

Nitrogen regulatory protein C-controlled genes of *Escherichia coli*: Scavenging as a defense against nitrogen limitation

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Nitrogen regulatory protein C (NtrC) of enteric bacteria activates transcription of genes/operons whose products minimize the slowing of growth under nitrogen-limiting conditions. To reveal the NtrC regulon of *Escherichia coli* we compared mRNA levels in a mutant strain that overexpresses NtrC-activated genes [*glnL*(Up)] to those in a strain with an *ntrC* (*glnG*) null allele by using DNA microarrays. Both strains could be grown under conditions of nitrogen excess. Thus, we could avoid differences in gene expression caused by slow growth or nitrogen limitation *per se*. Rearranging the spot images from microarrays in genome order allowed us to detect all of the operons known to be under NtrC control and facilitated detection of a number of new ones. Many of these operons encode transport systems for nitrogen-containing compounds, including compounds recycled during cell-wall synthesis, and hence scavenging appears to be a primary response to nitrogen limitation. In all, ≈2% of the *E. coli* genome appears to be under NtrC control, although transcription of some operons depends on the nitrogen assimilation control protein, which serves as an adapter between NtrC and σ^{70} -dependent promoters.

Enteric bacteria initially perceive external nitrogen limitation as a decrease in the internal concentration of glutamine (1). One consequence is activation of transcription of genes under control of nitrogen regulatory protein C (NtrC), the products of which ameliorate slowing of growth. The products of NtrC-activated genes have three roles: they mediate assimilation of ammonium into the two central intermediates glutamate and glutamine, yield these intermediates catabolically, or spare the requirement for them. Products of NtrC-activated genes include an ammonia transporter (AmtB), glutamine synthetase (encoded by *glnA*), and amino acid permeases and catabolic enzymes (see references in Table 1). NtrC also activates transcription of genes encoding regulatory proteins: *ntrB* and *ntrC* (*glnL* and *glnG*, respectively), *glnK*, and *nac* (2). The nitrogen assimilation control (Nac) protein activates transcription of σ^{70} -dependent genes, whereas NtrC activates transcription of σ^{54} -dependent genes. Thus, Nac, which has no known coregulator other than its DNA-binding sites (3), serves as an adapter between NtrC and σ^{70} -dependent operons.

Studies with DNA microarrays (4, 5), together with use of new computer tools, allowed us to detect all genes/operons known to be under NtrC control and a number of new ones. These studies also allowed us to assess which of the new operons depended on the adapter Nac. Some of the largest and most interesting responses on microarrays were confirmed by other means, including direct detection of proteins.

Materials and Methods

Strains, Growth Conditions, and Assays. Parental strain MG1655 (CGSC6300) was obtained from the *Escherichia coli* Genetic Stock Center. Unexpectedly, it differs in some regards from the strain that was sequenced (ref. 6; E.S., unpublished work). The *glnA1859* allele was introduced by P1vir-mediated transduction

based on linkage to *zih-102::Tn10* (7). The *glnL2302* allele [*glnL*(Up)] (8) and the *glnG10::Tn5* allele (9) then were introduced by transduction to *glnA*⁺, yielding NCM3292 and NCM3285, respectively. Transductants that had lost *zih-102::Tn10* were chosen. The *nac-28* disruption (2), which also deletes the *nac* promoter, was introduced directly into NCM3292 to yield the kanamycin-resistant strain NCM3596. The *glnL*(Up) and *glnG* alleles also were introduced into strain IADL310 (10) to yield strains NCM3700 and NCM3701, respectively. IADL310 carries at the λ attachment site a fusion of the *ddp* promoter-regulatory region (12–287 bp upstream of the translational start for *ddpX*) to *lacZ*.

Strains were grown with aeration at 37°C to midexponential phase on N⁻C⁻ minimal medium (11) with 0.4% glucose as carbon source and 10 mM NH₄Cl or 5 mM glutamine as nitrogen source (http://coli.berkeley.edu/~zimmer/PNAS_NtrC_2000). On NH₄Cl, *glnL*(Up) strains grew more slowly than *glnG* strains (representative doubling times of 90 and 75 min, respectively). The *nac* disruption suppressed the growth defect of *glnL*(Up) strains. The basis for slow growth and its suppression are not understood. On glutamine, *glnL*(Up) strains grew faster than *glnG* strains (representative doubling times of 110 and 180 min, respectively, after midexponential phase). Slow growth of *glnG* strains on glutamine is due, at least in part, to poor expression of the high-affinity glutamine transport system (*glnHPQ*).

