$^+$	186 150		0	46 3	7 20			0
5	KG2	+	MK9127	*glnA51 metB9 rbs-3*	Met$^+$	52	6	12					
6	MK9267	*glnA51*	MK9271	*ilvA1 metB6 rha-1*	Met$^+$	100		17	55				
7	MK9080	*ilvA3 argH2*	MK9341	*metB6 ppc-63 rha-1*	Met$^+$ Ppc$^+$	102 101			49 22	 74	69	57 89	28 53
8	MK9266	*glnA50*	MK9271	*ilvA1 metB6 rha-1*	Ilv$^+$	100		0	7	29			
9	KG2	+	MK9086	*ilvA3 rbs-2*	Ilv$^+$	150	0						
10	MK9052	*metB4*	MK9096	*ilvA3 ppc-63 rha-1*	Ppc$^+$	100			12	55			65
11	KG2	+	MK9013	*ilvA1 ppc-63*	Ilv$^+$	94					82		
12	KG2	+	MK9080	*ilvA3 argH2*	Arg$^+$ Ilv$^+$	104 104						 57	36
13	MK9052	*metB4*	MK9080	*ilvA3 argH2*	Arg$^+$	104				32			52
14	MK9020	*ilvA1 rha-1*	MK9080	*argH2*	Arg$^+$	50			8				

that *metB* is approximately 25% linked to *ilvA* and that *rha* is approximately 5% linked to *ilvA*. The order suggested by these results is *rbs-glnA-rha-metB-ilvA*.

Additional crosses were carried out to order these genes in relation to *ppc* and *argH*. Crosses 7, 10, and 11 show that *ppc* is linked to *metB* and *ilvA* more closely than to *rha*. Cross 7 shows close linkage of *ppc* to *argH*, and crosses 7 and 12 to 14 show that *argH* is more closely linked to *metB* and to *ilv*A than to *rha*. We can conclude on the basis of these results that *ppc* and *argH* are located between *metB* and *ilvA*.

We confirmed the order *glnA-rha-metB-ilvA* by the analysis of the recombinants obtained in a cross between strain MK9266 (*glnA50*) and strain MK9271 (*ilvA1*, *metB6*, *rha-1*) in which Met$^+$ transductants were selected (Table 6). We found approximately 15% of the transductants in class 4 (Ilv$^+$, Rha$^-$, Gln$^+$). This class can arise by a double crossover, when order A is the correct one, but only by a quadruple crossover if order B is the correct one (Fig. 2). The relatively frequent occurrence of recombinants of this class is therefore strong evidence for the order *glnA-rha-metB-ilvA*.

A similar procedure was used to establish the order of *ppc* and *argH* with respect to *ilvA* and *metB*. In this case, strain MK9080 (*ilvA3*, *argH2*) was crossed with strain MK9341 (*ppc-63*, *metB6*, *rha-1*). Transductants of the Ppc$^+$, Arg$^+$ phenotype were selected. Approximately 30% of the transductants are of class 1 (Ilv$^+$, Met$^+$, Rha$^+$) and another 30% are of class 2 (Ilv$^+$, Met$^+$, Rha$^-$) (Table 7). These classes could arise by double crossovers if order A were the correct one, but could only arise by quadruple crossovers if order B were the correct one (Fig. 3). On the other hand, only 1% of the transductants were of class 4 (Ilv$^-$, Met$^-$, Rha$^-$); transductants of this class could be formed as the result of a double crossover if order B were the correct one, but could arise only by a quadruple crossover if order A is the correct one. Thus, the frequent occurrence of recombinations leading to classes 1 and 2, and the rarity of recombinations leading to class 4, establish the order *rha-metB-ppc-argH-ilvA*.

The map of the region of the *K. aerogenes* chromosome near the *glnA* site, established by the experiments described in this section, is shown in Fig. 4A.

