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Regulation of Pyrimidine Biosynthetic Gene Expression in Bacteria: Repression without Repressors

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INTRODUCTION

The pyrimidine nucleotides UTP and CTP and their derivatives are essential for all living organisms, but pyrimidine bases and nucleosides, the transportable precursors of the nucleotides, are often unavailable as exogenous nutrients. It is not surprising, therefore, that all sequenced bacterial genomes, except certain intracellular parasites, encode the enzymes required for de novo biosynthesis of pyrimidine nucleotides. The enzymatic steps of the pyrimidine nucleotide biosynthetic pathway are the same in all bacteria. However, the genomic organization of the genes encoding the pyrimidine biosynthetic enzymes and the mechanisms controlling the expression of these genes vary greatly from gene to gene and across the phylogenetic spectrum. The study of mechanisms that regulate the expression of pyrimidine biosynthetic (*pyr*) genes, which has been a major focus of research in our laboratories for many years, has proven to be a rich source for the discovery of novel biochemical strategies for coordination of gene expression with the intracellular levels of pyrimidine nucleotides. We review here our current understanding of the mechanisms that regulate expression of *pyr* genes in bacteria. Studies of the regulation of *pyr* genes in *Escherichia coli* and *Bacillus subtilis* will be presented in most detail, because these systems have been by far the most thoroughly characterized. Examination of genomic sequences from many other bacteria, which will also be briefly presented here, indicates that the mechanisms found in *E. coli* and *B. subtilis* are operative, sometimes with variations, in a large number of other bacterial species.

It is a remarkable fact that, with rare exceptions, the many mechanisms known to regulate the expression of bacterial *pyr* genes do not involve the participation of a DNA-binding repressor or activator protein. Rather, as will be seen in this review, the information that specifies pyrimidine-responsive regulation of *pyr* gene expression is generally encoded within the promoter-leader region of the regulated downstream genes. (The leader region is defined as the DNA extending from the start of transcription to the first gene of an operon.) During transcription of the leader regions, alternative sequences and/or secondary structures in the leader-specified RNA determine whether transcripts will be prematurely terminated or fully elongated or, alternatively, whether an elongated transcript will be efficiently translated. In all cases except those involving the *pyr* mRNA-binding regulatory protein PyrR, the concentration of pyrimidine nucleotides is sensed directly by RNA polymerase. While the predominance of such mechanisms may result from their ancient evolutionary origins, their wide distribution and retention must also reflect their efficiency and sensitivity. The importance of the novel regulatory mechanisms described in this review extends beyond *pyr* genes, however. Their implications for the mechanism of transcription in bacteria in general and for the ways that transcription can be harnessed for regulation of other genes will be discussed in the course of this review.

REGULATORY MECHANISMS IN ENTERIC BACTERIA

History and Overview

From classic experiments in the 1950s, the operon model emerged to explain regulation of lactose utilization in *E. coli* and, optimistically, all gene regulation in living cells (61). In this model, the rate of protein synthesis was controlled by a repressor, later shown to be a protein (47), which was either inactivated (induction) or activated (repression) by specific metabolites. The active repressor bound to a DNA operator to prevent the synthesis of mRNA, which served as a short-lived intermediate that in association with a ribosome directed the synthesis of the encoded protein(s). The operon model was so compelling that scientists studying the regulation of many different genes in various bacteria in the 1960s and 1970s eagerly searched for their repressors. One of these early studies focused on *pyr* gene expression in *E. coli* and the closely related bacterium *Salmonella enterica* serovar Typhimurium (12, 128). These studies concentrated on the six operons encoding the enzymes required for the biosynthesis of UMP, the precursor of all pyrimidine nucleotides (Fig. 1). These operons, designated *carAB*, *pyrBI*, *pyrC*, *pyrD*, *pyrE*, and *pyrF*, were shown to be genetically unlinked and scattered on the chromosome (124). These operons were also found to be subject to complex regulation. Expression of the *pyrBI*, *pyrE*, and *pyrF* operons was repressed by a uridine nucleotide, whereas expression of the *pyrC* and *pyrD* operons was repressed predominantly by a cytidine nucleotide (81, 132, 145). Expression of the *carAB* operon, which is essential for both pyrimidine and arginine biosynthesis (Fig. 1), was subject to cumulative repression by a pyrimidine nucleotide and arginine (1, 132). These results suggested that at least two repressors controlled transcription of the pyrimidine biosynthetic operons. However, attempts to isolate mutants lacking the putative repressors failed (75, 127). Additional experiments showed that under conditions of pyrimidine limitation, derepression of pyrimidine biosynthetic

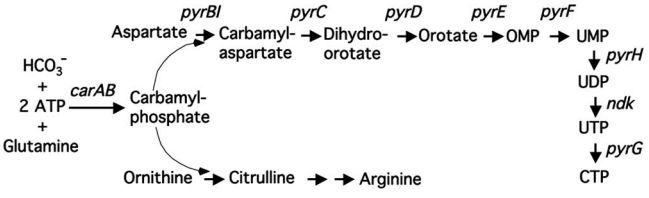


FIG. 1. Pyrimidine nucleotide biosynthetic pathway of *E. coli* and *Salmonella*. Gene names are used to represent the encoded biosynthetic enzymes. The genes shown in the figure and the encoded proteins are as follows: *carA*, glutaminase subunit of carbamylphosphate synthetase; *carB*, catalytic subunit of carbamylphosphate synthetase; *pyrB*, catalytic subunit of aspartate transcarbamylase; *pyrI*, regulatory subunit of aspartate transcarbamylase; *pyrC*, dihydroorotase; *pyrD*, dihydroorotate dehydrogenase; *pyrE*, orotate phosphoribosyltransferase; *pyrF*, OMP decarboxylase; *pyrH*, UMP kinase; *ndk*, nucleoside diphosphokinase; and *pyrG*, CTP synthetase.

operon expression was noncoordinate (124). This observation suggested that the expression of each operon was regulated by an independent mechanism.

By the early 1980s, the iconoclastic discoveries of activator proteins (35, 179) and attenuation control mechanisms of amino acid biosynthetic operons (175) in *E. coli* and *S. enterica* serovar Typhimurium raised the possibility that expression of the pyrimidine biosynthetic operons in these bacteria was controlled by novel mechanisms. However, nothing could have prepared us for the number of new mechanisms that would emerge. These mechanisms were elucidated once investigators began to focus on the regulation of individual operons. The first unique mechanism was attenuation control of *pyrBI* expression in *E. coli*, which employed a previously unrecognized method of controlling transcription termination at an attenuator (159). An analogous mechanism was also found to control *pyrE* expression in *E. coli* (13). Next was the discovery that *pyrC* expression in *E. coli* and *S. enterica* serovar Typhimurium was mediated by CTP-sensitive transcription start site switching, which produced alternative transcripts with different potentials for translation (153, 169). The expression of *pyrD* appeared to be similarly regulated (40). Perhaps the most surprising discovery was a second *pyrBI* control mechanism that employed the unusual reiterative transcription reaction during transcription initiation. This reaction results in the repetitive addition of UMP to the growing end of the nascent transcript. This transcript, with poly(U) at its 3′ end, can no longer be productively elongated and is eventually released from the transcription initiation complex (97). Reiterative transcription was then found to participate in the regulation of *carAB* expression (53) and that of the pyrimidine salvage operons *codBA* (137) and *upp-uraA* (157). The latter two mechanisms provided additional surprises, integrating two of the newly discovered paradigms.

ATTENUATION CONTROL BY COUPLED TRANSCRIPTION AND TRANSLATION

First Examples of Attenuation Control: a Mold To Be Broken

The concurrent pioneering studies of Charley Yanofsky, Bruce Ames, and their collaborators in the 1970s led to the discovery of transcription attenuation control mechanisms for the *trp* operon of *E. coli* and the *his* operon of *S. enterica* serovar Typhimurium (reviewed in reference 86). The hallmark of these regulatory mechanisms is control over transcript elongation at a conditional intrinsic transcription terminator, called the attenuator, within the leader region of each operon. An intrinsic transcription terminator specifies a G+C-rich RNA hairpin (stem-loop) followed typically by an eight-residue poly(U) tract, and termination requires that the hairpin form while RNA polymerase is completing the synthesis of the poly(U) tract (52, 150). In addition to the attenuator, the common regulatory elements in each leader region are a peptide-encoding open reading frame (ORF) that contains multiple adjacent codons for the regulating amino acid (i.e., tryptophan with the *trp* operon, etc.) and a leader transcript with segments capable of forming alternative RNA hairpins. (The leader transcript is defined as the RNA specified by the leader region of an operon.) The upstream-most hairpin (1:2 hairpin for *trp*) forms part of a transcription pause site used to synchronize leader transcription and translation, and the downstream-most hairpin (3:4 hairpin for *trp*) is the terminator hairpin specified by the attenuator. Formation of an alternative hairpin, called the antiterminator hairpin (2:3 hairpin for *trp*), precludes formation of the terminator hairpin, allowing transcription through the structural genes of the operon and production of the encoded enzymes. The peptide-encoding ORF of the leader transcript overlaps the upstream segment of the first hairpin (segment 1 for *trp*).

According to the model for regulation and using the *trp* operon as an example, transcription is initiated at the promoter and proceeds through the leader region specifying transcript segments 1 and 2, which then form the 1:2 hairpin. The transcribing RNA polymerase pauses at this point, permitting a ribosome to bind to the nascent transcript and initiate translation of a 14-codon ORF that encodes the leader peptide. Early in translation the ribosome releases the stalled RNA polymerase by disrupting hairpin 1:2, and then the ribosome proceeds to codons 10 and 11 of the leader ORF, which encode tandem tryptophan (Trp) residues. When Trp is limiting and the level of Trp-tRNA^{Trp} in the cell is low, the ribosome pauses at this site and covers transcript segment 1. During this time, the reengaged RNA polymerase continues transcription and synthesizes transcript segment 3, permitting formation of the 2:3 antiterminator hairpin. Continuing transcription extends the leader transcript through segment 4 and the poly(U) tract (without formation of the 3:4 terminator hairpin necessary for transcription termination) and eventually through the entire operon. Translation of the full-length *trp* mRNA produces the enzymes that increase the cell's capacity to make more Trp. On the other hand, when there is ample Trp and Trp-tRNA^{Trp} in the cell, the translating ribosome does not pause at the tandem Trp codons but proceeds to the stop codon at the end of the leader ORF. At this point, the ribosome covers transcript segments 1 and 2. As reengaged transcription continues, transcript segments 3 and 4 and the poly(U) tract are synthesized, allowing the 3:4 terminator hairpin to form and transcription termination to occur. As a consequence, the synthesis of more Trp biosynthetic enzymes is prevented when there is sufficient Trp-tRNA^{Trp} to support optimal cell growth. Regulation of the *his* operon occurs by an analogous mechanism in which seven adjacent histidine codons are used as the control codons in the leader region (72).

Soon after the elucidation of the attenuation control mechanisms of the *trp* and *his* operons, similar mechanisms were discovered for several other amino acid biosynthetic operons in enteric bacteria (86). Each example employed ribosome stalling at control codons as a regulatory signal and an alternative transcript secondary structure as a means of preventing terminator hairpin formation. These similarities raised the possibility that attenuation control was limited to amino acid biosynthetic operons and to a single mechanism for regulating transcription termination. However, this idea was soon dispelled by studies of the regulation of *pyrBI* expression in *E. coli*, which revealed an attenuation control mechanism that was fundamentally different from that described for the amino acid biosynthetic operons (159).

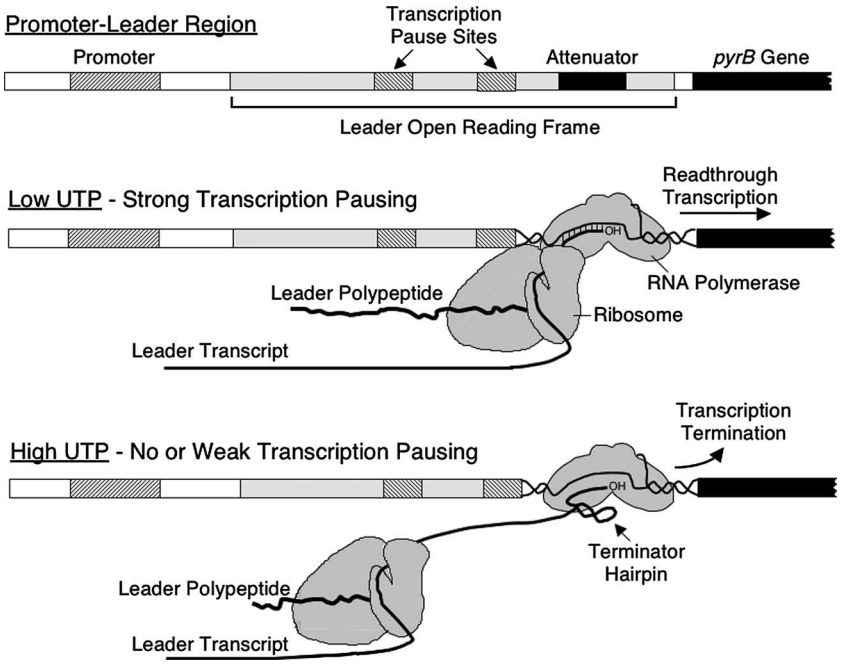


FIG. 2. Model for attenuation control of *pyrBI* expression in *E. coli*. The diagram shows the relative positions of RNA polymerase and the translating ribosome within the leader region when UTP concentrations are either low or high. See the text for additional details. (Modified from reference 86 with permission.)

UTP-Sensitive Attenuation Control of *pyrBI* Expression in *E. coli*

In the in vivo studies of *pyr* operon expression in enteric bacteria described below, conditions of pyrimidine excess and limitation were typically produced by growing a pyrimidine auxotroph (usually carrying a mutation in the *carAB* operon) in a phosphate-buffered minimal medium with uracil or UMP as the pyrimidine source (140). Under these conditions, uracil is a good pyrimidine source and allows the auxotrophic cells to maintain pyrimidine nucleotide levels similar to those found in wild-type cells. In contrast, UMP is a poor pyrimidine source because it must be dephosphorylated to produce uridine, which unlike UMP can be transported into the cell. However, dephosporylation of UMP is a slow process when cells are grown with ample phosphate in the medium, and thus uridine production is restricted (168). As a consequence, pyrimidine nucleotide levels are lower and cell growth is slower in comparison to the case for wild-type cells.

The *pyrBI* operon of *E. coli* encodes the catalytic (*pyrB*) and regulatory (*pyrI*) subunits of the allosteric enzyme aspartate transcarbamylase, which catalyzes the first committed step in the de novo synthesis of pyrimidine nucleotides (Fig. 1). Expression of the *pyrBI* operon is negatively regulated over a wide range by pyrimidine availability, specifically by the intracellular concentration of UTP (98, 158). The regulatory role of UTP was first established in studies employing an in vitro DNA-dependent coupled transcription-translation system in which the levels of nucleotides and other small molecules could be varied (158). The discovery that a substrate for transcription was a regulatory effector of *pyrBI* expression suggested that the *pyrBI* control mechanism did not sense UTP levels per se but instead detected the effects of these levels on the rate of transcription of a regulatory site within the operon. This regulatory site was most likely located within the leader region. For this and other reasons, several laboratories determined the DNA sequence of the *pyrBI* leader region (120, 141, 159).

These studies identified the sequence of a putative intrinsic transcription terminator, or more specifically an attenuator, located 23 base pairs (bp) before the *pyrB* structural gene. Additional in vitro studies indicated that *pyrBI* transcription was initiated at either of two promoters located upstream of the attenuator and that this transcription was efficiently (~98%) terminated at the attenuator (159). These results strongly indicated that *pyrBI* expression was regulated by a transcription attenuation control mechanism. However, the sequence of the *pyrBI* leader region revealed that the leader transcript could not adopt alternative secondary structures to regulate terminator hairpin formation, implying that attenuation control of *pyrBI* expression was mechanistically different from that described for the amino acid biosynthetic operons. The construction of a model for this new mechanism for attenuation control required the identification of two additional elements in the *pyrBI* leader region. The first was a 44-codon ORF that extends through the leader region and ends 6 base pairs before the *pyrB* gene (Fig. 2). In the leader transcript, this ORF is preceded by an apparent ribosome binding site, indicating that it can be translated. The second element was UTP-sensitive transcription pausing, i.e., pausing caused by low UTP levels, in the *pyrBI* leader region upstream of the attenuator. This pausing was detected in vitro (at 20 μM UTP) and initially appeared to be limited to a small cluster of sites at which UTP (or UMP after pyrophosphate release) was incorporated into the leader transcript (159). The pause site region was located approximately 20 nucleotides before the terminator hairpin.

Based on these features and assuming that only the downstream in vitro promoter was physiologically significant, which was subsequently confirmed (31, 91, 96), the following model was proposed for UTP-mediated regulation of *pyrBI* expression (Fig. 2) (159). Transcription is initiated at the *pyrBI* promoter and proceeds into the 158-bp leader region. When the intracellular concentration of UTP is low, RNA polymerase stalls at the UTP-sensitive transcription pause sites, which provides time for a ribosome to initiate translation of the leader transcript and translate up to the stalled polymerase. When the RNA polymerase eventually escapes the pause region and transcribes the attenuator, formation of the terminator hairpin by the nascent transcript is blocked by the presence of the adjacent translating ribosome. In this case, transcription termination at the attenuator is precluded, and RNA polymerase continues transcription into the *pyrBI* genes. In contrast, when the intracellular concentration of UTP is high, RNA polymerase transcribes the leader region without stalling at the UTP-sensitive pause sites. In this instance, there is insufficient time for a ribosome to establish tight coupling with RNA polymerase (or perhaps even bind to the leader transcript) before the formation of the terminator hairpin. The result is transcription termination at the attenuator and no transcription of the *pyrBI* genes. The hallmark of this regulatory mechanism is that tight coupling between transcription and translation in the leader region allows a translating ribosome to disrupt or preclude the formation of the terminator hairpin by steric hindrance. In this mechanism, the extent of coupling reflects the intracellular concentration of UTP. Overall, this regulatory mechanism coordinates the synthesis of aspartate transcarbamylase with the level of UTP needed by the cell for optimal growth.

For a decade after this model was proposed, numerous studies that confirmed its key features were conducted. The central role of transcription termination at the *pyrBI* attenuator was established by biochemical analysis of cellular *pyrBI* transcripts and characterization of deletion mutations in the *pyrBI* leader region (31, 91, 92, 96, 98, 140). These studies clearly showed that *pyrBI* transcripts, initiated at a single physiologically relevant promoter, were subject to UTP-sensitive termination at the *pyrBI* attenuator in vivo. These studies also indicated that attenuation control accounted for most, although not all, pyrimidine-mediated regulation of *pyrBI* expression. To determine the contribution of attenuation control to this regulation, *pyrBI* expression was measured in a mutant *E. coli* strain containing a 9-bp chromosomal deletion that removes the run of eight T · A base pairs at the end of the *pyrBI* attenuator plus an adjacent base pair to maintain the reading frame of the leader polypeptide (98). All intrinsic transcription termination is abolished at this mutant attenuator. When the mutant strain was grown under conditions of pyrimidine excess, *pyrBI* expression was approximately 50-fold higher than that in an isogenic *pyrBI*$^+$ strain. When growth of the mutant was limited for pyrimidines, operon expression increased an additional sevenfold. Growth of the *pyrBI*$^+$ strain under the same pyrimidine-limiting conditions resulted in a 300- to 350-fold increase in operon expression. These results indicate that attenuation control is responsible for pyrimidine-mediated regulation over a 50-fold range, while additional regulation occurs over a sevenfold range through another control mechanism. The latter mechanism, which involves reiterative transcription, will be described in detail below.

In the *pyrBI* attenuation control model, translation of the 44-codon ORF of the leader transcript plays a critical regulatory role. To confirm that the leader ORF was indeed translated in vivo, a gene fusion was constructed in which the *pyrBI* promoter-leader region through codon 11 was fused in frame to codon 9 of the *lacZ* gene. An *E. coli* strain carrying this gene fusion synthesized a β-galactosidase fusion protein with the amino-terminal sequence of the leader polypeptide (140). To show that regulation of the *pyrBI* operon requires translation of the leader ORF, the in vivo effects of mutations that either strongly inhibit translation initiation of the ORF or introduce stop codons early in the ORF, well before the attenuator, were measured (26, 139, 140). Each mutation greatly reduced operon expression, especially under conditions of pyrimidine limitation, and significantly reduced the range of pyrimidine-mediated regulation. Furthermore, mutant (i.e., *rpsL*) strains containing slowly translating ribosomes exhibited reduced *pyrBI* expression, apparently due to reduced coupling of transcription and translation in the *pyrBI* leader region (64). Although translation of the leader ORF is clearly important for regulation, the sequence of the encoded polypeptide is not. A mutant strain carrying a frameshift mutation that changes the sequence of the leader polypeptide, while still allowing translation of the entire leader region, exhibited essentially normal attenuation control (26).

