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Glutamine Auxotrophs with Mutations in a Nitrogen Regulatory Gene, *ntrC*, that is Near *glnA*

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Summary. Some mutations to glutamine auxotrophy in the 86 unit region of the *Salmonella* chromosome lie within the *nit*rogen *r*egulatory gene, *ntrC*, rather than the structural gene encoding glutamine synthetase, *glnA*. Assignment of mutations to *ntrC* is based on fine structure mapping by P22-mediated transduction and on complementation analysis. Strains with *ntrC* lesions that cause glutamine auxotrophy ($NtrC^{repressor}$) have very low levels of glutamine synthetase (lower than those of strains that completely lack *ntrC* function and comparable to those of strains that lack *ntrA* function). $NtrC^{rep}$ strains fail to increase synthesis of glutamine synthetase or several amino acid transport components under nitrogen limiting conditions. Thus, like *ntrA* strains, they appear to repress *glnA* transcription and fail to activate transcription of *glnA* or other nitrogen controlled genes. Mutations that suppress the glutamine requirement caused by $NtrC^{rep}$ lesions arise at high frequency; these mutations also suppress the glutamine requirement caused by *ntrA* lesions. Several suppressor mutations result in loss of function of *ntrC*.

Introduction

In *Salmonella typhimurium* synthesis of several proteins including glutamine synthetase [L-glutamate: NH_3 ligase (ADP-forming), EC6.3.1.2] and four periplasmic amino acid binding proteins is controlled by availability of nitrogen in the growth medium; synthesis of these proteins is increased under nitrogen-limiting conditions (Brenchley et al. 1975; Kustu et al. 1979b). This response appears to be mediated by products of nitrogen regulatory genes *ntrA* (previously called *glnF*), which lies at 68 units on the chromosomal map, and *ntrB* and *ntrC* (previously called "*glnR*"), which lie at 86 units and are closely linked to the structural gene encoding glutamine synthetase, *glnA* (Garcia et al. 1977; Kustu et al. 1979a; McFarland et al. 1981). A working model for function of the *ntr* gene products in nitrogen control is presented in Fig. 1 (McFarland et al. 1981). The *ntrB* and *ntrC* products can repress transcription of *glnA*, or activate transcription of this and other genes under nitrogen control[1]. Activation requires a functional *ntrA* product. Both the *ntrB* and *ntrC* products are required for repression of *glnA* expression.

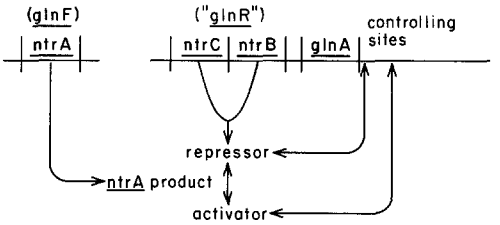

Fig. 1. Working model for control of *glnA* expression by the *ntrA* (previously called *glnF*) and *ntrB* and *ntrC* (previously called "*glnR*") products. The *ntrB* and *ntrC* products can repress transcription of *glnA* or activate transcription of *glnA* (and other genes under nitrogen control). The *ntrA* product leads to formation of activator. The model accommodates the possibility that the *ntrB* and *ntrC* products may work as a protein complex. In strains with an $NtrC^{rep}$ mutation transition from repressor to activator apparently does not occur even in the presence of a functional *ntrA* product. (This transition also fails to occur in *ntrA* strains)

The *ntrC* product can apparently activate *glnA* expression without need for the *ntrB* product. Mutations to loss of function of *ntrC* result in failure to activate or repress *glnA* expression, and thus in a low level of synthesis of glutamine synthetase[2], and in failure to activate expression of other nitrogen controlled genes (Kustu et al. 1979a; McFarland et al. 1981). We now describe mutations in *ntrC* [$NtrC^{rep(ressor)}$] which result in failure to activate expression of nitrogen-controlled genes and in permanent repression of *glnA* expression. In contrast to strains that lack *ntrC* function, which are glutamine independent, $NtrC^{rep}$ strains are glutamine auxotrophs. Their properties are very similar to those of *ntrA* strains (Garcia et al. 1977; Kustu et al. 1979a) (see Fig. 1). We compare the properties of $NtrC^{rep}$ strains with those of glutamine auxotrophs that have lesions in *glnA*.

Materials and Methods

Isolation of Strains. Strains were derived from *Salmonella typhimurium* (LT2) strain TA831 (*ΔhisF645*) and are listed in Table 1. Glutamine auxotrophs were selected in two ways: 1) by penicillin enrichment following diethylsulfate mutagenesis 2) by positive selection for spontaneous D-histidine utilizers (ability to utilize D-histidine to satisfy histidine auxotrophy) as described previously (Kustu and McKereghan 1975). The second selection was also modified by including low concentrations of glutamine or glycyclglutamine (0.2 mM) in the plates to

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1 Effects of *ntr* lesions on expression of β-galactosidase in *glnA*::Mud1 [Ap^R, *lac*, cts62] fusions indicate that *ntr* products act at the level of transcription (Rothstein et al. 1980; K. Krajewska-Grynkiewicz and S. Kustu, manuscript in preparation)

2 Residual expression of *glnA* in *ntrC* strains may be controlled by a regulatory mechanism other than the *ntr* mechanism or may be due to unregulated transcription by RNA polymerase alone

