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Activation of the *glnA*, *glnK*, and *nac* Promoters as *Escherichia coli* Undergoes the Transition from Nitrogen Excess Growth to Nitrogen Starvation

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The nitrogen-regulated genes and operons of the Ntr regulon of *Escherichia coli* are activated by the enhancer-binding transcriptional activator NRI~P (NtrC~P). Here, we examined the activation of the *glnA*, *glnK*, and *nac* promoters as cells undergo the transition from growth on ammonia to nitrogen starvation and examined the amplification of NRI during this transition. The results indicate that the concentration of NRI is increased as cells become starved for ammonia, concurrent with the activation of Ntr genes that have less-efficient enhancers than does *glnA*. A diauxic growth pattern was obtained when *E. coli* was grown on a low concentration of ammonia in combination with arginine as a nitrogen source, consistent with the hypothesis that Ntr genes other than *glnA* become activated only upon amplification of the NRI concentration.

Escherichia coli contains six operons that are known to be part of the Ntr regulon (*argThisJQMP*, *astCADBE*, *glnALG*, *glnHPQ*, *glnKamtB*, and *nac*). These operons require σ^{54}-RNA polymerase for expression and are activated by the phosphorylated form of enhancer-binding transcription factor NRI (NtrC) (reviewed in reference 16). A seventh operon, consisting of the *ygiG* gene, may also be a part of the Ntr regulon (16). *E. coli* also contains numerous additional genes that become activated or repressed upon nitrogen starvation (27).

The mechanism of activation by NRI~P at σ^{54}-dependent promoters has been studied in some detail (reviewed in reference 10). NRI~P binds to upstream enhancer elements, oligomerizes, and displays ATPase activity. This complex interacts with σ^{54}-RNA polymerase bound at the promoter to bring about formation of the open transcription complex. The interaction between NRI~P and σ^{54}-RNA polymerase requires the formation of a DNA loop, bringing the enhancer-bound activator and promoter-bound polymerase into proximity. In some cases, regulatory factors bind the intervening DNA and activate or repress transcription by topological alteration of the DNA.

The different nitrogen-regulated promoters contain distinct arrangements of NRI-binding sites that constitute their enhancer elements. The *glnAp2* promoter apparently contains the most potent enhancer, consisting of two adjacent high-affinity NRI-binding sites (14, 20). The *glnHp2* promoter, consisting of overlapping high-affinity sites, appears to be slightly less effective in vitro (4). The *Klebsiella pneumoniae nifLA* enhancer contains adjacent low-affinity NRI-binding sites and is only effective at high NRI~P concentrations in vitro (26). Similarly, the *nac* promoter of *Klebsiella aerogenes* has a weak enhancer that is only effective at high NRI~P concentrations in vitro; this enhancer consists of a high-affinity NRI-binding site and an adjacent site that is bound by NRI~P only at high concentration (7). Thus, in vitro transcription studies are consistent with the hypothesis that amplitude modulation of the NRI~P concentration results in the sequential activation of Ntr genes.

A considerable body of additional evidence also supports this hypothesis. The intracellular concentration of NRI rises dramatically in cells growing under nitrogen-limiting conditions (19), owing to the activation of the *glnAp2* promoter by NRI~P (15). Also, cells that have been genetically manipulated such that the NRI concentration is always low retain the ability to fully activate *glnAp2* but are unable to grow on arginine as a nitrogen source (15) or activate the *glnK* promoter (2). The inability to grow on arginine probably reflects the inability to activate the *astC* promoter (16, 23). Similarly, the activation of the *K. pneumoniae nifLA* promoter requires a high concentration of NRI~P in vivo (10). It seems reasonable that the *nac*, *glnK*, *astC*, and *nifLA* promoters should be activated by NRI~P only at high concentrations, since the products resulting from their activation are useful under starvation conditions (16–18).

Finally, the signal transduction system that regulates the NRI phosphorylation state is able to provide rheostat-like control of NRI~P in response to signals of nitrogen status (reviewed in reference 13). This, in combination with the observation that the NRI concentration is dramatically regulated in cells, suggests that cells may widely vary the concentration of NRI~P in response to changes in environmental conditions.