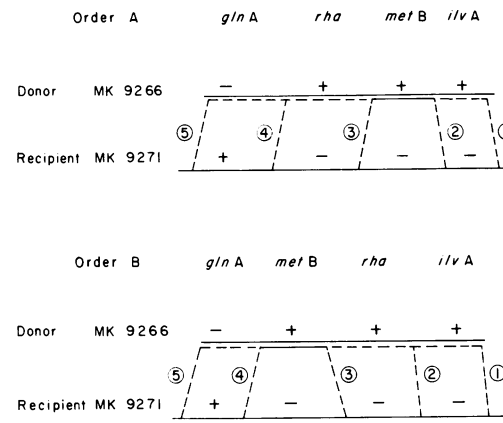

FIG. 2. *Representation of the possible crossover events occurring in the cross of MK9266 × MK9271 reflecting two possible orders of the markers. Data obtained from the cross are given in Table 6.*

TABLE 6. *Determination of the relative order of glnA, rha, metB, and ilvA in K. aerogenes*[a]

Class	Recombinant genotype			No. colonies	Recombinational events[b]	
	ilv	*rha*	*gln*		Order A	Order B
1	−	−	+	37	2 and 3	3 and 4
2	−	+	+	35	2 and 4	2 and 4
3	−	+	−	18	2 and 5	2 and 5
4	+	−	+	16	1 and 3	1 and 2 3 and 4
5	+	+	+	4	1 and 4	1 and 4

[a] The donor strain was MK9266 (*glnA50*) and the recipient strain was MK9271 (*ilvA1*, *metB6*, *rna-1*). The selected phenotype was Met$^+$, and 110 transductants were examined.

[b] Hypothetical recombination events are as in Fig. 2.

TABLE 7. *Determination of the relative order of ppc argH with respect to ilvA and metB in K. aerogenes*[a]

Class	Recombinant genotype			No. colonies	Recombinational events[b]	
	ilv	*met*	*rha*		Order A	Order B
1	+	+	+	33	3 and 6	2 and 3; 4 and 6
2	+	+	−	31	3 and 5	2 and 3; 4 and 5
3	+	−	−	29	3 and 4	2 and 3
4	−	−	−	1	1 and 2; 3 and 4	1 and 3
5	−	+	−	1	1 and 2; 3 and 5	1 and 3; 4 and 5
6	+	−	+	1	3 and 4; 5 and 6	1 and 3; 5 and 6

[a] The donor was 9080 *ilvA3*, *argH2*, and the recipient was 9341 *ppc-63*, *metB6*, *rha-1*. The selected phenotype was Ppc$^+$Arg$^+$, and 96 colonies were analyzed.

[b] Hypothetical recombination events are as shown in Figure 3.

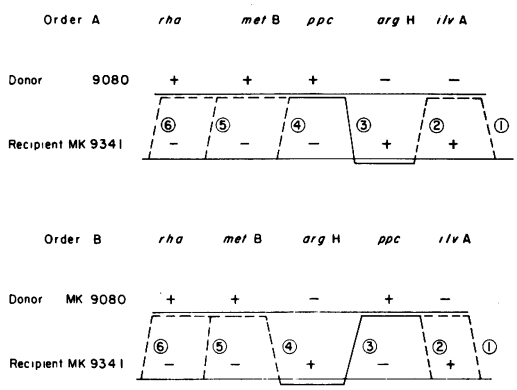

FIG. 3. *Representation of the possible crossover events occurring in the cross of MK9080 × MK9341 reflecting two possible orders of the markers. Data obtained from the cross are given in Table 7.*

Mapping of glnA in E. coli by transduction with phage P1. The map of the chromosomal region of *K. aerogenes* shown in Fig. 4A differs from the published map of the corresponding region of *E. coli*. Examination of the Taylor map (13) of the *E. coli* chromosome shows that *ilv* (75 min) is too far from *metB* (78 min) to show linkage by P1 transduction; on the other hand, *ilv* (75 min) and *rbs* (74 min) are close enough for co-transduction by P1.

We used P1 transduction to examine the linkage of a putative *glnA* mutant to other genes located in 74- to 78-min region of the *E. coli* chromosome. The results show that *glnA* is, as in the case of *K. aerogenes*, linked to *rha* and *metB*, but not *ilv* (Table 8, crosses 1 and 2). We confirmed that *metB* is not linked to *ilv* (crosses 1, 3, and 4), but is linked to *rha* (cross 3) and to *argH* and *rif* (cross 4). Finally, *argH* was shown to be linked more closely to *metB* and to *rif* than to *rha* (crosses 4 and 5). These results and the published findings summarized in the Taylor map give the gene order shown in Fig. 4B. It appears that the chromosomal segment containing *ilv* located between *rbs* and *glnA* in *E. coli* is located near *argH* in *K. aerogenes*.