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Table 1. Bacterial strains

Strain	Genotype	Mutagen	Phenotype	Apparent[a] proportion of glutamine independent derivatives
Strains constructed for this work				
TA831[b]	*ΔhisF645*	–	Gln^+ Aut^{+c} Dhu^{-d}	
SK95	*glnA71 hisF645*	diethylsulfate	Gln^- Aut^- Dhu^+	$\sim 10^{-8}$
SK96	*ntrC72 hisF645*	diethylsulfate	Gln^- (leaky) Aut^- Dhu^- ($NtrC^{rep}$)	$\sim 10^{-5}$
SK97	*ntrC73 hisF645*	diethylsulfate	Gln^- (leaky) Aut^- Dhu^- ($NtrC^{rep}$)	$\sim 10^{-5}$
SK98	*ntrC74 hisF645*	diethylsulfate	Gln^- (leaky) Aut^- Dhu^- ($NtrC^{rep}$)	$\sim 10^{-5}$
SK105	*ntrC90 hisF645*	diethylsulfate	Gln^- (leaky) Aut^- Dhu^- ($NtrC^{rep}$)	$\sim 10^{-5}$
SK107	*gln-91 hisF645*	diethylsulfate	Gln^- Aut^- Dhu^-	$< 10^{-8}$
SK109	*ntrC94 hisF645*	diethylsulfate	Gln^{+e}	
SK110	*ntrC95 hisF645*	diethylsulfate	Gln^- (leaky) Aut^- Dhu^- ($NtrC^{rep}$)	$\sim 10^{-5}$
SK429	*glnA188 hisF645*	spontaneous	Gln^- Aut^- Dhu^+	$\sim 10^{-7}$
SK454	*glnA192 hisF645*	spontaneous	Gln^- Aut^- Dhu^+	$\sim 10^{-7}$
SK459	*glnA197 hisF645*	spontaneous	Gln^- (leaky) Aut^- Dhu^+	$\sim 10^{-5}$
SK481	*glnA195 hisF645*	spontaneous	Gln^- (slightly leaky) Aut^- Dhu^+	$\sim 10^{-6}$
SK482	*glnA196 hisF645*	spontaneous	Gln^- (slightly leaky) Aut^- Dhu^+	$\sim 10^{-7}$
SK862	*ΔhisF645*	diethylsulfate	Gln^+ Aut^+ Dhu^- (revertant of SK97)	
SK874	*ntrC73 ntrC8 hisF645*	spontaneous (from SK97)	Gln^{+e} Aut^- Dhu^-	
SK879	*ntrC73 ntrC13 hisF645*	spontaneous (from SK97)	Gln^{+e} Aut^- Dhu^-	
SK883	*ntrC73 ntrB?17 hisF645*	spontaneous (from SK97)	Gln^{+e} Aut^- Dhu^-	
SK940	*ntrC73 galE542*[f] *metB869*::Tn10			
SK942	*ntrC73 ntrC8 galE542*[f] *metB869*::Tn10			
SK943	*ntrC73 ntrC13 galE542*[f] *metB869*::Tn10			
SK944	*ntrC74 ntrC28 hisF645*	spontaneous (from SK98)	Gln^{+e} Aut^- Dhu^-	
SK948	*ntrC74 ntrC29 hisF645*	spontaneous (from SK98)	Gln^{+e} Aut^- Dhu^-	
SK961	*ntrC74 galE542*[f] *metB869*::Tn10			
SK962	*ntrC74 ntrC28 galE542*[f] *metB869*::Tn10			
SK963	*ntrC74 ntrC29 galE542*[f] *metB869*::Tn10			
SK964	*ntrB243 galE1794*[g] *metB869*::Tn10			
SK965	*ntrB285 galE1794*[g] *metB869*::Tn10			
SK967	*ntrC302 galE1794*[g] *metB869*::Tn10			
Other strains				
SK35	*Δ(glnA–ntrB)60*[h] *hisF645*			
SK70	*rha182 galE1792*			
SK72	*Δ(glnA–ntrB)60 hisF645 galE1794*			
SK176	*Δ(glnA–ntrB)60 ntrA75 hisF645*			
SK574	*Δ(glnA–ntrB)60 hisF645 galE1794*/ F′100–12 $\lambda glnA_2$[i]			
SK576	*Δ(glnA–ntrB)60 hisF645 galE1794*/ F′100–12 $\lambda glnA_6$[i]			
SK611	*ntrB243 hisF645*		Gln^{+e} Aut^- Dhu^-	
SK615	*ntrC247 hisF645*		Gln^{+e} Aut^- Dhu^-	
SK622	*ntrB285 hisF645*		Gln^{+e} Aut^- Dhu^-	
SK628	*ntrC291 hisF645*		Gln^{+e} Aut^- Dhu^-	
SK653	*ntrB316 hisF645*		Gln^{+e} Aut^- Dhu^-	
SK659	*ntrC322 hisF645*		Gln^{+e} Aut^- Dhu^-	
SK691	*glnA192 galE1824*			
SK694	*glnA188 galE1825*			
SK763	*Δ(glnA–ntrB)60 hisF645 galE1794*/ F′100–12 $\lambda glnA_6$ *ntrB1*[i]			
SK767	*Δ(glnA–ntrB)60 hisF645 galE1794*/ F′100–12 $\lambda glnA_6$ *ntrC4*[i]			
SK835	*ntrC352*::Tn10 *hisF645*		Gln^{+e} Aut^- Dhu^-	
SK925	*rha182 galE1792 zig-206*::Tn10			
SK927	*glnA188 galE1825 zig-206*::Tn10			

Strain	Genotype	Mutagen	Phenotype	Apparent[a] proportion of glutamine independent derivatives
SK968	*Δ(glnA−ntrB)60 galE1794 metB869*::Tn10/ F′100–12 $\lambda glnA_2$[i]			
SK969	*Δ(glnA−ntrB)60 galE1794 metB869*::Tn10/ F′100–12 $\lambda glnA_6$[i]			
SK972	*glnA71 hisF645 galE1829 zig-206*::Tn10			

[a] Number of Gln^+ colonies (revertants or suppressors) when plates were seeded with 10^8 cells. Due to growth of leaky mutant strains this could be an overestimate of the proportion of Gln^+ derivatives by a factor of up to 10-fold

[b] Parent

[c] Aut = arginine utilization. Ability to use arginine as sole nitrogen source. Glucose was the carbon source

[d] Dhu = D-histidine utilization. Ability to use D-hisitidine to satisfy histidine auxotrophy

[e] Doubling time on minimal medium is ~25% longer than that of wild-type strain TA831

[f] The *galE542* mutation was introduced as follows: recipient strains were first transduced to tetracycline resistance with phage grown on a strain carrying *bio203*::Tn10. The resulting strains were transduced to Bio^+ with phage grown on a strain carrying the *gal* mutation and transductants were tested for Gal^-. Linkage of *galE* to *bio203*::Tn10 is ~15%