One of the major assumptions of the model is that under conditions of pyrimidine limitation, tight coupling of transcription and translation in the *pyrBI* leader region allows the ribosome to physically prevent the formation of the terminator RNA hairpin. To test this assumption, stop codons were individually introduced at numerous sites within the 44-codon leader ORF to determine the distance that a ribosome must translate to suppress transcription termination at the attenuator (139). Based on the size of the ribosome footprint on its RNA template, translation would have to proceed to a codon located within approximately 15 nucleotides of the terminator hairpin sequence to permit the ribosome to interact directly with this sequence (79, 154). Examination of the strains carrying separate stop codon mutations showed that translation termination at or before codon 24, which is 16 nucleotides upstream of the terminator hairpin, limited operon expression to approximately 5% of the wild-type level under pyrimidine-limiting conditions. In contrast, translation termination at codon 25, which should be the first stop codon at which ribosome binding overlaps the sequence of the terminator hairpin, allowed expression at 64% of the wild-type level. The level of operon expression generally increased to near-wild-type levels as the stop codon was moved further downstream, perhaps reflecting greater disruption of the terminator hairpin. These results provide strong support for the proposed role of the ribosome. In addition, the observation that *pyrBI* expression increased as the stop codon mutations were moved downstream of codon 25 suggests that *pyrBI* expression is enhanced by coupling of translation of the leader ORF and the *pyrB* cistron. In the wild-type *pyrBI* operon, it is likely that such coupling occurs due to the close proximity of these ORFs (159).

The model requires only a single round of translation of the

leader transcript to allow readthrough transcription, and more translation would presumably be wasteful. Such wasteful translation appears to be limited by the use of a relatively weak ribosome binding site preceding the leader ORF (86). In addition, sequences in the downstream half of the leader transcript are complementary to the leader ribosome binding site (120, 140). Formation of a secondary structure by these sequences could block multiple rounds of translation of readthrough transcripts and perhaps all translation of attenuated transcripts.

The discovery of UTP-sensitive transcription pausing in the *pyrBI* leader region was key in developing the attenuation control model. This pausing provided the regulatory sensor, equivalent to control codons in the amino acid biosynthetic operons, that responds to different levels of UTP in a way that influences transcription termination at the attenuator. In *E. coli*, the UTP concentration varies from approximately 50 μM in cells grown under conditions of severe pyrimidine limitation to 1 mM or slightly above in cells grown under conditions that provide ample pyrimidines (3, 124, 158). The first in vitro experiments to detect UTP-sensitive transcription pausing in the *pyrBI* leader region revealed only a small cluster of pause sites that correspond to a uridine-rich region located approximately 20 nucleotides before the terminator hairpin in the leader transcript (159). Subsequent in vitro transcription studies employing a more sensitive assay provided a different view of pausing in the leader region preceding the attenuator (32). Instead of one cluster of pause sites, there is a large number of sites throughout the leader transcript at which RNA polymerase pauses when the UTP concentration is low. Nearly all of these sites correspond to positions where UMP is added to the leader transcript. Pausing at these sites decreases with increasing UTP concentrations (from 20 to 200 μM) and is no longer detectable at a concentration of 400 μM. Although some degree of pausing apparently can occur before the addition of every UMP in the leader transcript at 20 μM UTP, the strength of individual pause sites is variable. This variability presumably reflects the effects of DNA sequence and RNA secondary structure (18, 19). In this regard, an upstream RNA hairpin enhances pausing within the originally identified cluster of UTP-sensitive transcription pause sites (86, 159). Although some pause sites within the leader region may be stronger than others, the large number of these sites indicates that the cumulative effect of pausing at multiple positions is the key factor in controlling coupling between RNA polymerase and the ribosome translating the *pyrBI* leader transcript. Consistent with this view, replacing all seven uridines in the originally identified pause cluster with adenines causes only a twofold reduction in the range of pyrimidine-mediated regulation of *pyrBI* expression (K. Mixter-Mayne and C. L. Turnbough, Jr., unpublished data).

In contrast to transcription pausing observed at low UTP concentrations, extensive pausing in the *pyrBI* leader region was not induced when the concentration of ATP, GTP, or CTP was low (i.e., 20 μM) (32). This difference appears to be due, at least in part, to a difference in the K_m values for these nucleotides during transcription elongation. The apparent K_m for UTP during elongation appears to be significantly higher than the K_m values for the other nucleoside triphosphates (NTPs) (66, 85). This higher K_m apparently results in nonsaturating binding of UTP to an elongating RNA polymerase at all physiological concentrations of UTP (i.e., in cells with limiting or ample pyrimidines). This situation appears to be unique because the physiological concentrations of the other NTPs are typically well above their K_m values for transcription elongation (124, 135). Thus, the rate of transcription elongation is uniquely sensitive to the intracellular concentration of UTP, which makes UTP an ideal regulatory effector for a control mechanism based on coupling of transcription and translation.

Additional noteworthy support for the proposed role of UTP-sensitive transcription pausing in attenuation control came from studies of *pyrBI* regulation in *S. enterica* serovar Typhimurium, which is similar to that in *E. coli* (see below). A mutant strain was isolated that carries an altered RNA polymerase that exhibits an approximately sixfold-higher K_m for the binding of UTP (and ATP) during transcription elongation (66). This mutant displayed constitutive expression of the *pyrBI* operon at high intracellular levels of UTP, indicating that transcription pausing during the addition of UMP (or another nucleotide) to the *pyrBI* leader transcript, and not the UTP level, is the key determinant in regulation. In related studies with *E. coli*, it was shown that the transcription elongation factor NusA enhances UTP-sensitive pausing within the *pyrBI* leader region in vitro and appears to be important in determining the level of *pyrBI* expression in vivo (3, 32). Presumably, NusA plays a key role in establishing a rate of transcription elongation that permits tight coupling of transcription and translation in cells limited for pyrimidines. These results indicate that the activity of NusA or of any factor that influences the rate of transcription elongation can affect the expression of the *pyrBI* operon or of similarly regulated operons.

Attenuation Control of *pyrBI* Expression in Other Enteric Bacteria

The earliest studies of pyrimidine biosynthetic gene expression in bacteria indicated that *pyrBI* expression was regulated similarly in *E. coli* and *S. enterica* serovar Typhimurium, which are closely related enteric bacteria. This similarity was confirmed with the determination of the sequence of the *pyrBI* operon of *S. enterica* serovar Typhimurium (117). The leader region of this operon is identical in length and very similar in sequence to that of *E. coli*, and it contains all the regulatory elements described above for UTP-sensitive attenuation control. Deletion of two T · A base pairs at the end of the *pyrBI* attenuator, which greatly reduces transcription termination efficiency, resulted in a 30-fold increase in *pyrBI* operon expression in *S. enterica* serovar Typhimurium, confirming the central regulatory role of transcription attenuation (117). The most notable difference between the *pyrBI* leader regions of *E. coli* and *S. enterica* serovar Typhimurium is that the latter contains a 33-codon ORF. This shorter ORF is due to a sequence difference that introduces an earlier in-frame stop codon in the leader transcript of *S. enterica* serovar Typhimurium. However, this stop codon is located near the middle of the sequence for the terminator hairpin, and translation to this point would still preclude formation of this hairpin. In fact, a mutation that introduces a stop codon at an equivalent site in the *pyrBI* leader transcript of *E. coli* allows for nearly normal levels of

expression and regulation (139). On the other hand, the shorter ORF in the *S. enterica* serovar Typhimurium *pyrBI* leader transcript may preclude translation coupling with the *pyrB* cistron. Such coupling, which likely occurs in *E. coli*, would presumably enhance *pyrBI* expression.

The attenuation control mechanisms of the *pyrBI* operons of *E. coli* and *S. enterica* serovar Typhimurium were elucidated the old-fashioned way, i.e., by doing many experiments. These experiments identified readily recognizable regulatory sequences. Today, it is possible to inspect a large number of bacterial genomes for these regulatory sequences and thereby identify other operons that are likely to be regulated by attenuation control mechanisms similar to those described above. Although many search formats can be used, even limited searches reveal interesting information about the prevalence of particular control mechanisms. For example, a BLAST search of currently available bacterial genome sequences using the amino acid sequence of the *pyrBI* leader polypeptide as the query (with CLUSTAL W alignment) produced 14 matches. All matches correspond to polypeptides encoded by the *pyrBI* leader regions of five strains of *E. coli* (i.e., K-12 MG1655, K-12 W3110, O157 EDL933, O157 Sakai, and CFT073), five strains of *Shigella* (i.e., *S. flexneri* 301 and 2457T, *S. dysenteriae*, *S. boydii*, and *S. sonnei*), and four strains of *Salmonella* (i.e., *S. enterica* serovar Typhimurium LT2, *S. enterica* serovar Typhi CT18 and Ty2, and *S. enterica* serovar Paratyphi A). All 10 of the *E. coli* and *Shigella* polypeptides contain 44 amino acids; eight of the polypeptide sequences are identical, and two (from the *S. flexneri* strains) contain a single amino acid difference. All four of the *Salmonella* polypeptides contain 33 amino acids, due to the shorter leader ORF described above, and their sequences are identical. These four sequences differ at only five residues compared to the other 10 polypeptides. These results and further inspection of leader sequences indicate that the 14 strains listed above employ an essentially identical attenuation control mechanism for pyrimidine-mediated regulation of *pyrBI* expression. The results are also consistent with the established evolutionary relationships among strains of *Escherichia*, *Shigella*, and *Salmonella* (41).

In the search for matches to the *E. coli pyrBI* leader polypeptide, the misses are as interesting as the hits. For example, no matches were found in the genome sequences of many other enteric bacteria. This result may indicate that the mechanisms for regulating *pyrBI* expression in these bacteria are different from that described for *E. coli*. However, inspection of selected "missed" enteric *pyrBI* operons indicates that they may still be regulated by an *E. coli*-like attenuation control mechanism—one that employs comparable regulatory elements with distinct sequences. This situation appears to be the case for *Yersinia pestis* CO92 and *Erwinia cartovora*, which have all the regulatory elements found in the *E. coli* leader region, including 41- and 40-codon ORFs, respectively. These ORFs encode leader polypeptides with no sequence similarity to the leader polypeptide of *E. coli* and modest sequence similarity with each other. However, the leader ORFs of *Y. pestis* and *E. cartovora* both stop at the same position near the middle of the sequence for the terminator hairpin, which is similar to the situation described for *S. enterica* serovar Typhimurium. Interestingly, the sequence of the leader polypeptide of *Y. pestis* is very similar (i.e., 57% identical) to that of a 37-amino-acid leader polypeptide encoded by the *pyrBI* leader ORF of *Serratia marcesens*. On the other hand, the leader region of *S. marcesens* does not appear to contain the sequence for an *E. coli*-like intrinsic transcription terminator, suggesting another regulatory twist. It should also be noted that the search for matches to the *E. coli pyrBI* leader polypeptide missed all nonenteric gram-negative bacteria. Nonetheless, inspection of selected genomic sequences, e.g., that of *Vibrio cholerae*, suggests again that *E. coli*-like regulation of *pyrBI* expression may occur but with divergent (and perhaps some new) regulatory elements.

Attenuation Control of *pyrE* Expression in *E. coli*

Early studies suggested that each *E. coli* pyrimidine biosynthetic operon would be regulated by an independent mechanism, which later research would show to be true. However, some of these independent control mechanisms are analogous. A case in point is the regulation of *pyrE* expression. The *pyrE* gene encodes the pyrimidine biosynthetic enzyme orotate phosphoribosyltransferase (Fig. 1). Expression of this gene is regulated over a 30-fold range almost entirely by an attenuation control mechanism that is analogous to that described for the *pyrBI* operon (13, 64, 134–136). However, there is a striking difference. The *pyrE* "leader ORF" contains 238 codons and is actually the *rph* gene, which encodes the tRNA-processing exoribonuclease RNase PH (129). Thus, the *pyrE* gene is the second gene of an *rph-pyrE* operon, and the cell uses UTP-sensitive transcription along with translation of the *rph* gene to control transcription termination at an attenuator preceding the *pyrE* gene. Another interesting contrast to the *pyrBI* story is that in the *rph-pyrE* transcript, the *rph* cistron ends 10 bases before the terminator hairpin sequence specified by the *pyrE* attenuator. Even so, based on the size of the ribosome footprint, translation to the end of the *rph* cistron would permit disruption of the terminator hairpin, thereby allowing readthrough transcription. Although it is now clear that the number of mechanistic variants used by bacteria to control gene expression by transcription attenuation is nearly endless (56, 86), especially with the recent discovery of riboswitches (50, 173), the studies of *pyrBI* and *pyrE* expression in enteric bacteria provided an exciting preview of coming attractions.

CONTROL OF TRANSLATION INITIATION VIA NUCLEOTIDE-SENSITIVE SELECTION OF TRANSCRIPTION START SITES

Promoters and Transcription Start Sites

Transcription of pyrimidine biosynthetic operons in enteric bacteria is initiated at promoters recognized by RNA polymerase containing the primary sigma factor σ^{70}. This sigma factor recognizes −10 and −35 regions for which the consensus sequences are 5′-TATAAT and 5′-TTGACA, respectively (118). The spacing between the −10 and −35 regions is typically 17 ± 1 bp, and transcription is usually initiated at one or more sites located 7 ± 1 bp downstream from the −10 region (55, 147). At about 75% of promoters, transcription is initiated with ATP or GTP (95). In some molecular genetics textbooks, this preference is used to imply that initiation with CTP or UTP is of little importance. However, initiation with pyrimidine NTPs is

often an essential element in gene expression. This fact was first demonstrated in studies of *pyrC* expression.

CTP-Sensitive Regulation of *pyrC* Expression

In *E. coli* and *Salmonella enterica* serovar Typhimurium, the *pyrC* gene encodes the pyrimidine biosynthetic enzyme dihydroorotase (Fig. 1). The primary pyrimidine regulatory effector of *pyrC* expression was identified as a cytidine nucleotide, probably CTP (145), and additional studies suggested that *pyrC* expression was regulated by the ratio of the intracellular concentrations of CTP and GTP (65). In early studies to define the mechanism controlling *pyrC* expression, it was found that the steady-state levels of *pyrC* transcripts and dihydroorotase activity changed coordinately in response to pyrimidine availability in *E. coli*, suggesting regulation at the transcriptional level (170). Furthermore, a highly conserved operator-like sequence was identified in the promoter regions of the *pyrC* and other pyrimidine biosynthetic (i.e., *pyrD* and *carAB*) operons whose expression appeared to be negatively regulated by CTP. This discovery suggested that *pyrC* expression was regulated by a pyrimidine repressor that employed CTP as a corepressor (170). However, subsequent studies provided different explanations for the circumstantial evidence for this model. The pyrimidine-mediated changes in the levels of *pyrC* transcripts were due not to changes in the rate of synthesis of these transcripts but to changes in their stability because of differential translation (99, 169). The operator-like sequence was in fact shown to be an operator but not one for a pyrimidine repressor. Instead, this operator was the binding site for the purine regulon repressor, PurR, which controls *pyrC* expression over a modest twofold range in response to purine availability in *E. coli* (25, 171) and *S. enterica* serovar Typhimurium (123). PurR is not involved in pyrimidine-mediated regulation of *pyrC* expression, which occurs over an approximately 15-fold range.

The experiments that eventually led to the correct mechanism for pyrimidine-mediated regulation of *pyrC* expression began with the determination of the sequence of the *pyrC* operon and primer extension mapping of its transcription start sites (9, 122, 170). Transcription initiation occurs at four adjacent sites in the initially transcribed region (ITR) of the promoter (170). The nontemplate strand sequence of these sites is 5′-TCCG, which is located 6 to 9 bp downstream from the −10 region (Fig. 3). These sites are designated T6, C7, C8, and G9, and the transcripts initiated at these sites are called the U6, C7, C8, and G9 transcripts, respectively (99). Inspection of the *pyrC* sequence also revealed a hyphenated dyad symmetry that includes the ITR of the promoter and a downstream region specifying part of the Shine-Dalgarno (SD) sequence of the *pyrC* ribosome binding site (Fig. 3) (100). This sequence indicates that U6 transcripts would form a hairpin with a 6-bp stem in which the upstream segment includes the first six nucleotides of the transcript and the downstream segment includes most of the *pyrC* SD sequence. Transcripts starting further downstream (i.e., at C7, C8, and G9) would form progressively shorter hairpins, with the shortest being a 3-bp hairpin formed by G9 transcripts. However, the calculated free energy of formation of the shortest possible hairpin suggests

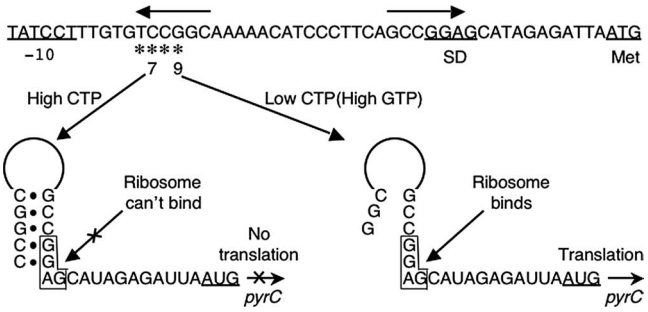


FIG. 3. Model for transcription start site switching and translational control of *pyrC* expression in *E. coli* and *Salmonella*. The nucleotide sequence of the *pyrC* promoter-regulatory region of *E. coli* is shown, with the −10 region, SD sequence, and *pyrC* initiation (Met) codon underlined and labeled. Asterisks indicate the four transcription start sites at the *pyrC* promoter, and the two major start sites, C7 and G9, are indicated. Inverted horizontal arrows indicate the region of dyad symmetry. The sequence and structure of transcripts initiated at start sites C7 (high CTP) and G9 (low CTP) are shown, with the SD sequence boxed. Only C7 transcripts form the hairpin that includes the SD sequence and prevents translation initiation.

that it would not be stable in cells (39, 111), a supposition that was later confirmed experimentally (99).

The final parts of the puzzle included the demonstration that point mutations in the hyphenated dyad symmetry, which were expected to destabilize the encoded hairpin, cause constitutive *pyrC* expression (82). In the same study, it was shown that expression of a transcriptional *pyrC*::*galK* fusion constructed with a short fragment of the *pyrC* operon is not regulated by pyrimidine availability, while expression of a translational fusion containing the same *pyrC* fragment is regulated. These observations led Kelln and Neuhard to propose that *pyrC* expression is regulated at the level of translation initiation through modulation of the secondary structure of the leader transcript (82). The regulatory input of intracellular CTP levels in this mechanism was suggested by the discovery that the selection of the *pyrC* transcription initiation site is affected by pyrimidine availability (153, 169, 170). Under conditions of pyrimidine excess, position C7 is the dominant start site; under conditions of pyrimidine limitation, the dominant start site is G9. This feature and those described above, which are identical in *E. coli* and *S. enterica* serovar Typhimurium, gave rise to the current model for regulation of *pyrC* expression (153, 169).

According to the model (Fig. 3), nucleotide-sensitive selection of transcription start sites is used to produce alternative transcripts with different potentials for translation. When the intracellular level of CTP is high (e.g., during growth with excess pyrimidines), C7 transcripts are synthesized predominantly. These transcripts are not translated, however, because they form a stable hairpin at their 5′ ends that blocks ribosome binding to the *pyrC* SD sequence. In contrast, when the CTP level is low and the GTP level is high, conditions found in cells limited for pyrimidines (142), G9 transcripts are synthesized primarily. The shorter G9 transcripts are unable to form the inhibitory hairpin and are readily translated. Thus, this mechanism allows the level of *pyrC* expression to change according to the cell's requirement for pyrimidine nucleotides. Furthermore, in this model changes in *pyrC* expression can be gradual

in response to incremental changes in the intracellular CTP (and GTP) concentrations.

The key aspects of the model have been confirmed. The importance of the hairpin at the 5′ end of the *pyrC* transcript was shown by using pairs of mutations in the hyphenated dyad symmetry of the *pyrC* leader region. Individually, these mutations cause constitutive *pyrC* expression. However, when a pair of complementary mutations capable of restoring complete base pairing in the leader transcript hairpin was introduced into a strain, it exhibited nearly normal levels of pyrimidine-mediated regulation of *pyrC* expression (153, 169). In related experiments, direct evidence for the predicted secondary structure at the 5′ end of C7 transcripts and the absence of this structure in G9 transcripts was obtained by chemical and enzymatic probing of *pyrC* transcripts isolated from cells grown under conditions of pyrimidine excess or limitation (151). The importance of start site switching was demonstrated by showing that a strain carrying a mutant *pyrC* promoter unable to switch start sites (e.g., containing a C7-to-A or C7-to-G mutation [see below]) fails to exhibit pyrimidine-mediated regulation of *pyrC* expression (99). In addition, nucleotide (CTP/GTP)-sensitive selection of transcription starts sites was demonstrated in vitro using a transcription assay containing only highly purified RNA polymerase, DNA template, NTP substrates, and salts. These results closely mimicked those observed in vivo, indicating that additional regulatory factors are not required for transcription start site switching at the *pyrC* promoter (169). One seemingly wasteful feature of the model is the synthesis of untranslated C7 transcripts. It was suggested that these transcripts would be prematurely terminated, as observed in polarity (82). Such a fate for C7 transcripts is indeed likely, because multiple Rho-dependent termination sites exist early in the *pyrC* ORF (J. Liu and C. L. Turnbough, Jr., unpublished data). Perhaps the most intriguing feature of the model for regulation of *pyrC* expression was nucleotide-sensitive selection of transcription start sites. Characterizing transcription initiation at mutant *pyrC* promoters provided rules for this selection process.