Differential rates of synthesis of β -galactosidase (units/ml per OD₆₀₀) were determined as described (12, 13). Levels of full-length mRNAs in *glnL*(Up) and *glnG* strains were assessed by priming first-strand cDNA synthesis in reverse transcriptase PCRs from the 3' ends of genes (Sigma *Genosys E. coli* ORFmer primer pairs). Periplasmic fractions were obtained by treating whole cells with chloroform essentially as described (11, 14).

Preparation of Microarrays and Data Analysis. Two sets of PCR products for *E. coli* ORFs were synthesized by using different sets of primers, and each was printed several times on glass slides as described (ref. 4 and http://coli.berkeley.edu/~zimmer/PNAS_NtrC_2000). Growth of cultures was quenched on ice that had been cooled to –80°C (15) or with 1/10 volume of 5%

Abbreviations: NtrC, nitrogen regulatory protein C; Nac, nitrogen assimilation control; DAP, diaminiopimelate.

See commentary on page 14044.

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Table 1. NtrC/Nac-activated operons

b no.	Coding strand	Name	Description	Control*	References for control	
					Nitrogen	Other
b0336-7	+	<i>codBA</i>	Cytosine transport , deaminase	Nac	(2)	(19) [†]
b0450-1	+	<i>glnK-amtB</i>	N-regulation and NH ₃ transport	NtrC	(13)	
b0652-5	–	<i>gltIJKL</i>	Glutamate transport	NtrC? [‡]	(11)	(20)
b0809-11	–	<i>glnHPQ</i>	Glutamine transport	NtrC	(21, 22)	(21–23)
b0854-7	+	<i>potFGHI</i>	Putrescine transport	NtrC		(24)
b0929	–	<i>ompF</i>	Outer membrane protein F	Nac? [§]		(25) [†]
b1006-12	–	<i>ycdGHIJKLM</i>	Hypothetical proteins	NtrC	¶	
b1217-8	+	<i>chaBC</i>	Cation transport regulator	NtrC? [‡]		
b1243-7	+	<i>oppABCDF</i>	Oligopeptide transport	Nac? [§]		(20, 26–29) [†]
b1440-4	+	<i>ycdSTUV</i>	Putrescine/spermidine transport (30)	Nac		(23)
b1483-8	–	<i>ddpXABCDE</i>	D-alanine-D-alanine dipeptide transport and dipeptidase	NtrC	¶	(10, 31)
b1744-8	–	<i>astCADBE</i>	Arginine catabolism	NtrC	(32–34)	(32)
b1783-4	+	<i>yeaGH</i>	Hypothetical proteins	NtrC?	¶	†
b1932	+	<i>yedL</i>	Hypothetical protein	Nac		
b1987	–	<i>cbl</i>	Regulator for sulfur metabolism	NtrC		(35)
b1988	–	<i>nac</i>	N-regulation	NtrC	(2, 36)	
b2306-9	–	<i>hisJQMP</i>	Histidine transport	NtrC**	(37–39)	(38, 39) [†]
b2310	–	<i>argT</i>	Basic amino acid transport	NtrC	(37–39)	(38, 39) [†]
b2393	+	<i>nupC</i>	Nucleoside transport	Nac		(40)
b2661-4	+	<i>gabDTPC</i>	γ-aminobutyric acid transport and catabolism	Nac	(41–43)	(23, 42, 43)
b3073	+	<i>ygjG</i>	Probable ornithine aminotransferase	NtrC		†
b3268-71	+	<i>yhdWXYZ</i>	Polar amino acid transport (30)	NtrC		
b3540-4	–	<i>dppABCDF</i>	Dipeptide transport	Nac? [§]		(20, 29, 44) [†]
b3868-70	–	<i>glnALG</i>	NH ₃ assimilation and N-regulation	NtrC	(45–47)	(47) [†]
b4207-8	+	<i>fkIB-cycA</i>	Peptidyl-prolyl isomerase; D-alanine, D-serine, gly transport	Nac		
Total: 25 operons; 75 genes						