[g] *ntrB* or *ntrC* mutations were introduced into strain SK749 [*Δ(glnA−ntrB)60 galE1794 hisF645*] by selecting Gln^+ transductants that had nitrogen regulatory defects of the *ntrB* or *ntrC* donor

[h] *Δ(glnA−ntrB)60* fails to recombine with *ntrB* point mutations including *ntrB243, ntrB285* and *ntrB316; ntrB243* and *ntrB285* recombine with *ntrB316*. The deletion does recombine with all *ntrC* point mutations that we have tested including *ntrC247, ntrC291, ntrC322,* and *ntrC352*::Tn10 and therefore may or may not extend into *ntrC*. The deletion is phenotypically $NtrC^-$ (McFarland et al. 1981)

[i] These strains also have λ helper phage on F′100–12. Both transducing phage and helper carry the cI857 and S7 mutations

allow isolation of strains with an absolute glutamine requirement. [High concentrations of glutamine inhibit D-histidine utilization by *glnA* strains (Kustu and McKereghan 1975)]. The *glnA* strains SK481 and SK482 were isolated from plates containing glutamine, strain SK459 from a plate containing glycyclglutamine, and strains SK429 and SK454 from a plate without glutamine.

Glutamine independent derivatives (revertants or suppressors) were isolated from glutamine auxotrophs with lesions in the *glnA* region. Fifty five spontaneous derivatives (from four independent cultures) and 40 diethylsulfate induced derivatives (from a single culture) were isolated from $NtrC^{rep}$ strains SK96, SK97, and SK98. These derivatives were tested for growth on arginine as nitrogen source, a nitrogen-controlled phenotype (Kustu et al. 1979a; Kustu et al. 1979b; McFarland et al. 1981). They were also tested for the presence of *glnA*-linked mutations that suppressed the glutamine requirement caused by an *ntrA* lesion as follows: P22 phage grown on strains carrying such suppressor mutations could be used to transduce strain SK176 [*Δ(glnA−ntrB)60 ntrA75*] to glutamine independence whereas phage grown on strains TA831, SK96, SK97, SK98 or $NtrC^+$ revertants of the latter three strains yielded no transductants.

Genetic Analysis. Recombinational analyses were done by P22-mediated transduction using phage P22 HT*int201* (Schmieger 1971), except as noted. Phage were cycled twice on the donor strain. Strains with lesions in the *glnA* region were identified as those that gave few glutamine prototrophic recombinants (<10% as many as the parent strain TA831) when used as donors in crosses with strain SK35 [*Δ(glnA−ntrB)60*]. Lesions in these strains were ordered with respect to other lesions in the *glnA* region [*Δ(glnA−ntrB)60, ntrB243, ntrB285,* and *ntrC247*] by reciprocal three-factor crosses. Lesions to loss of function of *ntrB* and *ntrC* were also ordered with respect to *glnA* and each other. In addition to *glnA* or *ntr* lesions donors for the crosses carried the *zig-206*::Tn10 mutation, which is approximately 40% linked to *glnA* by P22-mediated transduction. Selection was for ability to utilize arginine as nitrogen source (Aut^+), except as noted; Aut^+ transductants were scored for inheritance of tetracycline resistance, which is encoded by Tn10. [All Gln^- strains (including $NtrC^{rep}$ and *glnA* strains) are Aut^- because tests for utilization of arginine as nitrogen source are done in the absence of glutamine. Glutamine is not supplied because it serves as nitrogen source at the high concentrations (3 to 5 mM) required to satisfy auxotrophy. Strains with mutations to loss of function of *ntrB* or *ntrC* are Aut^- but Gln^+ (McFarland et al. 1981).] The *zig206*::Tn10 lesion was ordered with respect to *glnA* and *rha* by reciprocal three-factor crosses using P1-mediated transduction. Phage was P1*cml clr100*. Phage was grown and transductions were performed according to Miller (1972).

Complementation analyses were performed with the following *λglnA* transducing phages carrying the *E. coli glnA* region: 1) $\lambda glnA_2$ (*glnA*$^+$ *ntrB*$^-$ *ntrC*$^-$), which carries the *glnA* gene but not the adjacent *ntrB* or *ntrC* genes (McFarland et al. 1981), 2) $\lambda glnA_6$ (*glnA*$^+$ *ntrB*$^+$ *ntrC*$^+$), which carries the entire region 3) derivatives of $\lambda glnA_6$ (*ntrB*$^-$ or *ntrC*$^-$) with frameshift lesions in *ntrB* or *ntrC* (McFarland et al. 1981). These phages, lysogenized on an *E. coli* F′*gal* episome (F′100-12), were transferred to $NtrC^{rep}$, *glnA* and other recipient strains (Gal^-) by selecting Gal^+ transconjugants. Transconjugants were tested for glutamine independence and ability to utilize arginine as nitrogen source. In all cases segregation of episomes carrying transducing phages was demonstrated. $\lambda glnA_2$ (*ntrB*$^-$ *ntrC*$^-$) and derivatives of $\lambda glnA_6$ (*ntrB*$^-$ or *ntrC*$^-$) were transferred to SK72, which carries *Δ (glnA−ntrB)60* and to known *ntrB* and *ntrC* recipients (SK964 and SK967, respectively) and shown to confer the appropriate phenotype: all phages complement SK72 to Gln^+ but not to Aut^+ (McFarland et al. 1981); $\lambda glnA_6$ *ntrB1* complements *Salmonella ntrC* strains to Aut^+ and fails to complement *Salmonella ntrB* strains to Aut^+; $\lambda glnA_6$ *ntrC4* fails to complement either *Salmonella ntrB* or *ntrC* strains to Aut^+[3] (L.L. McCarter and M.K. McKinley, unpublished results).