Rules for Selecting Transcription Start Sites and a Revised Model for *pyrC* Regulation

In *E. coli* and *S. enterica* serovar Typhimurium growing exponentially in minimal-glucose or rich media, the intracellular concentrations of CTP and GTP are approximately 0.7 mM and 1.1 mM, respectively. These cells also contain approximately 1.4 mM UTP and 2.7 mM ATP (110, 124). When cells are grown under conditions that severely limit pyrimidine availability, the CTP and UTP levels decrease about 3-fold and 20-fold, respectively. In contrast, under these conditions the GTP and ATP levels each increase approximately threefold (142). These changes seem sufficient to explain the initial step in pyrimidine-mediated regulation of *pyrC* expression, namely, CTP/GTP-sensitive selection of transcription start sites. Assuming that CTP and GTP are competing initiating nucleotides, CTP would "win" when CTP and GTP concentrations were similar, and GTP would "win" when its concentration was much greater than the CTP concentration. However, this simple solution implies that CTP is a better initiating nucleotide than GTP. If this is true, then it seems peculiar that many more *E. coli* and *S. enterica* serovar Typhimurium transcripts are initiated with GTP than with CTP. These observations indicated that more experiments were needed to establish the basis for transcription start site selection. The *pyrC* promoter-leader region was well suited for use in quantitative primer extension mapping experiments to determine preferred initiating NTPs and transcription start sites (99).

The nontemplate strand sequence of the *pyrC* ITR is 5′-TCCGG, located 6 to 10 bases downstream of the −10 region (Fig. 3). Transcription at the wild-type promoter can occur at the first four positions, as described above. Therefore, if context effects are ignored and corrections are made for different transcript stabilities, the levels of C7 and C8 transcripts in cells can be used to calculate the frequency of in vivo transcription initiation at positions C7 and C8 (99). Such an experiment demonstrated that C7 was a fivefold better start site than C8. If a single-base deletion that removes the T residue immediately downstream of the −10 region is introduced into the *pyrC* promoter, the possible start sites are now CCGG at "new" positions 6 to 9. Repeating the experiment described above with the mutant promoter revealed that C7 was a much better start site than C6, with C6 transcript levels so low that they could not be measured. Likewise, it was possible to use the tandem G8/G9 sites to show that G8 was a 13-fold-better start site than G9. Additional mutant promoters were then constructed in which other deletions (i.e., ΔTT and ΔTTG) or a T insertion were introduced immediately downstream of the −10 region. These promoters created more possible positions for the CC and GG pairs, and the transcripts initiated at these sites were analyzed as described above. Combining all of the results permitted the assignment of the following preferences for start site positions: $7 > 8 > 6 > 9 > 10$. Similar analyses were performed to determine preferences for the initiating nucleotide, using a different set of mutant *pyrC* promoters. These promoters contain single-base substitutions at the best initiation position, 7, and at a relatively poor initiation position, 9. Specifically, C7 was changed to a T, G, or A, and G9 was changed to a C or A. Measuring the frequency of initiation at these sites revealed the following preferences for the initiating nucleotide: $\text{ATP} \geq \text{GTP} > \text{UTP} \gg \text{CTP}$. The actual difference between the initiation efficiencies of UTP and CTP was sixfold, making CTP the poorest initiating nucleotide by far. Although the experiments described above were done with *E. coli* (99), the same preferences were observed at wild-type and mutant *pyrC* promoters in *S. enterica* serovar Typhimurium (152).

The preferences or "rules" for selecting transcription start sites suggest a somewhat revised version of the model for *pyrC* regulation. Specifically, these rules provide the basis for nucleotide-sensitive start site switching at the wild-type *pyrC* promoter. The worst initiating nucleotide (CTP) is used to start transcripts at the best start location (position 7), and a good initiating nucleotide (GTP) is used to start transcripts at a weak start location (position 9). These combinations establish competition between initiation at positions C7 and G9, which can be influenced dramatically by changes in intracellular levels of CTP and GTP that reflect pyrimidine availability. The same rules restrict transcription initiation at positions T6 and C8, which utilizes the combination of a suboptimal start position and a poor initiating nucleotide.

It appears that the rules for selecting transcription start sites identified with the *pyrC* promoter apply in general to other σ^{70} promoters. Examination of several hundred well-characterized *E. coli* promoters shows frequencies for selecting initiating nucleotides (A [47%], G [28%], T [15%], and C [10%]) (57, 95) and start site positions (7 [40%], 8 [24%], 6 [11%], 9 [10%], and other sites [≤5%]) (55) that reflect the preferences identified above. These results suggest that most transcripts start with efficient initiating nucleotides and favored positions to maximize transcript synthesis. It also suggests that the use of inefficient initiating nucleotides and less favored positions is evolutionarily selected to reduce or control transcript synthesis.

Finally, the rules described above for selecting transcription start sites ignore context effects. However, the local DNA sequence can be an important factor in start site selection (17, 69, 93, 166). Of particular importance is the sequence at position +2 of the transcript, which accounts for the so-called second-nucleotide effect. It was demonstrated many years ago (113, 126), and again in a clear fashion during the analysis of mutant *pyrC* promoters (152), that high concentrations of both the first and second NTP substrates are required for highly efficient initiation of transcription. Apparently, after formation of the first internucleotide bond, the dinucleotide product stabilizes the transcription initiation complex. Lower concentrations of NTP substrates are required for transcript extension beyond position +2, though the relaxation of the requirement for high NTP concentrations may occur gradually until promoter clearance (2). Based on these observations and the fact that the +2 nucleotide in *pyrC* C7 transcripts is a C, it appears necessary to make a final modification to the model for *pyrC* regulation. Namely, synthesis of C7 transcripts is restricted at low CTP concentrations because of insufficient levels of the first and second NTPs required to initiate transcription.

CTP-Sensitive Regulation of *pyrD* Expression

In *E. coli* and *Salmonella enterica* serovar Typhimurium, the *pyrD* gene encodes the membrane-associated flavoprotein dihydroorotate dehydrogenase (88), which catalyzes the fourth step in the de novo pyrimidine nucleotide biosynthetic pathway (Fig. 1). Pyrimidine-mediated regulation of *pyrD* expression occurs over an approximately 20-fold range (124) through a mechanism analogous to that described for the *pyrC* gene (40, 152). The only noteworthy difference is that the nontemplate strand sequence of the *pyrD* transcription start region is 5′-CCCG (instead of 5′-TCCG). Transcription initiation at the *pyrD* promoter appears to occur primarily at positions C6 and C7 under conditions of pyrimidine excess and at position G9 under conditions of pyrimidine limitation. The longer C6 and C7 transcripts are capable of forming a stable hairpin at their 5′ ends that blocks ribosome binding to the *pyrD* SD sequence, while shorter G9 transcripts cannot form this hairpin and are readily translated (151). Also, as described for the *pyrC* operon, the purine repressor PurR controls *pyrD* expression over an approximately twofold range in response to purine availability (163).

Inspection of published bacterial promoter sequences reveals many other transcription initiation regions at which nucleotide-sensitive start site switching is predicted. Such switching can produce transcripts with minor differences in sequence at their 5′ ends, which produce major differences in the ability of the transcripts to be translated. This effect may be due to formation of secondary structures that inhibit translation initiation as seen with the *pyrC* and *pyrD* regulatory mechanisms. However, nucleotide-sensitive start site switching can generate sequence differences at the 5′ ends of transcripts that alter gene expression in many other ways, some of which were also discovered by studying genes of pyrimidine metabolism (see below).

REGULATION BY UTP-SENSITIVE REITERATIVE TRANSCRIPTION

Reiterative Transcription

Reiterative transcription, which is also known as pseudotemplated transcription, transcriptional slippage, and RNA polymerase stuttering, is a reaction catalyzed by a number of RNA polymerases, including bacterial, phage, viral, and eukaryotic enzymes (62, 68, 94, 107, 137). In this reaction, nucleotides are repetitively added to the 3′ end of a nascent transcript because of slippage between the transcript and DNA (or viral RNA) template. Typically, slippage occurs between a homopolymeric sequence in the transcript and at least three complementary bases in the template (23, 174). The mechanism apparently involves one or more rounds of a one-base upstream shift of the transcript so that the same nucleotide in the template specifies multiple residues in the transcript (10, 51). Reiterative transcription can occur during initiation or elongation, resulting in transcripts that can be immediately released from the transcription complex (11, 97) or extended by normal elongation after a switch to nonreiterative nucleotide addition (87, 164). Although reiterative transcription can involve the addition of any nucleotide, at least under certain conditions, addition of U or A residues appears to occur most frequently. This preference presumably reflects a requirement in the reaction for disruption of the RNA-DNA hybrid, which would be facilitated by relatively weak U · A or A · T base pairing (34).

Second Mechanism to Regulate *pyrBI* Expression in *E. coli*

As described above, characterization of the transcription attenuation control mechanism of the *pyrBI* operon of *E. coli* revealed that pyrimidine (UTP)-mediated regulation of *pyrBI* expression also occurs through a second mechanism, which independently controls operon expression over a sevenfold range. Several studies indicated that this second mechanism requires only the *pyrBI* promoter region and functions at the level of transcription initiation (31, 96, 98). Other observations suggested that this second mechanism involves a run of three T · A base pairs (nontemplate strand T residues) in the ITR of the *pyrBI* promoter. The *pyrBI* promoter region contains the sequence 5′-TATAATGCCGGACAATTTGCCG, with the −10 region and the in vivo transcription start site (A8) underlined (31). It was discovered that RNA polymerase forms heparin-resistant, transcription-competent initiation complexes at the *pyrBI* promoter in the presence of ATP but not with ATP and UTP. This result suggested that the synthesis of a nascent transcript with the sequence AAUUU (but not AA)

destabilizes the initiation complex or perhaps interferes with promoter clearance. It was proposed that this effect could be modulated by the intracellular concentration of UTP and thus contribute to pyrimidine-mediated regulation (31). These observations lingered, however, until a fortuitous encounter with a report of pseudotemplated transcription at a mutant *sar* promoter of phage P22 (63). This mutant promoter contained a G-to-T change at the transcription start site (+1), which created a run of four nontemplate strand T residues from −1 to +3 (i.e., TGTT to TTTT). Transcription from the mutant promoter in vitro produced poly(U) transcripts of various lengths, with abundance decreasing with length. The only requirement to detect the more abundant short poly(U) transcripts was separation of transcription products in a high-percentage polyacrylamide gel.

The sequence requirement for reiterative transcription at the mutant *sar* promoter, as well as at several other promoters (51, 54, 59, 106), appeared to be a short (i.e., ≥3-bp) tract in the ITR that specified a homopolymeric run in the nascent transcript. Thus, the run of three T residues at positions +3 to +5 in the ITR of the *pyrBI* promoter appeared to be a possible site for reiterative transcription. To investigate this possibility, the *pyrBI* promoter-leader region was transcribed in vitro in reaction mixes containing high (≥200 μM) or low (20 μM) concentrations of UTP, with high concentrations of [γ-^{32}P]ATP, GTP, and CTP. The transcripts produced were separated in a 25% polyacrylamide gel (a procedure never employed in the many previous analyses of *pyrBI* transcripts synthesized in vitro) and visualized by autoradiography. The results revealed a ladder of transcripts generated at high UTP concentrations, with the longest transcript containing over 30 nucleotides. Synthesis of this ladder was greatly reduced at the low UTP concentration. The sequences of the transcripts in the ladder were shown to be AAUUU$_n$ (with n = 1 to >30), which established that reiterative transcription indeed occurs at the T_3 tract within the *pyrBI* ITR. Furthermore, transcripts containing extra (i.e., >3) U residues were always released from the transcription initiation complex without switching to normal transcript elongation (which was also demonstrated in vivo), and synthesis of the AAUUU$_n$ transcripts inhibited the production of full-length *pyrBI* transcripts (97). These results suggested that reiterative transcription could be involved in UTP-sensitive regulation of transcription initiation at the *pyrBI* promoter.

To examine the role of reiterative transcription in regulation of *pyrBI* expression, base substitutions were introduced into the T_3 tract within the *pyrBI* ITR. Transcription in vitro of DNA templates carrying these substitutions showed that any change in the T_3 tract abolished reiterative transcription (L. Heath and C. L. Turnbough, Jr., unpublished data). Using a mutant strain carrying one of these base substitutions, it was shown that *pyrBI* expression was sevenfold greater that that observed in a *pyrBI*$^+$ strain when cells were grown under conditions of pyrimidine excess. When this base substitution was introduced into a strain carrying a defective *pyrBI* attenuator, pyrimidine-mediated regulation of *pyrBI* expression was effectively eliminated (97). These results demonstrate the regulatory role of reiterative transcription at the *pyrBI* promoter and show that UTP-dependent reiterative transcription and UTP-sensitive transcription attenuation are sufficient to account for all pyrimidine-mediated regulation of *pyrBI* expression in *E. coli*.

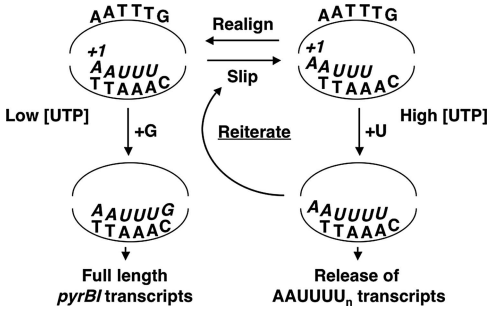


FIG. 4. Model for the regulation of *pyrBI* expression by UTP-sensitive reiterative transcription. DNA sequences in the transcription bubble are shown, and the sequence of the nascent transcript, starting at position +1, is italicized. For details, see the text.

According to these observations, the following model was proposed for regulation of *pyrBI* expression by reiterative transcription (Fig. 4) (97). After the synthesis of the nascent transcript AAUUU, weak base pairing between the transcript and its DNA template allows a rapid and reversible one-base upstream shift (or slip) of the nascent transcript. When the intracellular level of UTP is high and the transcript is in the "slipped" position, the last (i.e., 5′) A in the AAA tract in the DNA template efficiently directs the addition of another U residue to the 3′ end of the transcript. This transcript can be released from the transcription initiation complex or it can shift again. The cycle of slippage and U addition can occur repeatedly, resulting in transcripts with progressively longer runs of U residues. However, all AAUUUU$_n$ transcripts are eventually released from the initiation complex, thereby preventing productive transcription of the *pyrBI* operon. On the other hand, when the intracellular level of UTP is low, slippage (if it occurs) and correct repositioning of the AAUUU transcript—without addition of extra U residues—occurs predominantly. Correct positioning of the RNA-DNA hybrid permits the addition of a G residue to the 3′ end (i.e., position +6) of the transcript. Once this addition occurs, more stable base pairing between the transcript and template precludes further slippage. The AAUUUG transcript either is released from the initiation complex, as a simple aborted transcript, or is extended by the addition of a C residue, which apparently commits the transcription complex to the elongation mode (97). Therefore, high levels of full-length *pyrBI* transcripts are produced only when their encoded enzyme, aspartate transcarbamylase, is needed to synthesize more UTP. In this model, regulation of *pyrBI* expression can occur gradually, over a range of intracellular UTP concentrations, by corresponding adjustments in the efficiency of reiterative transcription.

Distribution of the "TTT Motif"

Comparison of the sequences of the *pyrBI* promoter and other promoters at which UTP-dependent reiterative transcription occurs (63, 71, 174) suggested that the only requirement for this reaction during transcription initiation is a run of at least three nontemplate strand T residues located at or very near the beginning of the ITR. If in fact these conditions were

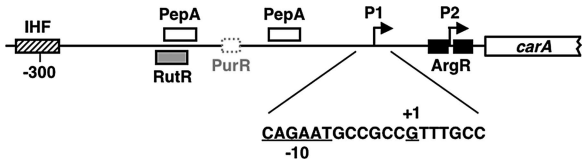


FIG. 5. Promoter-regulatory region of the *carAB* operon of *E. coli*. Promoters P1 and P2 and the binding sites for IHF, PepA, RutR, PurR, and ArgR are shown. The partial sequence of promoter P1 includes the −10 region and transcription start site (+1), which are underlined.

sufficient to permit reiterative transcription, it would seem likely that other operons with promoters containing the TTT motif would be subject to regulation similar to that described for the *pyrBI* operon. Inspection of approximately 500 well-characterized *E. coli* promoters (57, 95) revealed that approximately 10% of these contain a run of three to eight nontemplate strand T residues starting at positions +1 to +3 relative to the transcription start site. Interestingly, several of the promoters containing this motif are in operons involved in nucleic acid metabolism, some of which are negatively regulated by a pyrimidine effector. Included in this group is the *carAB* operon.

Regulation of *carAB* Expression in *E. coli*

The *carAB* operon encodes the two subunits of carbamylphosphate synthetase. This enzyme (and only this enzyme in enteric bacteria) catalyzes the formation of carbamylphosphate, an intermediate in both the pyrimidine nucleotide and arginine biosynthetic pathways (Fig. 1). Expression of the *carAB* operon is subject to cumulative repression by the end products of each pathway (27). Transcription of the operon is initiated at two tandem promoters designated P1 and P2 (Fig. 5). Initiation at promoter P2, the more downstream promoter, is negatively regulated by arginine-dependent binding of the hexameric arginine repressor, ArgR, to two operator sequences that flank the transcription start site (Fig. 5) (15, 133). The molecular details of the ArgR-operator interactions have been described (22, 167). Initiation at promoter P1, the more upstream promoter, is negatively regulated by pyrimidines and to a lesser extent by purines, with the latter occurring by PurR-mediated repression (15, 101, 133). The purine-mediated regulation and part of the pyrimidine-mediated regulation require a nucleoprotein complex that forms upstream of promoter P1 (30). This complex includes integration host factor (IHF), PepA (aminopeptidase A), and PyrH (UMP kinase). The binding site for IHF and two binding sites for PepA have been mapped upstream of promoter P1 (Fig. 5); UMP kinase appears to be recruited to the complex by protein-protein contacts (29). UMP kinase was initially assumed to be the pyrimidine sensor of the complex; however, recent results indicate that this role is played by a protein called RutR, which appears to be a uracil/thymine-binding master regulator for genes involved in pyrimidine synthesis and degradation (146). Apparently, low intracellular levels of pyrimidines allow RutR to bind upstream of promoter P1, at a site that overlaps one of the PepA binding sites (Fig. 5). Without PepA bound to this site, repression of transcription initiation at promoter P1 is prevented (146). In this mechanism, uracil and thymine act as regulatory surrogates for pyrimidine nucleotides.

Although IHF/PepA/PyrH/RutR-mediated regulation is unusually complex, pyrimidine-mediated regulation of *carAB* expression involves yet another independent control mechanism. As suggested by the presence of a TTT motif in the ITR of promoter P1, this other mechanism requires reiterative transcription. Promoter P1 contains the sequence 5′-CAGAATG CCGCCGTTTGCC, with the −10 region and the transcription start site (G7) underlined (53). Analysis of transcription initiation at promoter P1 in vitro demonstrated reiterative transcription within the T_3 tract of the ITR, which increased with higher concentrations of UTP, essentially as observed at the *pyrBI* promoter (53). The analysis of transcripts initiated at promoter P1 in vivo showed that transcripts containing one or more extra U residues (i.e., $GUUUU_n$, where $n \geq 1$) were not extended to include sequences specified by the *carAB* genes (53). Finally, +3T-to-G or +3T-to-C mutations were shown to prevent reiterative transcription at promoter P1 while increasing the production of normally elongated, full-length *carAB* transcripts. Each mutation also caused an approximately threefold reduction in pyrimidine-mediated regulation of *carAB* expression, which was independent of regulation involving IHF and PepA. Pyrimidine-mediated regulation involving IHF and PepA occurs over a six- to ninefold range (53).

Taken together, these results indicate that regulation of *carAB* expression by UTP-sensitive reiterative transcription occurs by a mechanism analogous to that described for the *pyrBI* operon (Fig. 4). In this mechanism, transcription is initiated at the G7 start site in a manner independent of the UTP concentration. After the nascent transcript is extended normally to include four bases and has the sequence GUUU, weak base pairing between the transcript and DNA template permits reversible one-base slippage. With a high UTP concentration and the nascent transcript in the slipped position, an extra U residue is added to the 3′ end of the transcript. Either this transcript can be released from the initiation complex or another round of slippage and U addition can occur. Repeating this cycle generates transcripts with long runs of U residues; however, these transcripts are excluded from the normal mode of transcription elongation. With a low UTP concentration, slippage (if it occurs) and correct repositioning of the GUUU transcript—without extra U addition—permit normal template-directed insertion of a G residue at position +5. This addition results in a more stable RNA-DNA hybrid and the loss of alternative alignments for the 3′ end of the transcript, which precludes further slippage. The GUUUG transcript is either released as a simple aborted transcript or extended downstream with a high probability that it will become a full-length *carAB* transcript. In this model, the level of *carAB* expression is inversely proportional to UTP-sensitive reiterative transcription, and the production of carbamylphosphate synthetase corresponds to the cell's need for pyrimidine nucleotides. Although not included in this (or the *pyrBI*) model, it is possible that intracellular GTP levels affect operon expression by influencing the addition of a U or G residue at position +5 (or +6 in the case of *pyrBI*) of the nascent transcript (70). Physiological conditions that allow GTP levels to modulate reiterative and productive transcription at the *carAB* P1 and *pyrBI* promoters remain to be established. However, this pos-

sibility seems likely because pyrimidine limitation typically results in both a decrease in the UTP level and an increase in the GTP level in the cell (142).

