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Metabolic Context and Possible Physiological Themes of σ^{54}-Dependent Genes in *Escherichia coli*

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INTRODUCTION AND OVERVIEW

Scope

The dissociable sigma subunits of RNA polymerase are responsible for specific binding to DNA and are therefore important determinants of differential gene expression (68, 170). σ^{54} was discovered during an analysis of glutamine synthetase and nitrogen assimilation in enteric bacteria (71, 77). Subsequent studies have confirmed its role in nitrogen assimilation but have also shown that it is involved in a variety of seemingly unrelated functions, such as carbon source utilization, certain fermentation pathways, flagellar synthesis, and bacterial virulence (97, 151). There have been several reviews about σ^{54} and σ^{54}-dependent activation, including recent ones (14, 28, 97, 110, 113, 148, 151). However, discussions of σ^{54} function usually consider genes from several organisms and therefore remove σ^{54} from the metabolic or physiological context of a single organism. The primary purpose of this review is to redress this imbalance and to discuss the functions of the known σ^{54}-dependent genes from a single organism, *Escherichia coli*. The most meaningful discussion of σ^{54} function requires a complete set of σ^{54}-dependent genes. Two sources provide information on likely σ^{54}-dependent promoters: a recent DNA microarray analysis of transcripts present during nitrogen-limited growth (176) and computer analysis of potential σ^{54}-dependent promoters, which are readily identified from the completed nucleotide sequence of the *E. coli* genome (23). Results of these analyses are integrated into the discussion of the function of the known σ^{54}-dependent genes. The central thesis of this review is that the σ^{54}-dependent genes of *E. coli* have only a few metabolic themes and that these themes may be related.

Sigma Subunits and Their Function in *E. coli*

E. coli has seven σ subunits, and each has a distinct function. σ^{70} is considered the primary sigma factor. Core RNA polymerase (E) associated with σ^{70} initiates transcription of housekeeping genes (68). $E\sigma^{70}$ also initiates the transcription of nonessential genes that are induced in specialized environments. σ^{S} has been called either an alternative sigma factor or a second primary sigma factor (69). Although $E\sigma^{S}$ binds the same sequences as $E\sigma^{70}$ (unpublished results cited in reference 69), σ^{S} is considered a general stress factor since it is associated with a variety of growth-impairing stresses: nutrient depletion, oxidative stress, high temperature, high osmolarity, acidic pH, or exposure to ethanol (69). σ^{32} and σ^{E} are also associated with stress. σ^{32} is required for the response to damage of cytoplasmic proteins, which is most commonly associated with heat shock, and σ^{E} controls the response to extracytoplasmic or extreme heat stress (174). σ^{FecI} and σ^{28} (FliA) are required for synthesis of the ferric citrate transporter and flagella, respectively (8, 115). As mentioned above, σ^{54} is usually associated with nitrogen assimilation.

Unique Features of σ54-Dependent Transcription

The σ factors in *E. coli* are homologous to σ^{70}, except for σ^{54} (110). Not surprisingly, σ^{54}-dependent transcription has several distinctive features (reviewed in reference 28). Core RNA polymerase (E) complexed to a σ^{70}-like factor can be sufficient for open promoter complex formation. In contrast, $E\sigma^{54}$ catalyzes strand separation only with the help of a distinct class of transcriptional activators. A consequence of this property is that transcription can be completely turned off. Such absolute control may account for the evolutionary persistence of σ^{54} (see "Physiological function of σ^{54}" below).

The activators of $E\sigma^{54}$-dependent genes are unusual. (The individual *E. coli* activators are discussed in a separate section.) Unlike most eubacterial transcriptional activators, the σ^{54}-dependent activators bind to sites that are effective regardless of distance and orientation (28, 29, 131). In this respect, the activator binding sites are analogous to eukaryotic enhancers, and the activators are often called enhancer binding proteins. However, the analogy to eukaryotic enhancer binding proteins is not precise, since the bacterial proteins do not enhance basal transcription but, instead, are absolutely required for any transcription. The activators interact with $E\sigma^{54}$ from these binding sites. This interaction sometimes requires a DNA bending protein. The DNA bending proteins that participate in σ^{54}-dependent gene expression in *E. coli* are integration host factor (IHF) and ArgR, the arginine repressor (72, 103). When transcription from a σ^{54}-dependent promoter does not require a DNA bending protein, DNA curvature facilitates the interaction between the activator and RNA polymerase (32). Another distinctive feature of σ^{54}-dependent activators is an essential ATPase activity (166).

Control of σ54-Dependent Promoters

The most important control of σ^{54}-dependent genes is through modulation of the activator's ATPase activity. The σ^{54}-dependent activators usually contain a regulatory domain that controls ATPase activity. Several mechanisms control the interaction of the regulatory domain with the ATP binding domain: phosphorylation, interaction with a low-molecular-weight ligand, or interaction with one or several regulatory proteins (148). Some control mechanisms are extremely com-

TABLE 1. *E. coli* operons with confirmed σ^{54}-dependent promoters

Operon	Regulator	Function	Promoter sequence[a]
argT-hisJQMP	NR_I-NR_{II}	Arginine, histidine transport	CAAGA **TGGC**A TAAGA CCTGC ATGAA AGAG
astCADBE	NR_I-NR_{II}-ArgR	Arginine catabolism	CTGGC **TGGC**A CGAAC CCTGC AATCT ACAT
atoDAEB	AtoC-AtoS	Acetoacetate catabolism	CATTC **TGGC**A CTCCC CTTGC TATTG CCTG
fdhF	FhlA	FDH	AAATG **TGGC**A TAAAA GATGC ATACT GTAG
glnALG	NR_I-NR_{II}	Nitrogen assimilation	AAAGT **TGGC**A CAGAT TTCGC TTTAT CTTT
glnHPQ	NR_I-NR_{II}	Glutamine transport	AAAAC **TGGC**A CGATT TTTTC ATATA TGTG
glnK-amtB	NR_I-NR_{II}	P_{II} paralog, NH_3 transport	ATTTC **TGGC**A CACCG CTTGC AATAC CTTC
hycABCDEFGHI	FhlA	Hydrogenase	AAAGT **TGGC**A CAAAA AATGC TTAAA GCTG
hydN-hypF	FhlA	Hydrogenase	AAAGA **TGGC**A TGATT TCTGC TGTCA GAAA
hypABCDE-fhlA	FhlA	Hydrogenase	AACAC **TGGC**A CAATT ATTGC TTGTA GCTG
nac	NR_I-NR_{II}	Nitrogen assimilation	AAAAC **TGGC**A AGCAT CTTGC AATCT GGTT
prpBCDE	PrpR	Propionate catabolism	AATTG **TGGC**A CACCC CTTGC TTTGT CTTT
pspABCDE	PspF	Phage shock and other stresses	AAAAT **TGGC**A CGCAA ATTGT ATTAA CAGT
rtcBA	RtcR	RNA terminal cyclase	TTTTC **TGGC**A CGACG GTTGC AATTA TCAG
ygjG	NR_I-NR_{II}?	Uncharacterized transaminase	CGGAG **TGGC**G CAATC CCTGC AATAC TTAA
zraP	ZraR-ZraS	Zinc tolerance	ATCGT **TGGC**A CGGAA GATGC AATAC CCGA
zraSR (hydGH)	ZraR-ZraS	Regulators of zinc tolerance	AAAGA **TGGC**A TGATT TCTGC TGTCA CCGA

[a] Residues that are conserved 100% are shown in bold type.

plex, such as that for PspF (discussed below). Variations in σ^{54} activity, either by ligand binding or by covalent modification, are not known in *E. coli*. Furthermore, the intracellular level of σ^{54} is apparently constant (87), and expression of *rpoN*, which specifies σ^{54}, appears to be constitutive (33). Strain W3110 contains about 700 molecules of σ^{70} per cell and 110 molecules of σ^{54}, whereas strain MC4100 may contain only about 285 molecules of σ^{70} and as few as 13 to 22 molecules of σ^{54} (87). The low level of σ^{54} could have regulatory implications. For example, it is possible that different σ^{54}-dependent operons compete for limiting σ^{54}. This possibility is even more plausible if there is some physiological relationship between the σ^{54}-dependent genes. One purpose of this review is to explore this issue.

COMMON FEATURES OF σ^{54}-DEPENDENT PROMOTERS

There are 11 σ^{54}-dependent promoters for which the transcription start site has been determined in vivo, in vitro, or both. These promoters precede *astCADBE*, *fdhF*, *glnALG*, *glnHPQ*, *hycABCDEFGHI*, *hydN-hypF*, *hypABCDE-fhlA*, *prpBCDE*, *pspABCDE*, *rtcBA*, and *ygjG*. It is virtually a certainty that *atoDAEB*, *glnK-amtB*, *nac*, *zraP*, and *zraSR (hydHG)* have a σ^{54}-dependent promoter, since their expression absolutely requires σ^{54} and their promoter regions contain an easily recognizable site for Eσ^{54}. Also, *argT-hisJQMP* has a verified σ^{54}-dependent promoter in *Salmonella enterica* serovar Typhimurium, and nitrogen limitation induces these genes in *E. coli*, which suggests that this operon possesses a σ^{54}-dependent promoter.

We will use the promoters for these 17 operons to characterize the σ^{54}-dependent promoters in *E. coli* (Table 1). These promoters suggest an apparent consensus of aaN_3TGGC AcN_6TGCNNt, where small letters indicate two to five mismatches, capital letters denote zero or one mismatch, and underlined bases have no mismatches. By similar criteria, the consensus derived from 186 promoters from a variety of organisms is N_5tGGcacN_5ttGC (14), which is similar but not identical to the apparent *E. coli* consensus. Figure 1 shows the locations of the σ^{54}-dependent operons within the context of neighboring genes, the size of the transcripts, and the direction of expression. Figure 2 shows the binding sites for Eσ^{54} in relation to the binding sites for the σ^{54}-dependent activators (when known) and the nearest upstream structural gene.

Several generalizations can be made concerning these promoters. First, all the known σ^{54}-dependent promoters are located outside the structural genes. This does not necessarily mean that an authentic binding site for Eσ^{54} will not be found within a gene, but it does mean that such binding sites will be the exception. Second, the activator binding sites are also outside the structural genes. The most spectacular example is the FhlA binding site for the *hyp* operon in the *hyp-hyc* regulatory region. The entire *hycA* gene is located between the binding sites for FhlA and RNA polymerase (Fig. 2). Even though the FhlA binding site for the *hyp* operon is within the *hyc* operon, it is not within a structural gene. Instead, it is completely located within the 130-base intergenic region between *hycA* and *hycB*. Third, the activator binding sites are almost always a significant distance from the adjacent upstream gene and therefore do not apparently interfere with expression of these genes (Fig. 2). The only exception is the binding site for PspF, which activates the *pspABCDE* operon. The PspF sites for activation overlap with the promoter for the *pspF* gene (Fig. 2), and it has been proposed that this has physiological significance (see "*psp* operon and phage shock response" below). The average size of the intergenic region that contains a known σ^{54}-dependent promoter is 267 $\pm$ 106 bases, with a range from 148 to 507 bases. This distance is apparently large enough for binding sites for both Eσ^{54} and an activator. Fourth, the distance from the 3′ end of the Eσ^{54} binding site to the nucleotides coding for the initiation codon is, on average, 50 bases. Such a short distance reduces the potential for RNA secondary structures or protein binding regions near the translational start site and therefore reduces the potential for translational control. Finally, the average A+T content of the 50 bases just upstream from the Eσ^{54} binding site is 70%, and no upstream region has an AT content less than 50%. Two rationales for such a bias can be suggested. Some σ^{54}-dependent promoters

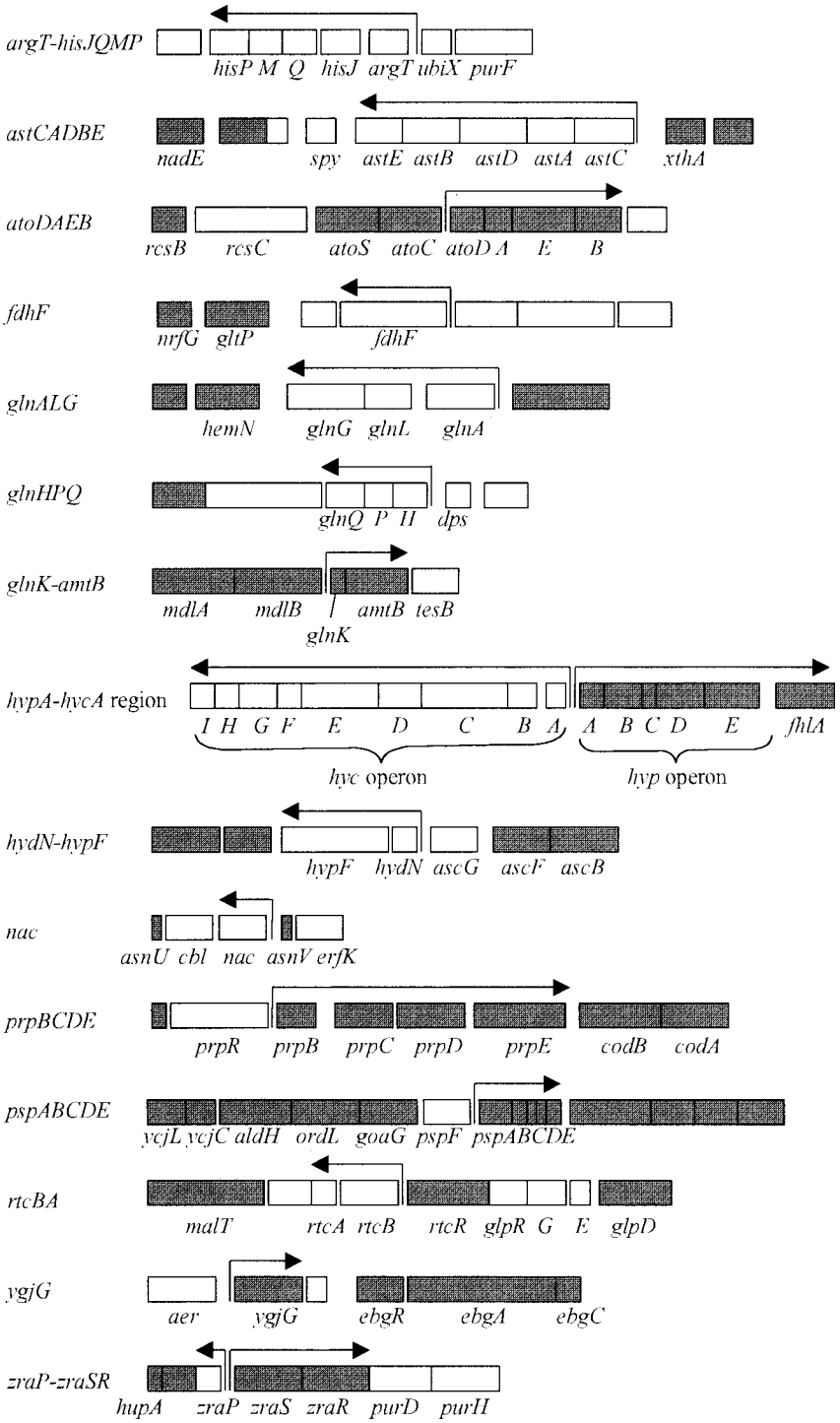

FIG. 1. σ^{54}-dependent genes in *E. coli*. The known σ^{54}-dependent operons and their transcripts are shown in the context of neighboring genes. The open and solid boxes indicate counterclockwise and clockwise transcription, respectively. If the gene has been assigned a function, this is indicated by the gene name underneath. Boxes without gene names indicate that the gene has an unknown function. The sizes of the genes and their intergenic regions are to scale.