Glutamine Synthetase and Binding Protein Assays. Glutamine synthetase activity was measured using the reverse γ-glutamyl transfer assay of Stadtman et al. (1970) with the modifications described (Kustu and

3 Since $\lambda glnA_6$ *ntrC4* complements *E. coli ntrB* lesions, failure to complement *Salmonella ntrB* lesions may be due to failure of the *E. coli ntrB* product to function with the *Salmonella ntrC* product. (See Discussion with regard to the possibility that the *ntrB* and *ntrC* products function as a protein complex). We have used $\lambda glnA_6$ *ntrC4* to exclude the possibility that *Salmonella* lesions which were complemented by λ $glnA_6$ *ntrB1* were in *glnA*, in which case they would also have been completed by λ $glnA_6$ *ntrC4*

McKereghan 1975; Bancroft et al. 1978). Glutamine synthetase activities were corrected for "blanks" in the absence of ADP and arsenate (Miller et al. 1974). Activities of the periplasmic glutamine and lysine/arginine/ornithine (LAO) binding proteins and amounts of binding protein and glutamine synthetase antigens were measured as described (Kustu et al. 1979b). Protein was determined by the method of Lowry et al. (1951) with bovine serum albumin as standard.

Polyacrylamide Gel Electrophoresis in the Presence of Sodium Dodecylsulfate (SDS-PAGE). SDS-PAGE was performed as described previously (Kustu et al. 1979b) at an acrylamide concentration of 9%.

Chemicals. L-[3,4-^{3}H]glutamine (42.5–47 Ci/m mol) and L-[2,3-^{3}H] arginine (15–28.2 Ci/m mol) were obtained from New England Nuclear Corp. Glycycl-L-Glutamine monohydrate was obtained from Vega-Fox Biochemicals, Tucson, Arizona.

Results

Isolation of Strains. Of eleven glutamine requiring strains isolated after mutagenesis with diethylsulfate and penicillin counter-selection, eight were found to have lesions linked to *glnA* (at 86U) by P22-mediated transduction as described in Methods. [Two had lesions in *ntrA* at 68U (Garcia et al. 1977) and one had a lesion in *glnD* at 7U (Bancroft et al. 1978).] We have studied six glutamine auxotrophs with lesions linked to *glnA* (SK95, SK96, SK97, SK98, SK105, SK110). [One glutamine auxotroph, strain SK107, has not been characterized. Another strain, SK109, appeared to have a lesion to loss of function of *ntrC* (Kustu et al. 1979a; McFarland et al. 1981).] All five glutamine auxotrophs isolated as spontaneous D-histidine utilizers (strains SK429, SK454, SK459, SK481, SK482) had lesions closely linked to *glnA*. [Selection for D-histidine utilization had been used previously to isolate strains with lesions demonstrated to lie within *glnA* (Kustu and McKereghan 1975); these strains produced glutamine synthetase with altered catalytic and physical properties or they produced glutamine synthetase antigen that was catalytically inactive.]

In preliminary tests glutamine auxotrophs with lesions linked to *glnA* could be divided into two groups on the basis of leakiness of the glutamine requirement, reversion of the glutamine requirement, and ability to utilize D-histidine. Several strains isolated by penicillin enrichment (NtrC^{rep} strains: SK96, SK97, SK98, SK105, SK110) had a "leaky" glutamine requirement (i.e. they grew slowly on plates in the absence of glutamine) and high apparent reversion rates (Table 1). However, a large number of glutamine-independent derivatives of NtrC^{rep} strains (88/95 for SK96, 88/95 for SK97, 95/95 for SK98) failed to utilize arginine as nitrogen source, unlike the wild-type, suggesting that these derivatives were not true revertants but contained suppressors of the original NtrC^{rep} lesion. NtrC^{rep} strains failed to utilize D-histidine. The degree of leakiness of the glutamine requirement of the remaining strains (*glnA*: SK95, SK429, SK454, SK459,

Table 2. Glutamine synthetase and binding protein activities of NtrC^{rep} and *glnA* strains

Strain	Glutamine synthetase μmol/min-mg		Glutamine-binding protein pmol/mg dry weight cells		LAO-binding protein[b] pmol/mg dry weight cells	
	N excess[a]	N limiting[a]	N excess	N limiting	N excess	N limiting
TA831 (parent)	0.14	1.4	3.5	10.3	1.4	18.2
SK96 (NtrC^{rep})	<0.01	<0.01	1.7	2.1	1.3[c]	0.1
SK97 (NtrC^{rep})	<0.01	<0.01	1.8	2.6	2.0[c]	0
SK98 (NtrC^{rep})	<0.01	0.01	0.3[d]	0.5[d]	0.6	0
SK105 (NtrC^{rep})	<.01	<0.01	0.3[d]	0.4[d]	0.4	0.2
SK110 (NtrC^{rep})	<0.01	<0.01	0.1[d]	0.8[d]	N.D.[e]	N.D.
SK862 (revertant of SK97)	0.14	1.1	N.D.	N.D.	N.D.	N.D.
SK95 (*glnA*)	0.27[f]	0.61	19.6	12.9	11.5	17.9
SK429 (*glnA*)	<0.01[g]	<0.01	17.4	15.2	12.2	12.2
SK454 (*glnA*)	0.01[g]	<0.01	17.7	19.8	14.4[c]	35.3[c]
SK459 (*glnA*)	0.33[f]	0.34	N.D.	N.D.	N.D.	N.D.
SK481 (*glnA*)	0.13[f]	0.27	N.D.	N.D.	N.D.	N.D.
SK482 (*glnA*)	0.01[g]	0.02	N.D.	N.D.	N.D.	N.D.
SK835 (*ntrC*::Tn10)	0.08	0.05	N.D.	N.D.	N.D.	N.D.
SK874 (NtrC^{rep} *ntrC*)	0.05	0.05	N.D.	N.D.	N.D.	N.D.
SK879 (NtrC^{rep} *ntrC*)	0.06	0.08	N.D.	N.D.	N.D.	N.D.
SK883 (NtrC^{rep} *ntrB*?)	0.02	0.06	N.D.	N.D.	N.D.	N.D.