The full range of pyrimidine-mediated regulation of *carAB* expression requires two independent control mechanisms that respond to the same or comparable (i.e., UTP and uracil) small-molecule effectors. Similar situations exist for UTP-sensitive regulation of *pyrBI* expression (i.e., transcription attenuation and reiterative transcription), Trp-sensitive regulation of the *trpEDCBA* expression (TrpR-mediated repression and transcription attenuation) (176), and numerous other operons in *E. coli* and other bacteria (48). The major advantage of such multiple control mechanisms is that regulation can respond to a wide range of concentrations of a particular effector molecule, with each control mechanism sensitive to a different range of effector concentrations. In the case of the *carAB* operon, it appears that IHF/PepA/RutR-mediated regulation occurs when UTP levels are relatively high (i.e., between 0.9 and 1.4 mM), while regulation by reiterative transcription occurs when UTP levels are lower (i.e., between 0.9 mM and 50 μM) (53). The lowest intracellular levels of UTP may be experienced by pyrimidine auxotrophs grown under pyrimidine-limiting conditions or by prototrophs following a shift from a pyrimidine-rich to a pyrimidine-poor environment.

Finally, an interesting difference between the reiterative transcription control mechanisms of the *carAB* and *pyrBI* operons is that the range of regulation provided by UTP-sensitive reiterative transcription at the *carAB* P1 promoter is smaller (by a factor of two to three) than that observed with the *pyrBI* promoter. This difference is presumably due to differences in the *carAB* and *pyrBI* promoter sequences. Mutational variants of the *carAB* P1 promoter were constructed to examine this assumption (X. Han and C. L. Turnbough, Jr., unpublished data). One variant showed that changing the G at the 5′ end of the transcript to an A increases the range of regulation nearly twofold. This result suggests that stronger G/C base pairing between the 5′ end of the transcript and the DNA template suppresses reiterative transcription and restricts the range of regulation. Another variant showed that changing the location of the transcription start site from position 7 to position 8 increases the range of regulation 2.5-fold. The reason for this enhancement is not obvious. However, the observations with the mutant P1 promoters indicate that promoter sequences and the architecture of the transcription initiation complex can significantly affect reiterative transcription. As a corollary, a TTT motif is necessary but not sufficient for UTP-dependent reiterative transcription. The dependence of reiterative transcription on additional promoter elements—sometimes an absolute dependence—was clearly established by the following examples of gene regulation in *E. coli*.

COMPOUND MECHANISMS FOR NUCLEOTIDE-SENSITIVE REGULATION

Salvage of Pyrimidine Bases

In addition to de novo synthesis, pyrimidine nucleotides can be synthesized from pyrimidine bases and nucleosides via salvage pathways in enteric bacteria (121). The pyrimidine salvage pathways can assimilate exogenous bases and nucleosides

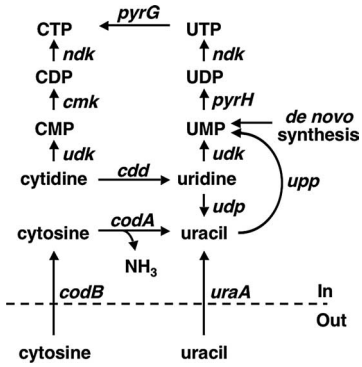


FIG. 6. Salvage pathways for uracil and cytosine. Gene names are used to represent the encoded proteins. Excluding those shown in Fig. 1, the genes and their encoded proteins are as follows: *cdd*, cytidine deaminase; *cmk*, CMP kinase; *codA*, cytosine deaminase; *codB*, cytosine permease; *udk*, uridine kinase; *udp*, uridine phosphorylase; *upp*, UPRTase; and *uraA*, uracil permease.

or can use bases and nucleosides produced inside the cell by normal nucleotide degradation. The pathways for uracil and cytosine salvage are shown in Fig. 6. Exogenous uracil and cytosine are transported into the cell by the cytoplasmic membrane proteins uracil permease and cytosine permease, respectively (5, 28). Intracellular uracil is converted directly to UMP by the enzyme uracil phosphoribosyltransferase. In contrast, intracellular cytosine is rapidly deaminated to uracil and ammonia by the enzyme cytosine deaminase. The uracil produced in this reaction is also converted to UMP by uracil phosphoribosyltransferase. The UMP formed by uracil and cytosine salvage is converted to UDP, UTP, and CTP as described for de novo nucleotide biosynthesis.

Regulation of *codBA* Expression in *E. coli*

In *E. coli*, the pyrimidine salvage proteins cytosine permease and cytosine deaminase are encoded by the *codB* and *codA* genes (Fig. 6), which are included in the *codBA* operon (28). Regulation of *codBA* expression is complex, including control by pyrimidine, purine, and nitrogen availability. Control by purines occurs through PurR-mediated repression (4, 84), and nitrogen control involves the Ntr system acting through the nitrogen assimilation control protein NAC (4, 119). Pyrimidine-mediated regulation occurs through a mechanism involving UTP-sensitive reiterative transcription, but this mechanism is fundamentally different from the original reiterative transcription mechanism described for the *pyrBI* operon (137).

The first indication that *codBA* expression might be regulated by UTP-sensitive reiterative transcription was the discovery that the *codBA* promoter contains a T_6 tract in its ITR, at a position that resembles the location of the T tract in the *pyrBI* promoter. The sequence of the *codBA* promoter region containing the ITR is 5′-TAGAATGCGGCGGATTTTTTGGG, with the −10 region and predicted transcription start sites underlined. However, the T tract in the *codBA* promoter is twice as long as the T tract of the *pyrBI* operon, which suggested that regulation of reiterative transcription at the *codBA* promoter would be different from that at the *pyrBI* promoter. Specifically, it was not clear how low UTP levels would inhibit

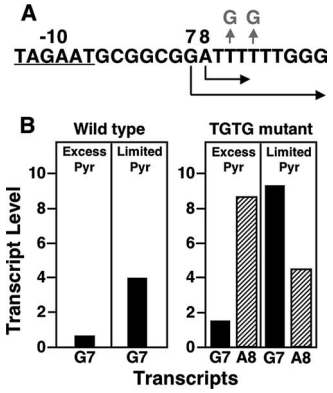


FIG. 7. Transcription from wild-type and mutant *codBA* promoters. (A) Sequence of the wild-type *codBA* promoter region with the −10 region underlined and the G7 and A8 start sites labeled with the number 7 or 8, respectively. Horizontal arrows indicate transcription initiation at the two start sites. The two T-to-G substitutions that created the TGTG mutant promoter are shown in gray. (B) Levels of *codB*::*lacZ* transcripts initiated at the wild-type and TGTG mutant promoters. Cells carrying either the wild-type or TGTG mutant *codB*::*lacZ* fusion were grown under conditions of pyrimidine excess or limitation, and fusion transcript levels were measured by quantitative primer extension mapping (137). Transcript levels are in arbitrary units. (Modified from reference 137 with permission from Elsevier.)

reiterative transcription. With six U residues specified by the *codBA* ITR and only three U residues required for reiterative transcription, it appeared that extra U residues would be added to essentially every nascent *codBA* transcript, regardless of the UTP level. Furthermore, if extra U addition prevented productive transcript elongation, as observed with nascent *pyrBI* transcripts, then transcription of the *codBA* operon would be precluded. Obviously, something was missing in this scenario.

To examine reiterative transcription at the *codBA* promoter, a DNA template containing the *codBA* promoter region was transcribed in vitro in reaction mixtures containing various physiological concentrations (from 20 μM to 1 mM) of UTP (137). Analysis of the transcription products showed that *codBA* transcripts are initiated at two sites: a G residue and an A residue located seven and eight bases downstream from the −10 region (and designated G7 and A8), respectively (Fig. 7A). Most transcripts initiated at position G7 appeared to be elongated normally (i.e., they did not engage in reiterative transcription). In contrast, all transcripts initiated at position A8 appeared to engage in reiterative transcription that produced $AUUUU_n$ (where $n = 1$ to >15) transcripts. These transcripts were released from the transcription initiation complex without further downstream extension. Varying the UTP concentration did not affect the extent of the reiterative transcript; i.e., $AUUUU_n$ transcript ladders were always comparable in length. Although the UTP concentration did not affect reiterative transcription, it had a major effect on start site selection: higher concentrations of UTP favored initiation at position A8. At 1 mM UTP, initiation at position A8 was strongly favored; below 100 μM UTP, nearly all initiation occurred at position G7. Thus, the UTP concentration controlled start site selection, even though UTP was not used as the initiating NTP (which will be explained below).

To investigate the role of reiterative transcription in pyrimidine-mediated regulation of *codBA* expression, a mutant *codBA* promoter was constructed in which the T_6 tract of the ITR was changed to TGTGTT (Fig. 7A) (137). This mutation was shown to eliminate UTP-dependent reiterative transcription initiated at the *codBA* promoter in vitro, which resulted in a sevenfold increase in the synthesis of full-length *codBA* transcripts at 1 mM UTP. To measure the effects of the mutant promoter on *codBA* expression and pyrimidine-mediated regulation in vivo, this promoter was incorporated into a *codB*::*lacZ* gene fusion, which was inserted into the chromosome of a pyrimidine auxotrophic (i.e., *car-94* Δ*codBA-lacZYA*) strain of *E. coli*. This strain and an isogenic control strain with a wild-type *codB*::*lacZ* fusion were grown under conditions of pyrimidine limitation and excess, and β-galactosidase levels in these cells were compared. Wild-type *codB*::*lacZ* expression was regulated over an approximately 30-fold range, while mutant *codB*::*lacZ* expression was unregulated (excluding ~1.5-fold pyrimidine-independent basal regulation). When cells were grown under conditions of pyrimidine excess, expression of the mutant *codB*::*lacZ* fusion was approximately 30-fold higher than that of the wild-type fusion. These results indicated that UTP-dependent reiterative transcription at the *codBA* promoter was required for all pyrimidine-mediated regulation of *codBA* expression.

Using the same four cultures (i.e., mutant and wild-type *codB*::*lacZ* fusion strains grown with limiting or excess pyrimidines), the steady-state levels and transcription start sites of the *codB*::*lacZ* transcripts were determined by quantitative primer extension mapping (137). The primer used in these experiments was complementary to *codB* sequences included in the *codB*::*lacZ* fusion. In the case of the wild-type strain, essentially all detectable transcripts were initiated at position G7, and the level of G7 transcripts in cells grown under conditions of pyrimidine limitation was at least 10-fold higher than that in cells grown with excess pyrimidines (Fig. 7B). Transcripts initiated at position A8 were not detected in cells grown under either condition. In the case of the mutant strain, in which reiterative transcription at the *codBA* promoter is precluded, both G7 and A8 transcripts were detected in cells grown under either condition (Fig. 7B). In both cultures, the levels of total (i.e., G7 plus A8) transcripts were similar, i.e., only 1.4-fold higher in cells grown under conditions of pyrimidine limitation. Also, the levels of total transcripts in the mutant cells (grown with either pyrimidine excess or limitation) were roughly threefold higher than that in wild-type cells grown with limiting pyrimidines, with a large part of this increase due to A8 transcripts. These results indicated that position A8 was a major transcription start site at the *codBA* promoter and that in the wild-type fusion strain, A8 transcripts were not detected because they were not extended downstream to include *codB* sequences. Presumably, these A8 transcripts were produced by nonproductive reiterative transcription and contain the sequence $AUUUU_n$. In addition, the results with the mutant strain showed a high level of pyrimidine-mediated switching between the G7 and A8 start sites. In mutant cells grown with excess pyrimidines, approximately 75% of the transcripts were initiated at position A8. In contrast, in mutant cells grown with limiting pyrimidines, the level of G7 transcripts was twice that of A8 transcripts (Fig. 7B). These responses suggest

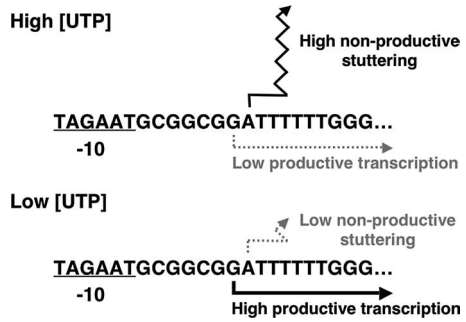


FIG. 8. Model for UTP-sensitive regulation of *codBA* expression. The model shows the effects of UTP concentration on productive transcription and nonproductive reiterative transcription (or stuttering), which occurs following transcription initiation at start sites G7 and A8, respectively. (Modified from reference 137 with permission from Elsevier.)

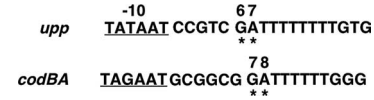


FIG. 9. Promoter region sequences of the *upp* and *codBA* operons. The −10 regions are underlined, and asterisks indicate the two transcription start sites at each promoter. The start sites are numbered according to their position downstream from the −10 region.

that in the case of the wild-type *codBA* promoter, pyrimidine (presumably UTP)-mediated switching between productive transcription initiation at position G7 and nonproductive transcription initiation at position A8 plays a major role in regulation.

Based on the in vitro and in vivo results, the following model involving both UTP-dependent reiterative transcription and UTP-sensitive transcription start site switching was proposed for pyrimidine-mediated regulation of *codBA* expression (Fig. 8) (137). When UTP levels are high, RNA polymerase initiates transcription primarily at position A8—the preferred start site—and synthesizes a nascent transcript with the sequence AUUU (or perhaps AUUUU). At this point, weak base pairing between the transcript and its DNA template allows slippage between the two strands, resulting in a one-base, upstream shift of the transcript. RNA polymerase then switches to the reiterative mode of transcription and adds an extra U residue, which thereafter excludes this transcript from normal elongation. This transcript can be released from the initiation complex or the slippage/extra U addition cycle can be repeated many times, producing longer $AUUUU_n$ transcripts that have a fixed probability of release after each cycle. Furthermore, synthesis of the $AUUU_n$ transcripts precludes initiation at position G7, resulting in a low level of productive transcription and operon expression. Alternatively, when UTP levels are low, initiation at position A8 is inefficient, which restricts the synthesis of $AUUU_n$ transcripts. This restriction allows efficient transcription initiation at the secondary start site G7, which results in the synthesis of high levels of full-length *codBA* transcripts. Translation of these transcripts provides the proteins required for cytosine uptake and conversion to pyrimidine nucleotides when the nucleotides are needed by the cell.

This model introduces two key regulatory elements that require additional explanation. The first element is UTP-sensitive selection of the transcription start site. This process apparently depends on two effects, namely, the inherent preference of RNA polymerase for position A8 as the start site (see above for rules) and the second-nucleotide effect (i.e., high concentrations of both the first and second NTP substrates are required for highly efficient initiation of transcription). The second-nucleotide effect is relevant in this case because UTP is the second nucleotide added to A8 transcripts. Furthermore, ample ATP is present in cells grown with excess pyrimidines (122, 135). Accordingly, high UTP levels support efficient initiation of A8 transcripts. On the other hand, when UTP levels are low, initiation at position A8 is restricted by the second-nucleotide effect and RNA polymerase selects the next best start site, position G7. Initiation of G7 transcripts can proceed relatively efficiently under these conditions because ATP, not UTP, is used as the second nucleotide. Additionally, initiation at position G7 might be facilitated by the two- to threefold increase in the GTP level that occurs in cells limited for pyrimidines (142). The second regulatory element in need of further explanation is the avoidance of reiterative transcription by G7 transcripts. The apparent explanation is that nascent G7 transcripts, from GAUUU through GAUUUUUU, form an RNA-DNA hybrid that is stable enough to preclude slippage and thus avoid reiterative transcription. Remarkably, this stability is imparted by the single G · C base pair formed by the first nucleotide of the nascent transcript.

Regulation of *upp-uraA* Expression in *E. coli*

In *E. coli*, the pyrimidine salvage proteins uracil permease and uracil phosphoribosytransferase are encoded by the *uraA* and *upp* genes, respectively (Fig. 6). These genes are included in the *upp-uraA* operon (5), which hereafter will be referred to as the *upp* operon for simplicity. Expression of the *upp* operon is negatively regulated over an approximately sixfold range by pyrimidine availability (7, 157). The sequence of the *upp* promoter region containing the ITR is 5′-TATAATCCGTCGATTTTTTTTGTG, with the −10 region and the initially reported transcription start site (7) underlined. This region is remarkably similar to the comparable region of the *codBA* operon, although there are two curious differences (Fig. 9). First, the T tract of the *upp* operon contains two more residues. The second difference is that in the *upp* promoter the GA residues preceding the T tract are one base closer to the −10 region. Thus, positions G6 and A7 in the *upp* promoter correspond to positions G7 and A8 in the *codBA* promoter. In spite of these differences, the strong similarities between the ITRs of the *codBA* and *upp* operons suggested that pyrimidine-mediated regulation of the operons would occur through analogous mechanisms.

The characterization of pyrimidine-mediated regulation of *upp* expression was essentially as described for the *codBA* operon (157). Initially, reiterative transcription at the *upp* promoter was examined in vitro, using reaction mixtures containing various physiological UTP concentrations. The results showed that *upp* transcripts are initiated at positions G6 and A7; most G6 transcripts were elongated normally, while all A7 transcripts appeared to engage in reiterative transcription

without further downstream extension. The UTP concentration did not affect the extent of reiterative transcription, which produced a ladder of $AUUU_n$ transcripts with $n = 1$ to >50. However, the UTP concentration had a major effect on start site selection, with lower concentrations favoring initiation at position G6. At 1 mM UTP, initiation at positions G6 and A7 was comparable, but at UTP concentrations of below 100 μM, all detectable initiation occurred at position G6.

The regulatory role of reiterative transcription at the *upp* promoter was investigated by constructing isogenic *E. coli* strains carrying a chromosomal *upp*::*lacZ* gene fusion with either a wild-type *upp* promoter or a mutant promoter in which the T tract was altered (e.g., T_8 to TTGTTTTT) to eliminate reiterative transcription. These strains were grown under conditions of pyrimidine limitation or excess, and *upp*::*lacZ* expression levels were measured. The results showed that wild-type *upp*::*lacZ* expression was regulated over a sixfold range and that this regulation was effectively abolished by the elimination of reiterative transcription. In addition, elimination of reiterative transcription at the *upp* promoter caused constitutive *upp*::*lacZ* expression. Quantitative primer extension mapping of *upp*::*lacZ* transcripts isolated from these cultures showed that in the case of the wild-type fusion strain, only G6 transcripts were detected and the level of G6 transcripts in cells grown with pyrimidine limitation was sevenfold higher than that in cells grown with excess pyrimidines. (Note that with the primer used here, $AUUUU_n$ transcripts could not be detected.) In the case of the mutant fusion strain, both G6 and A7 transcripts were detected in cells grown under either condition; however, pyrimidine availability dramatically affected the relative amounts of the two transcripts. For cells grown with excess pyrimidines, 40% of the transcripts were initiated at position G6 and 60% were initiated at position A7. For cells grown with limiting pyrimidines, 90% of the transcripts were initiated at position G6 and 10% were initiated at position A7. Under both conditions, the levels of total (G7 plus A8) transcripts were similar, i.e., only 1.4-fold higher in cells grown under conditions of pyrimidine limitation. Taken together, these results revealed UTP-sensitive selection of alternative transcription start sites and different fates of the transcripts initiated at these sites that mirror the key regulatory elements of the *codBA* operon. Thus, a model for pyrimidine-mediated regulation of *upp* expression was proposed that is completely analogous to that for *codBA* expression (Fig. 8).

Briefly, according to the model, when intracellular levels of UTP are high, RNA polymerase preferentially initiates transcription at position A7. The resulting nascent transcript is extended until it contains three or perhaps four U residues, at which point weak base pairing in the RNA-DNA hybrid permits the transcript to slip one base upstream. RNA polymerase then adds a U residue to the 3′ end of transcript, which irreversibly directs the transcript into a nonproductive transcription pathway. The transcript can be released from the initiation complex, or another round of slippage and U addition can occur. This process can be repeated many times, with a similar probability of transcript release after every U addition. Synthesis of the resulting $AUUUU_n$ transcripts occludes the promoter, thereby reducing the opportunity for initiation at position G6 and the production of full-length *upp* transcripts. In contrast, when intracellular levels of UTP are low, RNA polymerase initiates transcription almost exclusively at position G6. The resulting transcripts, in general, avoid reiterative transcription due to the formation of a more stable hybrid between the transcript and DNA template. These nonstuttering transcripts are elongated normally and can be extended downstream to generate translatable *upp* transcripts. Consequently, high levels of the proteins required for uracil salvage are produced only under conditions of pyrimidine limitation.

An Interesting Difference between the *upp* and *codBA* Operons

Although there are many similarities between the mechanisms of pyrimidine-mediated regulation of *upp* and *codBA* expression, there is one striking difference. The range of regulation observed with the *upp* operon (6-fold) is much smaller than that with the *codBA* operon (30-fold). One factor that could contribute to this difference is the length of the T tract in the ITR. For example, the longer T tract of the *upp* operon (eight versus six residues) could promote a higher level of reiterative transcription with G-initiated transcripts, thereby restricting the maximum level of productive transcription and the range of regulation. However, this explanation was excluded by examining the effects of systematic, single-base deletions in the T tract of the *upp* operon (23). Reducing the number of residues in the T tract from eight to six did not increase the range of pyrimidine-mediated regulation but decreased it from 5.7-fold to 4.0-fold. In fact, the range of regulation gradually decreased as the T tract was shortened from eight to three residues, at which point only basal (1.5-fold) regulation remained. The decreases in the range of regulation were due to increases in the fractions of both G6 and A7 transcripts that avoid reiterative transcription and are elongated normally. These results indicate that the long T tract of the *upp* operon and presumably the *codBA* operon is required to ensure a high level of nonproductive reiterative transcription with the A-initiated transcripts, which is necessary for the widest range of regulation. Furthermore, the results indicate that although the tract of three T residues in an ITR with the sequence ATTT is necessary for reiterative transcription, transcription of these residues does not ensure reiterative transcription.