Mapping of glnB in K. aerogenes by transduction with phage P1. In one of the glutamine-requiring mutants of *K. aerogenes*, the mutation responsible for this deficiency is not linked to *glnA* (8). We have shown in an earlier section that episome F'142 of *E. coli*, which carries *tyrA*, can complement *glnB3*. We therefore carried out the crosses summarized in Table 9 to determine the linkage of *glnB* to *tyrA*

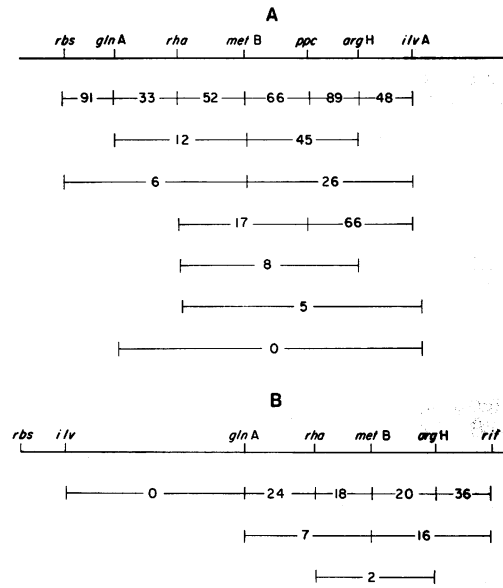

FIG. 4. *(A) Map of the glnA region of K. aerogenes derived from the data in Table 5. Numbers below the map represent the average cotransduction frequency of the markers. (B) Map of the glnA region of E. coli derived from the data in Table 8 and the Taylor map. Numbers below the map represent the average co-transduction frequency of the markers as obtained in this laboratory.*

TABLE 8. *Mapping of the glnA region of the E. coli chromosome*

Cross	Relevant genotype		Selected phenotype	No colonies analyzed	Donor character (%)					
	Donor	Recipient			Ilv	Gln	Rha	Met	Arg	Rif
1	LS519 *metB rha*	FS323 *glnA200*	Gln^+	51			24	4		
2	RS127 *ilv*	FS321 *glnA200 metB*	Met^+	104	0	8				
			Gln^+	97	0			10		
3	RS127 *ilv*	LS519 *metB rha*	Met^+	104	0		20			
4	RS127 *ilv*	LS518 *metB argH rif*	Met^+	55	0				20	16
5	LS519 *rha*	LS518 *argH rif*	Arg^+	50			2			36

TABLE 9. *Mapping of the glnB region of the K. aerogenes chromosome*

Cross	Relevant genotype		Selected phenotype	No. colonies tested	Donor character (%)			
	Donor	Recipient			Tyr	Nad	Gln	Gua
1	MK9020 +	MK9083 *glnB3 tyrA1*	Tyr$^+$	260			7	
2	MK9057 *glnB3*	MK9111 *tyrA2*	Tyr$^+$	80			11	
3	MK9011 +	MK9083 *glnB3 tyrA1*	Tyr$^+$	172			10	
4	MK9040 *glnB3*	MK9113 *nadB1*	Nad$^+$	204			26	
5	MK9083 *glnB3 tyrA1*	MK9113 *nadB1*	Nad$^+$	118	73		10	
6	MK9113 *nadB1*	MK9083 *glnB3 tyrA1*	Tyr$^+$	100		27	5	
7	MK9113 *nadB1*	MK9111 *tyrA2*	Tyr$^+$	104		56		
8	MK9057 *glnB3*	MK9110 *guaB1*	Gua$^+$	130			3	
9	MK9110 *guaB1*	MK9083 *glnB3 tyrA1*	Tyr$^+$	100		10		0

and to other genes found in this region of the *E. coli* chromosome. It can be seen that *glnB* is linked to *tyrA* (crosses 1 to 3, 6 and 9), *nadB* (crosses 4 and 5), and to *guaB* (cross 8). We also found that *tyrA* is closely linked to *nadB* (crosses 5 to 7), but is not linked to *guaB* (cross 9). The order suggested by these results is shown in Fig. 5.

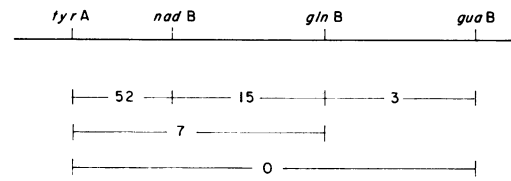

FIG. 5. *Map of the glnB region of K. aerogenes derived from the data in Table 9. Numbers below the map represent the average cotransduction frequency of the markers.*

Phenotypes of glnA mutants. The majority of mutations affecting the production of glutamine synthetase (16 of 19) that we have isolated and examined so far are in the chromosomal site we have called *glnA*. These mutations are all linked by transduction with phage P1 to *metB* (6 to 30% co-transduction) and to *rha* (25 to 46% co-transduction). The variation in the frequency also observed in duplicate crosses does not appear significant. Crosses between *glnA* mutants show that the mutations are approximately 99% linked.