require a DNA bending protein. Both of the DNA bending proteins that facilitate the activation of σ^{54}-dependent genes in *E. coli*, IHF and ArgR, have an AT-rich consensus sequence. Alternately, σ^{54}-dependent promoters that do not require a DNA bending protein often have an intrinsic bend between the activator and RNA polymerase binding sites (32), and this may favor AT-rich regions. In either case, the requirement for long-range protein-protein interactions appears to bias the base composition of DNA just upstream from the $E\sigma^{54}$ binding site.

Despite the reasonably uniform properties of the known

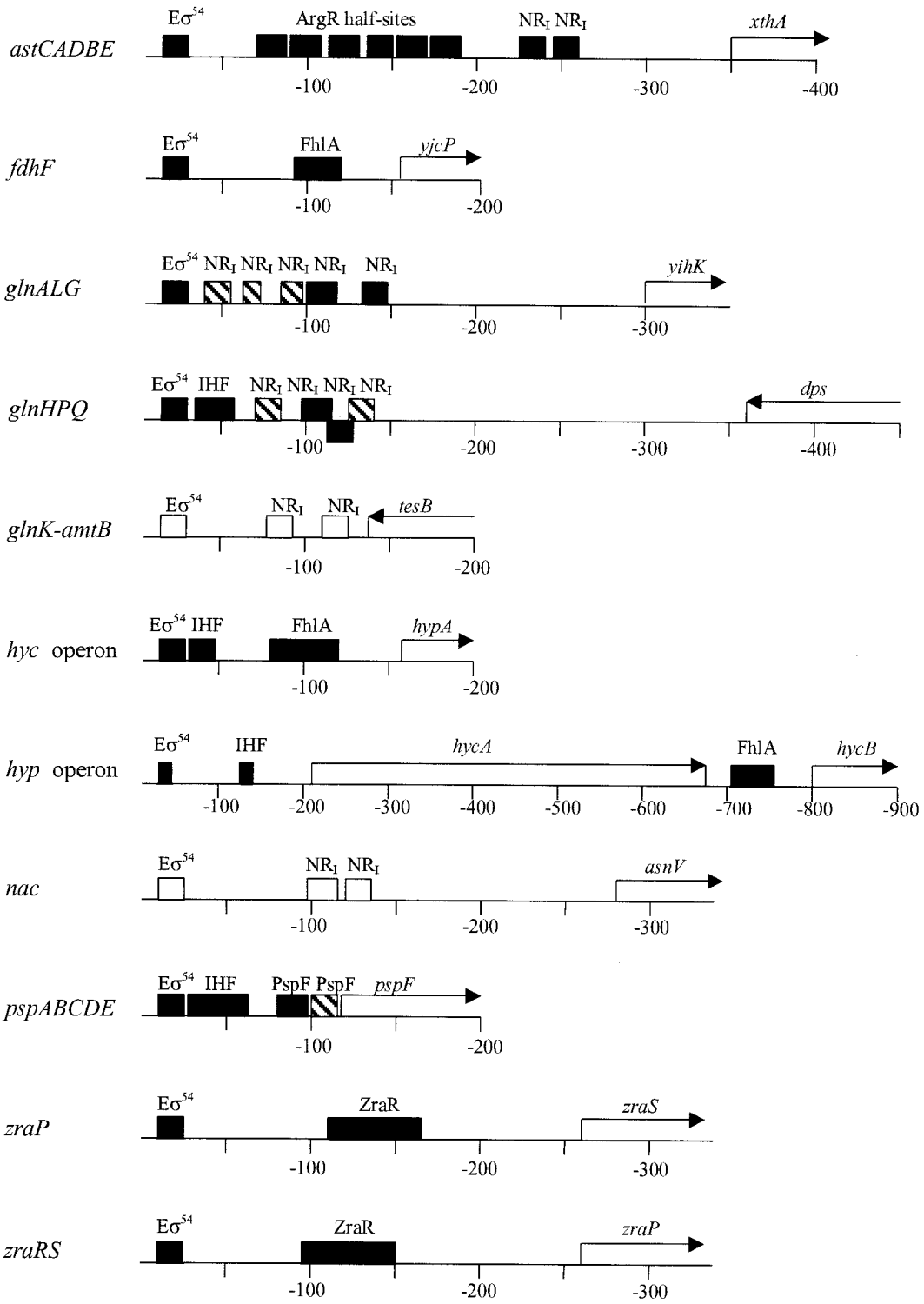

FIG. 2. Regulatory regions of the characterized σ^{54}-dependent genes. The following features are shown for each of the σ^{54}-dependent promoters: binding sites for activators, DNA bending proteins (when required), and RNA polymerase. Solid boxes indicate that the binding site has been demonstrated. A hatched box indicates a confirmed binding site, but binding is weak. An open box signifies a proposed binding site. The relative location and orientation of the nearest upstream gene are also shown. All diagrams are to the same scale, except for the *hyp* operon.

σ^{54}-dependent promoters, there is little useful information for promoter prediction. For uncharacterized promoters, the activator binding sites will probably not be known, and there may be uncertainty in the size of intergenic regions if the protein coding regions have been misidentified (which is not uncommon). A more useful predictor of potential promoters is based on computer identification of binding sites for $E\sigma^{54}$, which is described in the next section.

TABLE 2. Comparison of consensus sequence for σ^{54}-dependent promoters from known *E. coli* promoters and the SeqScan program[a]

Source	Sequence
E. coli promoters	A_{71} A_{71} NNN **T_{100}** **G_{100}** **G_{100}** **C_{100}** **A_{94}** C_{71} **$(A/G)_{94}$** NNNN T_{52} **T_{94}** **G_{94}** **C_{94}** **$(A/T)_{100}$** $(A/T)_{88}$ T_{71}
SeqScan program	**T_{90}** **G_{100}** **G_{100}** C_{81} A_{84} C_{70} $(A/G)_{87}$ NNNN T_{72} T_{86} **G_{99}** **C_{90}** $(A/T)_{86}$

[a] A base-by-base comparison of the frequency distributions of the consensus sequences is shown. N indicates any base. Residues with greater than 90% conservation are shown in bold type.

COMPUTER IDENTIFICATION OF POTENTIAL σ54-DEPENDENT PROMOTERS

Site Identification and the Problem of False Positives

The most meaningful analysis of the physiological function of σ^{54} requires a comprehensive set of σ^{54}-dependent genes. One method to help determine the complete set of such promoters is a computer analysis of potential σ^{54}-dependent promoters from the completed *E. coli* genome sequence. We used the SeqScan program (B. T. Nixon, Department of Biochemistry and Molecular Biology, Pennsylvania State University [http://www.bmb.psu.edu/seqscan]) for this analysis. This program uses 86 σ^{54}-dependent promoters from several organisms to define a consensus sequence that does not differ substantially from that derived from the 17 known *E. coli* promoters (Table 2). The program uses a weighted matrix to give sites a score from 0 to 100, and then reports sites with a score higher than 60.

The SeqScan program identified approximately 8,000 potential σ^{54}-dependent promoters from the *E. coli* genome, or about 1 for every 600 bases. Clearly, there are a number of false positives. We can use the properties of the known σ^{54}-dependent promoters to eliminate many of these false positives. We eliminated all the sites within structural genes, which reduced the number of potential sites over 30-fold. The 17 known σ^{54}-dependent promoters were in the set of 213 intergenic sites in which the potential promoter transcribed a gene in the correct direction. We found that 121 potential intergenic $E\sigma^{54}$ binding sites transcribe genes in the wrong orientation. Table 3 and its footnotes list all of the intergenic sites that could potentially transcribe a gene in the correct direction.

Predictive Value of the Promoter Scores

The distribution of scores for properly and improperly oriented sites is shown in Fig. 3. There are only 2 incorrectly oriented sites (1.6%), and there are 25 correctly oriented sites (11.7%) with a score of at least 76. Of the 25 high-scoring correctly oriented sites, 18 (72%) are known σ^{54}-dependent promoters or are induced by nitrogen limitation. Together, these results imply that properly oriented high-scoring sites are not common and are likely to contain an authentic σ^{54}-dependent promoter. In contrast, only 7 (3%) of 213 sites with a score lower than 76 are known or likely (i.e., nitrogen limitation-induced) σ^{54}-dependent promoters. Therefore, scores below 76 are a reasonably good, but not infallible, indicator that the site is not a promoter. Despite the correlation with promoter scores, the promoter ranking is not an exact indicator of promoter strength. For example, the *nac* promoter has a higher score than the σ^{54}-dependent promoter of the *glnALG* operon, even though the latter is stronger (54). Nonetheless, and this point cannot be emphasized too much, the computer program recognizes all known $E\sigma^{54}$ binding sites, which implies that the failure to detect such a site is a reliable indicator that such a site does not exist.

Estimating the Number of σ54-Dependent Promoters in *E. coli*

There are 17 σ^{54}-dependent promoters which either have been verified by direct evidence or whose expression requires σ^{54}; there are 7 other operons which are induced by nitrogen limitation (often associated with σ^{54}), appear to require a σ^{54}-dependent activator, and for which computer analysis suggests the presence of an appropriately located sequence for a σ^{54}-dependent promoter: b1012-b1006, *chaC*, *ddpXABCDE*, *gltIJKL*, *potFGHI*, *yeaGH*, and *yhdWXYZ*. Assuming that all of these genes have a functional σ^{54}-dependent promoter (which is unlikely) and that a few have been missed for various reasons (e.g., misidentified open reading frames), we estimate that *E. coli* contains about 30 σ^{54}-dependent promoters.

σ54-DEPENDENT GENES OF NITROGEN METABOLISM

Of the 17 known σ^{54}-dependent promoters, 7 are involved in nitrogen metabolism. In addition, microarray analysis has identified 7 other genes that are induced by nitrogen limitation (176), and these have an appropriately placed potential σ^{54}-dependent promoter. This section discusses the 14 operons that are involved in nitrogen metabolism and the functions of their products. To understand the metabolic context of these proteins, it is first necessary to discuss nitrogen assimilation and the response to nitrogen deprivation. Because there have recently been some major changes in our understanding of these topics, the next sections summarize our current knowledge.

Nitrogen Assimilation and Its Control

Nitrogen assimilation. Glutamate and glutamine are the major intracellular nitrogen donors, and they provide about 75 and 25% of the cell's nitrogen, respectively (calculated from numbers presented in reference 116). Nitrogen assimilation must therefore result in the synthesis of these two nitrogen donors.