Derived from Table 3. To avoid making the summary list artificially long, six operons (11 genes) in this table were excluded because it was difficult to assign their control and little is known about them. These are b1034–35, b1296, b1384 (*feaR*), b2875–76, b2882–85, and b3512. See Xi *et al.* (48) for recent information on b2875–76 and b2882–85. In addition, annotations (6) for the following operons have been changed: *gltIJKL*, *oppABCDF*, *yeaGH*, *hisJQMP*, *gabDTPC*, *yhdWXYZ*, and *fkIB-cycA*.

*Assessed as described in *Materials and Methods*.

[†]RNA levels in an *ntrC* strain were ≥ 2 -fold different for cells grown on glutamine versus ammonia (experiment *i*).

[‡]Some data from the second set of PCR products of poor quality; hard to assess NtrC versus Nac control.

[§]At least one gene in these operons also appears to be NtrC-controlled, which is unlikely biologically; Nac control is in accord with known forms of control for these operons (see references), which involve sigma factors other than σ^{54} .

[¶]Putative σ^{54} promoter (6, 49).

^{||}The *nac* promoter is NtrC-activated (2). The *cbl* gene is probably expressed from the *nac* promoter under nitrogen-limiting conditions.

**The NtrC-controlled promoter may be upstream of *argT* (38).

phenol in ethanol (16) and RNA was extracted with hot phenol/SDS (15). Synthesis of cDNAs containing fluorescent nucleotide analogues, hybridization to microarrays, and scanning and normalization were as described (4).

To visualize spots on microarrays in genome order, images representing the Cy3 and Cy5 fluorescence intensities of spots were cut from a portable network graphic (D.P.Z., unpublished work). The spots then were ordered from left to right and top to bottom according to their *b* numbers (6) to provide a new genome image. Genome images are 100 spot columns wide and 45 spot rows tall. Operons (6) under NtrC/Nac control were identified by the following criteria: (i) the mRNA level for at least one gene in the operon was ≥ 2.5 -fold higher (see *Results* for rationale) in the *glnL*(Up) than the *glnG* strain on ammonia for arrays printed with at least one set of PCR products [$(R/G)_{\text{median}}$ and $(R/G)_{\text{mean}} \geq 2.5$] (see Table 2, which is published as supplementary material on the PNAS web site, www.pnas.org; 99 genes in 43 operons); (ii) mRNA levels for genes that met the first criterion were also ≥ 2.5 -fold higher in the *glnL*(Up) strain on glutamine for the same set of PCR products (see Table 3, which is published as supplementary material on the PNAS web site, www.pnas.org; 86 genes in 31 operons). With the exceptions indicated in the

legend, operons containing genes that met both criteria are included in Table 1 (*Results*).

Eleven experiments (designated *a–k*) were performed with different strains under different growth conditions: *a* and *b*, *glnL*(Up) (Cy5) versus *glnL*(Up) *nac* (Cy3), NH₃, and glutamine as nitrogen source, respectively (second set of PCR products); *c* and *d*, *glnL*(Up) (Cy5) versus *glnG* (Cy3), NH₃, (first and second set of PCR products, respectively); *e* and *f*, same as *c* and *d* with glutamine; *g* and *h*, *glnL*(Up) *nac* (Cy5) versus *glnG* (Cy3), NH₃, and glutamine, respectively (second PCR); *i*, *glnG*, glutamine (Cy5) versus NH₃ (Cy3) (first PCR); *j*, MG1655, glutamine (Cy3) versus NH₃ (Cy5) (first PCR) (note reversal of dyes); and *k*, same as *j* with 0.4% glycerol as carbon source. Higher mRNA levels in the first strain in experiments *a–f* are indicative of Nac activation, whereas higher levels in the first strain in experiments *c–h* are indicative of NtrC activation. This qualitative statement was quantified as follows: For each gene in an operon we determined ratios of RNA levels between the two strains [$(R/G)_{\text{median}}$] in experiments *a–h* (see example in Fig. 2B). If all ratios in experiments *a–f* were >1 , we assigned Nac control, whereas if all were >1 in experiments *c–h*, we assigned NtrC control. If the majority of genes in an operon were Nac-activated, we assigned Nac control to the operon and similarly for NtrC