Nevertheless, there are a few significant gaps in our knowledge. The great instability of NRI~P has prevented its direct measurement in situ. Furthermore, most of the experiments with intact cells summarized above were conducted with log-phase cells growing under nitrogen excess or nitrogen-limiting

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TABLE 1. Strains used in this study

Strain	Relevant genotype	Construction or reference
YMC10	*endA1 thi-I hsdR17 supE44* Δ*lacU169 hutC*$_{klebs}$	3
X	*endA1 thi-1 hsdR17 supE44* Δ*lacU169 hutC*$_{klebs}$*amtB*::Cam^r	2
TE2680	*recD1903*::Tn*10 trpDC700*::*putPA130* [Kan^s Cam^r]	5
YMC10Φ	*endA1 thi-1 hsdR17 supE44* Δ*lacU169 hutC*$_{klebs}$ *trpDC700*::*putPA130* [Φ(*glnKp-lacZ*) Kan^r Cam^s]	2
MAAplac3	*recD1903*::Tn*10 trpDC700*::*putPA130* [Φ(*glnAp2-lacZ*) Kan^r Cam^s]	TE2680 × *Pst*I pglnAplac4
YMC10ApΦ2	*endA1 thi-1 hsdR17 supE44* Δ*lacU169 hutC*$_{klebs}$ [Φ(*glnAp2-lacZ*) Kan^r Cam^s]	YMC10 × MAAplac3 P1*vir*
TE2680NpΦ	*recD1903*::Tn*10 trpDC700*::*putPA130* [Φ(*nacp-lacZ*) Kan^r Cam^s]	TE2680 × *Pst*I pNacLacZ
YMC10NpΦ	*endA1 thi-1 hsdR17 supE44* Δ*lacU169 hutC*$_{klebs}$ [Φ(*nacp-lacZ*) Kan^r Cam^s]	YMC10 × TE2680NpΦ P1*vir*

conditions; our conclusions concerning transitions represent extrapolations from these results. Here, we focused on the growth of cells as their environment changes from nitrogen replete to nitrogen starved and measured the activation of the *glnA*, *glnK*, and *nac* promoters, as well as the amplification of the intracellular concentration of NRI. In addition, we examined the patterns of growth when *E. coli* was provided with ammonia and arginine as nitrogen sources.

MATERIALS AND METHODS

Bacteriological techniques. Luria-Bertani broth and W salts-based defined media, preparation of plasmid DNA, preparation of competent cells, transformation of cells with DNA, sequencing of plasmid DNA, PCR amplification of DNA, preparations of P1*vir* phage lysates, P1-mediated transduction, recombination of DNA onto the bacterial chromosome, and long-term storage of strains were by standard techniques or were as described previously (1, 2, 5, 8, 12, 22, 24). The bacterial strains, plasmids, and oligonucleotides used in this work are described in Tables 1 to 3. Turbidity of bacterial cultures was measured with a Beckman DU65 spectrophotometer.

Construction of *glnAp-lacZ* and *nacp-lacZ* fusions and recombination onto the bacterial chromosome. PCR was used to amplify the *glnA* control region with primers glnApD.S.5 and glnApU.S.2 (Table 3). Similarly, primers NacpLacZus2 and NacpLacZds2 were used to amplify the *nac* control region (Table 3). The amplification products were cleaved with *Eco*RI and *Bam*HI and then cloned into similarly cleaved pRS551 (25) to form a transcriptional fusion to *lacZ* bracketed by transcriptional terminators with flanking sequence homology to *trp* genes. The fusions were recombined onto the bacterial chromosome by electroporation of strain TE2680 with *Pst*I-digested plasmid DNA and selection for kanamycin-resistant, chloramphenicol-sensitive transformants (5). The recombinants were confirmed to have a new auxotrophic requirement for tryptophan, indicating correct recombination into the *trp* locus. The fusions were then moved into various genetic backgrounds by P1*vir*-mediated generalized transduction, with selection for the nearby kanamycin resistance marker.

GS and β-galactosidase assays. The γ-glutamyl transferase activity of glutamine synthetase (GS) was measured as described previously (21). Protein determinations were as described by Lowry et al. (11). Two cultures were used for each determination, and the experiments were repeated on three different occasions. Within a given experiment, values for duplicate cultures were within 10%, while the day-to-day reproducibility was ±20%. β-Galactosidase was measured by the Miller assay and expressed as Miller units, and sodium dodecyl sulfate and chloroform were used to disrupt the cells as described previously (24). In previous work, we measured the expression of the *glnKp-lacZ* fusion using cell sonicates (2). Comparison of the two methods indicated that the Miller assay reproducibly detects about one-fourth of the activity found in cell extracts (A. J. Ninfa and M. R. Atkinson, unpublished data).