We have reported previously that mutations affecting the production of glutamine synthetase also affect the ability of the organism to produce histidase in a medium containing glucose (4, 8). In such a medium, cells with normal glutamine synthetase produce histidase at a high level when the nitrogen source is limiting, but not when ammonia is present in excess (Cn$^+$; [4, 8]). The mutants either fail to produce histidase at a high level even when starved of a source of nitrogen (Cn^s), or produce it at a high level even when grown with excess ammonia (Cn^R).

On the basis of synthesis of active glutamine synthetase and the regulation of histidase production, we can recognize three distinct phenotypes among the strains with mutations in *glnA* (Table 10).

The gln$^-$, Cn^s phenotype characteristic of *glnA6*, *glnA5*, and *glnA20* is the inability to produce active glutamine synthetase and the inability to produce histidase at a high level during nitrogen starvation; the Gln$^-$, Cn^R phenotype, characteristic of *glnA10* and *glnA51*, combines the inability to produce active glutamine synthetase with the ability to produce histidase at a high level even when ammonia is present in excess. It is of interest that one of these strains, MK9267 (*glnA51*), produces a protein that reacts with anti-glutamine synthetase serum at a high level whether starved of nitrogen or grown with an excess of ammonia (5). The other Gln$^-$, Cn^R strain, MK9021 (*glnA10*), was not found to produce such a protein (5).

The mutants with the phenotypes discussed so far require glutamine for growth. The mutants with the phenotype GlnC$^-$, Cn^R, characteristic of the *glnA4* and *glnA29* mutations, do not require glutamine; in contrast to the Gln$^+$

TABLE 10. *Phenotypes of various glnA mutations in K. aerogenes*[a]

Mutation	Mutagen	Glutamine synthetase[a]		Regulation of *hut*[b]
		Enzyme	Antigen	
glnA$^+$		+[c]	+	Cn$^+$
glnA6	NTG	−[d]	−	Cn^s
glnA5	NTG	−	±	Cn^s
glnA20	SPONT	−	−	Cn^s
glnA10	ICR	−	−	Cn^R
glnA51	EMS	−	++	Cn^R
glnA4	SPONT	++[e]	++	Cn^R
glnA29	SPONT	++	ND[f]	Cn^R

[a] Strains containing the indicated marker were grown in minimal medium containing glucose, ammonia, and glutamine and were assayed for glutamine synthetase activity and glutamine synthetase antigen (5) and for histidase activity (8).

[b] Cn$^+$, Cn^s, and Cn^R are defined in the text.

[c] (+) Low enzyme (antigen) level.

[d] (−) Not detected.

[e] (++) High enzyme (antigen) level.

[f] ND, Not done.

strain, they produce glutamine synthetase and also histidase at a high level even when grown in a medium containing excess ammonia. Another characteristic property of GlnC$^-$ strains is their inability to produce glutamate dehydrogenase (3). A double mutant, in addition to having the GlnC$^-$ phenotype, lacks glutamate synthase (Asm$^-$) and therefore cannot use ammonia as source of nitrogen; it can only grow in a medium supplemented with glutamate.

Fine structure genetic analysis of glnA. We attempted to order the mutations resulting in the different phenotypes. Crosses between the mutants revealed that when a strain carrying *glnA6* is crossed with strains carrying *glnA5* or *glnA20*, no recombinants able to grow without glutamine are obtained; on the other hand, *glnA6* can recombine with *glnA10* and *glnA51*, and *glnA5* and *glnA20* can recombine with one another to give Gln$^+$ strains. It would therefore appear that *glnA6* is a small deletion covering *glnA5* and *glnA20*, but not *glnA10* or *glnA51*. This interpretation is supported by our failure to obtain Gln$^+$ revertants from a strain with the *glnA6* mutation.