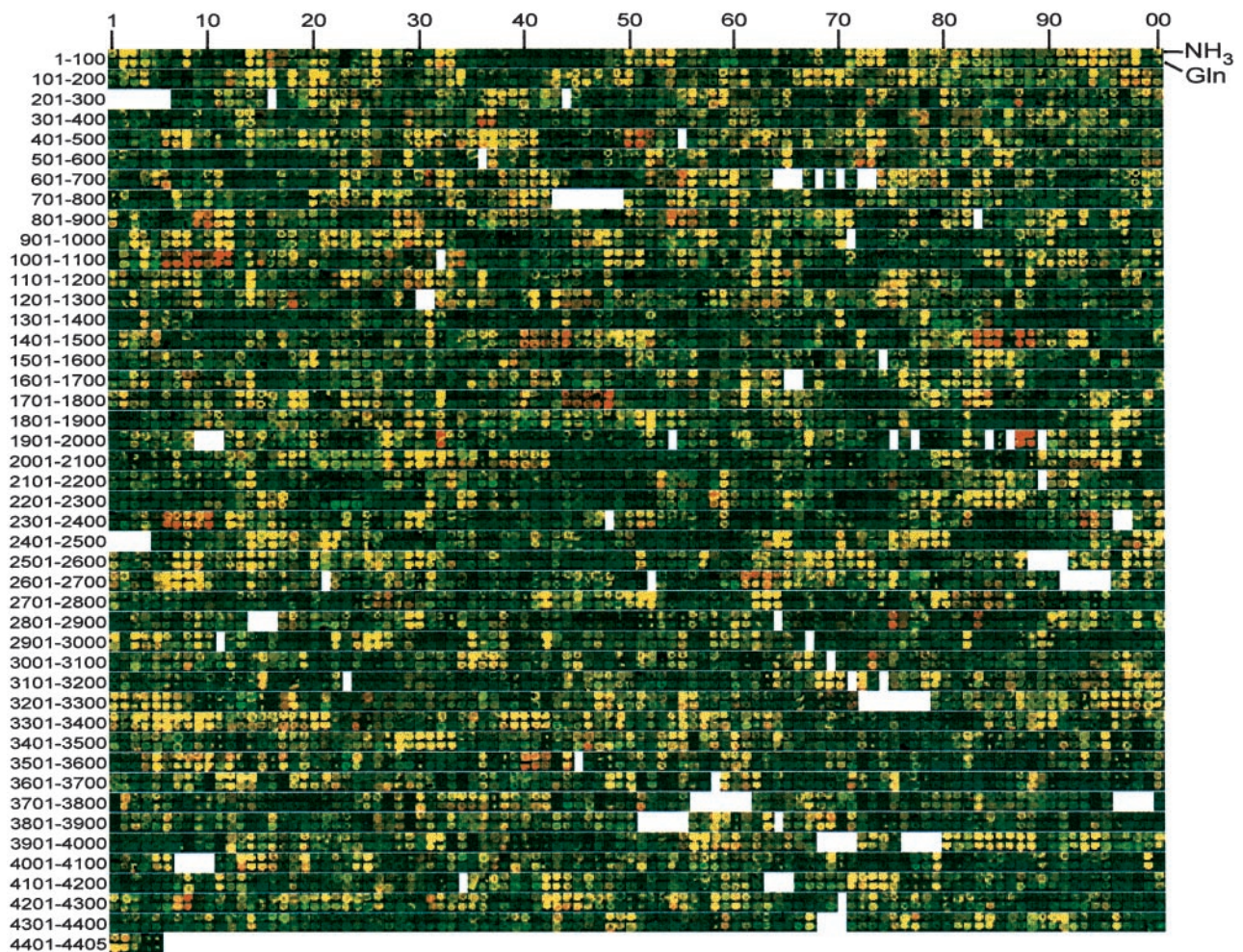


Fig. 1. Two aligned genome images. Microarrays were probed with mixtures of cDNAs from the *glnL*(Up) and *glnG* strains grown on ammonium (upper row of each pair, experiment c) or glutamine (lower row, experiment e) as nitrogen source. Spots from fluorescence scanning images of microarrays were rearranged in genome order. The *b* number centuries (6) are indicated to the left. Blanks represent either *b* numbers that do not correspond to ORFs or that no longer exist. Red spots can be seen for most operons in Table 1. For some highly expressed genes—e.g., *codBA* (b0336–37) in the upper row and *glnA* (b3870)—spots appear intense yellow rather than red because of image saturation.