Ammonia can be considered the focal point of nitrogen assimilation. There are two routes of ammonia assimilation (Fig. 4). For the first pathway, glutamate dehydrogenase assimilates ammonia and synthesizes glutamate. For the second pathway, glutamine synthetase (GS) assimilates ammonia, and glutamate synthase synthesizes glutamate. The former pathway is often associated with the presence of ammonia, and the latter pathway is associated with low ammonia levels or growth with a nitrogen source other than ammonia, since the K_m for ammonia for glutamate dehydrogenase is about 20-fold higher

TABLE 3. Computer ranking of intergenic σ^{54} sites[a]

Type[b]	Score	Gene	Ntr induction[c]	Distance[d]		Function
				Down	Up	
X	95.4	*rtcBA*	—	38	134p	RNA terminal phosphate cyclase
	92.8	*rpoH*	—	40	188t	Heat shock sigma factor
X	90.2	*glnK-amtB*	10–20	52	112t	Alternate Ntr regulator and ammonia transport
X	89.6	*prpBCDE*	—	46	176p	Propionate catabolism
(X)	88.6	*yhdWXYZ*	2–9	187	334t	Putative amino acid transport operon
X	88.6	*zraP*	2	34	196p	Zinc tolerance
(X)	88.0	*gltlJKL*	3–6	?	99t	Interrupted glutamate-aspartate transport genes
X	88.0	*hypABCDE-fhlA*	—	37	165p	Formate hydrogen lyase
	86.0	b2710-*ygbD*	—	47	48p	Flavodoxin, oxidoreductase
X	85.9	*atoDAEB*	?	45	134t	Acetoacetate metabolism
X	85.5	*astCADBE*	7–11	72	357p	Arginine degradation
	85.4	*kch*	—	264	20t	Potassium channel
X	85.2	*nac*	15–50	55	255p	Regulator of nitrogen assimilation genes
	83.9	*yfhKGA*	—	190	288t	Two-component regulatory system
(X)	83.8	*yeaGH*	2–4	103	316p	Possible operon, unknown function
X	83.3	*glnALG*	11–24	83	273p	Nitrogen assimilation and regulation of Ntr response
X	80.7	*glnHPQ*	5–9	54	333t	Glutamine transport
X	80.0	*pspABCDE*	1.5–2.5	51	84p	Phage shock
X	79.1	*hycABCDEFGHI*	—	36	159p	Formate hydrogen lyase
	78.8	*ybhK*	—	55	325p	RocR-like protein
	77.3	*yaiS*	—	33	656p	Hypothetical protein, 136 residues
(X)	76.8	b1012-1006	24–47	28	201p	Possible pyrimidine catabolic operon
(X)	76.6	*ddpXABCDE*	52–60	44	198t	D-Ala–D-Ala dipeptide transport and dipeptidase
	76.5	*topA*	—	201	162p	Topoisomerase I
X	76.2	*zraSR (hydHG)*	—	40	190p	Two-component regulatory system for zinc tolerance
X	72.6	*fdhF*	—	51	130t	Formate hydrogen lyase
X	72.3	*argT-hisJQMP*	10–18	68	181t	Arginine and histidine transport
X	70.7	*ygjG*	3–6	136	355p	An omega-transaminase
(X)	68.1	*chaC*	4–5	74	46t	Ca/H antiporter, in *chaBC* intercistronic region?
X	64.5	*hydN-hypF*	—	37	95t	Formate hydrogen lyase
(X)	63.9	*potFGHI*	5–6	59	275t	Putrescine transport
(X)	62.2	*ycjJ*	3	192	39t	Putative amino acid transporter

[a] There are over 200 sites with a computer score over 60. This table provides details of all sites with (i) verified σ^{54}-dependent promoters, (ii) potential sites before genes that are induced by nitrogen limitation, and (iii) sites with a score of 76 or higher (there are only seven such sites that fail to meet either criterion a or b). These criteria might exclude some authentic σ^{54}-dependent promoters. The following is a list of all the other intergenic sites. The following genes have sites with a score from 70.0 to 75.9 (from highest to lowest): *yigL*, *ygeW*, *b2343*, *b2878*, *yjcB-vacB*, *ytfJ*, *crl*, *acrD*, *ptrA*, *clpPX*, *mutH*, *csgBA*, *b2878*, *pyrG*, *sohA*, *yehR*, *rmf*, *mscL*, *mdoB*, *gcvTHP*, *intF*, and *yfaO*. The following sites have a ranking between 65.0 and 69.9: b1983, *b2670*, *yhcC*, *yabI*, *malK-lamB-malM*, *hyfABCDEFGHIR*, *sgcCQ*, *yehZYXW*, *yejH*, *metZ*, *yhfA*, *ybcLM*, *proP*, *purC*, *ygiF*, *b1440-1444*, *yeaQ*, *yigM*; b0833-0834, *yefM*, *gdhA*, *rfe-wzzE*, *emrD*, *cysDNC*, *gcvA*, *yhfZY*, *ychH*, *b2380-2382*, *caiF*, *xseB-ispA-dxs*, *hcaA1*, *xdhABC* (b2866-2868), *ndh*, *pgpB*, *b1722*, *tolC*, *galETKM*, *ymcC*, *degQ*, *yheB*, *b0540*, *aer*, *glyQS*, *cadCBA*, *b2374*, *arsR*, *mviN*, *yieP*, *b2420*, and *ygdP-ptsP*. The following sites have a ranking between 60.1 and 64.9: *ybgE*, *fumB*, *b0805*, *ompF*, *ygdH*, *accBC*, *yciK-btuR*, *brnQ*, *acs*, *yceC-yceF*, *purM*, *ybcLM*, *b1017*, *kbl-tdh*, *b1625*, *yjhQP*, *aroG*, *zipA*, *yiiP*, *cybC*, *yjjJ*, *dinG*, *prsA*, *ydcD*, *emrD*, *secE*, *mog*, *ybiH*, *b0836*, *ymdD*, *dnaA*, *sapABCDF*, *slyA*, *b1826*, *sanA*, *yjdE*, *intB*, *hyaABCDEF*, *yhcL*, *yjhA*, *b2444-2445*, *tktA*, *bglX*, *yafW*, *dsbB*, *yfjZ-ypjF*, *yicK*, *panD*, *ycbG*, *rplY*, *yigK*, *yafK*, *yahA*, *araE*, *dfp*, *yfcD*, *feaB*, *gnd*, *yhhI*, *ybiA*, *dicF*, *b3000-b2999*, *yhaM*, *ydiQ*, *yagA*, *yhfA*, *ilvGMEDA*, *adiY*, *yjgL*, *trpR*, *osmB*, *sgaT*, *yjhQ*, *ydiQ*, *yecH*, *upp-uraA*, *rfe-wzzE* (again), *map*, *ybiT*, *trpEDCBA*, *b1592*, *fepB*, *yrbG*, *cydA*, *ssrA*, *yhcC*, *yhgN*, *yhjR*, *metA*, *talB*, *lpxC*, *b1432*, *hisS*, *mreB*, *rrfF*, *xylB*, *pfkA*, *hslVU*, *yjhR*, *ahpF*, *b1506*, *yihG*, *sbp*, *nhaA*, *proBA*, *hrpA*, *nirC-cysG*, and *yigK*.

[b] X, the site is a verified σ^{54}-dependent promoter; (X), a probable σ^{54}-dependent promoter as suggested by computer identification of an appropriately located site and induction by nitrogen limitation.

[c] Ntr induction refers to the elevation of expression induced by nitrogen limitation as determined by microarray analysis. Dashes indicate no induction. The number indicates the range of the increase for the most highly induced gene of the operon.

[d] "Down" indicates the distance from the 3′ end of the σ^{54} site to the first base of the downstream structural gene. "Up" indicates the distance from the 5′ end of the σ^{54} site to the most proximal base of the nearest upstream gene. The notation p or t indicates whether the promoter or the terminator, respectively, of the adjacent upstream gene is nearest the σ^{54} site. The implication is that a promoter region may require a larger region. The size of the intergenic region is the sum of down plus up plus the 16 bases of the σ^{54} site.

than that for GS. However, the most important difference between the two pathways appears to be that the former does not consume ATP but the latter does. Helling has shown that the glutamate dehydrogenase pathway is physiologically advantageous during carbon- and energy-limited growth while the GS-glutamate synthase pathway is used whenever energy is readily available (66, 67). (The energy difference between the ammonia assimilation pathways can be calculated, and it is significant. A 1-g amount of *E. coli* requires the synthesis of about 57,000 μmol of ATP and contains about 10,500 μmol of nitrogen. The ATP requirement for glutamine synthesis depends on the pathway of ammonia assimilation. If glutamate dehydrogenase assimilates ammonia, the cell requires about 2,300 μmol of glutamine and a corresponding amount of ATP for its synthesis. If the GS-glutamate synthase route assimilates ammonia, glutamine is also the precursor for glutamate and the cell must synthesize an additional 8,070 μmol of glutamine and ATP, or 14% more energy.)

Control of ammonia assimilation and GS activity: role of glutamine. Ammonia assimilation involves the regulation of three enzymes. Little is known about the regulation of glutamate dehydrogenase activity or synthesis in *E. coli* (127). The leucine-responsive regulatory protein (Lrp) controls the synthesis of glutamate synthase (53). A discussion of the function

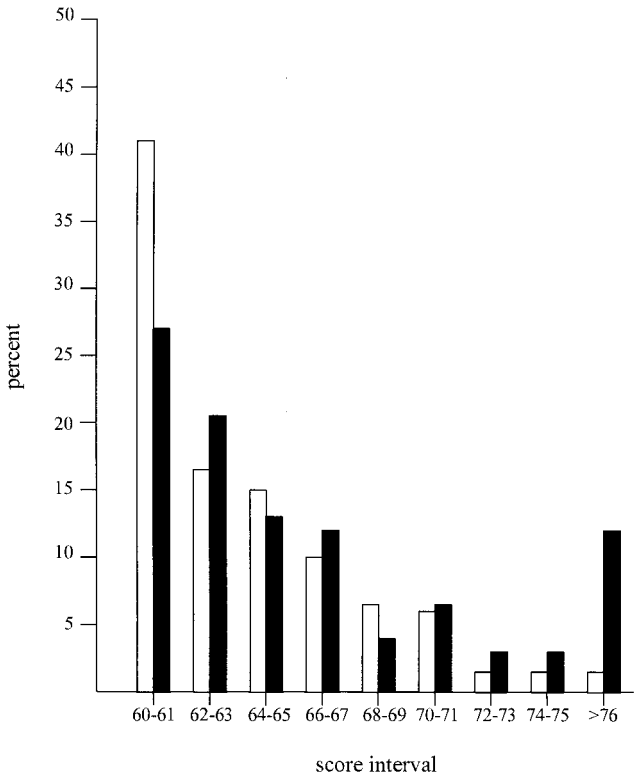

FIG. 3. Distribution of scores with properly and improperly oriented sites. The open bars indicate sites that are oriented toward the 3′ end of a gene; the solid bars indicate sites that are oriented toward the 5′ end.

of Lrp and Lrp-dependent regulation, which does not require σ^{54}, is beyond the scope of the review. Instead, we will focus on the control of GS activity.

Two different but related mechanisms control GS activity: cumulative feedback inhibition by metabolites that require glutamine for their synthesis and covalent adenylylation (127). GS is a dodecamer, and adenylylation inactivates the modified subunit and renders the remaining subunits sensitive to feedback inhibition (127). A major function of adenylylation is to determine the function of GS. When GS is highly adenylylated and subject to cumulative feedback inhibition, its primary function is glutamine synthesis. In this situation, GS is just active enough to supply glutamine but not to supply glutamate. *E. coli* requires about 2,310 μmol of glutamine for biosyntheses per g (dry weight). In contrast, unadenylylated GS, which is not subject to feedback inhibition, can assimilate enough ammonia to meet all the cell's need for organic nitrogen. In this situation, *E. coli* needs to synthesize about 10,300 μmol of glutamine per g. A second function of adenylylation is to prevent the depletion of intracellular glutamate during the transition to a nitrogen-rich environment (96).

A cascade of three proteins controls GS adenylylation: the uridylyltransferase (UTase)-uridylyl removing (UR) enzyme, which in turn controls the activities of P_{II} and adenylyltransferase (ATase). It has been a long-standing paradigm that the ratio of α-ketoglutarate to glutamine (a sensor of relative carbon-to-nitrogen sufficiency) controls UTase-UR activity and therefore GS adenylylation. This conclusion was based on the properties of partially purified UTase-UR (2), which were not confirmed with purified UTase-UR (83). Furthermore, metabolite measurements suggested that low intracellular glutamine levels might be sufficient to control the response to nitrogen limitation (discussed below), which UTase-UR also controls (79). These results suggest that glutamine is the primary effector of UTase-UR and therefore of GS adenylylation (Fig. 5). Low glutamine levels stimulate UTase activity, which uridylylates P_{II}. P_{II}-UMP interacts with adenylyltransferase, which now removes adenylyl groups from GS and activates GS activity. High glutamine (nitrogen excess) stimulates UR activity, which results in the formation of unmodified P_{II}, whose interaction with adenylyltransferase stimulates adenylylation and reduces GS specific activity. Even though α-ketoglutarate does not affect UTase-UR, it does control the activity of unmodified P_{II} (discussed below).

Nitrogen-Regulated (Ntr) Response

Nitrogen sources. Ammonia is considered the preferred nitrogen source for *E. coli* grown in a minimal medium because ammonia supports the fastest growth and its presence prevents the synthesis of several proteins of nitrogen metabolism (reviewed in references 127 and 129). In place of ammonia, *E. coli* and related organisms can utilize a small number of nitrogen sources, usually amino acids, nucleosides, nucleobases, and a few inorganic nitrogen sources, e.g., nitrite and nitrate, which are reduced to ammonia. Steady-state growth on alternate nitrogen sources is slower and is said to be nitrogen limited. Catabolism of the alternate nitrogen sources must produce ammonia for the synthesis of glutamine, one of the intracellu-

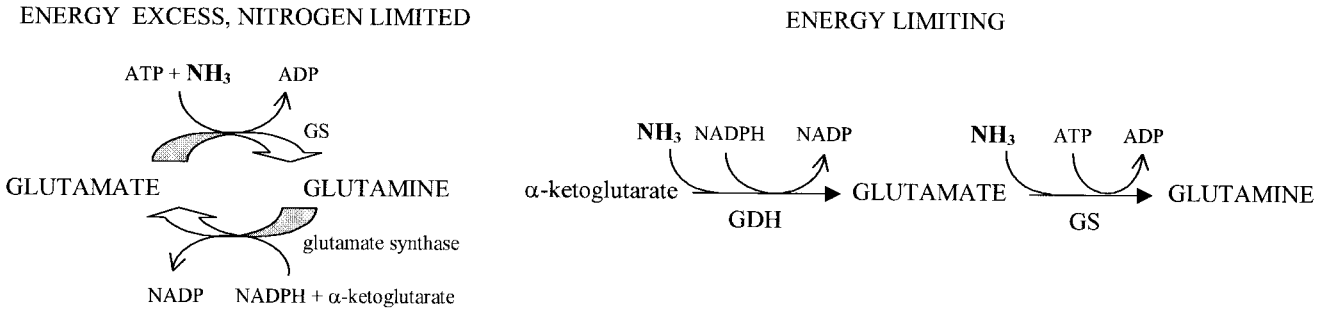

FIG. 4. Pathways of ammonia assimilation. GDH, glutamate dehydrogenase.