We ordered the mutations in *glnA* with respect to the outside marker *rha*, whose linkage to individual *glnA* mutations is approximately 40%. In these crosses, the recipient carried one *glnA* mutation and was Rha$^-$; the donor carried another *glnA* mutation and was Rha$^+$. Gln$^+$ recombinants were scored for the Rha character. Appearance of the donor Rha$^+$ phenotype would result from either a double crossover or a quadruple crossover, depending on the position of the mutations in *glnA* (Fig. 6). Reduction of the transfer of Rha$^+$ significantly below 40% would be the indication of a quadruple crossover. Wherever possible, we confirmed our analysis by carrying out reciprocal crosses (see Table 11).

Our first set of reciprocal crosses (Table 11, crosses 1 and 2) was between *A20* and *A10*. The results show clearly that *A10* is located between *A20* and *rha* (order A of Fig. 6). We next crossed *A20* and *A51* (crosses 3 and 4), and obtained the order *A20-A51-rha*. Finally, we crossed *A51* and *A10* (crosses 5 and 6), and obtained the order *A51-A10-rha*. Together the results of these three sets of reciprocal crosses establish the order *A20-A51-A10-rha*. In addition, the results of the experiments described at the beginning of this section locate *A5* and *A6* near *A20* and to the left of *A51*.

We attempted to confirm the location of *A5* by crosses 7-9 (Table 11). Unfortunately, *glnA5* reverts too readily to permit use of strains carrying this mutation as recipients in crosses where Gln$^+$ recombinants are selected; reciprocal crosses could therefore not be performed. Cross 7 indicates that *A51* is located between *A5* and *rha*. Cross 8 suggests the order *A5-A10-rha*. Thus, these crosses confirm the location of *A5* to left of *A51* and of *A10*. The results of cross 9 between *A5* and *A20* is somewhat ambiguous; the frequency of Rha$^+$ recombinants is higher than expected for the order *A20-A5-rha*, where a quadruple crossover is required. We favor this order because negative interference in crosses between such closely linked markers should favor recombination. Thus, we suggest that *A5* is located between *A20* and *rha*.

Additional crosses were performed to deter-

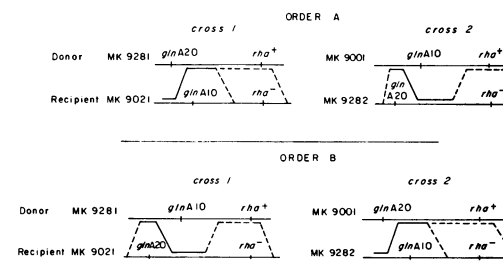

FIG. 6. *Representation of the possible crossover events occurring in the reciprocal crosses MK9267 × MK9021 and MK9001 × MK9203 reflecting the two possible orders of the glnA mutations glnA51 and glnA10 relative to rha. Data obtained from the crosses are given in Table 11, lines 5, 6.*

TABLE 11. *Fine structure analysis of glnA*

Cross	Relevant genotype		No. *gln*$^+$ transductants analyzed	Rha$^+$ (%)	Probable order
	Donor	Recipient			
1	MK9281 *glnA20*	MK9021 *glnA10 rha-1*	88	27	*glnA20-glnA10-rha*
2	MK9001 *glnA10*	MK9282 *glnA20 rha-2*	30	3	
3	MK9267 *glnA51*	MK9282 *glnA20 rha-2*	100	11	*glnA20-glnA51-rha*
4	MK9281 *glnA20*	MK9203 *glnA51 rha-4*	28	39	
5	MK9267 *glnA51*	MK9021 *glnA10 rha-1*	39	41	*glnA51-glnA10-rha*
6	MK9001 *glnA10*	MK9203 *glnA51 rha-4*	44	2	
7	MK9052 *glnA5*	MK9203 *glnA51 rha-4*	43	35	*glnA5-glnA51-rha*
8	MK9052 *glnA5*	MK9021 *glnA10 rha-1*	102	23	*glnA5-glnA10-rha*
9	MK9052 *glnA5*	MK9282 *glnA20 rha-2*	32	12	*glnA20-glnA5-rha*
10	MK9019 *glnA29*	MK9203 *glnA51 rha-4*	207	39	*glnA29-glnA51-rha*
11	MK9040 *glnA4*	MK9203 *glnA51 rha-4*	197	13	*glnA51-glnA4-rha*
12	MK9019 *glnA29*	MK9282 *glnA20 rha-2*	95	10	*glnA20-glnA51-rha*