(Table 1, *Results*). Ambiguities are noted in footnotes to Table 1. Log ratios were used in hierarchical clustering analysis (17).

Results

Initial Microarray Experiments. To determine members of the NtrC regulon of *E. coli* we first compared mRNA levels in a *glnL*(Up) strain, which is called *ntrB*(Con) in other bacteria (Cy5; red) to those in a *glnG* strain, which is called *ntrC* in other bacteria (Cy3; green) for cells grown in minimal medium with ammonium chloride as sole nitrogen source (see *Materials and Methods*). The GlnL (NtrB) protein is a histidine autokinase that donates phosphoryl groups to response regulator GlnG (NtrC) and thereby activates it (18). The autokinase is more active in *glnL*(Up) [*NtrB*(Con)] strains than in wild-type strains, and hence genes and operons under NtrC (GlnG) control are overexpressed even under conditions of nitrogen excess.

Data from DNA microarrays were displayed in genome order (Fig. 1), and genes for which mRNA levels in the *glnL*(Up) strain were at least 2.5-fold higher than those in the *glnG* strain were identified (Tables 1 and 2). The value of 2.5 was the minimum that yielded at least one gene in each operon known from previous biochemical and genetic studies to be under NtrC control in enteric bacteria [nine operons containing 27 genes

(Table 1)]. The spots that met our first criterion often fell in groups of spots that appeared to be red by visual inspection (Fig. 1). With few exceptions (see *Discussion*), the boundaries of such groups corresponded to operon borders (6). Despite the relatively high ratio required in the first criterion, the induced operons included a number of new ones. Among those whose substrate specificities were easily rationalized were the following: three operons (b0854–57, b1243–47, b3540–44; 15 genes) specifying ATP-binding cassette transporters for putrescine, oligopeptides, and dipeptides, respectively, and two operons (b2393 and b4208) encoding secondary ion-coupled transporters for nucleosides, and D-alanine/D-serine/glycine, respectively. A mixed transport and catabolic operon for cytosine (*codBA*; b0336–37) and a gene encoding a probable ornithine aminotransferase (b3073) were also in this group.

Putative NtrC-controlled operons included three specifying ATP-binding cassette transporters of unknown substrate specificity (b1440–44, b1483–88, and b3268–71) (6). Paulsen and colleagues (30) predicted that their substrates were spermidine, dipeptides, and an amino acid, respectively. Lessard and colleagues (10) implicated D-alanine-D-alanine as the dipeptide transported by the products of the b1483–88 operon, which also encodes a specific dipeptidase. Also putatively activated by NtrC

were: *cbl* (b1987), which encodes a regulator for sulfur metabolism (see below); *ompF* (b0929), which encodes a large outer-membrane pore (see below); and three operons of unknown function (b1006–12, b1783–84, and b1932). Finally, NtrC appeared to activate a group of genes (b3504–17) around the *hdeAB* (H-NS-determined expression) operon (b3509–10). Additional experiments indicated that the “*hdeAB* group” was not under NtrC/Nac control (see below).

As is the case for operons known to be activated by NtrC, most differences in mRNA levels between the *glnL*(Up) strain and the *glnG* (*ntrC*) strain seen with ammonia as sole nitrogen source were also apparent with glutamine (Fig. 1; Tables 1 and 3). Note that the relative growth rates of the two strains were reversed on glutamine (*Materials and Methods*). Genes of the *hdeAB* group were an exception. Because these genes did not meet the criterion that mRNA levels in the *glnL*(Up) strain be ≥ 2.5 times higher than those in the *glnG* strain on glutamine as nitrogen source, they and others like them are not included in Table 1. Hierarchical clustering (17) also indicated that the *hdeAB* group was not under NtrC/Nac control (http://coli.berkeley.edu/~zimmer/PNAS_NtrC_2000/). Additional differences between regulatory mutant strains seen only with glutamine as nitrogen source have not been analyzed in detail.