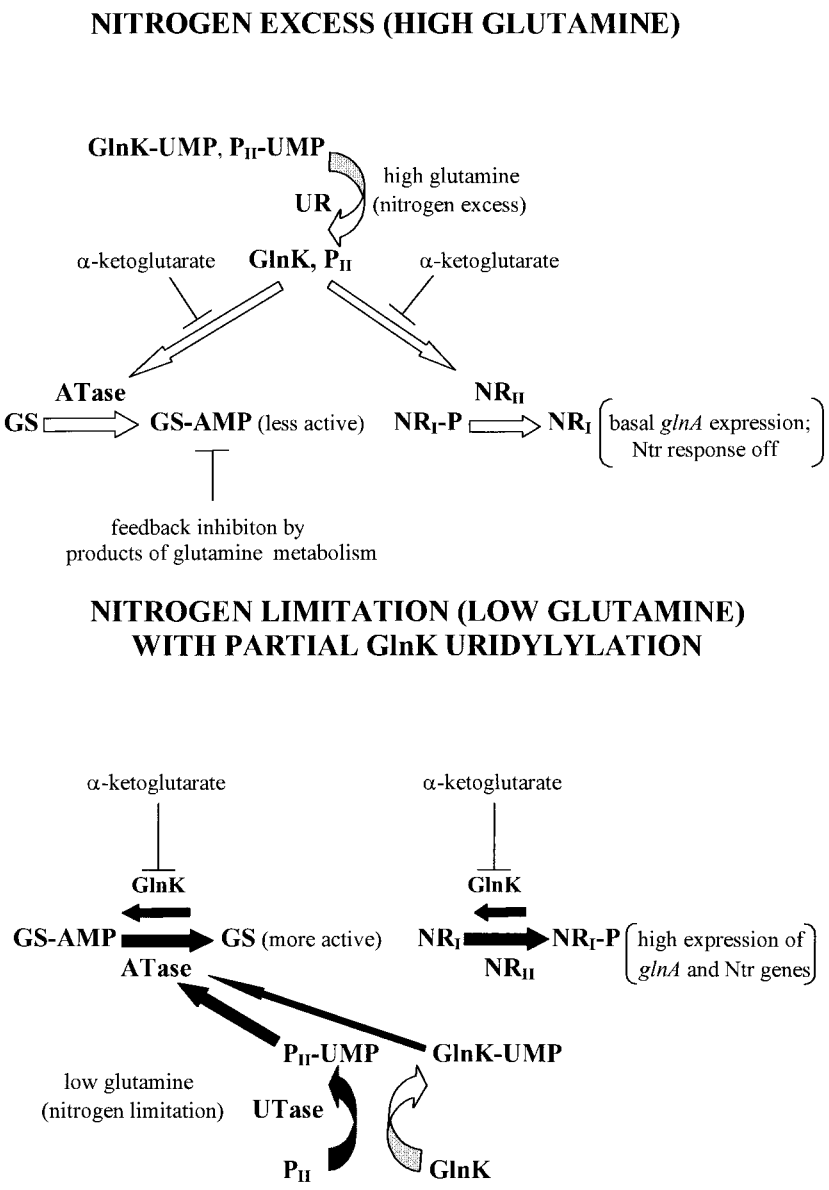

FIG. 5. Regulation of GS activity and the Ntr response. The pathways are shown for conditions of nitrogen excess (high glutamine) (top) and nitrogen limitation (low glutamine) with partial GlnK uridylylation (bottom). The open arrow in the bottom panel is meant to indicate that only partial uridylylation occurs. It is assumed that partial uridylylation occurs either during nitrogen limitation or during the transition to steady-state nitrogen-limited growth. The T-like symbol indicates an inhibition.

lar nitrogen donors. For growth with a nitrogen source that cannot transfer its nitrogen to glutamate by transamination (e.g., adenosine), ammonia becomes an obligatory intermediate for all cellular nitrogen. In these situations, GS is the primary enzyme of ammonia and nitrogen assimilation (Fig. 4). Nitrogen-limited growth results in maximal synthesis of GS and also induces proteins that transport and catabolize several nitrogen sources. The coordinated response to nitrogen limitation is called the nitrogen-regulated (Ntr) response.

Control of the Ntr response by glutamine. The two proteins that control GS adenylylation, UTase-UR and P_{II}, also control the Ntr response. High glutamine levels (nitrogen sufficiency) stimulate UR activity, which prevents uridylylation of P_{II}. Unmodified P_{II} interacts with nitrogen regulator II (NR_{II}, also called NtrB) and stimulates the dephosphorylation of nitrogen regulator I (NR_I, also called NtrC). The net effect is low expression of the *glnALG* operon and failure to activate Ntr genes. Low glutamine levels (nitrogen limitation) result in the formation of P_{II}-UMP, which is unable to interact with NR_{II}. In this situation, NR_{II} phosphorylates itself and transfers the activated phosphate to NR_I. NR_I-P then activates the expression of the *glnALG* operon and other Ntr genes.

α-Ketoglutarate counters the effects of unmodified P_{II}. Even though recent studies have suggested that the ratio of glutamine to α-ketoglutarate does not regulate UTase-UR activity, α-ketoglutarate does affect P_{II} activity (83–85). α-Ketoglutarate counteracts the effects of unmodified P_{II} (present when glutamine levels are high and nitrogen is in excess) and there-

fore stimulates *glnALG* expression and increases GS activity. In other words, the ratio of glutamine (via UTase-UR) to α-ketoglutarate (via P_{II}) appears to control nitrogen assimilation during relative nitrogen sufficiency. This leaves the question whether there is a mechanism to coordinate carbon and nitrogen metabolism during nitrogen-limited growth when P_{II} is uridylylated. It will be suggested elsewhere in this review that such coordination might be a function of GlnK, a P_{II}-like protein.

NR_I regulon. NR_I directly or indirectly controls the vast majority of Ntr genes. It is known to activate the expression of *glnALG* (GS and Ntr regulators), *astCADBE* (arginine catabolism), *glnK-amtB* (an alternate P_{II} and an ammonia transporter), *nac* (a σ^{70}-dependent transcriptional activator), and *glnHPQ* (glutamine transport) in *E. coli*. Several lines of evidence also suggest that it controls the expression of *argT-hisJMPQ* (arginine and histidine transport) and *gltIJKL* (glutamate-aspartate transport). In addition to these genes, microarray analysis suggests that NR_I might also activate b1012–b1006 (possibly for pyrimidine catabolism), *chaC* (calcium transport), *ddpXABCDE* (D-alanine–D-alanine metabolism), *potFGHI* (putrescine transport), *yeaGH* (unknown function), *ygjG* (a transaminase), and *yhdWXYZ* (amino acid transport) (176). In addition to activation, NR_I represses the two minor promoters, $glnAp_1$ and *glnLp*, of the *glnALG* operon.

Nac regulon. There are two majors regulators of the Ntr response: NR_I and Nac. NR_I activates σ^{54}-dependent promoters, while Nac activates σ^{70}-dependent promoters. Nac is homologous to LysR (114). Unlike LysR, Nac apparently does not bind a ligand, which implies that it is constitutively active (16, 63).

Nac has been most intensively studied in *Klebsiella aerogenes*, where it activates genes for histidine, proline, urea, and D-alanine catabolism and represses glutamate dehydrogenase (16, 80, 106). It does not regulate the same genes in *E. coli*. *E. coli* lacks *hut* and *ure* operons, and Nac does not regulate the *E. coli dad* operon (16, 109). Nac deficiency in *E. coli* results in a slight derepression of glutamate dehydrogenase synthesis, slightly slower growth with cytosine as the nitrogen source, and slightly faster growth with arginine (114). The effect on arginine utilization is undoubtedly indirect, since synthesis of arginine catabolic enzymes does not require Nac (114) (see "*astCADBE* operon and catabolism of arginine and ornithine" below). Microarray analysis suggests Nac-dependent induction of b1440-1444 (probably for putrescine transport), *codBA* (cytosine metabolism), *dppABCDF* (dipeptide transport), *fklB-cycA* (D-alanine, D-serine, and glycine transport), *gabDTP* (γ-aminobutyrate [GABA] metabolism), *nupC* (nucleoside transport), *ompF* (outer membrane protein F), *oppABCD* (oligopeptide transport), *yedL* (unknown function), and *yhiE* (unknown function) (176). Nac-dependent control has been directly verified for the *gab* operon (S. Ruback and L. Reitzer, unpublished observation) but not for the other genes.

Why are there two general regulators of the Ntr response? The main question concerning Nac is why there is a second Ntr regulator. Clearly, it is not necessary, since *S. enterica* serovar Typhimurium lacks it (114). We suggest that Nac-dependent control is important physiologically and serves a different function from NR_I-dependent control. NR_I-dependent genes respond to general nitrogen limitation, i.e., to intracellular glutamine, and not to specific induction mechanisms. The only known exception is the *ast* operon, which requires arginine-specific induction. In contrast, many Nac-dependent genes require both general and specific regulation: pyrimidines control the *codBA* operon by a complex process called reiterative transcription (126); GABA controls *gab* operon expression via the GabC repressor (Ruback and Reitzer, unpublished); and histidine and HutC control the *hut* operons in *K. aerogenes* (16). Nac may permit specific regulation, which may be difficult for σ^{54}-dependent promoters. This is illustrated in Fig. 6, which shows the regulatory sites for the single *hutUH* promoter of *K. aerogenes*. Other rationales for Nac have been proposed (16).

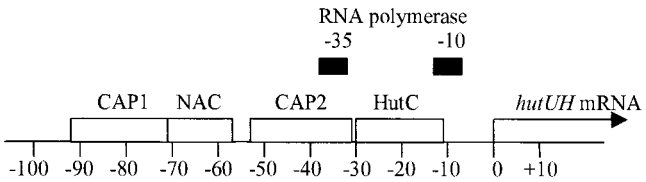

FIG. 6. Binding sites for regulatory proteins at the *hutUH* promoter.

Function of the Ntr response. It is likely that most of the σ^{54}-dependent Ntr genes have been identified using results from a microarray analysis and the complementary computer analysis of potential σ^{54}-dependent promoters. The Ntr genes can be divided into a few categories: GS, regulators, transport proteins, and catabolic enzymes.

Most Ntr genes specify transport proteins: *amtB* (ammonia), *argT-hisJMPQ* (arginine, lysine, ornithine, and histidine), b1006 (uracil?), b1440-b1444 (putrescine?), *codB* (cytosine), *cycA* (D-alanine, D-serine, and glycine), *ddpXABCDE* (D-alanyl–D-alanine), *dppABCDE* (dipeptides), *gabP* (GABA), *glnHPQ* (glutamine), *gltIJKL* (glutamate-aspartate), *nupC* (nucleosides), *oppABCD* (oligopeptides), *potFGHI* (putrescine), and *yhdWXYZ* (amino acids?). It has been suggested that a major function of the Ntr response is scavenging (176). However, Ntr proteins do not scavenge all amino acids. There are no Ntr-dependent transport systems for the aromatic amino acids, the branched-chain amino acids, threonine, methionine, or cysteine. An explanation for this pattern of expression may be that *E. coli* does not readily use these amino acids as nitrogen sources, which implies that their nitrogens are not readily available. In other words, *E. coli* generally has Ntr-dependent transport systems only for amino acids that can readily provide nitrogen for glutamate and glutamine synthesis.

Another class of Ntr genes specify enzymes for catabolic pathways. There are very few Ntr catabolic pathways, and unlike the transport genes, optimal synthesis usually requires specific induction. It should be noted that these catabolic pathways are not the major amino acid catabolic pathways, i.e., those that degrade amino acids that can be converted in one or two steps to intermediates of central metabolism, such as aspartate, glutamate, glutamine, serine, alanine, and glycine. An explanation for this observation is not apparent.

In summary, the function of the Ntr response is nitrogen assimilation when the intracellular glutamine level is low. This explains all the major aspects of the Ntr response: the regula-

tion of GS activity and synthesis by glutamine, the regulation of the Ntr response by glutamine, and the reason why there are so many Ntr transport systems that scavenge nitrogenous compounds that have readily utilizable nitrogen.

glnALG (*glnA-ntrBC*) Operon

The *glnALG* operon codes for GS, NR_{II}, and NR_I, respectively (127, 129). All three products of this operon are required for nitrogen assimilation and the Ntr response (discussed above). σ^{54} and NR_I~P are required for transcription from the major promoter, $glnAp_2$, which has a score of 83.3. Nitrogen limitation activates *glnA* expression 11- to 24-fold, but appears to have little effect on *glnL* or *glnG* transcription (176). However, direct measurements of NR_I indicate that nitrogen limitation induces NR_I synthesis 14-fold (128). Minor promoters, $glnAp_1$ and *glnLp*, ensure basal synthesis of the important products of this operon (130, 157).

glnK-amtB Operon

Nitrogen limitation and NR_I are required for expression of the *glnK-amtB* operon (10, 160). Although the transcription start site has not been directly determined, the promoter has a score of 90.2 and potential binding sites for both $E\sigma^{54}$ and NR_I (Fig. 2) (160). In addition, nitrogen limitation increases the *glnK* transcript at least 10-fold (176). Therefore, this operon undoubtedly contains an authentic σ^{54}-dependent promoter. Both products of the operon contribute to the response to nitrogen limitation.

GlnK: a P_{II} paralog. The existence of a P_{II} paralog was first suspected because of the rapid deadenylylation of GS in an *E. coli glnB* (P_{II}-encoding) mutant (160). The gene coding for this protein was cloned and called *glnK* (160). A *glnK* mutant has only a subtle phenotype (10). It has higher basal expression of an Ntr gene (*glnK* itself) in an ammonia-containing medium (even though the GlnK concentration should be low) and lower expression of an Ntr gene (again *glnK*) in a nitrogen-limiting medium. The mutant also has less of a lag during the transition to growth with arginine as a nitrogen source. This might result from higher basal expression of the *ast* operon, whose products degrade arginine. The phenotype of a *glnB glnK* double mutant, which lacks both P_{II} and GlnK, is more dramatic. It fails to grow in a nitrogen-rich minimal medium. The reason for this lethality is not known with certainty, but it may be related to uncontrolled phosphorylation of NR_I, which has been suggested to cause inappropriate overexpression of an Ntr gene (10). An alternate explanation is NR_I-dependent overexpression of a σ^{54}-dependent gene that is not normally regulated by NR_I.