Immunoblotting. A crude rabbit anti-NRI antibody was kindly provided by Lawrence Reitzer. Immunoblotting was performed with the Amersham ECL Western blotting system according to the manufacturer's directions.

In vitro transcription assays. The assay methods were identical to those described in reference 7. Transcription templates pTH8 and pLR100, containing the *glnAp* control region, were as described previously (7, 9, 14). The *glnKp* transcription template, pglnK13, was constructed by PCR amplification using primers 7159I and 4804J (Table 3), cleavage with *Bam*HI and *Hin*dIII, and ligation into similarly cleaved pTE103, essentially as described previously (9). The plasmid was sequenced to verify that no alterations were introduced to the *glnK* control region during these manipulations. NRI, NRII, core RNA polymerase, and σ^{54} were purified as described earlier (7, 14).

TABLE 2. Plasmids used in this study

Plasmid	Relevant features and construction	Reference
pRS551	*lac* fusion vector	25
pglnAplac4	*glnA* promoter fused at +165 to *lac* operon in pRS551	
pTH8	*glnA* promoter cloned into pTE103	9
pLR100	*glnA* promoter cloned into pTE103	14
pTE103	pUC8 multicloning site with phage T7 terminator	6
pglnK13	*glnK* promoter cloned into pTE103	
pNacLacZ	*nac* promoter fused to *lac* operon in pRS551	

RESULTS

Establishing conditions to study the transition from nitrogen excess growth to nitrogen starvation. Since the preferred nitrogen source for *E. coli* is ammonia, we examined the effect of growth in the presence of various concentrations of ammonia. We found that while cell yield depended on the ammonia concentration, the growth rate did not (Fig. 1). Apparently, *E. coli* is very effective in capturing ammonia, even when it is present at low concentration. These results call into question the validity of the common practice in which medium containing limiting ammonia is used to establish low steady-state rates of growth in chemostat experiments. The *amtB* gene, encoding a putative ammonia transporter, had no effect on the utilization of ammonia in our experiments, even at very low ammonia concentrations (Fig. 1).

To study the activation of the *glnA*, *glnK*, and *nac* promoters with high precision in dilute bacterial cultures, we engineered

TABLE 3. Primers used in this study

Primer	Sequence and description
glnApU.S.2	CCGGAATTCATCCTCCGCAAACAAGTATTGCAGAG; upstream primer for fusing *glnAp* to *lac* in pRS551 (*Eco*RI)
glnApD.S.5	CGCGGATCCTTACACCTGATGAGCAGGGATAGTGAC; downstream PCR primer to fuse *glnAp* to *lac* in pRS551 (*Bam*HI)
7159I	GCTGCAGGGATCCCATTGAGCGCCTGAATAGCGC; upstream PCR primer to clone *glnKp* into pTE103 (*Bam*HI)
4804J	GAAGCTTGAATGGTTTGATTATCACGGTCACC; downstream PCR primer to clone *glnKp* into pTE103 (*Hin*dIII)
NacpLacZus2	CCGGAATTCGCTTTCAATCTTATTGG; upst ream primer for fusing *nac* promoter to *lac* in pRS551 (*Eco*RI)
NacpLacZds2	CGCGGATCCTGCCGCCATTACTTACA; downstream primer for fusing *nac* promoter to *lac* in pRS551 (*Bam*HI)

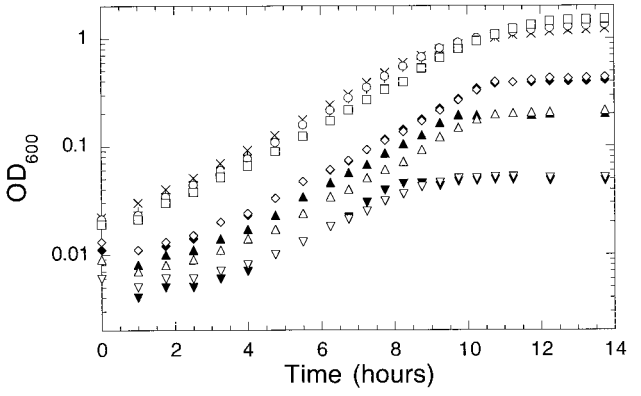

FIG. 1. Growth of YMC10 (wild type) and X (*amtB*::Cam^r) on various concentrations of ammonium sulfate. Overnight cultures grown at 30°C in 0.4% (wt/vol) glucose and 0.2% (wt/vol) ammonium sulfate were washed and resuspended in medium containing 0.4% (wt/vol) glucose and the following concentrations of ammonium sulfate: for YMC10, 0.2 (×), 0.05 (+), 0.01 (◆), 0.005 (▲), and 0.001% (▼); for X, 0.2 (○), 0.05(□), 0.01 (◇), 0.005 (△), and 0.001% (▽). OD_{600}, optical density at 600 nm.