mine the sites of the mutations responsible for the GlnC$^-$ phenotype, *A29* and *A4*. We took advantage of the fact that strain MK9203 carries, in addition to the *glnA51* mutation, a second mutation in *asm*. Cells combining the GlnC$^-$ and Asm$^-$ phenotypes cannot produce glutamate from α-ketoglutarate and ammonia under any condition. They lack glutamate synthase because of the mutation in the *asm* site. They lack glutamate dehydrogenase because of the mutation responsible for the GlnC$^-$ phenotype; the high level of glutamine synthetase brings about the repression of glutamate dehydrogenase (4). Consequently, an Asm$^-$, GlnC$^-$ double mutant can grow on the glucose-ammonia medium only when it is supplemented with glutamate (4). In a cross with MK-9203 as recipient and a GlnC$^-$ strain as donor, plating on glucose ammonia medium eliminates the recombinants with the GlnC$^-$ phenotype of the donor and thus selects for recombination between the mutations responsible for the GlnC$^-$ character and *glnA51* to give GlnC$^+$ transductants. The GlnC$^+$ character of these recombinants was confirmed by the test described above.

Cross 10 of Table 11 gives the order *A29-A51-rha*; cross 11 is somewhat ambiguous, but appears compatible with the order resulting from a quadruple crossover, *A51-A4-rha*. Finally, cross 12, between a GlnC$^-$ strain (strain *glnA29*) as donor, and *glnA20* as recipient, was carried out. Here, all Gln$^+$ recombinants were examined for their GlnC$^-$ character, and the rare GlnC$^+$ recombinants were then scored for their Rha character. The cross suggests the order *A20-A29-rha*.

Thus, the crosses in which the GlnC$^-$ strains with the mutations *A29* and *A4* were used as donors indicate the order *A20-A29-A51-A4-rha*. It is of interest that a mutation leading to nonrepressible synthesis of active glutamine synthetase *glnA29* is located between two mutations leading to inactive glutamine synthetase, *glnA20* and *glnA51*.

The map of the *glnA* site derived from the experiments described in this section is shown in Fig. 7. The mutations resulting in the Cn^s phenotype, *A5*, *A20*, and *A6*, lie distal to *rha* (region II), while the mutations leading to the Cn^R phenotype, *A4*, *A10*, *A51*, and *A29*, are on the side proximal to *rha* (region I).

DISCUSSION

The similarity of the organization of the genetic material in related enteric organisms allows the preliminary localization of mutations on the chromosome of *K. aerogenes* through complementation by the available episomes from *E. coli*. Using this technique, we found

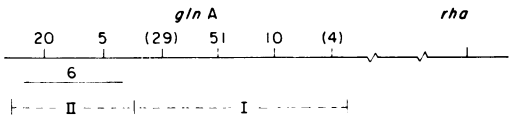

FIG. 7. *Map of the glnA site of K. aerogenes. Mutations in the glnA site were ordered by the data in Table 11. The phenotypes of the glnA mutations are discussed in Table 10. The glnA29 mutation has not been ordered relative to glnA5 but has been determined to lie between glnA51 and glnA20. Mutation glnA6 appears to be a small deletion covering glnA5 and glnA20.*

that *glnA*, the structural gene for glutamine synthetase (5), is located approximately in a position corresponding to 77 min on the Taylor map of the *E. coli* chromosome (13); similarly, *glnB* was located at a position corresponding to 48 min on the Taylor map of the *E. coli* chromosome.

These positions could be confirmed by determination of the linkage of the *gln* genes to other genetic sites by transduction with phage P1. The *glnB* gene was found to be linked to *tyrA* (49½ min), *nadB* (49 min), and *guaB* (47 min). The order is shown in Fig. 5. Similarly, *glnA* was found to be linked to *rha* and *metB* (78 min); however, it was also found to be closely linked to *rbs* (74 min). Closer study of this chromosomal region in both *K. aerogenes* and *E. coli* revealed an interesting difference; the section of the *E. coli* chromosome located between *rbs* and *glnA*, which contains the *ilv* locus, is found on the *K. aerogenes* chromosome at the other side of *glnA*, beyond *argH* (Fig. 4). Our finding that *glnA* of *E. coli* is linked to *metB* and *rha* confirms an earlier observation by E. P. Mayer, O. H. Smith, W. W. Fredricks, and M. A. McKinney (Abstr. Annu. Meet. Amer. Soc. Microbiol. 1973, G8, p. 27).