Purified GlnK and P_{II} have similar activities, but the regulation of these activities is different (9, 58, 160, 161). However, the physiological relevance of many differences has not been established and is sometimes refuted by mutant phenotypes. The only safe basis for discussing the relevant properties of GlnK is when they account for the phenotype of mutants. One aspect of the mutant phenotype is the higher basal expression of an Ntr gene in a nitrogen-rich environment, which implies that GlnK suppresses this expression. One property of purified GlnK that accounts for this suppression is the relatively slow uridylylation of GlnK compared to that of P_{II} (9). This property is accentuated by the formation of GlnK-P_{II} heterotrimers (58, 161) and the inactivation of P_{II}-UMP by GlnK in such heterotrimers (161). The net effect is enhanced dephosphorylation of NR_I~P and lower expression of Ntr genes. The second aspect of the phenotype of a *glnK* mutant is lower induced expression of an Ntr gene in a nitrogen-limited environment, which implies that GlnK stimulates Ntr expression. This is consistent with one property of purified GlnK. Although GlnK can efficiently stimulate the dephosphorylation of NR_I~P via NR_{II}, α-ketoglutarate is more efficient in inhibiting the activity of GlnK than of P_{II} (9).

Why have GlnK? The lower induction of an Ntr gene and higher basal expression in a *glnK* mutant suggest that GlnK sharpens the response to nitrogen availability. Perhaps the responsiveness of GlnK to α-ketoglutarate partially explains this effect. In this case, GlnK essentially restores the coordination of carbon and nitrogen metabolism (the responsiveness to the ratio of α-ketoglutarate to glutamine) that is lost when P_{II} is completely uridylylated, i.e., during nitrogen-limited growth, and is no longer responsive to α-ketoglutarate. A second function for GlnK has been found, but not in *E. coli*. GlnK is required for control of NifL, which inhibits the activity of NifA in *Klebsiella* species (65). NifA is the transcriptional activator required for expression of the nitrogenase gene cluster in *Klebsiella* species. Uridylylated and nonuridylylated GlnK can relieve repression. It is not known how GlnK mediates shutoff of the *nif* genes when ammonia is added, but it may interact with other proteins.

Product of *amtB* and ammonia transport. The second gene of the operon, *amtB*, codes for an ammonia transporter. None of the phenotypes of the *glnK* mutant could be attributed to polar effects on *amtB* expression (10). An *amtB* mutant of *S. enterica* serovar Typhimurium has only a subtle phenotype (149). It is unable to utilize a low concentration of ammonia if the pH is less than 7. This phenotype implied that uncharged NH_3, not NH_4^+, is transported. It was proposed that AmtB did not concentrate ammonia but only facilitated equilibrium across the membrane. This mechanism of transport might have significant physiological implications, which are discussed in "Physiological Function of σ^{54}" (below).

nac

Although *E. coli*, *S. enterica* serovar Typhimurium, and *K. aerogenes* are closely related, they differ in their ability to utilize certain nitrogen sources and in the regulation of some genes of nitrogen metabolism. For example, nitrogen limitation strongly represses glutamate dehydrogenase in *K. aerogenes* but not in *E. coli* (16, 127). The transcriptional regulator Nac accounts for many of these differences in regulation. Nac has been most extensively studied from *K. aerogenes* but has also been studied from *E. coli*. In contrast, *S. enterica* serovar Typhimurium lacks Nac (114).

The *nac* operon is monocistronic (114, 145). Transcription initiated from the *K. aerogenes nac* promoter requires σ^{54} and NR_I~P (16, 54, 106). Nitrogen limitation induces *E. coli nac* (114, 176), and computer analysis indicates a likely binding site for $E\sigma^{54}$ with a score of 85.2, which is very high. These results suggest that NR_I and $E\sigma^{54}$ are also required for *E. coli nac* expression. Nac negatively modulates its own synthesis in *K.*

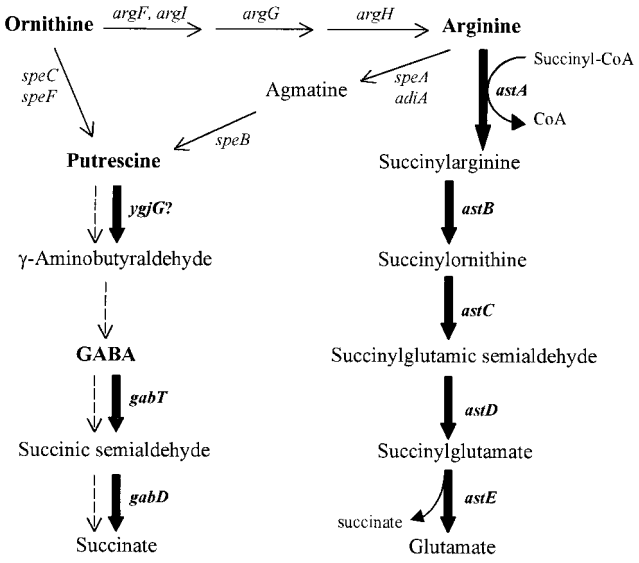

FIG. 7. Metabolic relationships between ornithine, arginine, putrescine, and GABA. A thick black arrow indicates that nitrogen limitation induces the enzyme indicated. A reaction catalyzed by two (or more) enzymes is indicated by two arrows. The genes that specify the enzymes are shown when they are known. A dashed arrow indicates that the gene has yet to be identified.

aerogenes by interfering with the interaction between NR_I and RNA polymerase (55, 114), and results from the DNA microarray analysis are consistent with such regulation in *E. coli* (176).

Catabolism of Arginine, Agmatine, Ornithine, Putrescine, and γ-Aminobutyrate

Arginine (via agmatine) and ornithine are both precursors for putrescine, which can be metabolized to GABA, and then to succinate (Fig. 7). Nitrogen limitation induces enzymes of GABA catabolism (175). Therefore, it was reasonable to propose that Ntr regulators affect the catabolism of all of these compounds, and some evidence is consistent with this regulation (147). However, recent studies with mutants containing targeted gene disruptions have indicated unsuspected pathways and a surprising complexity and redundancy of pathways and regulators. Only the enzymes of arginine and GABA catabolism require σ^{54}, while the enzymes of putrescine catabolism may not.

***astCADBE* operon and catabolism of arginine and ornithine.** The five-step arginine succinyltransferase pathway catabolizes arginine (144). The pathway is named after the first reaction, which is the succinylation of the α-amino group of arginine. The *astCADBE* operon codes for the proteins of the pathway (144). Disruption of the operon in *E. coli* prevents growth with arginine as a nitrogen source and impairs but does not eliminate growth with ornithine (60, 144). It has been proposed that AstC (which catalyzes the deamination of succinylornithine) is one of at least two transaminases that can deaminate ornithine, which generates an intermediate of proline catabolism (144). The identity of the second transaminase is unknown.

Expression of the *astCADBE* operon in *E. coli* and *S. enterica* serovar Typhimurium requires either nitrogen limitation or entry into stationary phase (12, 60, 103, 144). There are two transcription start sites, which are separated by five bases (A. Kiupakis and L. Reitzer, unpublished results). Expression from the Ntr promoter requires NR_I~P and σ^{54}, while expression from the other promoter requires $E\sigma^S$. SeqScan gives the σ^{54}-dependent promoter a score of 85.5. Some evidence suggests that transcription from one promoter prevents transcription from the other (60). One unusual feature of the *ast* operon in *E. coli* is that ArgR binds to the region between the $E\sigma^{54}$ and NR_I binding sites and has been proposed to stimulate the interaction between the two proteins (103). ArgR is required for optimal transcription of the *E. coli ast* operon but is not absolutely necessary (Kiupakis and Reitzer, unpublished). In contrast, ArgR appears to be required for the *S. enterica* serovar Typhimurium *ast* operon (103). The *ast* operon contains the only known *E. coli* NR_I-dependent promoter that also requires specific induction. Microarray analysis indicates that general nitrogen limitation (i.e., without arginine induction) increases *ast* transcription 7- to 11-fold (176), which is consistent with the results of a direct assay of the gene products (144). Arginine induces the enzymes three- to fourfold further (144), which is consistent with in vitro results (Kiupakis and Reitzer, unpublished). Another unusual aspect of *ast* expression is that a strain with a *glnL* (*ntrB*) deletion cannot utilize arginine as a nitrogen source (L. Reitzer, unpublished observation) but can still activate *glnA* expression, albeit not as rapidly (130). Expression of the *ast* operon requires phosphorylation of NR_I by both NR_{II} and small phosphodonors (B. L. Schneider, D. Fewell, and L. J. Reitzer, unpublished observation).

GABA and putrescine catabolism and the *gabDTPC* operon. *E. coli* can utilize GABA as a nitrogen source (H. Kasbarian, S. Ruback, and L. Reitzer, unpublished results), although earlier studies indicated otherwise (50). A mutant with a *gabDT* deletion cannot utilize GABA as a nitrogen source (Ruback and Reitzer, unpublished). The distance between genes suggests the existence of a *gabDTPC* operon. Furthermore, transcript analysis indicates the presence of only one promoter for these genes, a promoter just upstream from *gabD*. Each gene of the putative operon has been implicated in GABA catabolism. GabT is a transaminase that deaminates GABA to succinic semialdehyde. GabD is an NADP-specific succinic semialdehyde dehydrogenase that oxidizes succinic semialdehyde to succinate. (An NAD-dependent succinic semialdehyde dehydrogenase is specified by the *sad* gene, and GABA or a product of GABA metabolism induces its synthesis [49].) GabP specifies a GABA permease (92). GabC appears to be a specific repressor since a deletion of *gabC* stimulates growth with GABA as a nitrogen source (Kasbarian et al., unpublished). Either nitrogen limitation or entry into stationary phase activates *gab* operon expression (12, 175; Kasbarian et al., unpublished). Expression of the *gab* operon requires Nac during nitrogen-limited growth, and Nac is required to reconstitute transcription in vitro (H. Kasbarian, A. Kiupakis, S. Ruback, and L. Reitzer, unpublished results). σ^S is required for expression during stationary phase.

GABA (via γ-aminobutyraldehyde) is a presumed intermediate in putrescine catabolism. Unexpectedly, a strain with a deletion of *gabDT* grew normally with putrescine or agmatine as a nitrogen source (Kasbarian et al., unpublished). Even

more surprising is the observation that an *rpoN* mutant (σ^{54} deficient) grew normally with putrescine as a nitrogen source (Kiupakis and Reitzer, unpublished). These results would suggest that nitrogen limitation is not required for induction of putrescine catabolic genes, but this is not the case. Microarray analysis suggests that nitrogen limitation activates the expression of two different putrescine transport operons: *potFGHI* (five- to sixfold) and b1440-1444 (five- to sevenfold) (176). In addition to these transport systems, *E. coli* possesses two σ^{54}-independent transport systems, the products of *potABCD* (which preferentially transport spermidine but also transport putrescine) and of *potE* (78). The genes of putrescine catabolism and the physiological function of the four transport systems have yet to be established.

***ygjG*.** Nitrogen limitation results in a three- to fivefold increase in the levels of steady-state *ygjG* transcripts (176). Expression of this gene requires σ^{54}, and the transcription start site has been determined (A. Kiupakis, R. Ye, and L. Reitzer, unpublished result). The score for this promoter is 70.7, which is low for an authentic σ^{54}-dependent promoter. The gene specifies a putative ω-transaminase which either removes the amino group from compounds with terminal primary amines (e.g., putrescine or ornithine), or adds amino groups to compounds with an aldehyde group (e.g., *N*-acetylglutamic semialdehyde, an intermediate in ornithine formation). Putrescine and compounds metabolized to putrescine activate *ygiG* expression, which suggests a possible role in putrescine catabolism. However, a mutant with a disruption of *ygjG* grows normally with putrescine as a nitrogen source, which suggests that YgjG is a redundant transaminase (C. Pybus and L. Reitzer, unpublished observation).

σ^{54}-dependent Amino Acid Transport Systems

More than half of the genes activated by nitrogen limitation in *E. coli* code for transport systems. Such activation usually does not require specific induction. Such regulation combined with the observation that mutational inactivation of the genes for σ^{54}-dependent amino acid transport system does not prevent growth on the respective amino acid suggests a scavenging function. In this section, the σ^{54}-dependent amino acid transport systems are considered within the context of the multiple transport systems for these amino acids.

Arginine. (i) The three transport systems. *E. coli* has three characterized arginine transport systems (described below), while *S. enterica* serovar Typhimurium has at least two (98). An early study suggested the presence of three periplasmic arginine binding proteins in *E. coli* (136). No strain has been constructed with mutations in all the characterized systems. Therefore, it is conceivable that there are other transport systems.

Only one of the three *E. coli* arginine transport systems requires σ^{54} for its synthesis. It contains the periplasmic ArgT protein, also called the LAO protein, which binds lysine, ornithine, and arginine with high affinity (136). The *argT* gene and its product have been extensively studied in *S. enterica* serovar Typhimurium but not in *E. coli*. ArgT interacts with HisP of *S. enterica* serovar Typhimurium (98). HisJ, the periplasmic histidine binding protein, also interacts with HisP (5). An *S. enterica* serovar Typhimurium *hisP* mutant grows much more slowly with arginine as the nitrogen source than an *argT* mutant does, which suggests that HisP also interacts with an arginine binding periplasmic protein other than ArgT (98).

σ^{54}-independent arginine transport systems have been studied only in *E. coli*. One system contains AbpS, also called the arginine-ornithine protein, which binds arginine and ornithine in the periplasm with lower affinity than the LAO protein does (34). Early reports refer to this protein as a low-affinity, arginine-specific protein (41, 136). The *abpS* gene has been approximately mapped to min 63.5 of the most recent *E. coli* map (35). AbpS has been purified, and its size and amino acid composition have been determined (36). However, no gene near min 63 specifies a protein with the published amino acid composition. The nearest matches to this amino acid composition in the *E. coli* genome, in descending order, are the products of *artI*, *artJ*, and *hisJ*, which are located at min 19.4, 19.4, and 52.3, respectively. It is possible that the sequenced MG1655 does not contain *abpS*. The second σ^{54}-independent system consists of the *artPIQM-artJ* operons, which are at min 19 of the *E. coli* chromosome (169). ArtJ is a periplasmic protein that binds arginine but not ornithine (169). ArtI is another putative periplasmic binding protein, but it does not detectably bind any amino acid. Mutants with mutations in the ArtJ system do not exist, but overexpression increases arginine transport, which is consistent with a proposed function in arginine transport (169). Promoters precede *artP* and *artJ*, and neither appears to require σ^{54} (169).