transcriptional fusions of these promoter regions to *lacZ*. These fusions were placed onto the chromosome in single copy within the *trp* operon (25) (see Materials and Methods). The *glnKp-lacZ* fusion was described previously (2); construction of the *glnA* and *nac* fusions is described in Materials and Methods. Expression of the *glnAp-lacZ* fusion was observed to parallel the expression of GS in adapted log-phase cells growing under nitrogen-rich and nitrogen-limiting conditions (data not shown).

Activation of *glnAp*, *glnKp*, and *nacp* as cells growing on ammonia became nitrogen starved. A comparison of the activation of *glnAp* and *glnKp* as cells underwent the transition from growth on ammonia to nitrogen starvation is shown in Fig. 2. A significant difference in the patterns of expression of *glnKp* and *glnAp* was observed. When grown on low concentrations of ammonium (0.005 [A] or 0.01% [B] ammonium sulfate), expression of *glnAp* was approximately one-third to one-fourth of the maximum level observed during exponential growth, while *glnKp* was essentially silent (Fig. 2A and B). In both cases, *glnKp* became highly expressed as the cells ran out of ammonia and growth ceased (Fig. 2A and B). In contrast, at 0.2% ammonium sulfate, where cell yield was not limited by ammonia, *glnAp* was expressed at approximately one-ninth of its maximum level, while *glnKp* was silent even after the cells stopped growing (Fig. 2C). Thus, the regulation of *glnAp* and *glnKp* was different in that under certain conditions (i.e., when cells reached stationary phase before exhaustion of the ammonia) *glnAp* was the only promoter expressed. Although the growth rate was not significantly altered at different concentrations of ammonia (provided as ammonium sulfate), the level of *glnA* transcription was clearly affected.

In similar experiments, we observed that the *nac* promoter, like *glnK*, became activated only when growth became limited by ammonia starvation. To better focus on the relationship between the *nac* and *glnK* promoters, we compared their activation side by side in experiments where cells became limited for ammonia at two different optical densities, owing to different initial concentrations of ammonium sulfate. These experiments indicated that *nac* and *glnK* were activated at about the same point, with *nac* perhaps lagging slightly behind *glnK* (Fig. 3).