Other factors that could contribute to the different ranges of *upp* and *codBA* regulation are the different positions of the transcription start sites and the preferences for selecting these sites (99). The G and A start sites are located at positions 6 and 7 in the *upp* promoter and at positions 7 and 8 in the *codBA* promoter. At the *upp* promoter, the G6 start site is predicted to be a much weaker start site than the A7 site. In contrast, at the *codBA* promoter, both start sites are predicted to be highly efficient. Therefore, much stronger competition between the start sites should occur at the *codBA* promoter. Consistent with this prediction, the observed pyrimidine-mediated start site switching at the *codBA* promoter is much more extensive than that at the *upp* promoter, which should permit a wider range of regulation. To examine this explanation, a mutant *upp*::*lacZ* operon was constructed by inserting a C residue into the sequence between the −10 region and transcription start sites of the *upp* promoter (Fig. 9), which changes the wild-type sequence CCGTC to CCGTCC. This +C mutation changes the

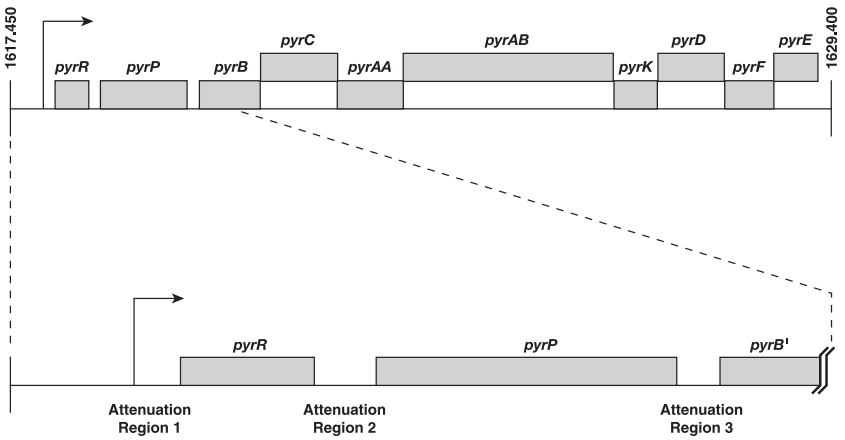


FIG. 10. Map of the *B. subtilis pyr* operon. Shaded bars indicate ORFs, and the bent arrow denotes the *pyr* promoter. The proteins encoded by the genes shown were identified in Fig. 1, except as follows: *pyrR*, *pyr* mRNA-binding attenuation regulatory protein; *pyrP*, uracil permease; *pyrAA*, glutamine-utilizing subunit of carbamylphosphate synthetase equivalent to *carA*; *pyrAB*, catalytic subunit of carbamylphosphate synthetase equivalent to *carB*; *pyrK*, electron-transferring accessory protein to dihdroorotate dehydrogenase. Numbers indicate the positions of nucleotides in the *B. subtilis* genome. (Modified from reference 155 with permission from Elsevier.)

positions of the start sites from G6 and A7 to G7 and A8, which are the same as the start sites in the *codBA* promoter. According to the previous arguments, the +C mutation should change the range of pyrimidine-mediated regulation of *upp* expression from 6-fold to 30-fold. However, the +C mutation did something quite unexpected: it completely eliminated pyrimidine-mediated regulation of *upp*::*lacZ* expression. The loss of regulation occurred because transcription was initiated almost exclusively at position G7 (i.e., without competition from position A8) in cells grown with either limiting or excess pyrimidines (E. Várady and C. L. Turnbough, Jr., unpublished data). Interestingly, partial regulation—and competition from the A8 start site—could be restored by a second mutation in the −10 region of the *upp* promoter. These surprising results suggest that interactions between distinct promoter elements can alter the conformation of the transcription initiation complex in ways that strongly influence the selection of transcription start sites. Additional studies are needed to provide the rules for these interactions.

REGULATORY MECHANISMS IN GRAM-POSITIVE BACTERIA

History and Overview

Only the gram-negative enteric bacteria *E. coli* and *Salmonella* were intensively studied in early investigations of the mechanisms of gene regulation, and for a time it was thought that the regulatory mechanisms found in these species were applicable to all bacteria. Subsequent research has shown that this is rarely the case. While the biochemical pathways of central metabolism are generally the same in gram-negative and gram-positive bacteria, the mechanisms adopted to regulate the expression of the genes for these pathways are usually quite different. Investigations with gram-positive bacteria have uncovered a fascinating variety of novel regulatory mechanisms (37, 172). The mechanisms for regulation of *pyr* gene expression described in this section of the review have been intensively studied in gram-positive bacteria only; however, as described under the heading Phylogenetic Distribution of *pyrR* Genes and Mechanisms of PyrR-Mediated Gene Regulation, variations on these mechanisms are found in gram-negative phyla.

The study of pyrimidine biosynthetic gene regulation in *B. subtilis* in the Switzer laboratory originated from an interest in the regulation of aspartate transcarbamylase activity by intracellular proteolysis (112). *B. subtilis* aspartate transcarbamylase differs from its enteric homolog in that it contains no regulatory subunit capable of mediating allosteric control of enzyme activity (16); allosteric regulation of de novo UMP biosynthesis is exerted at the level of a pyrimidine-repressible carbamylphosphate synthetase (131). The *B. subtilis pyrB* locus was sequenced to determine the amino acid sequence of aspartate transcarbamylase (90). The DNA sequences flanking the *pyrB* gene showed that, in contrast to the case in enteric bacteria, the *pyrB* gene was part of a 10-gene *pyr* operon that encoded all of the enzymes required for the de novo synthesis of UMP (Fig. 10) (89, 138, 161). The sequence of the *pyr* operon revealed three putative intrinsic transcription terminators in the promoter-proximal region, and the positions of these terminators suggested their involvement in a transcription attenuation control mechanism (Fig. 10). The characterization of this mechanism is described in detail in this review. The *B. subtilis pyrG* gene, which as in enteric bacteria encodes CTP synthetase, is not located in the multigene *pyr* operon. Regulation of *B. subtilis pyrG* expression is also regulated by an attenuation control mechanism, but this mechanism, which is described below, is quite different from that of the *pyr* operon.

TRANSCRIPTION ATTENUATION BY PyrR, AN mRNA-BINDING PROTEIN

Regulation of the *Bacillus subtilis pyr* Operon

In *B. subtilis*, the genes encoding all enzymes required for de novo synthesis of UMP lie in a single coordinately regulated *pyr* operon (130). The structure of this operon (Fig. 10) was established by determination of its nucleotide sequence (138,

161). The functions of its individual genes were assigned by comparison of their sequences to those of *pyr* genes of known function (138) and were confirmed by complementation of *E. coli pyr* mutations (89). The functions of three *B. subtilis pyr* genes, *pyrR*, *pyrP*, and *pyrK* (previously called *pyrDII*), were not immediately recognized from their sequences. Subsequent analysis demonstrated that these genes encode the regulatory protein for the *pyr* operon (161), a uracil permease that is homologous to the permease encoded by *uraA* in *E. coli* (44, 161), and an electron-transferring accessory subunit of dihydroorotate dehydrogenase (77, 78), respectively. The last eight genes of the operon overlap by 1 to 32 base pairs, and the operon contains three short noncoding segments that are involved in regulation of operon expression.

The *B. subtilis pyr* operon is transcribed from a single promoter, which appears to be constitutive (105, 161). Operon expression is controlled by an attenuation mechanism in which transcription termination at three sites in the 5′ region of the operon is regulated by uridine and guanosine nucleotides via the PyrR regulatory protein. It was evident from the earliest studies that each of the three untranslated segments of the operon contains a typical intrinsic transcription terminator, which specifies a terminator hairpin (the 3:4 stem-loops in Fig. 11, left) followed immediately by a run of U residues in the mRNA. These untranslated segments are the 5′ *pyr* leader (attenuation region 1, 151 nucleotides), the *pyrR-pyrP* intercistronic region (attenuation region 2, 173 nucleotides), and the *pyrP-pyrB* intercistronic region (attenuation region 3, 145 nucleotides) (Fig. 10 and 11). Clearly, if the action of these terminators is not suppressed, little expression of the downstream genes, which include all of the enzymes of de novo pyrimidine biosynthesis, can occur. It was also recognized that the RNA from all three untranslated segments is capable of folding into an alternative secondary structure, which is a large hyphenated stem-loop (Fig. 11, right) (161). These structures were named antiterminators because they prevent formation of the downstream terminator hairpin by sequestering residues of the 5′ segment of the terminator stem-loop via base pairing with upstream sequences. The key to regulation of the *pyr* operon lies in the ability of PyrR to favor formation of the terminator hairpins, which results in premature termination of transcription and reduced expression of the downstream genes. PyrR does this by binding to *pyr* mRNA when the protein is activated by binding of uridine nucleotides. Furthermore, binding of PyrR to *pyr* mRNA is antagonized by guanosine nucleotides, which effectively activates *pyr* operon expression. The segment of *pyr* mRNA to which PyrR binds was first identified from conserved regions within the nucleotide sequences of the three *B. subtilis* attenuator regions. These conserved regions were always found in the upstream segment of the antiterminator structure, so that binding by PyrR would be predicted to prevent base pairing in the antiterminator RNA and allow the downstream segment of the antiterminator to fold into the alternate terminator hairpin (Fig. 11).

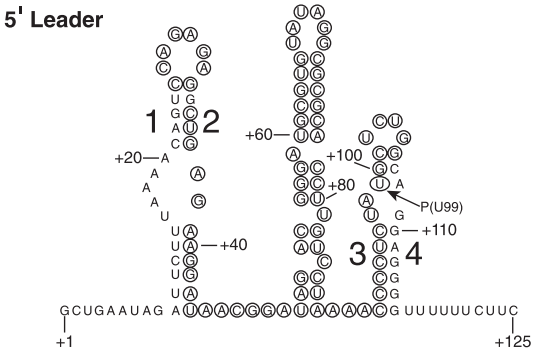
Further analysis of the potential secondary structures formed by the attenuator region RNAs led to the prediction that the conserved PyrR binding sequences were embedded in a third stem-loop, which was named the antiantiterminator or the binding loop (104) (Fig. 11, left, stem-loops labeled 1:2). This stem-loop is formed by base pairing of nucleotides from the upstream strand of the antiterminator stem with sequences that lie still further upstream. Formation of the binding loop disrupts the lower stem of the antiterminator and allows its 3′ strand to fold into the terminator stem-loop. Thus, the regulation of the *B. subtilis pyr* operon can be conceived as involving two alternative conformations of each of the three attenuator RNA segments. One is the antiterminator conformation, which predominates when PyrR is not bound (Fig. 11, right) and leads to transcription readthrough of the downstream *pyr* genes. The other is the PyrR-stabilized antiantiterminator-plus-terminator conformation (Fig. 11, left), which results in termination and reduced expression of the downstream genes. The binding of PyrR to the binding loop within this conformation is crucial to regulation of the *pyr* operon. Since the binding of PyrR is stimulated by UMP and UTP (14, 103) and is antagonized by GMP, GDP, and GTP (14, 21, 73), an effective means of metabolic regulation of transcription of the *pyr* operon by the ratio of uridine to guanosine nucleotides is provided. This mechanism is illustrated in schematic form in Fig. 12.

The secondary structure of the binding loop is conserved (Fig. 11). In its 5′ strand, it contains a purine-rich tract within an internal loop or bulge. The beginning of the internal loop or bulge contains the conserved sequence 5′-UUUAA. In addition, the consensus sequence 5′-ARUCCNGNGAGGYU is located in the terminal stem and loop of the binding loop. Experimental evidence that this secondary structure forms and that the regions of conserved sequence are important for PyrR binding to the RNA is presented below.

Regulatory Function of PyrR: Genetic Evidence

The first clues that the product of the *B. subtilis pyrR* gene acts as a regulatory protein for *pyr* gene expression were obtained from studies with high-copy-number plasmids carrying a fusion between either the *pyr* promoter-leader region or the *pyr* promoter-leader region plus *pyrR* and a downstream reporter gene (161). When transformed into *B. subtilis*, the plasmid carrying just the *pyr* promoter-leader region caused elevated expression of both the reporter gene and the chromosomal *pyrB* gene. The elevated expression was not repressible by exogenous uracil. However, when the plasmid carrying the *pyr* promoter-leader region plus *pyrR* was transformed into cells, regulation of both the reporter gene and the chromosomal *pyrB* gene was normal and repressible. This result suggested that multiple copies of the *pyr* leader region, or more likely its specified RNA, sequestered the PyrR formed from the chromosomal *pyrR* gene, thereby eliminating PyrR-mediated repression. Furthermore, normal regulation was restored by producing elevated levels of PyrR from the high-copy-number plasmid. Subsequent studies confirmed that the plasmid-specified *pyr* leader transcripts were responsible for the titration of PyrR (102). In fact, within a limited range there was a correlation between the amount of *pyr* transcript specified by any of the three noncoding/attenuation regions of the *pyr* operon and the extent of depression of the chromosomal *pyr* operon (102).

A second line of genetic evidence for a role of PyrR in regulation of the *pyr* operon came from characterization of 12 point mutations that caused constitutive expression of the *pyr* operon (46). All of the mutations were located in or near the


5' Leader
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2
3
4
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+40
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+80
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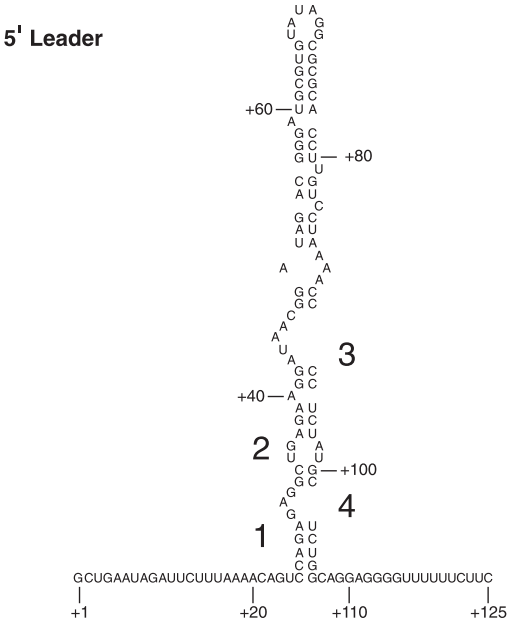
+1
+125


5' Leader
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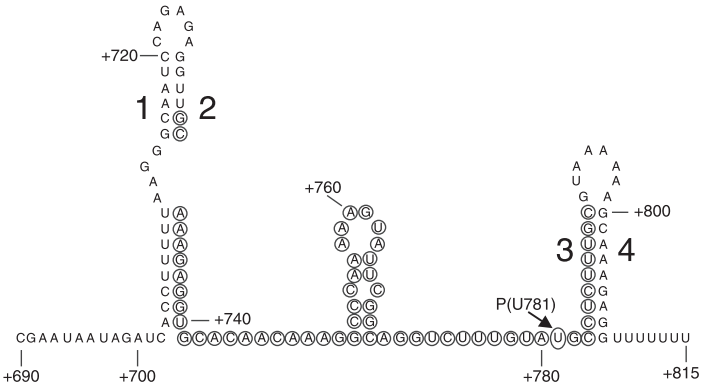
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2
3
4
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+740
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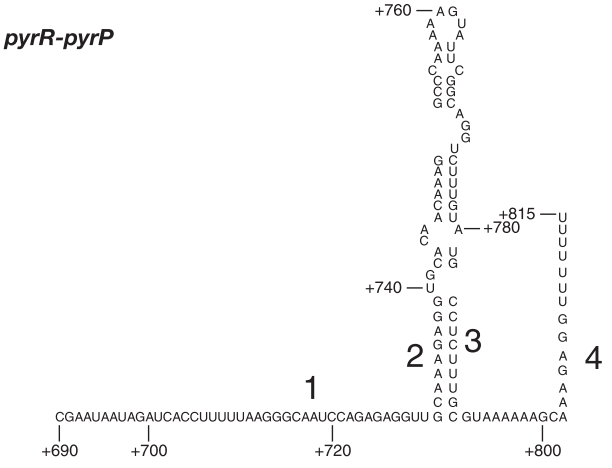
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+760
+780
+800
+815
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2
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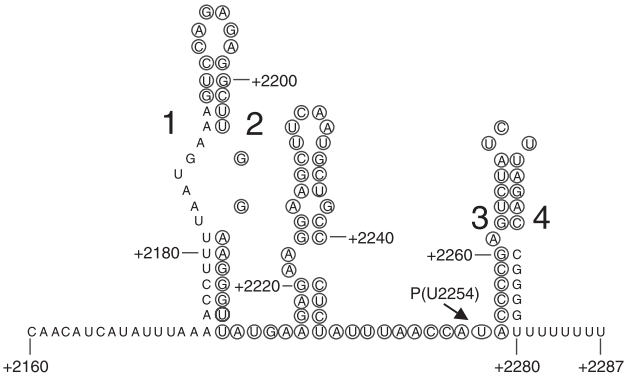
+800


pyrP-pyrB
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2
3
4
+2180
+2200
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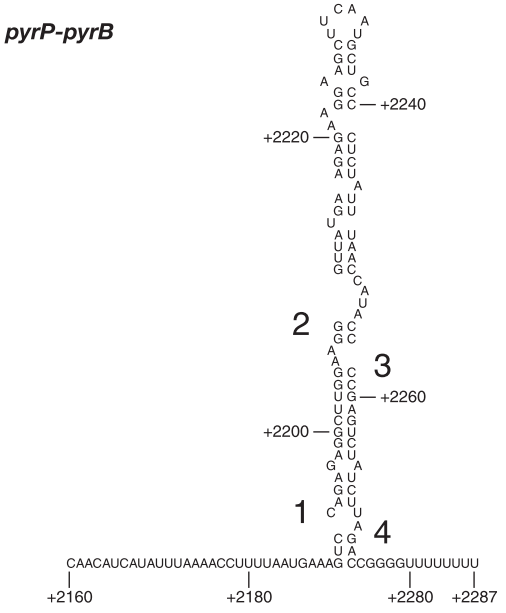
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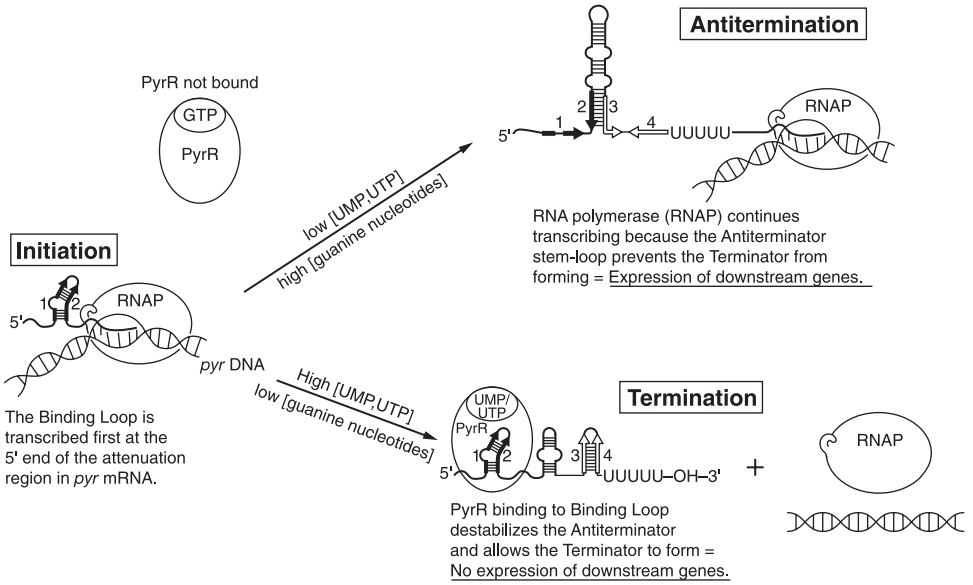

FIG. 12. Mechanism of PyrR-mediated transcription attenuation control of *pyr* operon expression in *B. subtilis*. For simplicity, only transcription of the *pyr* 5′-leader attenuation region is shown. For details, see the text. (Modified from reference 178.)

pyrR gene. Two were premature chain termination mutations, one altered the *pyrR* ribosome binding site, and the others were missense mutations in the *pyrR* gene. The most direct demonstration of the crucial role of PyrR in the regulation of *pyr* operon expression came from the analysis of a *B. subtilis* strain carrying an in-frame deletion in the chromosomal *pyrR* gene (161). In the mutant strain, expression of the *pyrB* gene was 250-fold greater than the repressed levels found in *pyrR*$^+$ cells, and the elevated expression was completely refractory to repression by exogenous pyrimidines. The regulation of *pyrB* expression in the mutant strain was restored to normal by the introduction of a plasmid-borne copy of *pyrR*.