[a] N excess: Minimal glucose medium with 20 mM NH_4^+ and 3 mM glutamine as nitrogen sources. N limiting: 3 mM glutamine as sole nitrogen source. Both media were supplemented with histidine and with 0.2 mM arginine, uracil, and hypoxanthine (and 2 μM thiamine), major end products that contain a nitrogen atom derived from glutamine, to stimulate the growth rate of mutant strains

[b] Lysine/arginine/ornithine-binding protein, calculated as described (Kustu et al. 1979b). Total arginine binding (by the LAO and other arginine-binding proteins) under N excess and N limiting conditions was: TA831, 16.5 and 32.3; SK96, 29.2 and 10.2; SK97, 24.1 and 10.8; SK98, 12.8 and 7.0; SK105, 4.0 and 4.2; SK95, 27.8 and 27.3; SK429, 27.1 and 28.2; SK454 48.5 and 59.3

[c] In this experiment total arginine binding and LAO-binding by TA831 were 25.2 and 0, respectively, under N excess conditions and 48.4 and 26.3, respectively, under N-limiting conditions

[d] In this experiment glutamine binding by TA831 was 0.8 under N excess and 6.0 under N limiting conditions

[e] N.D., not determined

[f] The reverse γ-glutamyltransfer assay (Stadtman et al. 1970) is a nonphysiological assay which is suitable for measurements of total glutamine synthetase activity and approximate degree of adenylylation in crude extracts. However, activity for strains with mutationally altered glutamine synthetase does not necessarily correlate with biosynthetic activity (see Results) or leakiness of the glutamine requirement in growth tests (Table 1)

[g] Strains SK429 and SK482 produced glutamine synthetase antigen whereas strain SK454 had no detectable antigen

SK481, SK482) was allele-specific and these strains generally had lower reversion rates than NtrC^{rep} strains (Table 1). The mutants in this group, including strain SK95, were able to utilize D-histidine.

Levels of Nitrogen Controlled Proteins – Glutamine Synthetase. NtrC^{rep} strains had very low levels of glutamine synthetase activity and antigen (< 5% the maximal amount of antigen in wild-type) under nitrogen limiting as well as nitrogen excess growth conditions (Table 2). Strain SK862, a revertant of NtrC^{rep} strain SK97, had normal levels of glutamine synthetase (Table 2). Three *glnA* strains (SK429, SK454, SK482) also had very low glutamine synthetase activity; two of these (SK429, SK482) had detectable glutamine synthetase antigen whereas one (SK454) had not detectable antigen (< 5% the maximal amount of wild-type). Thus, levels of glutamine synthetase antigen in these strains were allele-specific. Three other *glnA* strains (SK95, SK459, SK481) had detectable glutamine synthetase activity (in the reverse γ-glutamyl transfer assay). Glutamine synthetase activity in these strains, like that in several previously characterized *glnA* strains (Kustu and McKereghan 1975), showed an altered response to divalent cations: activity was strongly inhibited by Mg^{++} even when adenylyl groups were removed from the enzyme by treatment with snake venom phosphodiesterase (Kustu and McKereghan 1975) (data not shown). Glutamine synthetase from strain SK95 had no biosynthetic activity (K. Krajewska-Grynkiewicz, unpublished results) in a Mg^{++}-dependent biosynthetic assay (Elliott 1955) and presumably lacked activity in vivo since SK95 is a tight glutamine auxotroph. The presence of altered glutamine synthetase activity or inactive antigen in *glnA* strains provided evidence that they carried lesions within the structural gene for glutamine synthetase. (Of the *glnA* strains, only strain SK454 lacked both activity and antigen.)

Levels of Nitrogen Controlled Proteins – Amino Acid Binding Proteins. NtrC^{rep} strains had low levels of the periplasmic glutamine and lysine/arginine/ornithine-binding proteins under nitrogen limiting as well as nitrogen excess growth conditions, whereas *glnA* strains had high levels of these proteins (Table 2). Effects of NtrC^{rep} and *glnA* mutations on expression of nitrogen controlled binding proteins are shown visually in Fig. 2. Periplasmic fractions of NtrC^{rep} strains SK96 and SK98 prepared from cells grown with limiting nitrogen lacked four protein bands with mobilities corresponding to those of binding proteins for glutamate, histidine, lysine/arginine/ornithine and glutamine (in order of decreasing molecular weight). These bands were present in periplasmic fractions of *glnA* strain SK95 and the parent strain TA831.

Fine-Structure Mapping. Reciprocal three-factor crosses by transduction yielded the following results: 1) NtrC^{rep} lesions and *glnA* lesions are located on opposite sides of *Δ(glnA−ntrB)60* (Table 3, lines 1–9). 2) NtrC^{rep} and *glnA* lesions are located on opposite sides of *ntrB* (Table 3, lines 10–14 and Table 4). 3) *ntrB*, *ntrC* and NtrC^{rep} lesions are located on the same side of *glnA* (Table 3, lines 1–5, lines 11–20) and *ntrB* lesions are located between *glnA* and *ntrC* [Table 3, line 21; also, *ntrC* mutations including *ntrC247*, *ntrC291*, *ntrC322* and *ntrC352*::Tn10 recombine with *Δ(glnA−ntrB)60*]. 4) NtrC^{rep} lesions are located between *ntrB* lesions and *ntrC247* (Table 3, lines 1–5, 10, 22–24 and Table 4). 5) The *zig206*::Tn10 lesion, which was used to order other markers, is on the *rha* proximal side of *glnA* (clockwise of *glnA*) (Table 5). Based on results 1) and 2) NtrC^{rep} lesions and *glnA* lesions must lie in different genes. Based on results