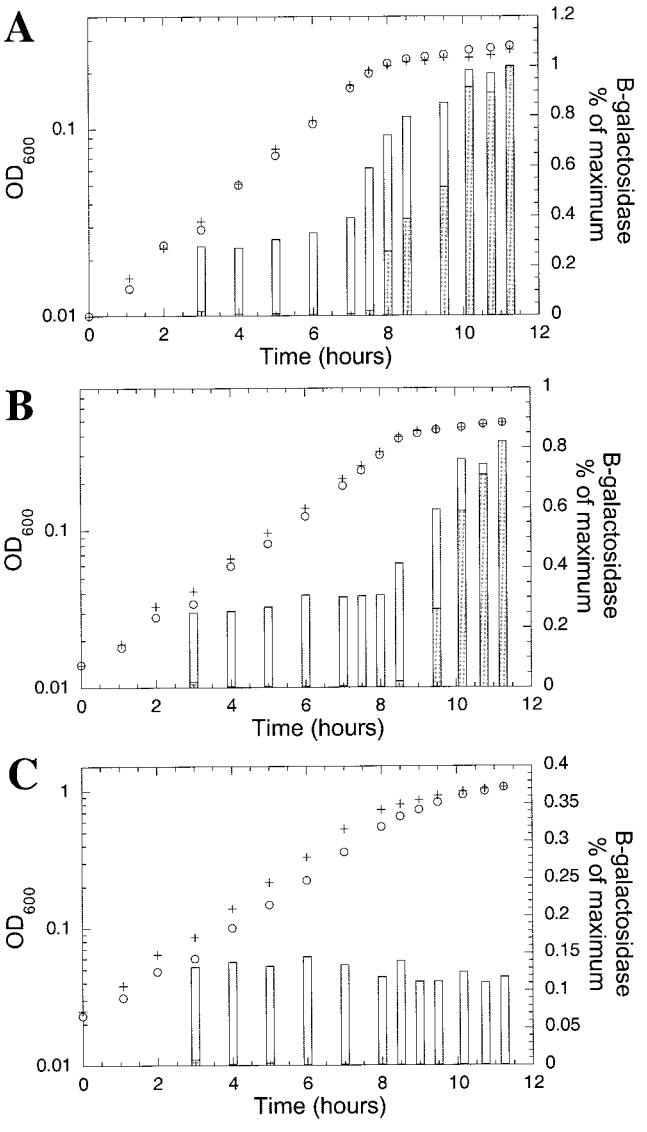

FIG. 2. Induction of *glnAp* and *glnKp* in response to nitrogen starvation. Isogenic cells, wild type except that they contained either *trp*::Φ(*glnAp-lacZ*) (YMC10ApΦ2; +) or *trp*::Φ(*glnKp-lacZ*) (YMC10Φ; ○) were grown at 30°C in defined media containing 0.004% tryptophan and 0.4% glucose with either 0.005 (A), 0.01 (B), or 0.2% (C) ammonium sulfate. At the indicated times, samples were removed and assayed for β-galactosidase. Symbols: + and o, growth (optical density at 600 nm [OD_{600}]); bars, β-galactosidase expression (white bars, YMC10ApΦ2; grey bars, YMC10Φ). Maximum expression was 2,640 Miller units for YMC10ApΦ2 and 1,680 Miller units for YMC10Φ.

We directly examined the intracellular concentration of NRI as cells transitioned from growth on ammonia to nitrogen starvation in immunoblotting experiments (Fig. 4). Reitzer and Magasanik had previously shown that the level of NRI in log-phase cells grown on nitrogen-limiting glucose-glutamine medium was ~10-fold higher than that in cells grown on nitrogen-rich glucose-ammonia-glutamine medium (20). We ob-

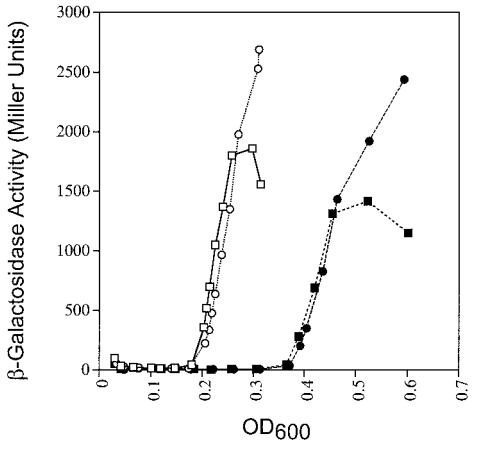

FIG. 3. Comparison of the activation of *glnKp* and *nacp* in response to nitrogen starvation. Isogenic cells, wild type except that they contained either *trp*::Φ(*glnKp-lacZ*) (YMC10Φ; ○ and ●) or *trp*::Φ(*nacp-lacZ*) (YMC10NpΦ; □ and ■) were grown at 30°C in defined media containing 0.004% tryptophan and 0.4% glucose with either 0.005 (○ and □) or 0.01% (● and ■) ammonium sulfate. The growth curves in both experiments for the two different strains were identical. At the indicated culture densities, samples were removed and assayed for β-galactosidase. OD_{600}, optical density at 600 nm.

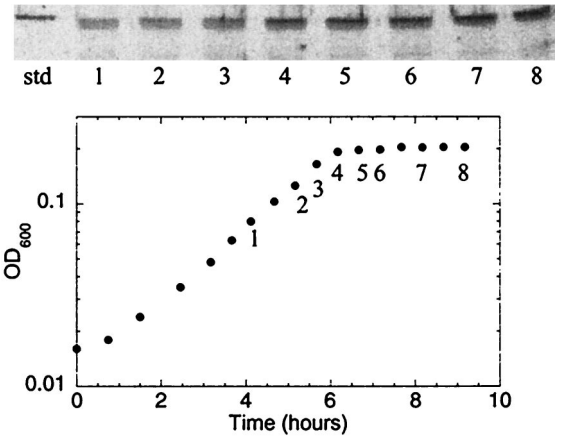

FIG. 4. Immunoblotting analysis of the NRI concentration during growth on ammonia and the transition to nitrogen starvation. Strain YMC10 (wild type) was grown at 30°C on defined medium containing 0.4% glucose and 0.005% ammonium sulfate. Samples were harvested for Western blot analysis at the indicated times. The standard lane (std) contains 6 ng of purified NRI. Each sample lane contains 5 μg of crude protein extract. OD_{600}, optical density at 600 nm.

served that a low and fairly constant level of NRI was present in cells growing on ammonia (Fig. 4). As the ammonia became depleted and cell growth stopped, the concentration of NRI increased (Fig. 4). The increase in NRI concentration occurred concurrently with the activation of *glnK* and *nac* (compare Fig. 2 and 4).