Regulatory Function of PyrR: Biochemical Evidence

To demonstrate directly the regulatory function of PyrR, highly purified protein was included in an in vitro transcription system consisting of *B. subtilis* DNA templates containing the *pyr* promoter fused to each attenuation region, purified *B. subtilis* RNA polymerase, and the four ribonucleoside triphosphates. In this system RNA polymerase initiated transcription at the *pyr* promoter and produced transcripts that either were terminated at an attenuator or were extended to the end of the template. As would be predicted if PyrR mediates repression of *pyr* operon expression in the presence of pyrimidines, addition of PyrR plus UMP or UTP substantially increased the fraction of terminated transcripts. (GMP did not affect termination in these experiments, probably because the high concentrations of GTP used as a transcription substrate obscured its effects.) Regulation of transcription termination by PyrR plus uridine nucleotides was demonstrated with templates from all three *pyr* attenuation regions. However, the quantitative effects of PyrR on attenuation region 1 (the *pyr* leader) most closely recapitulated the effects observed in vivo with a chromosomal *pyr*::*lacZ* fusion containing a selected attenuator region (105). This result may indicate that trailing ribosomes translating upstream ORFs (i.e., *pyrR* and *pyrP*) alter the behavior of attenuation regions 2 and 3 in vivo.

Purified *B. subtilis* PyrR has been shown to possess two other properties required by the regulatory model: it binds with high specificity to *pyr* binding loop RNA sequences, and its affinity for these RNAs is increased by UMP and UTP (14, 160). More recent studies with purified PyrR from *Bacillus caldolyticus* led to the previously unrecognized finding that guanosine nucleotides cause greatly reduced affinity of PyrR for *pyr* RNA and antagonize the effects of uridine nucleotides (21, 73). Similar opposing effects of uridine nucleotides and guanosine nucleotides were observed in the allosteric regulation of the *pyrAA*/*pyrAB*-encoded carbamylphosphate synthetase from *B. subtilis* (131). Such effects have been proposed to provide a means of coordinating rates of pyrimidine biosynthesis with the size of intracellular purine nucleotide pools (21, 73).

Importance of RNA Secondary Structures in Regulation

The secondary structures of the RNAs from the three *pyr* attenuation regions shown in Fig. 11 were derived from computer-based folding programs (180). The parameters of these

FIG. 11. Predicted secondary structures of the regions of *pyr* transcripts specified by attenuation regions 1 (5′ leader), 2 (*pyrR-pyrP*), and 3 (*pyrP-pyrB*). Nucleotides are numbered from the start of transcription (i.e., +1). (Left side) RNA is shown folded into the binding loop, formed by base pairing of segments 1 and 2, and the terminator hairpin, formed by base pairing of segments 3 and 4, conformation. Bases involved in the formation of the alternative antiterminator stem-loop conformation are circled. (Right side) RNA is shown folded into the antiterminator stem-loop conformation. (Modified from reference 178.)

programs are updated from time to time, so the structures in Fig. 11 should be regarded as approximate; numerous variant, but functionally equivalent, structures can exist. Biochemical structural mapping of these RNA segments has been carried out only in the case of the binding loop from attenuation region 2, as described below (14). However, there can be little doubt that all binding loop, terminator, and antiterminator stem-loop structures form and play the regulatory roles assigned to them. The most convincing evidence for these roles came from studies of the effects of antisense oligodeoxynucleotides on the frequency of transcription termination within the three *pyr* attenuation regions in vitro (104). Oligodeoxynucleotides that were designed to disrupt each of the stem-loops by base pairing with their upstream segments consistently had the predicted effects on transcription. For example, disruption of the antiterminator stem-loop caused greater termination at the downstream attenuator, whereas disruption of the binding loop or terminator stem-loop caused increased readthrough transcription. Interestingly, only oligodeoxynucleotides that base pair with the upstream strands of the target stem-loops were effective; oligodeoxynucleotides of equal length that base pair with their downstream strands had little effect. This observation indicates that stem-loop formation occurs very rapidly in solution and that intramolecular base pairing to form the stem-loop competes very effectively with intermolecular base pairing. This led to the suggestion that kinetic aspects of transcription and RNA folding are important to the proper functioning of this attenuation control mechanism. Specifically, the binding loop hairpin must fold and bind PyrR before the synthesis of downstream sequences that direct folding of the more stable antiterminator stem-loop.

The properties of a set of deletion mutations introduced into a gene fusion in which the *pyr* promoter-leader region was joined to *lacZ* also illustrated the importance of RNA secondary structures in regulation (161). Deletion of the terminator in the leader region resulted in high and constitutive *lacZ* expression, whereas deletion of the antiterminator region eliminated expression under all growth conditions. A detailed, systematic deletion analysis of the *pyr* leader region has not been conducted, however.

As noted above, the secondary structure of binding loop 2 of the *pyrR-pyrP* intercistronic attenuation region has been studied by nuclease digestion (14). The patterns of cleavage by single-strand-specific and double-strand-specific nucleases were consistent with the secondary structure shown in Fig. 13B, although these results do not establish this structure unequivocally. Weak cleavage of the RNA in the terminal loop regions provided an indication that this loop may fold into a relatively compact structure, as is known for some other terminal RNA loops (38, 58). Unexpectedly strong single-strand cleavages in the region of A · U base pairs upstream of the bulge also suggested that the single-stranded segment of the lower 5′ strand of the stem-loop is longer than predicted from computer analysis of RNA folding. The other predicted RNA secondary structures shown in Fig. 11 have not been subjected to nuclease digestion analysis.

Regulation of *pyr* Operon Expression as Deduced from *pyr*::*lacZ* Fusions

The model of PyrR-mediated regulation of the *B. subtilis pyr* operon predicts that four *pyr* transcripts will be formed from the operon under derepressing conditions (161). These transcripts are 0.12, 0.65, 2.3, and 12 kb in length and correspond to termination at attenuation region 1, attenuation region 2, attenuation region 3, and the terminator at the end of the operon, respectively. All except the first transcript are predicted to become less abundant when cells are grown with exogenous pyrimidines. The cumulative diminution of readthrough transcription at the multiple attenuators should produce larger reductions in the levels of the longer transcripts. Presumably, the 12-kb transcript is formed and translated largely as a single unit, because the genes that it encodes have been shown to be coordinately regulated in vivo (130). The qualitative predictions of this model were confirmed by Northern hybridization analysis (161). All four *pyr* transcripts were detected, and the abundance of all but the 0.12-kb transcript decreased sharply in cells grown with pyrimidines. However, this approach was not suitable for quantitative analysis of *pyr* transcription.

For the quantitative analysis of *pyr* transcription, Lu et al. analyzed a series of chromosomal *pyr*::*lacZ* fusions expressed in cells under conditions that repress or derepress *pyr* operon expression (105). The first set of transcriptional fusions included the *pyr* promoter followed by DNA containing either attenuator region 1, attenuator region 2, or attenuator region 3. For the latter two fusions, the native intervening upstream attenuators and ORFs were excised. Expression of all fusions was repressed to about the same extent by exogenous pyrimidines. Repression was approximately 4-fold relative to that in cells grown on minimal medium and 20-fold relative to that in cells starved for pyrimidines by slow growth on orotate, a poor pyrimidine source. Repression was completely dependent on a wild-type *pyrR* gene. In addition, the expression levels of the three *pyr*::*lacZ* fusions were similar. In another set of transcriptional fusions, two or three attenuators were linked in tandem. In these cases, repression of *lacZ* expression was cumulative. That is, the extents of repression by excess uracil relative to that in cells starved for pyrimidines were 18-fold, 136-fold, and 200-fold when the fusions contained one, two, and three attenuators, respectively. These observations were entirely consistent with the proposed regulatory model. Studies with other *pyr*::*lacZ* fusions provided clear evidence that the DNA sequences upstream of the *pyr* promoter are not involved in regulation of operon expression and that possible translation of a small ORF in the *pyrP-pyrB* intercistronic region is not required for attenuation control.

Occurrence and Significance of Transcription Pausing in the *pyr* Operon

Our studies with antisense oligodeoxynucleotides suggested a key role for rapid folding of the RNA secondary structures involved in attenuation control of the *pyr* operon. To examine this possibility, Zhang and Switzer analyzed the pausing during the transcription of the *pyr* attenuation regions and attempted to determine whether pausing was important for regulation

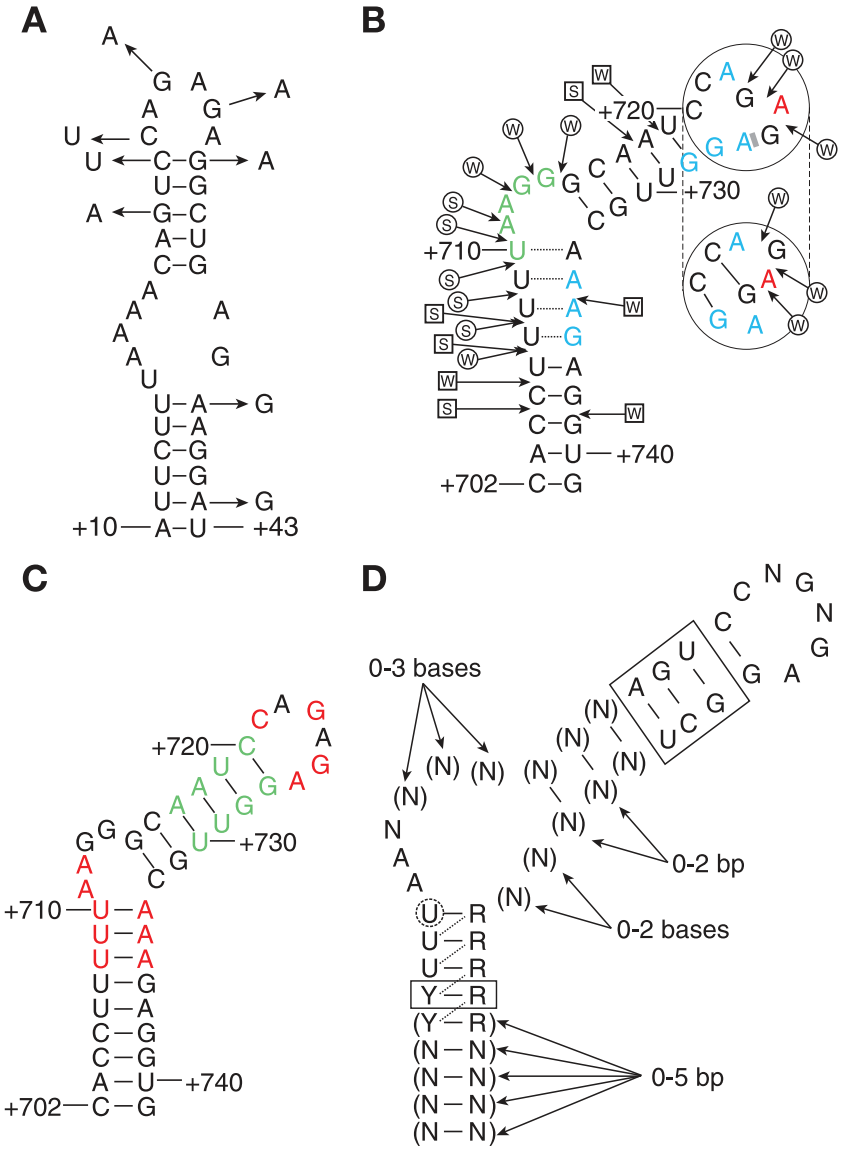

FIG. 13. Elements in the *pyr* binding loop RNA that are important for PyrR binding and *pyr* operon regulation. Nucleotides are numbered as in Fig. 11. (A) Mutations in the *pyr* 5′ leader RNA that cause a loss of repression by pyrimidines. (B) Protection against hydroxyl radical cleavage of binding loop 2 RNA by PyrR and mapping of secondary structure by nuclease digestion. Sites adjacent to nucleotides shown in blue were strongly protected against hydroxyl radical cleavage, the site adjacent to the nucleotide in red was moderately protected, and sites adjacent to nucleotides in green were weakly protected. Arrows with circles indicate sites of cleavage by a single-strand-specific nuclease (RNase I), arrows with squares indicate sites of cleavage by a double-strand-specific nuclease (RNase V_1), and S and W denote strong and weak cleavage, respectively. Suggested alternative structures for the terminal loop of the RNA hairpin are shown in circles. (C) Specificity of PyrR binding to binding loop 2 RNA determined by gel mobility shift analysis. Residues shown in red cannot be replaced without loss of binding, residues in green can be replaced as long as the secondary structure of the RNA is preserved, and residues in black can be replaced or deleted. (D) Consensus sequence and structure of the PyrR binding site. The consensus structure was derived from 20 binding loops identified in *pyr* operons of gram-positive bacteria. Secondary structures were predicted by MFOLD (R = A or G, Y = U or C, and N = any nucleotide). Parentheses indicate nucleotides present in only some species. In 8 of 20 examples, the U nucleotide shown in a dashed circle is part of the internal loop, which directs the predicted alternative base pairing shown with dashed lines. The sequences and base pairs shown in boxes are highly but not universally conserved. (Panel A is reprinted from reference 155 with permission from Elsevier; panels B and D are reprinted from reference 14 with permission from Oxford University Press.)

(178). According to the model for attenuation control, transcription pausing at a site or sites in the downstream strand of the antiterminator stem-loop would allow the time needed for the antiantiterminator stem-loop to form and bind to uridine nucleotide-activated PyrR. Such binding would then preclude formation of the antiterminator stem-loop. Pausing within the three *pyr* attenuation regions was measured in vitro by using a two-step, single-round transcription assay developed to examine the kinetics of transcript elongation. With each DNA template carrying a particular attenuation region, one or more discrete, NusA-enhanced pause sites were detected. In each case, the site of pausing was located within the upstream region of the antiterminator sequence, at a position that would allow pausing to play its proposed role in the timing of RNA folding

and PyrR binding. These findings demonstrated that transcription pausing could play an important role in the regulation of the *pyr* operon, but they did not demonstrate that it actually does so in vivo.

In a subsequent study, Zhang et al. attempted to evaluate the function of transcription pausing in the *pyr* operon in vivo by constructing and analyzing mutations that greatly reduce pausing (177). The mutations changed selected pyrimidine nucleotides to purine nucleotides near pause sites, which was shown to substantially reduce transcription pausing at the mutant site in vitro. These mutations were then incorporated into *pyr*::*lacZ* fusions, and their effect on pyrimidine-mediated regulation of *pyr*::*lacZ* expression was determined. No consistent correlation between elimination of transcription pausing in vitro and defects in cellular regulation was observed. A major complication in these experiments was the inability to separate the effects of the mutations on transcription pausing from their effects on transcription termination at an attenuator.

BIOCHEMICAL CHARACTERIZATION OF PyrR

PyrR Is a UPRTase

An unexpected property of PyrR, first discovered by Ghim and Neuhard (44) in studies of *B. caldolyticus pyrR*, is that it catalyzes the uracil phosphoribosyltransferase (UPRTase) reaction. This activity was surprising because *Bacillus* species also produce a highly active UPRTase of the ubiquitous *upp* family (108, 155). Outside of a short segment in the phosphoribosyl pyrophosphate(PRPP)/UMP binding site, PyrR homologs have no significant sequence similarity to other phosphoribosyltransferases, including the *upp*-encoded UPRTases (155). Both *pyrR*-encoded and *upp*-encoded UPRTase activities are functional in vivo (108), but the affinity of PyrR for uracil is much lower than the affinity of *upp*-encoded UPRTases for this substrate (160). For this reason it is unlikely that PyrR plays an important role in uracil salvage in vivo.

The three-dimensional structure of PyrR demonstrates that the protein is a member of the type I phosphoribosyltransferase structural family in spite of its low sequence relatedness to them (149). The kinetic mechanism of the UPRTase reaction catalyzed by *B. subtilis* PyrR has been characterized (49). In most respects, this enzyme is a typical phosphoribosyltransferase. Other than the requirement for the binding of nucleotides to the UPRTase active site, there is no obvious relationship between the protein's enzymatic and regulatory activities. Mutant forms of PyrR that fail to bind RNA and regulate *pyr* transcription, but which have normal UPRTase activity, have been described (143). A mutant PyrR that has lost catalytic activity while retaining the ability to regulate *pyr* expression has not been isolated, but it has been reported that *Lactococcus lactis* PyrR lacks UPRTase activity (109). Thus, it is unlikely that PyrR must be able to catalyze this reaction to exert its regulatory function. We believe that the UPRTase activity of PyrR reflects its evolutionary origin as a phosphoribosyltransferase that later acquired the ability to bind to RNA. Of all the members of the type I phosphoribosyltransferase family, PyrR most strongly resembles, in both sequence and tertiary structure, hypoxanthine guanine phosphoribosyltransferase (76). This similarity suggests that PyrR did not evolve from the *upp* family (156). The recent observation of a dimer of *B. caldolyticus* PyrR with GMP in one active site and UMP in the other reinforces the idea that the protein is evolved from the hypoxanthine guanine phosphoribosyltransferases (21). The purine repressor PurR of *B. subtilis* provides another example of a phosphoribosyltransferase structural domain that has acquired a regulatory function (148). The binding of PurR to *pur* operator DNA is regulated by PRPP, which binds to the conserved phosphoribosyltransferase active site of the protein. PurR apparently does not catalyze an enzymatic reaction, however, and the nucleic acid binding site resides in an additional helix-turn-helix domain that is not found in PyrR or other phosphoribosyltransferases (148).

RNA Sequence and Structure Required for PyrR Binding

Several experimental methods have been used to identify the RNA sequence and secondary structure required for tight binding of *B. subtilis* PyrR. In an early genetic study, Ghim and Switzer isolated *cis*-acting mutations that resulted in constitutive expression of *pyr*::*lacZ* fusions (45). Most of the mutations were mapped to a short region of conserved sequence in the terminal loop and upper stem of the binding loop RNA (Fig. 13A), which implicated this region in the regulation of *pyr* operon expression. A reasonable conclusion was that this region is involved in PyrR binding, but this was not directly demonstrated by these studies.

Hydroxyl radical footprinting experiments with an RNA specifying *B. subtilis* binding loop 2, which is known to bind tightly to PyrR, were used to map the portions of the RNA that were protected by PyrR (14). Three segments were protected from hydroxyl radical cleavage (Fig. 13B). The terminal loop and upper stem were strongly protected, as were three nucleotides at the top of the lower stem opposite the conserved element that initiates the purine-rich bulge. The purine-rich bulge itself was weakly protected.

Electrophoretic gel mobility shift experiments were used to examine the binding of purified PyrR to 37 structural variants of *B. subtilis* binding loop 2 (14). The results are summarized in Fig. 13C. The requirements for tight PyrR binding to *pyr* RNA appeared to be quite exacting. Numerous single-nucleotide substitutions or deletions led to a reduction in the apparent affinity by as much as three orders of magnitude. The importance of maintaining the RNA secondary structure shown in Fig. 13C was documented. The requirement for specific nucleotide residues in positions in the upper stem and loop and in the lower stem just below the purine-rich bulge was confirmed, as was the requirement for the bulge itself. The smallest RNA that bound well to PyrR contained the 28 nucleotides from position 708 to 735 in Fig. 13C (14). Recently, examination of the specificity of PyrR binding to all three *B. subtilis* binding loop RNAs in vivo using a yeast three-hybrid method confirmed the generalizations shown in Fig. 13C (60).

The results of genetic, footprinting, and RNA binding studies yielded a consistent picture of the PyrR binding site. However, several aspects of PyrR binding suggested by gel shift assays were puzzling. Specifically, PyrR binding to binding loops 1 and 3 appeared to be much weaker and less affected by nucleotides than binding to binding loop 2. These differences were inconsistent with the essentially equivalent regulation in

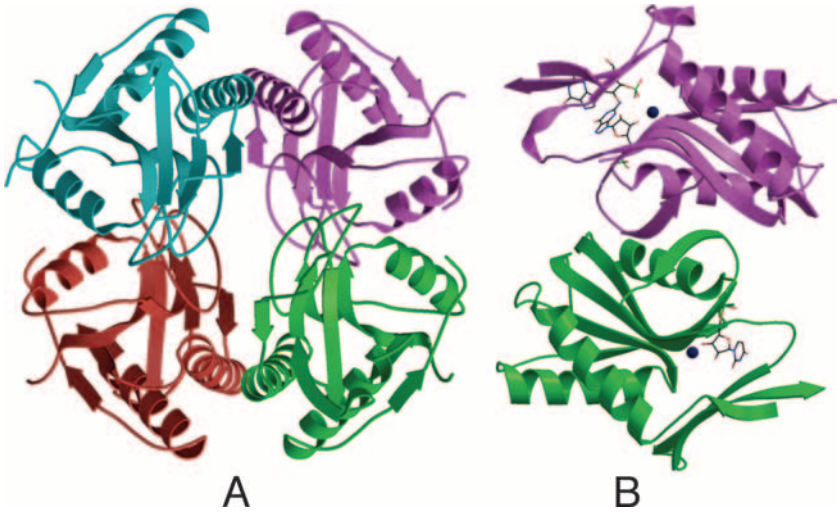

FIG. 14. Ribbon diagram of the crystal structure of PyrR from *B. caldolyticus*. Each polypeptide chain is shown in a distinct color. (A) The native tetrameric structure. (B) One of the two identical dimeric structures that combine to form the tetramer. Very similar dimeric structures are found in PyrR from other species. The black circles indicate the location of bound Mg^{2+} ions. The stick structures indicate the locations of 5′-UMP bound to the active site of the green subunit, 5′-GMP bound to the active site of the purple subunit, and 3′-GMP bound in a crystal contact lattice. (Reprinted from reference 21.)

vivo of *pyr*::*lacZ* fusions containing individual attenuation regions. For this reason, a detailed study of the binding of *B. caldolyticus* PyrR to the three *B. caldolyticus pyr* attenuation regions was undertaken using a filter binding assay (73). (For technical reasons, *B. subtilis* PyrR binding to RNA could not be measured with this assay.) The apparent dissociation constants (0.1 to 1 nM) for PyrR binding and the effects of nucleotides on this binding were similar for all three binding loops. These results were consistent with regulation of all three attenuators by physiological concentrations of nucleotides. The ratio of uridine to guanine nucleotides appeared to be the primary determinant of PyrR binding, a conclusion supported by the effects of exogenous uridine and guanosine on *pyr* operon expression in growing cells of *B. subtilis*. Recent studies by Jørgensen et al. revealed a requirement for Mg^{2+} in the gels used for gel shift assays, which may have led to substantial artifacts in the previous determinations of binding constants for PyrR binding to *B. subtilis* attenuation regions 1 and 3 (73). Furthermore, some of the conclusions from the study of RNA structural variants summarized in Fig. 13C should be reexamined, especially those results with variants that appeared to bind very poorly to PyrR. Many of the conclusions of Bonner et al. (14) concerning the RNA sequence and secondary structure required for binding to PyrR were confirmed, however.