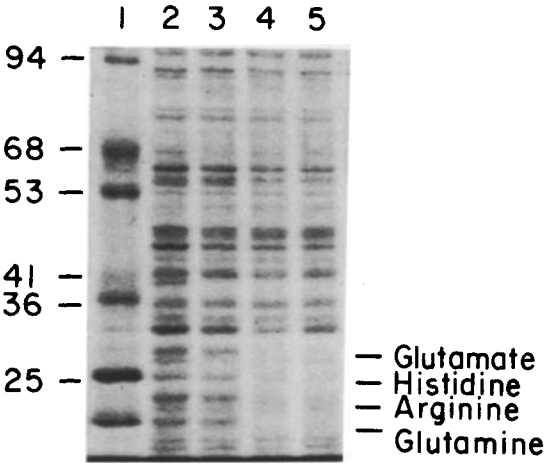

Fig. 2. Polyacrylamide gel electrophoresis of periplasmic fractions from NtrC^{rep} and *glnA* strains. Electrophoresis of ~2 μg protein in each case was performed in the presence of sodium dodecylsulfate. Periplasmic fractions were prepared from cells grown with limiting nitrogen (3 mM glutamine as sole nitrogen source). Strains were TA831 (parent) in well 2; SK95 (*glnA*) in well 3; SK96 (NtrC^{rep}) in well 4 and SK98 (NtrC^{rep}) in well 5. Well 1 contained the following molecular weight standards (the numbers in parentheses are the respective subunit molecular weights as in Ames and Nikaido 1976): phosphorylase b (94,000), bovine serum albumin (68,000), beef liver catalase (60,000), bovine liver glutamic dehydrogenase (53,000), horse liver alcohol dehydrogenase (41,000), rabbit muscle glyceraldehyde 3-phosphate dehydrogenase (36,000), *Salmonella* histidine-binding protein J (25,000), and *Salmonella* glutamine-binding protein. Since there were no protein bands of molecular weight higher than phosphorylase b, the top portion of the gel is not included in the figure. Positions of binding proteins for glutamate (Kustu et al. 1979b), histidine [J protein (Lever 1972)], arginine [lysine/arginine/ornithine (Kustu et al. 1979b)] and glutamine (Kustu et al. 1979b) are indicated by arrows at the right

3) and 4), ntrC^{rep} lesions lie in *ntrB* or *ntrC*. Based on results 1)–5), the order of markers in the *glnA* region is

$$(polA)\ldots ntrC247\ \text{NtrC}^{rep}\quad \frac{\Delta(ntrB-glnA)60}{(ntrB243,\ 285)}\quad \begin{pmatrix} glnA71,\ 188 \\ 192,\ 195, \\ 196,\ 197 \end{pmatrix}\ldots zig206::\text{Tn10}\ldots rha$$

Complementation Analysis. Strains SK940 and SK961, which carry NtrC^{rep} mutations isolated in SK97 and SK98, respectively, were complemented to (glutamine independence and) growth on arginine as nitrogen source by *λglnA$_6$ ntrB1* (*glnA$^+$ ntrB$^-$ ntrC$^+$*) and were not complemented by *λglnA$_6$ ntrC4* (*glnA$^+$ ntrB$^+$ ntrC$^-$*). These results indicated that Ntrc^{rep} lesions do not lie in *ntrB* or *glnA* and, together with results of three-factor crosses, suggested that NtrC^{rep} lesions lie in *ntrC*.

Strains SK972 and SK691, which carry the *glnA* mutations isolated in SK95 and SK454, respectively, were complemented to (glutamine independence and) growth on arginine as nitrogen source by *λglnA$_2$* (*glnA$^+$ ntrB$^-$ ntrC$^-$*). Glutamine synthetase levels in a merodiploid derived from strain SK691 were subject to nitrogen regulation (McFarland et al. 1981). These results indicated that the *ntrB* and *ntrC* genes in SK691 (which lacks detectable glutamine synthetase antigen) were intact and could mediate nitrogen regulation of both *glnA* expression and expression of genes required for growth on arginine.

Table 3. Ordering of NtrC^{rep} and *glnA* lesions with respect to *ntrB* lesions and *ntrC247*

Recipient	Donor phage[a]	% Tet^r [b]	Recipient	Donor phage[a]	% Tet^r [b]
1. SK35 [*Δ(glnA−ntrB)60*]	SK96 (NtrC^{rep})	− (0/4)[c]	SK96	SK35	0 (0/50)
2. SK35	SK97 (NtrC^{rep})	44 (16/36)	SK97	SK35	0 (0/50)
3. SK35	SK98 (NtrC^{rep})	32 (10/31)	SK98	SK35	0 (0/27)
4. SK35	SK105 (NtrC^{rep})	30 (15/50)	SK105	SK35	0 (0/100)
5. SK35	SK110 (NtrC^{rep})	N.D.[d]	SK110	SK35	0 (0/50)
6. SK35	SK95 (*glnA*)	0 (0/24)	SK95	SK35	28 (11/39)
7. SK35	SK454 (*glnA*)	6 (3/50)	SK454	SK35	34 (17/50)
8. SK35	SK459 (*glnA*)	4 (2/45)	SK459	SK35	32 (8/25)
9. SK35	SK481 (*glnA*)	2 (2/100)	SK481	SK35	40 (20/50)
10. SK611 (*ntrB243*)	SK97 (NtrC^{rep})	32 (16/50)	SK97	SK611	8 (4/50)
11. SK611 (*ntrB243*)	SK454 (*glnA*)	4 (2/50)	SK454	SK611	46 (27/50)
12. SK653 (*ntrB316*)	SK454 (*glnA*)	17.5 (7/40)	SK454	SK653	30 (15/50)
13. SK611 (*ntrB243*)	SK429 (*glnA*)	0 (0/50)	SK429	SK611	18 (3/17)
14. SK653 (*ntrB316*)	SK429 (*glnA*)	0 (0/30)	SK429	SK653	18 (3/17)
15. SK615 (*ntrC247*)	SK454 (*glnA*)	6 (3/50)	SK454	SK615	58 (29/50)
16. SK628 (*ntrC291*)	SK454 (*glnA*)	6 (3/50)	SK454	SK628	64 (32/50)
17. SK659 (*ntrC322*)	SK454 (*glnA*)	12 (4/33)	SK454	SK659	38 (19/50)
18. SK615 (*ntrC247*)	SK429 (*glnA*)	0 (0/31)	SK429	SK615	42 (21/50)
19. SK628 (*ntrC291*)	SK429 (*glnA*)	N.D.[d]	SK429	SK628	46 (23/50)
20. SK659 (*ntrC322*)	SK429 (*glnA*)	0 (0/50)	SK429	SK659	28 (14/50)
21. SK611 (*ntrB243*)	SK615 (*ntrC247*)	34 (17/50)	SK615	SK611	14 (7/50)
22. SK615 (*ntrC247*)	SK97	4 (4/100)	SK97	SK615	22 (22/100)
23. SK615	SK98	4 (4/100)	SK98	SK615	32 (32/100)
24. SK615	SK105	5 (5/100)	SK105	SK615	18 (18/100)