Activation of *glnAp*, *glnKp*, and *nacp* as cells grow on glutamine as the sole nitrogen source. Most studies of Ntr gene activation are focused on the expression of genes in log-phase cultures growing on glutamine as the sole nitrogen source. Here, we examined *lac* fusion expression as adapted cells grow on glutamine, providing a view of *glnA*, *glnK*, and *nac* regulation under these conditions (Fig. 5). When cells grew on glutamine, a reduction in the rate of growth occurred in mid-log phase, at an optical density at 600 nm (OD_{600}) of ~0.5 (Fig. 5B). Prior to this reduction in growth, fusion expression was similar to that in cells growing on ammonia, namely, *glnA* was partially activated and *glnK* and *nac* were not activated (Fig. 5A). At the point where the growth rate was reduced, fusion expression was similar to that seen in cells that depleted a limiting ammonia concentration, namely, all three promoters were sharply activated. The sharp transition between these two states occurred in mid-log phase; thus, use of mid-log phase glutamine-adapted cultures for assessment of Ntr gene expression levels is somewhat risky.

In vitro transcription from the *glnK* promoter. Since the *glnAp2* and *nac* promoters had been examined in vitro but the *glnK* promoter had not, we examined the NRI~P dependence of transcription from this promoter by using purified components. NRI~P stimulates the isomerization of the closed promoter-polymerase complex to the open complex, competent for transcription initiation. The formation of the open complex may be assayed by examining the rate at which uninitiated complexes are formed in the presence of ATP alone or by measuring the formation of short initiated complexes in the absence of a single nucleotide. Because complexes of the latter type are very stable, their formation permits assessment of activation at promoters where the open complex is unstable (7). In our transcription assays, as before (7), we controlled the concentration of NRI~P by adding various concentrations of NRI in the presence of excess NRII (NtrB). As templates we used supercoiled plasmids containing a strong transcriptional terminator positioned downstream from the promoter of interest (7, 9, 14).

As expected, activation of *glnAp2* required a lower concentration of NRI~P than did activation of *glnKp* when single promoters were examined, as well as when both promoters were present in the same transcription reaction mixture (Fig. 6). In contrast, two templates containing the *glnAp2* promoter positioned different distances from the transcriptional terminator were simultaneously activated as the concentration of NRI~P was increased (Fig. 6). The relative behaviors of the promoters in the in vitro transcription system were the same regardless of whether open complexes (Fig. 6A) or initiated complexes (Fig. 6B) were assayed; thus, the open complexes formed at these two promoters may have similar stabilities. The open complex at the *glnK* promoter seemed to be considerably more stable than the corresponding open complex at the *nac* promoter since we could see evidence of their formation in experiments where heparin challenge preceded initiation (7) (Fig. 6B).

Patterns of growth on the combination of ammonia and arginine. Arginine can serve as the sole nitrogen source for *E. coli*, supporting a doubling time of ~4 h in defined medium with excess glucose. Growth on arginine requires the presence of NRI, as the *astCADBE* operon is part of the Ntr regulon (16, 23). We examined the growth of *E. coli* when both ammonia at low concentration and arginine at high concentration were provided. The rate of growth on ammonia plus arginine was indistinguishable from that observed with ammonia alone until all the ammonia was consumed. At that point, the cells began