How well do the studies of *B. subtilis* PyrR binding to attenuation regions predict the binding requirements of PyrR proteins from other species? A mutational analysis of PyrR binding to attenuation regions in *Lactobacillus plantarum* indicates that the required RNA features are similar to those found in *B. subtilis* (125). A phylogenetic comparison of 20 known or probable PyrR binding loops from nine different species suggests that there is little variation in PyrR selectivity among these species (14). As seen in Fig. 13D, the loops vary in the lengths of the upper and lower stems and the size of the bulge/internal loop but not in the overall secondary structure or identity of critical nucleotides. The structure of the binding loop can vary as much from attenuator to attenuator within a given species (e.g., in *B. subtilis* or *L. lactis*) as it does from species to species. We have relied on this conservation of structure and sequence to identify likely modes of action of PyrR in other bacteria (see below).

High-Resolution Structures of PyrR and PyrR Complexes with Nucleotides

A detailed understanding of how PyrR binds to RNA and how the binding of nucleotides alters the affinity of PyrR for RNA requires the determination of the structure of PyrR with and without nucleotides and RNA bound to it. Considerable progress has been made toward that goal. High-resolution structures of unliganded PyrR from *B. subtilis* (156) and *B. caldolyticus* (21) have been obtained by X-ray diffraction analysis of crystals. The structure of *B. caldolyticus* PyrR with bound nucleotides has been obtained (20, 21), as have the structures of unliganded *Mycobacterium tuberculosis* PyrR (80) and *Thermus thermophilus* PyrR (PDB code 1UFR).

PyrR folds into a core domain consisting of a curved central sheet formed by five parallel β-strands and flanked by three α-helices (Fig. 14B). A small subdomain called the "hood," which is made up of three antiparallel β-strands, caps the major core domain. The core domain strongly resembles the architecture found in many other type I phosphoribosyltransferases (149). The conserved amino acid residues of the PRPP/nucleotide binding site in all type I phosphoribosyltransferases are located in the central β-strand of the core domain and the following α-helix; PyrR obeys this generalization. Binding of a sulfate ion in this site in unliganded *B. subtilis* PyrR and subsequent determination of the location of nucleotides bound to *B. caldolyticus* PyrR confirmed this identification of the nucleotide binding site (21). The "hood" domain varies greatly from one phosphoribosyltransferase to another; residues in this do-

main are involved in determining the specificity of nucleotide binding.

B. subtilis PyrR was crystallized in dimeric and hexameric forms. In the hexameric form, dimeric units that are virtually identical to the dimeric crystal are arranged around a threefold central axis with a small solvent-filled central cavity. Both unliganded and liganded *B. caldolyticus* PyrR crystallized as a tetramer (Fig. 14A) made up of dimeric units with a structure that is very similar to that of the *B. subtilis* dimer. The structure of unliganded *M. tuberculosis* PyrR is very similar to that of PyrR of *B. caldolyticus*. PyrR from *T. thermophilus* is also a tetramer made up of dimeric units that are very similar to those of the *Bacillus* PyrR proteins. However, the mode of association of the dimeric units is very different from that for *B. caldolyticus* PyrR. The finding of three different quaternary structures indicates that PyrR probably functions as the dimeric form. The strong interactions between subunits in the dimer make its dissociation to native monomers unlikely. Recently, the stoichiometry of RNA binding to *B. caldolyticus* PyrR was shown by analytical ultracentrifugation to correspond to one RNA to two PyrR monomers (i.e., one RNA binds per PyrR dimer), as predicted (73).

Crystals of *B. subtilis* PyrR with UMP bound could not be obtained under conditions in which unliganded PyrR crystallizes, and the addition of UMP to PyrR crystals led to dissolution of the crystals (J. L. Smith, personal communication). These observations suggest that substantial conformational changes accompany binding of uridine nucleotides to PyrR. Such conformational changes are of particular interest because they might help to explain how the binding of nucleotides increases the affinity of PyrR for RNA. Crystals of *B. caldolyticus* PyrR with nucleotides bound were first obtained inadvertently in attempts to obtain a PyrR-RNA cocrystal (21). An RNase contaminant led to degradation of the RNA, and PyrR was obtained with UMP in the active site of one monomer of the dimeric unit and with GMP in the active site of the other monomer (Fig. 14B). These nucleotides interact with Mg^{2+} and with conserved active-site amino acid residues in a manner that differs in interesting ways from that observed with other phosphoribosyltransferases (21). Specifically, the magnesium ions in the active sites of PyrR are not ligated to the phosphate moiety or to the vicinal 2′, 3′ hydroxyls of the nucleotide, as is usual in the substrate complexes of other phosphoribosyltransferases. UMP and GMP are bound in a very similar manner to the active sites; the same amino acid residues are hydrogen bonded to the uracil and guanine bases. A third nucleotide, probably 3′-GMP, was found located between tetramers in the crystal lattice. More recently, the structure of *B. caldolyticus* PyrR with only 5′-UMP bound to the active sites was solved (20). The structure of this form of the protein was identical to the structure of the unliganded (but sulfate-bound) state and the UMP- plus GMP-bound state of PyrR. Curiously, no protein conformational changes among unliganded and two nucleotide-bound PyrR crystal structures were detectable, so the structures do not reveal how nucleotide binding alters the affinity of PyrR for RNA. Perhaps the association of PyrR subunits in the tetrameric state in the crystal obscures the changes that are induced by nucleotides in the dimeric RNA binding form of PyrR.

Characterization of the RNA Binding Site of PyrR

The electrostatic surface potential map of *B. subtilis* PyrR revealed a large concave surface on the dimer that is lined with positively charged and hydrophilic residues (156). It was proposed that this surface was the most likely site for binding of the negatively charged *pyr* binding loop RNA. A similar surface is also found on the *B. caldolyticus*, *T. thermophilus*, and *M. tuberculosis* PyrR dimers, but otherwise the electrostatic surface potential maps of these PyrR homologs are quite different from one another (21). This observation reinforces the suggestion that the concave basic surface of PyrR is the RNA binding site. In the hexameric state of *B. subtilis* PyrR and the tetrameric states of *B. caldolyticus* and *M. tuberculosis* PyrR, the basic surface is located in a central cavity that is too small to accommodate the *pyr* binding loop. Only the dimeric forms of these proteins would be capable of binding this RNA, which is consistent with the idea that the PyrR dimer is the physiologically functional form.

Site-directed mutagenesis of *B. subtilis* PyrR was used to test the hypothesis that conserved amino acid residues whose side chains lie on the concave basic surface are required for RNA binding and regulation of the *pyr* operon (143). Glutamine substitution mutations in four residues that lie in this surface (Thr18, His22, Arg141, and Arg146) clearly identified them as involved in normal RNA binding and *pyr* regulation; the mutants had no detectable loss of UPRTase activity or structural integrity. Two other residues (Arg27 and Lys152) were similarly implicated, with the reservation that small changes in their average apparent native molecular weight were observed, which might indicate that these mutant proteins did not fold into fully native tertiary structure (143). These six residues are generally conserved in bacterial PyrR sequences. It seems likely that they form part of the RNA binding surface, although one cannot conclude that they interact directly with RNA. The elucidation of a detailed map of PyrR-*pyr* RNA interactions must await a high-resolution X-ray diffraction analysis of PyrR-RNA cocrystals.

PHYLOGENETIC DISTRIBUTION OF *pyrR* GENES AND MECHANISMS OF PyrR-MEDIATED GENE REGULATION

Distribution of *pyrR* Genes

The sequences of *pyrR* genes are well conserved, so the identification of species carrying *pyrR* has rapidly followed the flood of new genome sequences. As of October 2007, genes believed to encode PyrR proteins had been identified in 245 discrete bacterial species. No doubt more will be found as additional genome sequences are reported. In addition, probable PyrR binding sequences in RNA can be readily identified, as can likely attenuator and antiterminator sequences upstream of *pyr* genes. Probable modes of PyrR action deduced from this information fall into several classes. However, it should be noted that genetic or biochemical experiments implicating PyrR in the regulation of *pyr* genes have been reported only for *B. subtilis* (155, 161), *B. caldolyticus* (21, 44), *Enterococcus faecalis* (43), *L. lactis* (109), *L. plantarum* (125), *Mycobacterium smegmatis* (36), and *M. tuberculosis* (C. J. Fields and R. L. Switzer, unpublished data).

PyrR-Mediated Transcription Attenuation of *pyr* Operons

In all 15 species in the genus *Bacillus* for which genomic sequences are available, *pyr* genes are organized in the same order within a single *pyr* operon. All but one of these operons contain three attenuation regions that are located as described for the *pyr* operon of *B. subtilis* (Fig. 10). The one exception, the *pyr* operon of *Bacillus clausii*, lacks the *pyrP-pyrB* intercistronic attenuation region. Presumably, in each case the *pyr* operon is regulated in the same way as described for *B. subtilis*. The *pyr* operon in *E. faecalis* is organized in a similar fashion, but the *pyrR-pyrP* and *pyrP-pyrB* intercistronic regions are very short and do not contain PyrR binding sequences, attenuators, or antiterminators (43). In this case, only a single attenuation region in the 5′ leader is used to control *pyr* operon expression, but the attenuation mechanism is still the same as in *B. subtilis*. *L. plantarum pyr* genes are also found in a single operon in the same order as in *Bacillus*, but the *pyrP* (uracil permease) gene is absent and only two attenuation regions are found (5′ leader and *pyrR-pyrB* intercistronic regions) (33); the mechanism of regulation by PyrR is essentially the same in *L. plantarum* as in *B. subtilis* (125). Thus, *pyr* operons can apparently be adequately regulated in arrangements involving one, two, or three attenuation regions. Without further study it is impossible to conclude whether a significant physiological advantage is conferred by *pyr* operons containing multiple attenuation regions. Organization of *pyr* genes into a single PyrR-regulated operon has been deduced from the genome sequences of a number of other low-G+C gram-positive species (e.g., *Listeria monocytogenes* and *Listeria innocua*). In many of these species, one or two genes, usually *pyrP*, *pyrK*, *pyrD*, or *pyrE* and *pyrF*, are located elsewhere on the chromosome and often appear not to be regulated by PyrR.

PyrR-Mediated Transcription Attenuation of Unlinked *pyr* Genes

The clustering of all of the pyrimidine biosynthetic genes into a single operon is limited to a small number of low-G+C gram-positive genera. More commonly in the gram-positive organisms, *pyr* genes are scattered around the chromosome in multiple operons. It appears that most, but not all, of the unlinked operons are regulated by PyrR-mediated transcriptional attenuation. *L. lactis* presents the best-characterized example. The *pyr* genes of *L. lactis* are scattered in at least five transcription units. Four of these, *pyrRPB-carA*, *pyrKDbF*, *pyrEC*, and *carB*, have obvious attenuation regions in their 5′ leader regions; there is evidence that PyrR regulates them in much the same way as shown for *B. subtilis* (83, 109). The fifth gene, *pyrDa*, encodes a second dihydroorotate dehydrogenase, which may be involved in catabolism of orotate, a pyrimidine that is abundant in bovine milk (6). Kilstrup et al. reviewed the organization and regulation of unlinked *pyr* operons in lactic acid bacteria and their close relatives (83); numerous variations on the patterns found in *Bacillus* and *Lactococcus* are seen, but the fundamental mechanism of regulation by PyrR appears to be the same in all of the species. On the other hand, the nature of regulatory mechanisms governing expression of *pyr* genes that are not subject to PyrR-dependent attenuation in these species is unknown. Future investigations of these mechanisms are likely to yield novel findings.

The chromosomes of *L. plantarum* and a number of other members of the *Lactobacillus* genus contain two *pyrR* genes (8). The product of one of these, $PyrR_1$, mediates regulation of the *L. plantarum pyr* operon in response to pyrimidines, as shown for *B. subtilis* and other species described above (125). Recently, the product of the second gene, $PyrR_2$, was shown to regulate expression of the *L. plantarum pyr* operon and the unlinked *pyrP* operon in response to the CO_2/HCO_3^- level in the medium (8). The two PyrR proteins operate independently and respond to different physiological signals. The biochemical mechanism of $PyrR_2$ action is not yet clear. Two possible mechanisms have been discussed (8). $PyrR_2$ could act by forming heterodimers with $PyrR_1$ that are unable to repress *pyr* expression, or $PyrR_2$ could interact with *pyr* RNA regulatory sequences to promote antitermination instead of favoring termination, the known action of $PyrR_1$. This discovery of dual regulation of *pyr* expression in lactobacilli adds a fascinating new chapter to the study of PyrR function in metabolic regulation.

PyrR as an Inhibitor of *pyr* Gene Translation

Analysis of the genomes of a number of bacteria has led to the suggestion that PyrR acts in some species as a translational repressor (155). In mycobacteria, for example, *pyr* operons contain a *pyrR* gene at their 5′ end, and this gene is preceded by a consensus PyrR binding sequence that overlaps the putative *pyrR* ribosome binding site. No attenuator or antiterminator sequences are found in the 5′ leader region of the operon (i.e., near the predicted PyrR binding site). This arrangement suggests that PyrR could act to inhibit translation of the *pyr* operon by occluding the translation initiation site when intracellular uridine nucleotides are elevated. This binding would inhibit translation of the *pyrR* gene, and probably translation of the downstream *pyr* genes if their translation is coupled to translation of upstream genes. Experimental evidence for this conclusion has now been published (36). Plasmids containing translation fusions that join the *M. smegmatis pyr* promoter-leader region to *lacZ* (i.e., those that link the mycobacterial *pyrR* ribosome binding site to the *lacZ* ORF) were repressed by exogenous uracil in *M. smegmatis*, but transcription fusions, in which the *lacZ* ribosome binding site is retained, were not repressed. Repression by uracil was shown to require both the *M. smegmatis pyrR* gene and an intact PyrR RNA binding loop sequence. Furthermore, PyrR proteins from *M. tuberculosis* and *M. smegmatis* have been purified and shown to bind specifically to the predicted *pyr* RNA sequences; binding is enhanced by uridine nucleotides and antagonized by guanosine nucleotides. These results demonstrate that PyrR from mycobacteria is biochemically capable of regulating *pyr* operon expression in response to nucleotide levels. Further characterization of this system is in progress (C. J. Fields and R. L. Switzer, unpublished experiments).

The combination of *pyrR* genes and PyrR binding sequences that overlap the ribosome binding sites for *pyr* genes, which we take to be suggestive of translational repression of those genes by PyrR, is quite widespread in other bacterial species. Numerous species have been identified in which ORFs for puta-

tive *pyrP* (*uraA*) genes and/or another putative transport protein of the major facilitator protein superfamily fit this pattern (C. J. Fields, unpublished data). In some species (e.g., *Bacillus*, *Clostridium*, *Lactobacillus*, and *Streptococcus* spp.), this arrangement coexists with regulation of *pyr* operon expression regulated by PyrR-mediated transcription attenuation. In other cases, translational repression of the genes for transport proteins appears to be the only mode of PyrR regulation (e.g., in *Haemophilus influenzae* and *Pasteurella maltocida*).

A very interesting hybrid regulatory mechanism appears to exist in *Thermus* strain ZO5 and related *Thermus* species (162). In *Thermus* strain ZO5 an ORF for short leader polypeptide precedes the *pyrR* gene and other downstream genes of a *pyr* operon. The ribosome binding site in the mRNA for the leader polypeptide overlaps a consensus PyrR binding sequence and could be subject to translational repression by PyrR. The RNA encoding the leader polypeptide is also capable of forming a transcription terminator. Van de Casteele et al. (162) have suggested that attenuation at this terminator is regulated by the rate of translation of the leader polypeptide, which is in turn responsive to pyrimidines via PyrR-mediated translational repression. Furthermore, 6 of 28 codons in the leader polypeptide encode arginine, which might account for stimulation of *pyr* gene expression in *Thermus* strain ZO5 by this amino acid. This interesting model has not yet been tested by genetic or biochemical means, however.

Species in Which the Function of PyrR Is Unclear

There are a few species in which genes encoding PyrR homologs are clearly recognizable but in which the function of PyrR is obscure. In the genomes of the cyanobacteria *Synechocystis* and *Synechococcus*, *pyrR* genes are found isolated from *pyr* genes, but no predicted RNA structures have been found that implicate PyrR in regulation of the *pyr* or other genes. Cho et al. have reported that a plasmid-borne copy of *pyrR* from *H. influenzae* could complement a *B. subtilis* Δ*pyrR* mutant strain but that *pyrR* from *Synechocystis* sp. strain PCC 6803 could not (24). This result indicates that *H. influenzae* PyrR is capable of binding to *B. subtilis pyr* RNA in a pyrimidine-dependent manner; we have suggested above that it may do so to regulate genes for uracil transport proteins. In the case of *Synechocystis*, the role of PyrR in regulation, if any, remains obscure. It is conceivable that PyrR serves only as a phosphoribosyltransferase in some species, even though they also have *upp* genes.

The function of the PyrR homolog in *Pseudomonas* presents a particularly interesting unsolved problem. In the *Pseudomonas aeruginosa* and *Pseudomonas putida* genomes, a *pyrR* gene lies at the 5′ end of the operon *pyrRBC′*. Genetic evidence indicates that the product of the *pyrR* gene possesses UPRTase activity and is involved in repression of *pyrB* by uracil (A. P. Kumar, C. J. Fields, and G. A. O'Donovan, personal communication). However, none of the RNA structures involved in PyrR binding or transcription attenuation as in *B. subtilis* can be identified in the *pyrRBC′* operon. Furthermore, the deduced sequences of *Pseudomonas* PyrR homologs differ from that of *Bacillus* PyrR in numerous residues that are thought to be important for RNA recognition and binding. If PyrR is involved in the regulation of *pyr* gene expression in *Pseudomonas*, its mechanism of action must be quite different from the previously characterized mechanisms. In preliminary studies by O'Donovan and colleagues, it was proposed that the *P. putida* PyrR acts as a DNA-binding protein to activate *pyr* gene expression, but detailed experiments have not been published. Future research on this system will be of great interest.

Species in Which *pyrR* Genes Are Not Identifiable

pyrR genes are readily identifiable in most gram-positive bacteria and are somewhat unpredictably scattered among many gram-negative phyla. A *pyrR* gene has been found so far in only one mycoplasma species, *Mycobacterium penetrans*. *pyrR* genes have not yet been identified in the sequenced genomes of enteric bacteria, bacteroides, alphaproteobacteria, epsilonproteobacteria, spirochetes, chlamydiae, or any archaea or eukaryotes.

REGULATION OF *pyrG* EXPRESSION BY A NOVEL MECHANISM BASED ON CTP-SENSITIVE REITERATIVE TRANSCRIPTION

pyrG Is Regulated by CTP Levels

CTP synthetase, which catalyzes the glutamine- and ATP-dependent amination of UTP to form CTP, is encoded by the *pyrG* gene (Fig. 1), which in *B. subtilis* is not part of the *pyr* operon and is not regulated by PyrR (115). It is not surprising that this gene would be regulated by the end product CTP, but such regulation is not readily demonstrated with wild-type strains for three reasons. First, the only exogenous cytosine-containing metabolite that can be used to increase internal CTP pools is cytidine, and cytidine is readily converted to uridine by cytidine deaminase. Specific repression of *pyrG* by cytidine can be demonstrated in mutants in which cytidine deaminase is inactive (115). Second, repressive effects of cytidine are small because the formation of CTP by the endogenous biosynthetic pathway maintains significant CTP pools. Only when de novo biosynthesis of pyrimidine nucleotides is impaired in mutants that are defective in the *pyr* operon or in *pyrG* can full derepression of *pyrG* be demonstrated (115). The third problem in characterizing *pyrG* regulation is that it is extremely difficult to assay CTP synthetase activity in crude extracts, a circumstance that has required the use of *pyrG*::*lacZ* transcription fusions (115). Such fusions are derepressed by 15- to 20-fold in pyrimidine auxotrophs that were grown on orotate, a poor pyrimidine source, compared to the same strain grown with excess cytidine. Similar studies with mutants in which interconversions between uridine and cytidine nucleotides were blocked, or in which cytidine uptake was inhibited, demonstrated that cytidine or a metabolite derived from cytidine, most likely CTP, specifically regulates *pyrG* expression (115).