The order of markers indicated by the crosses is *ntrC247* NtrC^{rep} *Δ (ntrB−glnA)60 glnA ... zig206*::Tn10

[a] Donors were derivatives of the strains listed which carried *zig206*::Tn10 in addition to markers indicated in Table 1

[b] Selection was for Aut$^+$ (utilization of arginine as nitrogen source), which required a crossover between the *glnA* or *ntr* lesion in the donor and that in the recipient. Scoring was for Tet^r (tetracycline resistance) encoded by *zig206*::Tn10 in the donor. *zig206*::Tn10 is located to the right (clockwise) of *glnA* (between *glnA* and *rha*) (Table 5) and is ~40% linked to *glnA* by P22-mediated transduction in two-factor crosses. A 40% frequency of Tet^r is expected in three-factor crosses if the lesion in the donor is to the left of that in the recipient whereas a much lower frequency is expected if the lesion in the donor is to the right of that in the recipient

[c] Few transductants were obtained because strain SK96 is phage resistant

[d] Not determined

Table 4. Ordering of NtrC^{rep} lesions with respect to *ntrB*

Donor[a] / Recipient	SK611 (*ntrB*) SK97 (NtrC^{rep})	SK622 (*ntrB*) SK97 (NtrC^{rep})	SK611 (*ntrB*) SK98 (NtrC^{rep})	SK622 (*ntrB*) SK98 (NtrC^{rep})	SK97 (NtrC^{rep}) SK611 (*ntrB*)
Unselected markers[b]					
ntrB NtrC^{rep}	8% (4/50)	1.8% (1/56)	10% (5/49)	4% (2/48)	4.4% (2/45)
ntrB ntrC$^+$	32% (16/50)	12.5% (7/56)	20% (10/49)	27% (13/48)	46.6% (21/45)
ntrB$^+$ NtrC^{rep}	60% (30/50)	84% (47/56)	67% (33/49)	68% (33/48)	29% (13/45)
ntrB$^+$ *ntrC*$^+$	0% (0/50)	1.8% (1/56)	2% (1/49)	0% (0/48)	20% (9/45)

The order of markers indicated by the crosses is NtrC^{rep} *ntrB zig205*::Tn10

[a] Donors were derivatives of the strains listed which carried *zig205*::Tn10 in addition to markers indicated in Table 1. Selection was for tetracycline resistance encoded by *zig205*::Tn10, which is located to the right (clockwise) of *glnA* (between *glnA* and *rha*) (Table 5). Values are in percent co-inheritance of unselected markers

[b] Phenotypes were: *ntrB* NtrC^{rep}, Gln$^+$ Aut$^-$ Aln$^-$ (unable to utilize alanine as nitrogen source) (L.L. McCarter, unpublished results); *ntrB ntrC*$^+$, Gln$^+$ Aut$^-$ Aln$^+$ (L.L. McCarter, unpublished results); *ntrB*$^+$ NtrC^{rep}, Gln$^-$ Aut$^-$ Aln$^-$; *ntrB*$^+$ *ntrC*$^+$, Gln$^+$ Aut$^+$ Aln$^+$

Suppressors of NtrC^{rep} Mutations. Several glutamine-independent derivatives (13/14 tested) of the NtrC^{rep} strain SK97 were found to have suppressor mutations in the 86U region that suppressed the glutamine requirement caused by an *ntrA* lesion, as well as that caused by the NtrC^{rep} lesion, as described in Materials and Methods. (The remaining strain, SK862, was characterized as an NtrC$^+$ revertant). Glutamine synthetase levels in three suppressor strains, SK874, SK879, and SK883, were similar to those of strains that lack *ntrC* function (Table 2). Mutations in two of these suppressor strains (SK874 and SK879) as well as those in two additional suppressors derived from strain SK98 (SK944 and SK948)[4] were complemented for growth

4 Strains used for complementation (SK942, SK943, SK962 and SK963) carried the original NtrC^{rep} lesions as well as suppressor mutations

Table 5. Ordering of *zig206*::Tn10 with respect to *glnA* and *rha*

Cross	Recipient	Donor phage[a]	Selected marker	Unselected markers	% co-inheritance
1	SK927 (*glnA188 zig206*::Tn10)	SK70 (*rha182*)	Gln^+	Tet^S Rha^+	50.4 (61/121)
				Tet^R Rha^+	41.3 (50/121)
				Tet^S Rha^-	4.9 (6/121)
				Tet^R Rha^-	3.3 (4/121)
2	SK925 (*rha182 zig206*::Tn10)	SK694 (*glnA188*)	Rha^+	Tet^S Gln^+	12.3 (9/80)
				Tet^R Gln^+	78.7 (63/80)
				Tet^S Gln^-	7.5 (6/80)
				Tet^R Gln^-	2.5 (2/80)

The order of markers indicated by the crosses is *glnA zig206*::Tn10 *rha*. (Results of cross 1 are not consistent with the alternate order *glnA rha zig206*::Tn10 and results of cross 3 and of two-factor crosses[a] are not consistent with the alternate order *zig206*::Tn10 *glnA rha*)

[a] Phage was P1 *cml clr100*. In two-factor crosses by P1-mediated transduction *rha* was ~33% linked to *zig206*::Tn10 and ~10% linked to *glnA*. In two factor crosses by P22-mediated transduction *rha* was apparently unlinked to *zig206*::Tn10 or *glnA*. *zig206*::Tn10 was ~40% linked to *glnA* (G.R. Wei, unpublished results)

on arginine as nitrogen source by $\lambda glnA_6$ *ntrB1* and were not complemented by $\lambda glnA_6$ *ntrC4*; glutamine synthetase levels and complementation data suggested that these suppressor mutations, like the original $NtrC^{rep}$ lesions, were in *ntrC* and that they were mutations to loss of function of the *ntrC* product.