Most differences between mRNA levels in regulatory mutant strains also were seen in the congenic wild-type strain MG1655 when it was grown under two different conditions, namely with glutamine (Cy3) or ammonia (Cy5) as sole nitrogen source (Fig. 2; Tables 2 and 3). Although the reasons are not obvious, growth on glutamine is known to elevate expression of genes under NtrC control (50).

Assessment of Nac Involvement. To determine which of the operons under NtrC control depended on the NtrC-Nac regulatory cascade, we compared mRNA levels in the *glnL*(Up) strain to those in a *glnL*(Up) *nac* strain on ammonia and on glutamine as nitrogen source (genes under Nac control detected). In addition, we compared mRNA levels in the *glnL*(Up) *nac* strain to those in the *glnG* (*ntrC*) strain under the same conditions (genes directly under NtrC control detected). These comparisons (*Materials and Methods*; Table 1) indicated that Nac activated transcription of the putative spermidine transport operon (b1440–44) (Fig. 2), the b1932 operon (ORF), the nucleoside transport operon *nupC* (b2393), the amino acid transport operon *fkIB-cycA* (b4207–08), and the *codBA* operon (b0336–37). The latter was in accord with slow growth of a *nac* strain on cytosine as sole nitrogen source (2). Nac activation of other operons, e.g., *ompF* and *opp*, was less clear and may be caused by indirect effects. These operons are known to be subject to a plethora of other controls (Table 1). NtrC apparently activated transcription of the putrescine transport operon (b0854–57), the b1006–12 operon (ORFs), the transport and catabolic operon for D-alanine-D-alanine (b1483–88) (Fig. 2), and the probable ornithine aminotransferase operon (b3073), in addition to operons known or thought to be under its control (Table 1). Although we did not focus on repression, we noted that Nac apparently repressed transcription of the *serA* (b2913) and *gtBDF* (b3212–14) operons by ≥ 3 -fold. Only repression of *gtBDF* was known (R.A.B., unpublished work).

NtrC Control of a *ddp-lacZ* Transcriptional Fusion. Previous studies indicated that a *ddp-lacZ* transcriptional fusion placed at the λ attachment site of *E. coli* was transcribed in the late exponential phase of growth on enriched medium (to 150 Miller units) and that expression depended on σ^S (10). Having observed a large and reproducible difference between the mRNA levels for the *ddp* operon in a *glnL*(Up) and a *glnG* (*ntrC*) strain (up to 60-fold for *ddpX*; Figs. 1 and 2), we tested for NtrC control of the *ddp-lacZ* fusion. The differential rate of synthesis of β -galacto-