These conclusions, which were based on studies with *B. subtilis* (115), were confirmed and extended in experiments performed with *L. lactis* by Jørgensen et al. (74). These investigators used similar genetic methods to manipulate nucleotide pools, and they employed *pyrG*::*lacLM* fusion strains to measure *pyrG* expression and to determine nucleotide pool levels. Their studies demonstrated that *pyrG* expression was directly correlated with the intracellular concentration of CTP.

pyrG Is Regulated by Transcription Attenuation

The *pyrG* promoter of *B. subtilis* has been mapped to the region between the similarly oriented *rpoE* and *pyrG* genes (115). The 5′ leader region of the *pyrG* operon contains 189 base pairs. This leader region contains an intrinsic transcription terminator, which also serves as the transcription terminator for the upstream *rpoE* operon. The analysis of deletions in the comparable *pyrG* leader regions of *B. subtilis* and *L. lactis* revealed that the intrinsic terminator is required for regulation; i.e., it is an attenuator (74, 115). However, leader sequences that could specify an antiterminator stem-loop could not be identified, and most of the *B. subtilis* leader region could be deleted without loss of normal regulation of *pyrG* expression (114, 115).

Comparison of the *pyrG* leader regions of several low-G+C gram-positive bacteria yielded valuable clues concerning the mechanism of attenuation control of *pyrG* expression (115). Only three short nucleotide sequences are conserved. These leader sequences specify GGGCUC at the 5′ end of the *pyrG* transcript and two complementary sequences, GCUCCC and GGGACG, at the bottom of the stem of the terminator hairpin of the attenuator. The nucleotides between the conserved sequences apparently do not play a role in regulation because they can be deleted (114). Systematic mutagenesis of the *pyrG* leader region of *B. subtilis* indicated that the conserved sequences 5′-GGGC at the start of the transcript and 5′-GCUCCC in the upstream segment of the terminator hairpin are the only *cis*-acting elements required for normal *pyrG* regulation (114). The lack of a requirement for an antiterminator stem-loop led Meng and Switzer to suggest that a regulatory protein might bind to critical transcript sequences at low CTP levels and prevent formation of the terminator hairpin (114). However, a search for the gene encoding such a protein by transposon mutagenesis was unsuccessful. Furthermore, Jørgensen et al. demonstrated that the regulation of the expression of *pyrG*::*lacLM* fusions in *L. lactis* was essentially the same whether the fusions were present as a single chromosomal copy or carried on a multicopy plasmid (74). This observation indicated either that a regulator protein was not titrated by multiple copies of *pyrG* mRNA or that no such protein was involved in regulation.

Regulation of *pyrG* by CTP-Sensitive Reiterative Transcription

A peculiar property of *pyrG* transcription was revealed by primer extension mapping of the 5′ ends of *pyrG* transcripts of *B. subtilis* (116). When cells were grown with excess cytidine, nearly all of the transcripts started with the sequence 5′-GGGCUC. However, when cells were grown under pyrimidine-limiting conditions, a ladder of transcripts from 1 to ~10 nucleotides longer than the 5′-GGGCUC. . . transcripts was also detected. Copying of these extended transcripts by reverse transcription, followed by cloning and sequencing, demonstrated that these longer transcripts contained 5′-end extensions produced by the addition of a variable number of G residues (116).

To identify the source and function of the longer transcripts, mutant *pyrG* promoters were constructed in which one of the

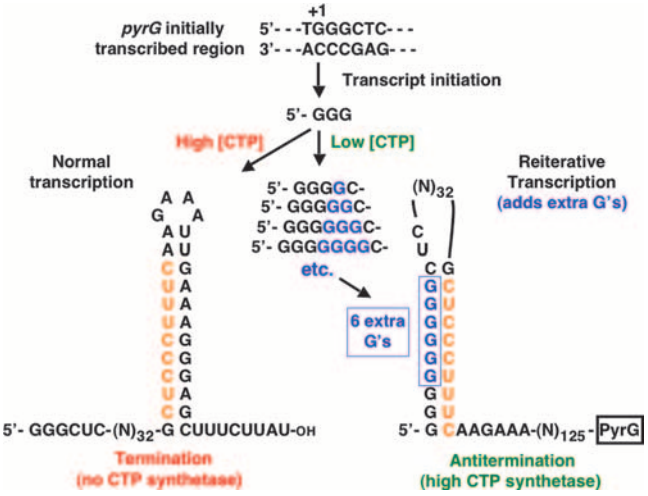

FIG. 15. Model for CTP-mediated regulation of *pyrG* expression in *B. subtilis*. The figure shows the effects of CTP concentration on the fate of the *pyrG* transcript after the first three G residues have been incorporated into the nascent transcript. A high CTP concentration allows normal transcript elongation until intrinsic termination occurs in the *pyrG* leader region. A low CTP concentration induces a transcription pause that allows reiterative transcription and the addition of extra G residues, which participate in the formation of an antiterminator hairpin. The extra G residues are boxed. The figure shows the insertion of six extra G residues, but as many as 10 extra residues can be added. (Modified from reference 116 with permission of the publisher. Copyright 2004 National Academy of Sciences, U.S.A.)

first four residues in the ITR (i.e., GGGC) was replaced by another base. Analysis of transcripts initiated at the mutant promoters in cells grown under conditions of pyrimidine excess and limitation revealed that substitution of any residue in the GGG sequence eliminated the formation of poly(G) extensions. Furthermore, the elimination of poly(G) extensions caused very low and essentially unregulated *pyrG* (actually *pyrG*::*lacZ*) expression (116). Analysis of a mutant promoter in which the C at position +4 in the ITR (i.e., +4C) was changed to a T (specifying a U in the transcript) showed that *pyrG* expression and regulation were similar to those observed with the wild-type *pyrG* promoter. However, a +4C-to-A substitution in the *pyrG* promoter abolished regulation. Finally, a mutant *pyrG* promoter was constructed in which four G residues were inserted after position +G3 in the ITR, creating a promoter with an ITR with the sequence 5′-GGGGGGGCUC. A *pyrG*::*lacZ* fusion containing this mutant promoter exhibited constitutive expression (116).

These observations are accounted for by conditional reiterative transcription at the *pyrG* promoter, which provides the basis for the current model for CTP-sensitive regulation of *pyrG* expression in *B. subtilis* (Fig. 15). When the intracellular level of CTP is high, *pyrG* transcripts are faithful copies of the DNA template and transcription elongation continues until termination at the attenuator. Therefore, when CTP is plentiful, transcription of the *pyrG* gene is suppressed (Fig. 15). On the other hand, when the intracellular level of CTP is low, *pyrG* transcription pauses after the synthesis of the nascent transcript 5′-GGG (and before position +4C) because of insufficient substrate. This pause provides time for the nascent transcript to slip upstream (relative to the DNA template) and

allow an extra G residue to be added to the nascent transcript. This process can be repeated multiple times (e.g., up to at least 10 times) until eventually a C residue is inserted. The transcript is then elongated normally until RNA polymerase transcribes the attenuator sequence that specifies the upstream segment of the terminator hairpin. The sequence of this segment in *B. subtilis* is 5′-GCUCCCUUUCAA, which includes a tract of nine pyrimidines. Because both C and U residues base pair with G residues, the run of pyrimidines will immediately base pair with the poly(G) tract at the 5′ end of the transcript, forming an antiterminator stem-loop. As RNA polymerase continues to elongate the *pyrG* transcript, formation of the terminator hairpin is precluded by the antiterminator secondary structure and full-length *pyrG* transcripts are formed (Fig. 15). These transcripts are translated to make CTP synthetase, which is needed to overcome the CTP deficiency. Although the model describes *pyrG* expression at high and low intracellular concentrations of CTP, regulation can occur continuously over a wide range of CTP concentrations that control the extent of pausing at position +4.

This model also accounts for the effects of the +4C-to-T or +4C-to-A and the G_4 insertion mutations. In the case of the +4C-to-T mutation, regulation of *pyrG* expression is nearly normal. Under the conditions used to measure regulation, the intracellular levels of both CTP and UTP vary in parallel. Therefore, the transcription pausing required to allow reiterative transcription can be induced at the mutant promoter at low UTP levels just as it is by low CTP levels at the wild-type promoter. Conversely, the +4C-to-A mutation abolishes regulation because pyrimidine starvation does not cause a decrease in the intracellular level of ATP, which would be needed to induce pausing before the addition of the nucleotide at position +4. Additionally, the constitutive expression caused by the G_4 insertion mutation is consistent with the model, which predicts that extra G residues at the 5′ end of the *pyrG* transcript, and not reiterative transcription per se, is required to prevent transcription termination at the attenuator. Therefore, permanently adding the extra G residues leads to suppression of transcription termination regardless of the state of pyrimidine availability. Furthermore, the model predicts that it is base pairing between the poly(G) and polypyrimidine tracts that precludes the formation of the terminator hairpin and transcription termination. In support of this prediction, a mutation that introduces two G residues into the polypyrimidine tract (i.e., the mutant sequence is 5′-CUC**GG**UUUC) greatly reduces *pyrG* expression to similar low levels in cells grown with excess and limiting pyrimidines (114). (Note that in this experiment, the two residues in the downstream segment of the terminator hairpin that normally base pair with the two mutated positions in the polypyrimidine tract were changed to maintain complete base pairing in the stem of the terminator hairpin.) Finally, the model provides a clear explanation for why the *pyrG* attenuator functions as a nonconditional transcription terminator for *rpoE* transcripts: these transcripts do not contain poly(G) tracts.

Reiterative transcription is a central element in the regulation of several pyrimidine biosynthetic and salvage operons in enteric bacteria, as described above, but the reiterative transcription reaction in these examples is fundamentally different from the reiterative transcription reaction that occurs at the *pyrG* promoter of *B. subtilis*. Specifically, in the case of the enteric promoters, all transcripts produced by reiterative transcription are aborted during the initiation phase of transcription, while transcripts that undergo reiterative transcription at the *pyrG* promoter switch to the normal mode of elongation, which allows transcription through the *pyrG* gene. A major part of this difference clearly involves the substrate for reiterative transcription, as discussed above. Reiterative transcription with UTP produces aborted transcripts, while reiterative transcription with non-UTP substrates produces transcripts that can be productively extended. The ability to make this distinction appears to be an intrinsic property of all RNA polymerases, although not much else is known about it.

The conserved sequences required for regulation of *pyrG* expression in *B. subtilis*—namely, the GGGC sequence that starts the ITR and the leader sequence specifying the polypyrimidine tract in the leader transcript—are found in the *pyrG* leader regions of at least 17 low-G+C gram-positive bacteria, including the genera *Bacillus*, *Listeria*, *Lactococcus*, *Enterococcus*, *Staphylococcus*, and *Streptococcus* (116). While reiterative transcription that produces runs of G residues has been demonstrated only for the *pyrG* operon of *B. subtilis* and CTP-sensitive regulation of *pyrG* expression has been shown only for the *pyrG* operons of *B. subtilis* and *L. lactis*, it seems highly likely that many species of the genera listed above will share these capabilities. On the other hand, the *pyrG* operons of enteric bacteria, which also encode CTP synthetase, possess leader regions that do not contain these conserved elements and are regulated by other mechanisms.

Further Characterization of *pyrG* Regulation

CTP-sensitive reiterative transcription at the *pyrG* promoter of *B. subtilis* and poly(G)-mediated suppression of transcription termination at the *pyrG* attenuator do not require any protein other than RNA polymerase. These processes have been recapitulated in vitro using a minimal assay for transcription, requiring only *B. subtilis* RNA polymerase, *pyrG* template DNA, ribonucleoside triphosphates, salts, and a buffered reaction mixture (67). Reiterative transcription producing poly(G) tracts and suppression of transcription termination at the *pyrG* attenuator were specifically induced at low CTP concentrations but not at low concentrations of any other NTPs. Mutations in the *pyrG* template that altered reiterative transcription and attenuation in vivo caused comparable changes in the in vitro system (67). These findings provide strong support for the major regulatory elements in the model for regulation of *pyrG* expression in *B. subtilis*.

To determine the minimum number of G residues at the 5′ end of the *pyrG* transcript that is required to prevent formation of the terminator hairpin, a set of mutant *pyrG* promoters was constructed with the number of G residues in the ITR varied systematically (34). The effects of these mutations on *pyrG* expression and CTP-sensitive regulation were measured, with decreases in the range of regulation used to indicate the inability of the terminator hairpin to form. The range of regulation with a promoter containing four G residues was slightly less than half of that with the wild-type promoter (i.e., 5.6-fold instead of 14-fold). Regulation with a promoter containing five or more G residues was essentially absent, and *pyrG* expression

was constitutive. These phenotypes indicate that five G residues are sufficient to form the antitermination stem—with the exclusion of the terminator hairpin—in essentially every *pyrG* transcript. The mutant *pyrG* promoters were also used to determine the number of G residues in the ITR that are needed to support maximum reiterative transcription in vivo. The results showed that a minimum of three G residues (as in the wild-type promoter) is required for reiterative transcription and that promoters with three or four G residues exhibit comparable levels of reiterative transcription. Compared to these levels, reiterative transcription is slightly reduced at a promoter with five G residues. In contrast, reiterative transcription is severely reduced or eliminated at promoters with six or more G residues, even in cells starved for pyrimidines. Apparently, an $rG_6 \cdot dC_6$ RNA-DNA hybrid is too stable to permit transcript slippage, even with extensive transcription pausing. Taken together, the results of this analysis reveal that the wild-type *pyrG* promoter, with its G_3 tract in the ITR, permits the widest range of regulation by the reiterative transcription mechanism.

It is interesting to note that recent studies with yeast mitochondrial RNA polymerase suggest that progressively lower concentrations of NTP substrates are required for active-site binding as the nascent transcript is extended from position +3 through +11 during transcription initiation (2). The end of this gradient at position +11 apparently reflects the transition to the elongation phase of transcription, when even lower concentrations of NTP substrates are needed. If these findings can be generalized to the *B. subtilis* RNA polymerase—which seems likely—they suggest that the sequence of the *pyrG* ITR, which directs CTP to position +4 of the transcript, was selected to maximize the sensitivity of reiterative transcription to the intracellular concentration of CTP. If the site for reiterative transcription in the ITR were followed by a C residue located downstream of position +4, the affinity of RNA polymerase for CTP might be too great to permit pausing even at low levels of CTP in the cell, and reiterative transcription producing poly(G) tracts would be prevented.

CONCLUSIONS AND SPECULATION

This review describes seminal research on the regulation of *pyr* gene expression in bacteria that extends over more than 30 years. A hallmark of these studies is that expectations and attractive ideas were often proven wrong. However, negative results provided opportunities for the discovery of new regulatory mechanisms and concepts. These discoveries frequently followed unpredictable paths along which intuition and happenstance guided the formation of hypotheses. To emphasize this process, our descriptions of the mechanisms that regulate *pyr* gene expression were presented in an historical context. These descriptions also include the rigorous and critical experimental testing that confirm proposed regulatory models. These models describe simple and economical mechanisms designed to provide gradual and sensitive control of *pyr* gene expression that reflects the metabolic needs of the cell.

Although these models are unlikely to be modified significantly in the future, many questions remain regarding fundamental processes that underlie the regulatory mechanisms. For example, some of the mechanisms involve transcription start site switching, which to a first approximation can be predicted from promoter sequences. However, the rules for start site selection do not take into account context effects that can significantly influence the process, as illustrated with mutant *upp* promoters of *E. coli*. Clearly, more work is required to understand these context effects. In addition, some regulatory mechanisms involve reiterative transcription, but this reaction can have quite different outcomes. Reiterative transcription at the *pyrBI* promoter of *E. coli* produces $AAUUUU_n$ transcripts that are always released from the initiation complex, whereas reiterative transcription at the *pyrG* promoter of *B. subtilis* produces $GGGG_n$ transcripts that can return to the normal mode of transcription elongation. The basis of these alternative outcomes is presently a mystery. Furthermore, the extent of reiterative transcription at a promoter can be influenced by the location of the transcription start site, as observed with a mutant *carAB* promoter. Apparently, there are undefined architectural features of the transcription initiation complex that modulate the reiterative transcription reaction. Finally, the structure-function relationships of the PyrR-RNA complex remain undefined. In future studies, it will be important to describe amino acid-RNA interactions, conformational changes that occur in both PyrR and its RNA substrate upon binding, and the structural basis for the uridine nucleotide-mediated increase and guanosine nucleotide-mediated decrease in the affinity of PyrR for RNA. Analysis of high-resolution structures of PyrR with RNA and nucleotides bound would likely accomplish most of these goals.

In this review, we have emphasized that the mechanisms and concepts elucidated by the study of *pyr* gene regulation can serve as useful guides in the analysis of unknown regulatory mechanisms. One interesting example is the study of pyrimidine (CTP)-mediated regulation of *pyrG* expression in *E. coli*. It appears that the regulatory mechanism, which is clearly different from the mechanism controlling *pyrG* expression in *B. subtilis*, requires CTP-sensitive start site switching in much the same way as described for the *pyrC* and *pyrD* regulatory mechanisms (T. Bedekovics and C. L. Turnbough, Jr., unpublished data). However, the mechanism by which *pyrG* transcripts initiated at neighboring start sites are differentially expressed remains to be determined. Other intriguing examples are the unresolved and apparently novel mechanisms of regulation by PyrR. Included in this list are the mechanism of $PyrR_2$-mediated regulation of *pyr* gene expression in response to inorganic carbon in *L. plantarum* and PyrR-mediated regulation of *pyr* gene expression in the absence of recognizable PyrR binding sequences in *Pseudomonas* species.

Importantly, the information garnered from the study of *pyr* gene regulation can also facilitate the analysis of regulatory mechanisms that are unrelated to pyrimidine biosynthesis. In fact, this information has already contributed to the discovery of such mechanisms. Two noteworthy examples are the regulation of expression in *E. coli* of the rRNA operons (42, 144) and the *fis* operon (165) by the concentration of the initiating NTP. In these cases, promoters contain sequences that restrict initiation to a single "unfavorable" position (e.g., A9 in the case of most rRNA operons), as defined by the rules established with mutant *pyrC* promoters. As a consequence, the efficiency of transcription initiation at the rRNA and *fis* promoters can be determined by the intracellular concentration of

the initiating NTP (i.e., ATP or GTP for the rRNA promoters and CTP for the *fis* promoter). Furthermore, the concentrations of the initiating NTPs vary under different growth conditions in a manner that causes appropriate levels of rRNA and *fis* operon expression, providing simple yet elegant regulation of gene expression.

Many other bacterial operons that are unrelated to pyrimidine biosynthesis contain elements that are critical components in the mechanisms of *pyr* gene regulation described in this review, so it seems likely that at least some of these operons will employ these features in comparable ways. However, the number of combinations and permutations of these elements is large, and thus there may be a high degree of flexibility in the assembly of regulatory mechanisms. In addition, it seems highly likely that hybrid mechanisms have evolved that combine features described above with a different set of regulatory elements. For example, many of the *pyr* gene regulatory mechanisms rely on nucleotide-sensitive reiterative transcription, start site switching, or transcription pausing as the key regulatory event in conditional gene expression. It is certainly conceivable, especially with the nuances described in this review, that factors other than the intracellular levels of nucleotides could also modulate these phases of transcription. Activating or inactivating these other factors by a cellular metabolite or condition would then make gene expression responsive to a nonnucleotide signal. On the other hand, the analysis of bacterial genome sequences reveals many *pyr* genes that do not appear to be regulated by any of the mechanisms discussed in this review. It is not known whether expression of these genes is altered in response to intracellular nucleotide pools, but this seems to be a likely possibility. The identification and examination of such regulated *pyr* genes offers rich possibilities for the discovery of novel modes of metabolic control.

Bioinformatic analysis of genomic DNA sequences was a useful tool in the development of models for many of the regulatory mechanisms discussed in this review, particularly in the identification of transcription terminators, antiterminators, and transcription pause sites that play roles in these mechanisms. However, a major limitation to this approach was illustrated by the mechanism of *pyrG* regulation in *B. subtilis*. In this mechanism, the antiterminator RNA hairpin contains an essential tract of nucleotides that was added by reiterative transcription and could not have been predicted from the sequence of the *pyrG* operon. In general, regulatory mechanisms involving reiterative transcription rely on subtle features in the DNA sequence of the promoter-leader region. These features are not readily identified by current bioinformatic analyses.

It should also be noted that many of the regulatory features described in this review involve only the basic transcriptional machinery of the cell and simple DNA sequences. Therefore, it is reasonable to suspect that some of the mechanisms and regulatory elements described here are also operative in eukaryotic cells. In fact, it would be surprising if they were not. The obstacle in their discovery is likely to be the education of more investigators about the surprises provided by the study of pyrimidine nucleotide biosynthesis in bacteria.

Finally, this review is yet another example of the importance of collaboration with other scientists. The combination of independent, intelligent minds that bring divergent experiences and ways of analyzing problems, when brought to bear on an unsolved scientific puzzle, is almost always more likely find a solution that survives the most thorough experimental testing. We also emphasize our shared pleasure in discovery. The more unexpected the answer, the greater the joy in finding it.

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