The examination of the fine structure of the *glnA* site suggests that mutations in the *rha*-distal and *rha*-proximal segments lead to different phenotypes. Three mutations in the *rha*-distal end, *glnA5*, *glnA6*, and *glnA20*, result in the failure to produce any material antigenically related to glutamine synthetase, or in production of only a small amount of such material. Strains carrying these mutations have Cn^s character, that is, they fail to produce the agent required for the activation of histidase synthesis, even when starved for a source of nitrogen. Mutants in the *rha*-proximal segment of *glnA* all have the Cn^R character, that is, they produce the agent required for the activation of histidase synthesis even during growth in ammonia excess. The mutations in this segment of *glnA* site may or may not result in the failure to produce enzymatically active glutamine synthetase. Of the four mutations we have studied, two, *glnA4* and *glnA29*, result in the production of active enzyme at a high level, even in the presence of an excess of ammonia. One mutation, *glnA51*, results in the production of enzymatically inactive glutamine synthetase antigen at a high level in the presence or absence of an excess of ammonia (5). Finally, in the strain carrying the *glnA10* mutation, neither active enzyme nor antigen could be detected (5).

It is of particular interest that the mutation *glnA29*, affecting the control of formation of glutamine synthetase, but not its enzymatic activity, appears to be located between two mutations, *glnA20* and *glnA51*, that have resulted in a loss of enzymatic activity. This finding is most easily explained by the assumption that glutamine synthetase controls the expression of its own structural gene by repression.

According to this hypothesis, glutamine synthetase is converted in cells growing with an excess of ammonia to a molecular form capable of repressing the synthesis of glutamine synthetase. Mutations in the structural gene *glnA* could result in the formation of glutamine synthetase molecules with normal enzymatic activity but unable to be converted to the repressive configuration; other mutations could result in the formation of molecules devoid of enzymatic activity as well as of the ability to assume the repressive conformation. These altered glutamine synthetase molecules would retain their ability to activate the synthesis of histidase. Finally, some mutations could cause the formation of a glutamine synthetase molecule permanently locked in the repressive configuration. This is keeping with the observation reported in the preceding paper (5) that the strain carrying the *glnA5* mutation produces the same small amount of enzymatically inactive glutamine synthetase antigen whether or not grown with an excess of ammonia.

We can exclude the possibility that the mutations resulting in the nonrepressed synthesis of glutamine synthetase are in an operator site. We have already discussed the fact that one of these mutations, *glnA29*, is located between two mutations, *glnA5* and *glnA51*, resulting in the formation of enzymatically inactive glutamine synthetase antigen. Moreover, we have found that this mutation is recessive to a wild-type allele; in a merodiploid strain with *glnA29* on the chromosome and the normal *glnA* gene of *E. coli* carried on an F′ episome, the synthesis of

glutamine synthetase is repressed by an excess of ammonia (S. L. Streicher, A. B. DeLeo, and B. Magasanik, unpublished data).

We cannot at present state categorically that all the mutations in the *glnA* site we have studied are in the structural gene for glutamine synthetase. Although we favor this view, we consider it possible that only the *rha*-distal end of the *glnA* gene, where mutations *glnA5*, *glnA6*, and *glnA20* are located, specifies the amino acid sequence of the enzyme. The *rha*-proximal segment may be the structural gene for an enzyme capable of modifying the subunit of glutamine synthetase to endow it with enzymatic activity. This modifying enzyme or the modified glutamine synthetase may be the repressor. In that case, it may be that cells with mutations affecting this hypothetical modifying enzyme might produce active or inactive glutamine synthetase not subject to repression.

The results of the experiments described in this paper shed a little light on the function of the product of the *glnB* gene. The fact that a merodiploid strain with a *glnB* mutation on the chromosome and a normal *E. coli glnB* gene on the F′ episome does not require glutamine indicates that $glnB^+$ is dominant. This fact suggests that the *glnB* product is required for the normal expression of the *glnA* gene. Mutations in the *glnA4* site (8) or the *glnA29* site (S. L. Streicher and B. Magasanik, unpublished data) abolish the dependence of glutamine synthetase formation on the product of the *glnB* gene. In the strains carrying the *glnA4* or *glnA29* mutations, glutamine synthetase formation is not subject to control by ammonia, irrespective of the presence or absence of functional *glnB* product. These results are best accommodated by the assumption that the product of the *glnB* gene prevents excessive repression of glutamine synthetase by glutamine synthetase.

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