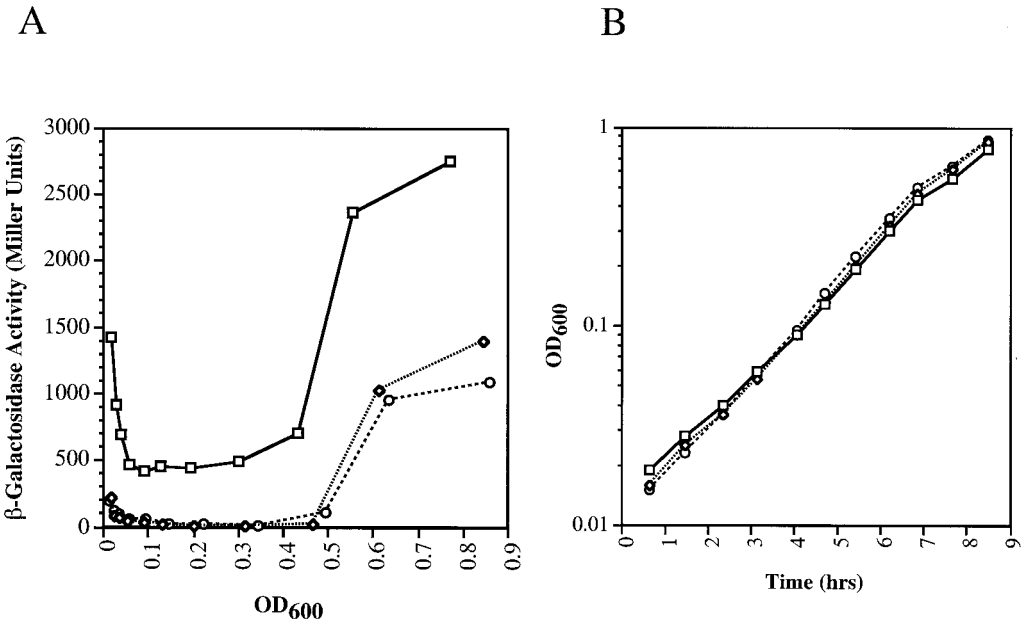

FIG. 5. Activation of *glnA*, *glnK*, and *nac* promoter fusions in cells growing on glutamine as the sole nitrogen source. Overnight cultures were grown in 0.4% glucose–0.2% glutamine to stationary phase. Cells were diluted into similar medium, except that it contained 0.1% (wt/vol) glutamine, and incubated at 30°C. (A) Expression of β-galactosidase. Symbols: □, *glnA-lacZYA*; ◇, *glnKp-lacZYA*; ○, *nacp-lacZYA*. (B) Growth of the three cultures. Symbols are as in panel A.

growing at the rate characteristic of cells using only arginine. In some cases, a short lag was detected. Thus, the pattern of growth on the mixture of arginine and ammonia was diauxic (Fig. 7).

DISCUSSION

The Ntr regulon has some resemblance to a developmental gene cascade, in that genes are sequentially activated in re-