(ii) Repression by arginine. Arginine represses all three *E. coli* transport systems (41, 137, 169). A possible mechanism of repression would involve ArgR, which mediates arginine repression for the enzymes of arginine synthesis. A computer analysis of ArgR sites in *E. coli* identified two sites in the *art* operon: one preceding *artP* and one preceding *artJ* (111). However, a missense mutation in *argR* had no effect on the kinetically detectable arginine transport systems (39). Instead of ArgR, ArgP and ArgK have been proposed to mediate arginine repression. ArgP is a transcriptional regulator required for synthesis of ArgK, which is required for arginine transport. Mutations in *argP* and *argK* affect both the ArgT and AbpS systems (40, 41, 137). Only one gene separates *argP* and *argK*, but they appear to be independently expressed. (*argK* is currently not listed in either GenBank or the latest *E. coli* genetic map. However, *argK* is *ygfD*, also called b2918 in GenBank.) ArgP is a LysR-type regulator that activates *argK* expression in the absence of arginine (37). ArgP complexed with arginine fails to activate *argK* expression and represses its own synthesis. ArgK has an ATPase activity that is apparently required for transport activity (158). ArgK also phosphorylates the periplasmic ArgT and AbpS (36), although this phosphorylation is not required for transport (38). ArgP is the previously characterized IciA, an inhibitor of the initiation of DNA replication (37, 154). An *iciA* mutant has no obvious phenotype, except for difficulty during dilution into fresh growth medium (154). It is conceivable that ArgP/IciA is a sensor of amino acid sufficiency that couples DNA synthesis with metabolism in some environments.

(iii) Transport and activation during nitrogen limitation. Nitrogen limitation induces ArgT in *E. coli* and *S. enterica* serovar Typhimurium, and this induction does not require arginine (98, 176). The genetics and regulation of arginine trans-

port have been studied in *S. enterica* serovar Typhimurium, and it is assumed that they will be similar in *E. coli*. In *S. enterica* serovar Typhimurium, loss of ArgT reduced but did not eliminate the binding of arginine to periplasmic proteins and an *argT* mutant grew normally with arginine as a nitrogen source, which implies a second transport system in *S. enterica* serovar Typhimurium during nitrogen-limited growth (98).

argT is adjacent to the *hisJQMP* operon, which codes for components of a histidine transport system. Transcript mapping in wild-type *S. enterica* serovar Typhimurium, not in an extensively studied *dhuA1* mutant which appears to have a mutationally created promoter, shows a σ^{54}-dependent promoter immediately preceding *argT* but not immediately preceding *hisJ* (6). This is consistent with expression studies with reporter gene fusions, which failed to identify an Ntr promoter preceding *hisJ* (142, 143). The potential σ^{54}-dependent promoter preceding *argT* in *E. coli* has a score of 72.3. There is only one binding site for NR_I, and it is in the *argT-hisJ* intercistronic region and not upstream from *argT* (6). The function of this site is not clear. It does not appear to be necessary for expression, which would imply that NR_I activates the *argT* promoter without binding to DNA (143). There is precedent for NR_I-dependent transcription that does not require a DNA binding site (131, 171).

Histidine. The genes and regulation of the HisJ transport system were discussed in the preceding section because of their relation to arginine transport. It is not known whether there are other histidine transport systems.

Glutamine. Glutamine transport has been studied in both *E. coli* and *S. enterica* serovar Typhimurium. The kinetically dominant system requires GlnH, a high-affinity glutamine-specific binding protein in the periplasm (162). The *glnHPQ* operon specifies GlnH and two membrane proteins, which presumably interact with GlnH (119). Loss of GlnH unmasks a low-affinity glutamate-inhibitable glutamine transport system in *E. coli* (162) but has no effect on growth in *S. enterica* serovar Typhimurium (98). These results suggest a second glutamine transport system. Kinetic assays of glutamine transport also suggest two transport systems (11, 168). It is possible that the glutamate-inhibitable system requires the periplasmic glutamate-aspartate binding protein (see the next section), which also binds glutamine (167).

Expression of *glnHPQ* requires nitrogen limitation but not glutamine (18, 98, 168). Nitrogen limitation increases the production of *glnHPQ* transcripts five- to ninefold (176). The operon contains two promoters (118). Transcription from the downstream promoter, $glnHp_2$, requires σ^{54} and NR_1 and is enhanced by IHF (44). The promoter has a score of 80.7. The factors that control the upstream promoter, $glnHp_1$, have not been examined.

Glutamate-aspartate. Schellenberg and Furlong defined five transport systems for glutamate and aspartate in *E. coli* by a combination of genetic and biochemical experiments (140). There are no studies with gene fusions; therefore, the regulation and functions of the individual systems are poorly understood.

Nitrogen limitation induces a periplasmic protein that binds both glutamate and aspartate in *S. enterica* serovar Typhimurium (98). Such a protein has been purified and characterized from *E. coli* (167). The closest match in the entire SWISS-PROT database with the published amino acid composition of the glutamate-aspartate binding protein is *gltI* (also called *ybeJ*) from *E. coli*. The *gltI* product was the only protein in *E. coli* with the correct pI, size, and number of cysteines. *gltI* might be part of a *gltIJKL* operon. Despite the annotation of *gltIJKL* as part of a glutamate-aspartate transport system, no published evidence supports this possibility (17).

There are no potential σ^{54}-dependent promoters preceding *gltI* with a score greater than 60. However, the gene preceding *gltI* specifies an IS*5* transposase, and the promoter region for the transposase gene contains a possible σ^{54}-dependent promoter with a score of 88, which is very high. Microarray analysis indicates a three- to sixfold increase of *gltI* transcription during nitrogen limitation, and NR_I-dependent activation (176). These results suggest that the σ^{54}-dependent promoter preceding the transposase gene can initiate *gltIJKL* transcription.

***ddpXABCDE* operon.** Microarray analysis shows that general nitrogen limitation (i.e., no specific induction) induces the *ddpXABCDE* operon 52- to 60-fold, which is more than any other operon. Expression appears to require NR_I (176). A potential σ^{54}-dependent promoter with a score of 76.6 precedes the operon. DdpX is a zinc-containing D-alanyl–D-alanine dipeptidase, while the other products of the operon appear to code for components of a dipeptide permease (100). There are two sources of D-alanyl–D-alanine: it is an intermediate in peptidoglycan synthesis, and it may be released during cross-linking of two diaminopimelic acids. Peptidoglycan cross-linking occurs in the stationary phase (156). Entry into the stationary phase induces the *ddpXABCDE* operon, and this induction requires σ^S (100). It is not known whether peptidoglycan remodeling also occurs in nitrogen-limited cultures. It has been proposed that the function of the *ddpXABCDE* products is to scavenge D-alanyl–D-alanine (176).

Peptide transport and *ompF*. *E. coli, S. enterica* serovar Typhimurium, and other gram-negative bacteria digest peptides intracellularly after their passage through the outer membrane and transport via periplasmic binding protein-dependent transport systems. Nitrogen limitation activates the expression of *dppABCDF* and *oppABCDF* (176). The products of these operons are the major peptide transporters in *E. coli* and *S. enterica* serovar Typhimurium (120), and the periplasmic components of these systems, DppA and OppA, are among the most abundant proteins in the periplasm (1, 70, 121). In addition to its function as a transport protein, DppA is required for chemotaxis to peptides (108). Neither peptide transport operon contains a potential σ^{54}-dependent promoter. The apparent 10-fold *dppABCDF* induction appears to require Nac (176), which is consistent with the absence of a σ^{54}-dependent promoter. In contrast, microarray analysis provides no evidence for Nac-dependent regulation for the four- to sixfold *oppABCDF* induction and equivocal evidence for NR_1-dependent regulation (176). Induction by nitrogen limitation is most evident in medium with glutamine as the nitrogen source but not in medium with ammonia as the nitrogen source for strains with constitutively active Ntr regulatory proteins. These results suggest that induction may require specific nitrogen-containing compounds. This is consistent with the known regulators of *oppABCDF* expression, Lrp and the *gcvB* transcript (a regulatory RNA), which respond to leucine (or alanine) and glycine,

respectively (31, 117, 159). (The *gcvB* transcript also controls *dppABCDF* expression.) Based on these considerations and the absence of a potential σ^{54}-dependent promoter, the effect of nitrogen limitation on *oppABCDF* may be indirect, i.e., independent of NR_I or Nac.

The first step in peptide transport is passage through the outer membrane, and nitrogen limitation results in 25-fold higher transcription of *ompF*, which codes for an outer membrane channel (176). The microarray analysis suggests very modest control by NR_I (two- to threefold), and stronger control by Nac (perhaps ninefold) (176). NR_I-dependent control cannot be ruled out since there is a weak potential σ^{54}-dependent promoter with a score of 64.4. However, like *oppABCDF* expression, the level of induction in mutants with constitutively active NR_{II} is dramatically stronger with glutamine (25-fold) than with ammonia (4-fold) (176), and Lrp has been implicated in *ompF* regulation (57). Perhaps nitrogen limitation controls *ompF* indirectly, as was suggested for *oppABCDF*.

Potential σ^{54}-Dependent Genes That Are Induced by Nitrogen Limitation

Nitrogen limitation results in a 20-fold increase in the expression of the putative b1012-1006 operon (176). The potential $E\sigma^{54}$ binding site preceding b1012 has a score of 76.8. The last gene of the putative operon codes for a possible uracil permease, which may suggest that this operon codes for enzymes of pyrimidine catabolism. This is consistent with the observation that [^{14}C]uracil or [^{14}C]thymine catabolism yields $^{14}CO_2$, even though *E. coli* cannot degrade these compounds as sole nitrogen sources (13).

Nitrogen limitation induces b2875-76 and b2882-85. Some of the genes in this region have been studied, and homology searches have suggested that they might participate in purine catabolism (172). The *xdhA* gene (b2866), which codes for one subunit of a recently discovered xanthine dehydrogenase, appears to have two promoters, and one of them might be σ^{54} dependent (172). *E. coli* can utilize intermediates of purine catabolism, such as allantoin, as nitrogen sources anaerobically but not aerobically (46). The potential σ^{54}-dependent promoters preceding *xdhA* (b2866), *ygeW* (b2870), and b2878 have low scores (66.0, 75.2, and 71.5, respectively), but they might contribute to purine catabolism during anaerobic growth.

Nitrogen limitation activates the putative *yhdWXYZ* operon two- to ninefold, and appears to be NR_I dependent (176). The potential promoter for this operon has a score of 88.6. The products of this operon have homology to transport proteins for polar amino acids.

Nitrogen limitation activates the *yeaGH* operon two- to fourfold, but it is not clear whether regulation requires NR_I or Nac (176). The potential $E\sigma^{54}$-binding site has a score of 83.8. However, homology searches provide no clue to the function of the products.

σ^{54}-DEPENDENT GENES THAT ARE NOT INVOLVED IN NITROGEN METABOLISM

Formate Catabolic Genes and the FhlA Regulon

Formate metabolism. The products of several σ^{54}-dependent operons contribute to formate metabolism during glucose fermentation. The products of glucose fermentation in *E. coli* in terms of total carbon from glucose are CO_2 (14.7%), ethanol (16.6%), acetic acid (12.2%), lactic acid (40%), formic acid (0.4%), succinic acid (7.2%), and cell constituents (~10%) (reviewed in reference 24). Formate is a major intermediate even though it does not accumulate. Formate formation is linked to pyruvate metabolism because pyruvate formate lyase cleaves pyruvate to formate and acetyl coenzyme A (acetyl-CoA) during anaerobic growth. The production of fermentation products that require acetyl-CoA as a precursor, acetate and ethanol, necessarily generates a stoichiometric amount of formate. Therefore, about 14% of glucose carbon (8.3% concomitant with ethanol formation plus 6.1% with acetic acid formation) is converted to formate during fermentation.

The formate hydrogenlyase (FHL) complex catalyzes the disproportionation of formate to CO_2 and H_2, which accounts for the CO_2 (0.88 mol per mol of glucose) and H_2 (0.75 mol per mol of glucose) produced during fermentation. (Hydrogenases 1 and 2 recycle some of the H_2 as an electron acceptor. However, the final ratio of H_2 to CO_2 suggests that only about 15% of the H_2 is recycled.) The primary function of the FHL complex is pH homeostasis during fermentation (24). During the initial stages of fermentation, formate is excreted from the cell. As the acidic fermentation products accumulate and lower the pH, formate is imported into the cell, which induces FHL synthesis. FHL consumes all the formate produced and increases the pH. The FHL complex contains a formate dehydrogenase, hydrogenase 3, and intermediate electron carriers (24). The formate dehydrogenase component is termed FDH_H. (*E. coli* possesses two other formate dehydrogenases, which use electron acceptors other than protons [139].) FDH_H contains selenocysteine and binds iron, molybdenum, and cobalt. FDH_H is associated with the [Ni-Fe]-containing hydrogenase 3, which catalyzes electron transfer to protons. This electron transfer does not result in energy conservation.

The four confirmed σ^{54}-dependent operons of formate metabolism. The requirement for σ^{54} has been established by examination of mutant phenotypes, *lacZ* fusions, transcript analysis from mutant and wild-type strains, and transcription with purified components (20, 21, 73, 104, 107). The four confirmed σ^{54}-dependent operons code for components required for the FHL complex. The monocistronic *fdhF* specifies one subunit of FDH_H. The divergently transcribed polycistronic *hyc* and *hyp* operons code for components of FDH_H, hydrogenase 3, proteins required for processing of hydrogenase 3 and other hydrogenases, and two transcriptional regulators (24). The *hydN-hypF* operon specifies a protein required for FDH_H activity (possibly a component of electron transport) and a second protein required for processing of several hydrogenases (107). The promoters for the *hyp*, *hyc*, *fdhF*, and *hydN-hypF* operons have scores of 88.0, 79.1, 72.6, and 64.5, respectively. The last two are among the lowest scores for confirmed σ^{54}-dependent promoters.