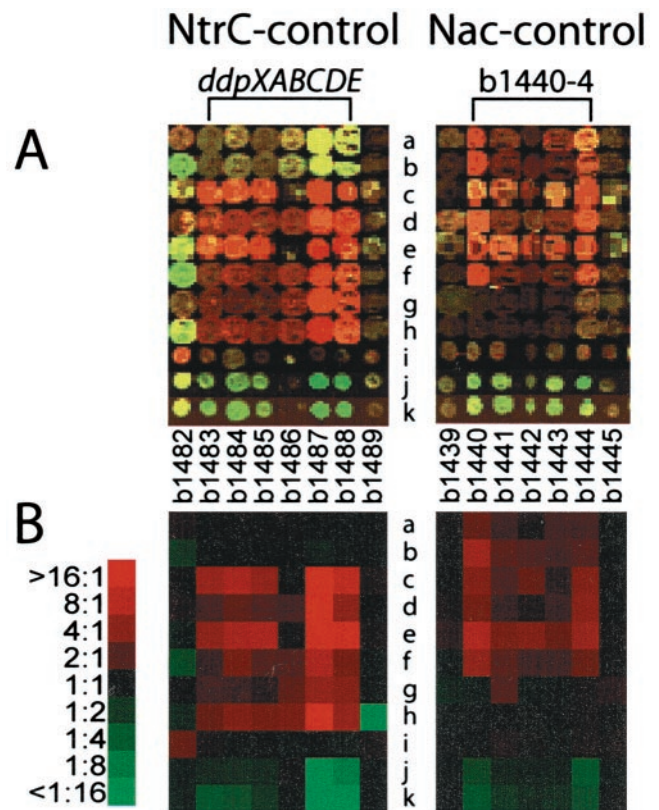


Fig. 2. Determination of NtrC versus Nac control. (A) Spot images for genes of the b1440–44 operon and the *ddp* (b1483–88) operon and adjacent genes in experiments a–k. (B) Profiles (log ratios) for these genes visualized as red and green intensities by using the program TREEVIEW (17) (<http://rana.stanford.edu/software/>). The patterns expected for Nac or NtrC activation are red in experiments a–f or c–h, respectively. For either Nac or NtrC control green spots are expected in experiments j and k. If there is no control other than NtrC/Nac, yellow or black spots (A) or black squares (B) are expected in experiment i. Note that genes flanking each operon have expression patterns different from those of the operon and that the first PCR product for b1486 (experiments c, e, i, j, and k) was apparently bad. Red and green intensities representing induction and repression ratios, respectively, are indicated to the left of B.

sidase in a strain carrying the fusion and the *glnL*(Up) mutation was >230 -fold higher than that in the congenic strain carrying the *glnG* (*ntrC*) mutation when they were grown on minimal medium with ammonium chloride as nitrogen source (2,300 versus <10 units). It was >390 -fold higher with glutamine as nitrogen source (3,900 versus <10 units). In support of NtrC control, there is a putative σ^{54} promoter for the *ddp* operon located 53 bp upstream of the translational start for *ddpX* (6, 49) and a putative NtrC-binding site 140 bp upstream. Both are present in the fusion construct.

Analysis of Periplasmic Fractions. Analysis by SDS/PAGE indicated that the periplasmic fraction from the *glnL*(Up) strain grown on ammonia contained several proteins not present or present in much lower amounts in the same fraction from the *glnG* strain (Fig. 3). Analyses of periplasmic fractions by HPLC and MS indicated that binding protein components of the transport systems for D-alanine-D-alanine (*ddpA*) and arginine (*argT*) and of the putative transport system for polyamines (b1440) were among the proteins most highly elevated in the *glnL*(Up) strain over the *glnG* strain (R. W. Corbin and D. Hunt, personal communication).

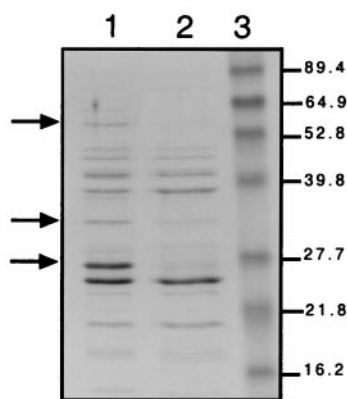


Fig. 3. Periplasmic fractions of the *glnL*(Up) strain (lane 1) and the *glnG* strain (lane 2) on ammonia. After SDS polyacrylamide electrophoresis (2 μ g protein; 12% gel), the gel was stained with Coomassie blue. Molecular weights of the standard proteins [GIBCO Benchmark Prestained Protein Ladder] in lane 3 are indicated to the right. Three bands of notably higher intensity in the *glnL*(Up) strain are indicated with arrows to the left.

Physiological Studies of Cbl. Whereas many of the new operons/genes detected by probing for NtrC-activated genes on DNA microarrays were easily rationalized, the *cbl* (CysB-like) gene (b1987), which lies just downstream of *nac* (b1988), was not. The difference in mRNA levels—up to 30-fold higher in the *glnL*(Up) strain—was one of the largest we observed. Although we could not demonstrate a clear physiological role for Cbl in nitrogen metabolism, it activates transcription of operons whose products catabolize nitrogen- and sulfur-containing compounds (51). The *cbl* mRNA in *glnL*(Up) strains appeared to be full length (not shown), and we hope to determine whether it is translated to yield protein.