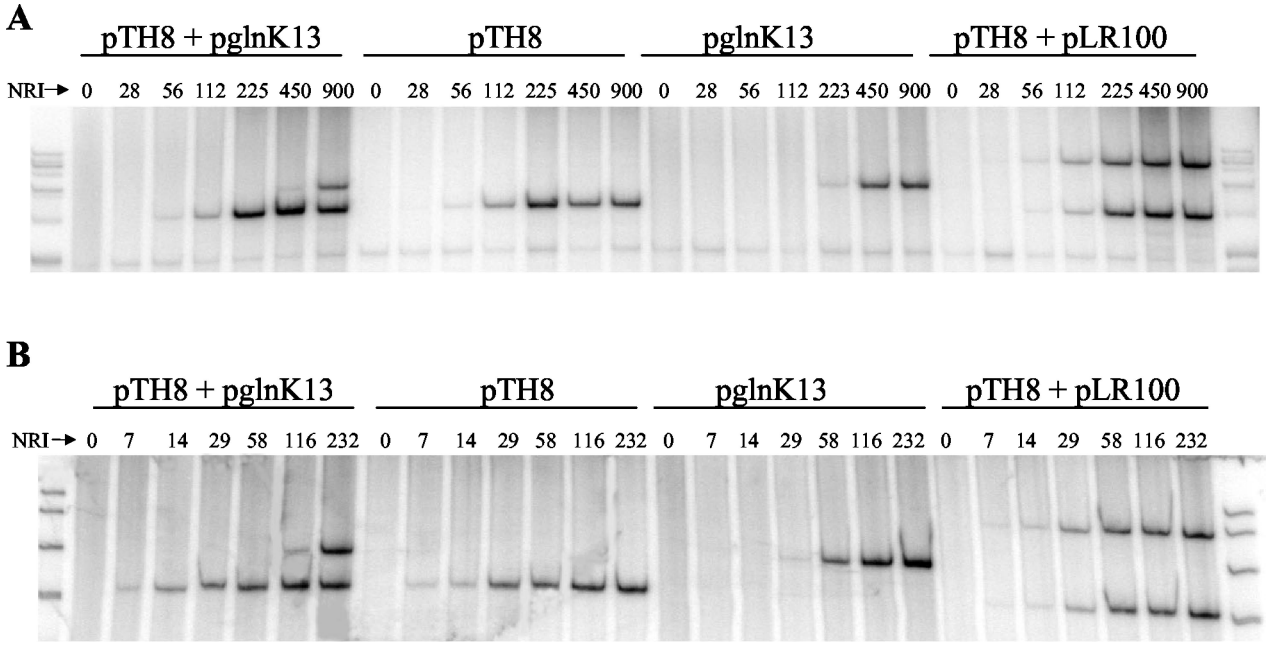

FIG. 6. Transcription from *glnAp2* and *glnKp* by purified components of *E. coli*. Transcription reaction mixtures contained the indicated supercoiled templates (10 nM each), core RNA polymerase (100 nM), σ^{54} (200 nM), NRII (100 nM), and the indicated concentration of NRI (nanomolar units). Reaction mixtures were incubated in the presence of ATP for the formation of open complexes (A) or in the presence of ATP, CTP, and GTP for the formation of short initiated complexes (B). Complex formation was stopped by addition of heparin, complexes were extended by addition of the missing nucleotide(s), and transcripts were recovered by phenol extraction and ethanol precipitation, subjected to electrophoresis on sequencing gels, and detected by autoradiography as described previously (7). Transcripts were labeled by use of [α-^{32}P]UTP. Templates pTH8 and pLR100 contained the *glnA* promoter positioned different distances upstream from the phage T7 terminator in the pTE103 vector. Template pglnK13 contained the *glnK* promoter positioned upstream from the phage T7 terminator in the pTE103 vector.

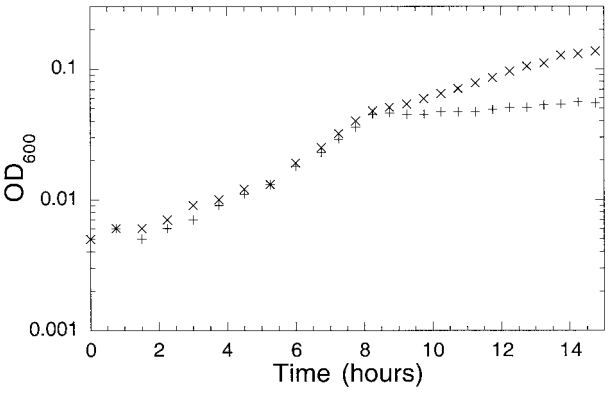

FIG. 7. Growth of *E. coli* on limiting ammonium sulfate and excess arginine is diauxic. Growth of YMC10 (wild type) at 30°C on defined minimal medium containing 0.4% glucose and 0.001% ammonium sulfate (+) or 0.001% ammonium sulfate and 0.2% arginine (×). OD_{600}, optical density at 600 nm.

sponse to an environmental stimulus. Our results support the hypothesis that, in the Ntr system, temporal staging of gene expression results from amplitude modulation of the phosphorylated form of the enhancer-binding activator protein. This amplitude modulation gives rise to the sequential activation of transcription because of the distinct features of the enhancers and promoter architecture at the various regulated promoters.

In cells growing on ammonia, the *glnAp* promoter was partially activated, while the *glnK* and *nac* promoters were not. This suggests that, when cells grew on defined glucose-ammonia medium, the level of NRI~P was sufficient to permit significant expression of *glnA* while *glnK* and *nac* remained silent. The modest (approximately threefold) regulation of *glnA* expression by ammonia concentration that we observed probably reflects the fine regulation of the NRI~P level when it is at the low end of its physiological range. Our immunoblotting analysis of NRI did not detect a significant increase in NRI as the ammonia concentration of the medium was reduced by consumption. The fairly uniform growth rate of bacteria irrespective of ammonia concentration suggests that this fine control of NRI~P at the low end of its physiological range and the attendant fine control of GS expression and activity enable cells to grow optimally without recourse to activation of the other Ntr genes.

The *glnK* and *nac* promoters became strongly activated when cells stopped growing due to ammonia starvation, suggesting that at this point the NRI~P concentration was significantly increased. Our immunoblotting analysis confirmed that NRI concentration was increased as cells became starved. Experiments examining the use of arginine as a nitrogen source suggested that the *astCADBE* operon (16, 23) was not expressed when cells had ammonia available but rather was only activated as ammonia became depleted. Thus, the *glnK*, *nac*, and *astC* promoters define a group of promoters that are regulated differently from *glnAp*.

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