Discussion

We have described a new type of allele, $NtrC^{rep}$, of the nitrogen regulatory gene, *ntrC*. $NtrC^{rep}$ alleles, like loss of function alleles [caused by ICR-induced mutations or insertions of the Tn10 transposon (Kustu et al. 1979a; McFarland et al. 1981; J.E. Stern, unpublished results)], result in failure to activate expression of nitrogen-controlled genes, including *glnA*. $NtrC^{rep}$ alleles differ from loss of function alleles in that $NtrC^{rep}$ also results in permanent repression of *glnA* expression, whereas loss of function alleles result in loss of repression (Kustu et al. 1979a; McFarland et al. 1981). Due to repression at *glnA*, the altered allele causes a more extreme phenotype – glutamine auxotrophy – than the loss of function allele, which causes approximately a 25% increase in doubling time in minimal medium (Kustu et al. 1979a). Properties of $NtrC^{rep}$ strains, which are very similar to those of *ntrA* strains (Garcia et al. 1977; Kustu et al. 1979a) (see Fig. 1) provide further evidence that the *ntrC* (and *ntrB*) products have negative as well as positive regulatory character.

We have hypothesized that the *ntrC* and *ntrB* products may function as a protein complex to regulate expression of nitrogen-controlled genes (McFarland et al. 1981) (see Fig. 1). If so, $NtrC^{rep}$ alleles would result in a protein complex that functions as a repressor of transcription at *glnA* and fails to be converted to an activator of transcription by the *ntrA* product. Consistent with the idea that the *ntrC* and *ntrB* products function as a protein complex, the glutamine requirement of $NtrC^{rep}$ strains can be suppressed by a non-polar mutation to loss of function of *ntrB*. (L.L. McCarter and G.R. Wei, unpublished results); this indicates that the *ntrB* product, as well as the altered *ntrC* product, is required for repression of *glnA* expression in $NtrC^{rep}$ strains. It will be of interest to determine whether $NtrC^{rep}$ alleles are dominant to the wild-type allele in homologous *Salmonella* merodiploids since this may provide information as to whether repression of *glnA* transcrption by the *ntrC* and *ntrB* products blocks their function in activation of transcription of this gene.

Although both $NtrC^{rep}$ and *glnA* lesions cause glutamine auxotrophy, the two types of lesions can be clearly distinguished on the basis of fine structure mapping and complementation analysis. Phenotypes caused by the two types of lesions also differ in some respects. The several $NtrC^{rep}$ strains we have studied are leaky glutamine auxotrophs (like *ntrA* strains); all have a high apparent reversion rate due to frequent occurrence of suppressor mutations. By contrast, the degree of leakiness and the reversion rate of *glnA* strains is allele specific. All $NtrC^{rep}$ strains have low levels of glutamine synthetase activity and antigen, which are correlated. Levels of glutamine synthetase activity and antigen in *glnA* strains are not necessarily correlated and are allele specific. $NtrC^{rep}$ strains fail to synthesize several amino acid transport proteins as well as glutamine synthetase, indicating that they are generally unable to activate expression of nitrogen controlled genes. By contrast, *glnA* strains (non-polar) are able to synthesize high levels of transport proteins, indicating that they have intact *ntr* genes and are therefore able to activate expression of nitrogen controlled genes. [*glnA* strains synthesize binding proteins at high levels even when they are grown with excess ammonia; this may be due to the fact that glutamine auxotrophs are "nitrogen starved" internally because they fail to transport glutamine sufficiently rapidly (Bender and Magasanik 1977a; Garcia et al. 1977). High synthesis of the histidine binding protein and other components of the high affinity histidine transport system may account for ability of *glnA* strains to utilize D-histidine; amounts of these transport components are known to be rate-limiting for growth on D-histidine (Ames and Lever 1970; Krajewska-Grynkiewicz et al. 1971) and to be subject to nitrogen control (Kustu et al. 1979b).]

Several years ago Magasanik and his colleagues postulated that glutamine synthetase was a central genetic regulatory element for nitrogen control – specifically that it directly controlled transcription of nitrogen regulated genes (reviewed in Magasanik 1976; Tyler 1978). One line of evidence for this hypothesis was that nitrogen regulatory mutations were found to lie between mutations that caused glutamine auxotrophy and were therefore assigned to *glnA*, the structural gene encoding glutamine synthetase (Bender and Magasanik 1977a and 1977b; Gaillardin and Magasanik 1978; Streicher et al. 1975). Since it is now clear that there are mutations to glutamine auxotrophy in *ntrC* ($NtrC^{rep}$) as well as *glnA*, regulatory mutations that lie between them do not necessarily lie in *glnA*, but may lie in *ntrC* or in *ntrB*, a second nitrogen regulatory gene located between *glnA*

and *ntrC* (Table 3; Fig. 1). The regulatory mutations described by Magasanik were of two types, those that caused low level expression of all nitrogen controlled genes including *glnA* (Gaillardin and Magasanik 1978) (the "GlnR phenotype") and those that caused high level expression of nitrogen controlled genes (Brenchley et al. 1973; Prival et al. 1973) (the "GlnC phenotype"). We have found that a number of mutations that cause low level expression (Kustu et al. 1979a; McFarland et al. 1981) are mutations to loss of function of *ntrC* (Kustu et al. 1979a; McFarland et al. 1981) or are mutations to loss of function of *ntrB* which are polar on *ntrC* (L.L. McCarter and M.K. McKinley, unpublished results). We have recently found by complementation analysis that mutations which cause high level expression of nitrogen-controlled genes (Kustu et al. 1979b) lie within *ntrB* (L.L. McCarter, M.K. McKinley and S.G. Kustu, manuscript in preparation). Thus, many nitrogen regulatory lesions in the *glnA* region which lie between mutations to glutamine auxotrophy lie in *ntrB* (or *ntrC*) rather than *glnA*.

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