The primary activator of these operons is FhlA (24). FhlA activates its own expression from the major promoter of the *hyp* operon, but secondary promoters ensure basal synthesis (24, 105). FhlA is homologous to other σ^{54}-dependent activators (reference 141 and references therein). Footprinting experiments have established the binding sites for FhlA (73).

Like other σ^{54}-dependent activators, it can activate when bound to distant sites (104).

Several factors regulate FHL complex synthesis (22, 125). Low pH and formate induce its synthesis, while oxygen, nitrate, and glucose repress it (138). The formate and oxygen control are regulated via FhlA. Formate stimulates the ATPase activity of FhlA (74) and is required for in vitro transcription, which implies that formate binds FhlA (73). Oxygen control may be mediated by OxyS, which is induced by oxidative stress (3). OxyS is an abundant stable untranslated RNA that binds to the FhlA mRNA and blocks its translation (4). Glucose, pH, nitrate, and additional oxygen control is probably indirect, i.e., via regulation of the synthesis of the major formate transport system, which is part of the *pfl* (pyruvate formate-lyase) operon (93, 138). HycA is a regulator that antagonizes the activation of FhlA (unpublished results cited in reference 24). The mechanism of this regulation has not been characterized. Mo availability also regulates expression of the *hyc* operon (146). ModE is a sensor of intracellular Mo, and a ModE-Mo complex represses the *modABCD* operon, which specifies components of a Mo transport system. ModE-Mo also stimulates transcription of the *hyc* operon by binding to a site centered 190 bases from the start site of transcription (146). (The FhlA binding site is centered 100 bases from the transcription start site [73]). ModE-Mo is not required for expression, but its presence accounts for a two- to threefold stimulation. In addition, the MoeA protein, a component of Mo metabolism, stimulates *hyc* expression two- to threefold by an unknown mechanism (146). The multiplicity of regulators might strengthen the binding of $E\sigma^{54}$ to FhlA-dependent promoters and may account for their low scores.

Hydrogenase 4. A 12-gene *hyf* operon at min 56 contains a possible σ^{54}-dependent promoter with a score of 68.7, and upstream sequences suggest the presence of FhlA binding sites. The expression of this operon has not been characterized or established. Homology analysis suggests that the products of the operon code for a putative hydrogenase 4, which catalyzes the same reactions as the FHL complex, and for proteins of respiration-linked proton translocation. Therefore, it was proposed that the products of the *hyf* operon specify an energy-conserving hydrogenase 4. The putative product of one gene of the operon, *hyfR*, is homologous to FhlA and may bind the same sites as FhlA because of conservation of the DNA binding residues (7).

ato Operon and Acetoacetate Catabolism

Loss of the *ato* operon results in failure to utilize acetoacetate as a carbon and energy source (reviewed in reference 43). The transcription start site has not been examined. Nonetheless, it is likely that this operon requires σ^{54} for several reasons. First, an *rpoN* mutant (σ^{54} deficient) cannot utilize acetoacetate as a carbon source (C. Pybus and L. Reitzer, unpublished). Second, expression of the *ato* operon requires AtoC, which is homologous to other activators of σ^{54}-dependent promoters (42, 43). Proteins homologous to AtoC usually activate σ^{54}-dependent promoters, although two activate σ^{70}-dependent promoters (59, 113, 173). The latter activators lack an essential region of the domain that interacts with $E\sigma^{54}$. The domain of AtoC that interacts with RNA polymerase is homologous throughout its length to the corresponding region of other σ^{54}-dependent activators (113). Therefore, it is likely that AtoC activates from a σ^{54}-dependent promoter. Finally, the score of the putative σ^{54}-dependent *ato* promoter is 85.9, which is very high.

The genes of the *atoDAEB* operon code for proteins of acetoacetate catabolism (42, 81). It has been proposed that *atoE* codes for an acetoacetate-specific transport system (42). The *atoD* and *atoA* genes specify the subunits of acetyl-CoA: acetoacetyl-CoA transferase, which catalyzes the transfer of CoA from acetyl-CoA to acetoacetate. AtoB specifies thiolase II, which catalyzes the formation of two molecules of acetyl-CoA from CoA and acetoacetyl-CoA (43). (Thiolase I is an enzyme in fatty acid β-oxidation.)

E. coli can utilize short-chain fatty acids (C_4 to C_6) such as butyrate (C_4) and valerate (C_5) as a carbon source and such catabolism requires the *ato* operon (43, 124). Butyrate catabolism requires the formation of butyryl-CoA by acetyl-CoA: acetoacetyl-CoA transferase, followed by dehydrogenation of the saturated fatty acid, hydration, and oxidation, which results in the formation of acetoacetyl-CoA. These reactions require enzymes of fatty acid degradation, products of the *fadR* regulon. Acetoacetyl-CoA is then degraded as described above. Butyrate does not induce either the *ato* or the *fad* genes. Therefore, growth with butyrate as the sole carbon source requires constitutive expression of both sets of genes (43).

AtoC is required for expression of the *atoDAEB* operon (82, 124). The nucleotide sequence of this region suggests that *atoC* is the second of two genes in an *atoSC* operon that is just upstream from the *atoDAEB* operon. AtoC is homologous to response regulators such as NR_I (NtrC), and the putative AtoS is homologous to sensor kinases, such as NR_{II} (NtrB). Acetoacetate or a product of acetoacetate metabolism probably binds AtoS and stimulates AtoC phosphorylation. Other aspects of regulation have not been examined. The possibility of IHF sites upstream from the *atoD* promoter has been suggested (42). Glucose blocks expression of the *ato* operon, and possible cyclic AMP sites that have been identified upstream from the *atoD* promoter may interfere with the AtoC-$E\sigma^{54}$ interaction (42).

prpBCDE Operon and Propionate Catabolism

Environmental propionate is the end product of several different fermentation pathways and can also result from β-oxidation of odd-chain fatty acids. Propionate is a membrane-permeable anion that can alter the internal pH of bacteria. The products of the *prp* operon degrade propionate. Most of the genetics of propionate catabolism and analysis of gene expression has been studied with *S. enterica* serovar Typhimurium. Expression of this operon requires σ^{54} in *S. enterica* serovar Typhimurium, although the transcription start site has not been identified (122). In addition to σ^{54}, expression requires IHF and PrpR, which is homologous to NR_I (122). 2-Methylcitrate or a product of its metabolism has been proposed to bind PrpR and induce the operon (155). It is assumed that regulation in *E. coli* is similar. The putative *E. coli* promoter has the third highest score, 89.6, of known σ^{54}-dependent promoters.

The propionate catabolic pathway is called the methylcitric

acid cycle (76, 153). The first reaction is the addition of CoA to propionate by PrpE, propionyl-CoA synthetase (75). In addition to PrpE, acetyl-CoA synthetase and possibly an enzyme of acetoacetate catabolism can catalyze this reaction (75, 133). The second reaction is catalyzed by methylcitrate synthase, the product of *prpC*, which generates the presumed inducer methylcitrate. Methylcitrate synthase also reacts with acetyl-CoA, although propionyl-CoA is the preferred substrate (76). The next reactions are a dehydration and hydration to form methylisocitrate with methylaconitate as an intermediate. An aconitase-like activity could conceivably catalyze these reactions, and PrpD might catalyze one or both of these reactions, but PrpD is not homologous to known aconitases (76). The last reaction is cleavage of methylisocitrate to succinate and pyruvate, which is catalyzed by PrpB, methylisocitrate lyase (76). The methylcitric acid cycle requires regeneration of oxaloacetate. The most obvious source of oxaloacetate is succinate. However, some strains of *E. coli* require the glyoxylate shunt for this oxaloacetate formation (153), and it has been proposed that *S. enterica* serovar Typhimurium generates oxaloacetate from pyruvate by the combined actions of phosphoenolpyruvate synthetase and phosphoenolpyruvate carboxylase (56). Several aspects of propionate catabolism are unusual. Strains lacking glutathione cannot utilize propionate as a carbon source (135). Strains lacking DNA polymerase I also fail to utilize propionate, which suggests that propionate or a product of propionate catabolism damages DNA (134). Finally, propionate is toxic in the absence of enzymes of the methylcitric acid cycle (64).

psp Operon and Phage Shock Response

The *pspABCDE* operon is unusual, and its regulation is perhaps the most complicated of the σ^{54}-dependent operons (see reference 112 for a review). This system was first discovered and studied by Peter Model and colleagues, who noticed that overexpression of a filamentous phage protein resulted in massive PspA synthesis in *E. coli* (25). It was subsequently shown that several different stresses also induce PspA synthesis: filamentous-phage infection, overexpression of some filamentous-phage proteins, overexpression of some outer membrane proteins (especially mutant forms), heat shock, ethanol, hyperosmotic shock, nutritional downshifts (passage into the late stationary phase of the growth cycle), proton ionophores and other uncouplers of oxidative phosphorylation (free fatty acids), and hydrophobic organic solvents (95, 112, 165). Various proteins are required to sense these stresses, and it is unlikely that there is a single inducing effector.

Mutants lacking PspA, PspB, or PspC have no dramatic phenotype during exponential growth. However, these mutants survive poorly in stationary phase in an alkaline environment (165). These mutants also have greater motility and slower protein translocation (112). PspA appears to maintain the proton motive force in stressed cells, and it has been proposed that this is the major function of PspA (94).

All the inducing stresses result in transcription from a single promoter that requires σ^{54} (163). The promoter has a score of 80.0 (Table 3). Activation from the *psp* promoter requires PspF, which is specified by a gene adjacent to, but divergently transcribed from, the *pspABCDE* operon (91). PspF and IHF bind cooperatively to the *psp* promoter (89). One function of IHF is to increase the specificity of activation by preventing an interaction with RNA polymerase by other activators (52, 91). However, PspF (and possibly other σ^{54}-dependent activators) can activate the *psp* operon without the PspF binding site (the enhancer) during hyperosmotic shock (90). PspF lacks an amino-terminal regulatory domain, and it activates transcription without phosphorylation or an activating ligand (91). Instead, as described below, PspA controls PspF activity.

An unusual aspect of the *psp* operon is that four of the five genes specify regulators that control *psp* expression. PspA binds to PspF, which blocks its ability to activate transcription (51). PspA also inhibits at least one other σ^{54}-dependent activator, NR_I, and perhaps others (51). It is not known whether this inhibition of NR_I is important in vivo. A region of PspA has homology to the RNA polymerase binding region of σ^{54}-dependent activators, which suggests that the mechanism of inhibition may involve a nonproductive interaction with RNA polymerase (88). PspA is peripherally associated with the inner membrane. PspB and PspC are also components of the inner membrane, and they cooperate in activating transcription, probably by antagonizing the effects of PspA (112, 163). PspD may similarly antagonize PspA (unpublished results cited in reference 112). No function is apparent for PspE (112). The induction mechanism is stimulus specific. Induction by the gene IV product of the filamentous phage f1 requires PspB, PspC, and PspD, whereas induction by heat shock requires none of these proteins. Other stimuli may require one or more of these regulatory proteins (112). The IHF dependence also varies with the stimulus (164).

PspF negatively autoregulates its own synthesis by binding to the same sites that activate *psp* operon expression (88). PspA, PspB, or PspC does not affect this autoregulation, which implies that these proteins do not affect the binding of PspF to DNA (88).

Proteins of the heat shock response also affect *psp* induction. Many but not all of the stimuli that induce the *psp* operon also induce the heat shock response. However, loss of σ^{32}, the heat shock sigma factor, results in higher and longer expression of the *psp* operon (25, 26, 112). The mechanism of this regulation is not known.

rtcBA Operon

The promoter for the *rtcBA* operon has the highest score (95.4) of known σ^{54}-dependent promoters. The existence of the promoter was shown by primer extension in wild-type *E. coli* and by failure to observe the transcript in an *rpoN* mutant (62). The σ^{54}-dependent transcript was the only detectable transcript in primer extension experiments. Possible IHF binding sites were identified between 46 and 68 bases upstream from the start site of transcription. Expression of the *rtcBA* operon appears to require the divergently transcribed *rtcR*, which has a deduced product that is homologous to other σ^{54}-dependent activators. Detectable expression of the operon requires artificial overproduction of RtcR. Deletion of its amino-terminal domain also increases expression, which suggests that this domain inhibits the activity of RtcR. The stimulus that controls the activity of RtcR is not known.

RtcA catalyzes the ATP-dependent formation of 2′,3′-cyclic

phosphodiester from an RNA with a 3′ phosphate at its 3′ end. The function of this activity is unknown, although such cyclic intermediates may be required for RNA ligation reactions (19). This enzyme is found from *E. coli* to HeLa cell extracts. An *E. coli* strain with 90% of *rtcA* deleted had no phenotype when grown in Luria-Bertani or minimal M9 medium (62). The activity of RtcB is not known.

zraSR (*hydHG*), *zraP*, and the Response to Zn^{2+} and Pb^{2+}

The products of *zraSR* (previously called *hydHG*) are a membrane-associated sensor kinase and a response regulator, respectively. They were initially implicated in the control of hydrogenase 3 synthesis (150), but this control was observed only in an *fhlA* mutant and was subsequently shown to be nonspecific (99). The gene divergently transcribed from *zraSR, zraP*, had been implicated in tolerance to high Zn^{2+}; therefore, the effect of Zn^{2+} on gene expression was examined (99). In response to high Zn^{2+} or Pb^{2+} concentrations, ZraR and ZraS specifically activated *zraP*, which is divergently transcribed from *zraSR*, and also autogenously activated *zraSR* expression. Purified ZraR bound in the *zraP-zraSR* intergenic region. Metal-induced expression required σ^{54} in vivo, and potential binding sites for Eσ^{54} were readily identifiable for *zraP* and *zraSR*, with scores of 88.6 and 76.2, respectively. In addition to these promoters, *zraSR* appeared to have a weak constitutive promoter, which ensures basal synthesis of the sensor and response regulator (99).