Discussion

The Extended NtrC/Nac Regulon. NtrC appears to control almost 2% of *E. coli* genes (~75) (Table 1). Transcription of most is apparently activated by NtrC in conjunction with σ^{54} -holoenzyme, but transcription of some (\approx 9 operons; 25 genes) appears to depend on the adapter Nac in conjunction with σ^{70} -holoenzyme.

The most striking observation about the extended NtrC/Nac regulon is the number of transport operons it contains. Almost 2/3 (45 genes) of the genes activated by NtrC/Nac are members of transport operons, which were expressed even in the absence of specific inducing compounds in the medium. Clearly, *E. coli* uses its capacity to scavenge for nitrogen-containing compounds as a first line of defense against nitrogen starvation. It uses both multicomponent ATP-binding cassette transporters, which often have high affinity for their substrates, and lower affinity ion-coupled transporters, usually encoded by a single gene. Not only does *E. coli* scavenge for compounds that may be found in the human intestine or other environments, but also for compounds it releases into the periplasmic space during cell growth and division. The latter include D-alanine (D-ala) and the D-alanine-D-alanine dipeptide (D-ala-D-ala) released during murein synthesis. The *cycA* gene (b4208), which encodes a transporter for D-ala, is under Nac control and the *ddp* operon (b1483–88), which encodes a transporter and a specific dipeptidase for D-ala-D-ala (10), is under NtrC control.

Although there has been no investigation under nitrogen-limiting conditions, under other conditions of limitation/starvation, *E. coli* increases cross-links between diaminopimelate (DAP) moieties in the murein, otherwise a minor proportion of the total (10, 52). This

increases release of the D-ala-D-ala dipeptide (as opposed to D-ala, which is released when the more common D-ala-DAP cross-links are formed) and could enhance recovery of nitrogen. To the degree that preexisting murein can be remodeled to convert the common D-ala-DAP cross-links to DAP-DAP cross-links, additional D-ala would be released. Interestingly, increased DAP-DAP cross-bridges and other changes in murein composition that occur upon nutrient limitation are correlated with greater resistance of this layer to destruction (10, 52). Presumably, both scavenging and toughening of the murein would enhance survival under nitrogen-limiting conditions.

Data Analysis. To make it more likely that the effects we observed were due directly to NtrC or Nac, we analyzed experiments done with pairs of regulatory mutant strains grown under the same conditions rather than the wild-type strain grown under two different conditions. The latter would be complicated by less direct responses. In fact, NtrC/Nac-controlled operons (Table 1) constitute a subset of those whose expression changed when the wild-type strain was grown on glutamine rather than ammonia. Obtaining stronger evidence that effects of NtrC and Nac on the newly identified operons in Table 1 are direct—or demonstrating that they are not—will require *in vitro* analysis and/or genetic dissection of promoter-regulatory regions. Although we have detected some putative NtrC binding sites by sequence analysis, we have not yet identified the pairs of sites that usually constitute functional enhancers (53).

Analysis of data from *E. coli* microarrays was facilitated by the use of genome images, i.e., the display of spot images in genome order (Fig. 1). This allowed easy recognition of differences in expression of multigenic operons, groups of contiguous genes that are transcribed together. We note, however, that differences in mRNA levels between our pairs of regulatory mutant strains were not uniform across operons (Fig. 2, Tables 2 and 3, and http://coli.berkeley.edu/~zimmer/PNAS_NtrC_2000/). In some instances lack of coordinacy may have a biological explanation—e.g., there are large differences in stability for portions of mRNA encoding periplasmic and membrane components of ATP-binding cassette transporters (54, 55). In other cases, however, we know or suspect that apparent lack of coordinacy was an artifact of differences in the quantity or quality of PCR products (e.g., see b1486 in Figs. 1 and 2). Use of genome images also assisted in eliminating false signals caused by antisense RNA [e.g., see *tesB* (b0452) in Fig. 1] and in recognizing supraoperonic organization [e.g., see *nac-cbl* (b1987–88) and the *hdeAB* group (b3504–17)]. For all of the reasons discussed above, visualization of microarray data in genome order should be generally useful for bacteria, archaea, and their viruses. In addition, it should facilitate detecting differences in expression of large regions of eukaryotic chromosomes (56).

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