The most important system for Zn^{2+} tolerance is the *zntA-zntR* system, which codes for a Zn^{2+} efflux protein and a Zn^{2+} binding MerR-like transcriptional activator, respectively (15, 27, 132). Its loss results in Zn^{2+} hypersensitivity (132). In contrast, loss of the *zraP-zraSR* system is observable only in a longer lag during the transition to medium with Zn^{2+} (99). The precise physiological function of the *zraP-zraSR* system has yet to be determined. However, it is possible that it acts as a sensor of extracellular Zn^{2+} while the ZntR system responds to intracellular Zn^{2+} (99). It is not known whether the regulatory circuits of the ZntR and ZraR systems overlap.

OTHER GENES WITH HIGHLY RANKED POTENTIAL Eσ^{54} BINDING SITES

Computer analysis identified 25 properly oriented intergenic sites with a score greater than 76, which is a good indicator of an Eσ^{54}-dependent promoter (discussed above). This section briefly describes the seven sites and the operons they potentially control, if they have not been discussed already. Nitrogen limitation does not affect the expression of any of these operons (176).

The site preceding *rpoH*, which codes for the heat shock sigma factor, has a score of 92.8, which is the second highest computer score for a Eσ^{54} binding site. It is normally spaced relative to the *rpoH* structural gene and the adjacent upstream gene. Furthermore, it is conserved among bacteria (123). There is an obvious rationale for having a σ^{54}-dependent promoter for *rpoH*. σ^{H} activates the synthesis of several proteases, which could transiently supply amino acids. However, we have been unable to demonstrate the existence of a transcript from the putative σ^{54}-dependent promoter from nitrogen-limited cells, which assumes, perhaps erroneously, that NR_I is the activator (A. Kiupakis and L. Reitzer, unpublished).

A possible b2710-*ygbD* operon has a potential σ^{54}-dependent promoter with a score of 86. BLAST analysis suggests that b2710 codes for a flavodoxin or a rubredoxin, a redox protein, and that YgbD has homology to oxidoreductases, such as rubredoxin reductase. The gene divergently transcribed from the b2710-*ygbD* operon is *ygaA*, and it specifies a potential σ^{54}-dependent activator that might regulate b2710-*ygbD* expression. Nothing else is known about this operon and its expression.

The site preceding the potential *yfhKGA* operon has a score of 83.9. The *yfhKGA* operon codes for a potential sensor kinase, a protein of unknown function, and a potential σ^{54}-dependent activator, respectively. The putative *yfhKGA* operon is upstream from and transcribed in the same direction as *glnB*, which specifies an important regulator of the Ntr response, P_{II} (102). *glnB* is not part of an operon containing *yfhKGA*, since the major *glnB* promoter precedes the *glnB* structural gene (102). Furthermore, *glnB* on a plasmid complemented the altered *glnALG* regulation in a *glnB* mutant, which implies that defects in the putative *yfhKGA* operon do not contribute to the altered regulation of the *glnB* mutant (102). Nothing else is known about the putative *yfhKGA* operon.

The potential Eσ^{54} binding sites preceding *kch, topA*, and *yaiS* have scores of 85.4,76.5, and 77.3, respectively. *kch* codes for a potassium channel, *topA* codes for topoisomerase I, and *yaiS* specifies a protein of unknown function. The distance between the putative Eσ^{54} binding site and the translational start site for *kch* (264 bases) or *topA* (201 bases) is larger than that for any known σ^{54}-dependent promoter (the range is from 34 to 136 bases). Furthermore, only 20 bases separate the 5′ end of putative Eσ^{54} binding site for *kch* from the adjacent upstream gene, which probably excludes the possibility of an activator binding site. The *yaiS* intergenic region (706 bases) is larger than that for any intergenic region containing an authentic σ^{54}-dependent promoter (the range is from 148 to 507 bases). The large intergenic regions for these three genes suggest that these sites are false positives.

The site preceding *ybhK* has a score of 78.8. Its product is homologous to RocR, a regulator of arginine catabolism in *Bacillus subtilis*, which is homologous to NR_I (30, 61). However, the homology does not extend to the σ^{54} binding domain, which implies that YbhK is not an activator of σ^{54}-dependent genes.

σ^{54}-DEPENDENT ACTIVATORS

A discussion of the physiological function of σ^{54} will account for all of the functional σ^{54}-dependent activators. The signature of these activators is a highly conserved activation domain that binds and hydrolyzes ATP and interacts with Eσ^{54}. Almost all σ^{54}-dependent activators also have an amino-terminal regulatory domain and a small carboxy-terminal DNA binding domain. To identify σ^{54}-dependent activators, we used PspF (which lacks an amino-terminal regulatory domain) and the central activation domain of NR_I as probes for BLAST searches. Both probes identified the same proteins: AtoC, the product of b1201, FhlA, HyfR, NR_I, PrpR, PspF, RtcR, YfhA,

TABLE 4. Activators of σ^{54}-dependent promoters in *E. coli*

Activator	Function	Genes activated
AtoC	Acetoacetate catabolism	*atoDAEB*
b1201	Dihydroxyacetone catabolism?	b1200-b1199-*ycgC*?
FhlA	FHL	*hyp* and *hyc*, operons, *fdhF*, and *hydN-hypF*
HyfR	Hydrogenase 4 control?	*hyf* operon?
NR_I	Nitrogen assimilation	*argT-hisJQMP, astCADBE*, b1012-b1006, *ddpXABCDE, glnALG, glnHPQ, glnK-amtB, gltlJKL, nac, potFGHI, yeaGH, ygjG*, and *yhdWXYZ*
PrpR	Propionate catabolism	*prpBCDE*
PspF	Phage shock	*pspABCDE*
RtcR	RNA terminal cyclase	*rtcBA*
YfhA	?	?
YgaA	?	?
YgeV	Purine catabolism control?	?
ZraR	Zinc tolerance	*zraP, zraSR*

YgaA, YgeV, and ZraR (HydH). The search also identified TyrR, an activator of σ^{70}-dependent genes of aromatic amino acid synthesis (45). It lacks a region of the activation domain that has been implicated in the interaction with $E\sigma^{54}$ (113). All the other proteins are homologous throughout the activation domain, which suggests that they activate σ^{54}-dependent promoters and not σ^{70}-dependent promoters. The functions of 10 of these proteins have already been discussed. The genetic contexts for the remaining two, the product of b1201 and YgeV, provide clues to their possible function and are discussed in this section. Table 4 summarizes the functions of these proteins and the genes that they activate. Curiously, if autogenous regulation is excluded (for ZraR), then only two of these regulators, NR_I and FhlA, are known to activate more than one operon.

The b1201 gene specifies an apparent σ^{54}-dependent activator. Genes flanking b1201 are transcribed in the opposite direction, which implies that b1201 is monocistronic. The b1201 gene is divergently transcribed from three genes, b1200-b1199-*ycgC*, which might form an operon. BLAST analysis suggests that b1200 and b1199 are homologous to a dihydroxyacetone kinase whereas YcgC is homologous to components of the phosphoenolpyrurate-dependent phosphotransferase system. Dihydroxyacetone kinase is an enzyme in the oxidative branch of glycerol fermentation (24). *Klebsiella pneumoniae* can ferment glycerol, but *E. coli* cannot. Nonetheless, a triply mutated *E. coli* can convert glycerol to dihydroxyacetone, which is initially excreted and subsequently metabolized (152). Furthermore, wild-type *E. coli* can use dihydroxyacetone as the sole carbon source (as long as the phosphate concentration is kept low) (86). Dihydroxyacetone kinase has yet to be assayed from *E. coli*. In *Streptococcus faecalis*, phosphotransferase system-dependent phosphorylation stimulates dihydroxyacetone kinase activity (48). Similarly, YcgC may be required for kinase activity in *E. coli*. In *Citrobacter freundii*, dihydroxyacetone kinase synthesis requires σ^{54} (47). Therefore, it would not be surprising if similar control was found for the *E. coli* genes. Unfortunately, computer analysis does not identify a potential σ^{54} promoter in the vicinity, perhaps because the σ^{54}-dependent promoter has been lost.

YgeV is a potential σ^{54}-dependent activator. Several genes in the vicinity of *ygeV* appear to code for enzymes of purine metabolism (172). Strains with a disruption of YgeV grow faster with aspartate as the sole nitrogen source without exogenous purines (H. Xi and L. Reitzer, unpublished observation). A strain with a disruption of the *xdhABC* operon, which codes for subunits of a xanthine dehydrogenase, has a similar phenotype (172). *xdhABC* appears to have a σ^{54}-dependent promoter and a σ^{54}-independent promoter (172). Since strains with a disruption of *ygeV* or *xdhA* have similar phenotypes, it is possible that YgeV is required for transcription from the σ^{54}-dependent *xdhA* promoter.

PHYSIOLOGICAL FUNCTION OF σ^{54}

Possible Relationship between the σ^{54}-Dependent Genes

With a nearly complete set of σ^{54}-dependent genes (verified or potential), it is appropriate to ask whether the physiological themes of these genes are related. Many σ^{54}-dependent genes are involved in nitrogen assimilation. These genes specify GS, the regulators NR_I and Nac, several transport systems, and a few catabolic operons. Is there a relationship between the σ^{54}-dependent genes of nitrogen metabolism and the other σ^{54}-dependent genes? One possibility is that there is no relation between them. This possibility makes one interesting prediction: σ^{54} is present in excess, and expression of one σ^{54}-dependent gene will not affect any other. Considering the low level of σ^{54} found in some strains (87), it is reasonable to question whether this is the case.

Another explanation for the apparent diversity of the σ^{54}-dependent genes is that certain conditions might make nitrogen assimilation very difficult, and several products of σ^{54}-dependent genes might remedy the problem. It has been suggested that genes of the FhlA regulon may have coevolved with the genes of nitrogen assimilation (101). pH homeostasis provides a rationale for such coevolution. The function of the FhlA regulon is to increase the pH in an acidic environment. This could help nitrogen assimilation, because of the mechanism of ammonia transport. The AmtB protein catalyzes facilitated diffusion of NH_3 but not NH_4^+ (149). pH determines the extent of ionization. If the external environment is acidic relative to the cytoplasm, NH_3 will leak out of the more basic cytoplasm (either with the AmtB carrier or without the carrier, because NH_3 is membrane permeable). Subsequent protonation of the external NH_3 will make it difficult to bring NH_4^+ back into the cell. The FhlA regulon, which increases the external pH, perhaps just locally, might alleviate this problem and facilitate nitrogen assimilation.

The *psp* operon may also alleviate a situation that impairs nitrogen assimilation. Since the *psp* operon responds to energy or nutrient limitation, it has been proposed that the ATP concentration controls *pspABCDE* expression (112, 165). The conditions that induce the *psp* operon may also modulate the expression of genes that require NR_I, which itself hydrolyzes ATP (166) and is severely inhibited by ADP (D. Fewell and L. Reitzer, unpublished observation). Another mechanism by which *psp* expression can affect nitrogen assimilation is based

on the proposal that PspA maintains the proton gradient, whose collapse would impair energy generation. Under such conditions, energy-consuming nitrogen assimilation might be inappropriate. (It has been estimated that 1 g of *E. coli* requires about 57,000 μmol of ATP and that if all nitrogen is assimilated via GS, then the GS reaction itself consumes about 10,500 μmol of ATP. This is obviously a major strain on cellular resources.) In this context, it should be noted that the expression of the *psp*, *ato*, and *prp* operons are probably linked. Fatty acids can collapse the proton gradient and presumably induce the *psp* operon, and the subsequent products of fatty acid catabolism will induce the *ato* and *prp* operons.

If certain conditions make nitrogen assimilation difficult, then not only might the more active σ^{54}-dependent genes that are not directly involved in nitrogen assimilation alleviate these conditions, but also their expression might downwardly modulate the expression of the σ^{54}-dependent genes of nitrogen assimilation by competing for σ^{54}. This is especially plausible for strains with a low level of σ^{54}, such as strain MC4100, which may contain as few as 20 molecules of σ^{54} per cell (87). Furthermore, PspA may have the ability to inactivate all σ^{54}-dependent transcription. If this is the case, expression of the *psp* operon might lower the availability of σ^{54} and also inactivate the activators of σ^{54}-dependent genes. The inhibition of the activators may be important even if σ^{54} is present in excess.

In summary, if the genes of the FhlA regulon and the *psp*, *ato*, and *prp* operons alleviate conditions that are detrimental to nitrogen assimilation, the vast majority of σ^{54}-dependent genes in *E. coli* have a function that is related to nitrogen assimilation.

Evolutionary Persistence of σ^{54}

The mechanism of σ^{54}-dependent transcription is complex and requires a large regulatory region. This raises the question why such cumbersome transcriptional control has been evolutionarily maintained. One advantage of σ^{54}-dependent control is the wide range of activity. PspA and GS (both products of σ^{54}-dependent operons) can become a few percent of the proteins of *E. coli*. Furthermore, expression of these operons can be completely suppressed. This could be important for enzymes of nitrogen assimilation, which consume energy and withdraw intermediates from central metabolic pathways, especially the citric acid cycle. The advantage of such absolute control may be to prevent the rapid and catastrophic depletion of resources. The potential for such a loss has been demonstrated by removal of just one layer of nitrogen assimilation control, the adenylylation system for GS, which can result in glutamate depletion (96).

The size constraints of σ^{54}-dependent promoters (the need for binding sites for $E\sigma^{54}$, distant activators, and perhaps a DNA bending protein) may counterbalance the potential advantages of σ^{54}-dependent promoters and also minimize the number of such promoters in a single organism. Such reasoning could account for the seemingly limited number of σ^{54}-dependent promoters in *E. coli*, which we estimate to be about 30 (discussed above). σ^{54} is widespread among bacteria, and the σ^{54}-dependent operons code for proteins with a variety of functions (151). The sheer diversity of these functions suggests that these genes are not always associated with nitrogen assimilation. Nonetheless, the possible evolutionary pressure to maintain few σ^{54}-dependent promoters within a single organism may limit the function of σ^{54}-dependent proteins to a few physiologically related themes.

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