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CTP Limitation Increases Expression of CTP Synthase in *Lactococcus lactis*

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CTP synthase is encoded by the *pyrG* gene and catalyzes the conversion of UTP to CTP. A *Lactococcus lactis pyrG* mutant with a cytidine requirement was constructed, in which β-galactosidase activity in a *pyrG-lacLM* transcriptional fusion was used to monitor gene expression of *pyrG*. A 10-fold decrease in the CTP pool induced by cytidine limitation was found to immediately increase expression of the *L. lactis pyrG* gene. The final level of expression of *pyrG* is 37-fold higher than the uninduced level. CTP limitation has pronounced effects on central cellular metabolism, and both RNA and protein syntheses are inhibited. Expression of *pyrG* responds only to the cellular level of CTP, since expression of *pyrG* has no correlation to alterations in UTP, GTP, and ATP pool sizes. In the untranslated *pyrG* leader sequence a potential terminator structure can be identified, and this structure is required for regulation of the *pyrG* gene. It is possible to fold the *pyrG* leader in an alternative structure that would prevent the formation of the terminator. We suggest a model for *pyrG* regulation in *L. lactis*, and probably in other gram-positive bacteria as well, in which *pyrG* expression is directly dependent on the CTP concentration through an attenuator mechanism. At normal CTP concentrations a terminator is preferentially formed in the *pyrG* leader, thereby reducing expression of CTP synthase. At low CTP concentrations the RNA polymerase pauses at a stretch of C residues in the *pyrG* leader, thereby allowing an antiterminator to form and transcription to proceed. This model therefore does not include any *trans*-acting protein for sensing the CTP concentration as previously proposed for *Bacillus subtilis*.

All living organisms need purine and pyrimidine nucleotides as building blocks for synthesis of DNA, RNA, and several coenzymes. The de novo synthesis of the pyrimidine ribonucleotide CTP occurs in *Lactococcus lactis*, as in all other organisms investigated so far, through a linear pathway starting with synthesis of carbamoyl phosphate and ending with an amination of UTP to form CTP (Fig. 1). The *pyrG* gene is not linked to any other gene involved in pyrimidine metabolism (35). It encodes the CTP synthase, the terminal enzyme in this pathway, which is an amidotransferase that converts UTP, glutamine, and ATP to CTP, glutamate, and ADP. A mutation in the *pyrG* gene makes an otherwise pyrimidine prototrophic organism dependent on a supply of cytidine from the medium. The availability of pyrimidines controls expression of pyrimidine biosynthetic genes in gram-positive bacteria such as *Bacillus subtilis*, *Enterococcus faecalis*, and *L. lactis*, since growth in the presence of a pyrimidine source reduces expression of the *pyr* genes (9, 22, 29). Regulation of the pyrimidine biosynthetic genes has been investigated in great detail in *B. subtilis*. It has been shown that PyrR, an RNA binding protein that senses the concentration of UMP in the cell, regulates *pyr* gene expression through an attenuator mechanism (33). When the UMP pool is high, PyrR and UMP form a complex that binds to a specific site in the mRNA leader of the *pyr* genes (17, 18). The leader sequences can form both terminator and antiterminator structures, and binding of the PyrR-UMP complex destabilizes the antiterminator, thus resulting in termination of *pyr* transcription (34). In the presence of low UMP concentra-

tions, PyrR does not bind the mRNA, giving transcriptional readthrough. PyrR is also found in a number of other gram-positive organisms, including *L. lactis* (22).

Little is known about the regulation of *pyrG* in gram-negative bacteria. For *Salmonella enterica* serovar Typhimurium, however, it has been shown that CTP synthase activity is decreased 11-fold when cytidine is included in the growth medium (37). Similar observations with cytidine repression have been made for the gram-positive bacterium *B. subtilis* (2, 25). By monitoring gene expression from a *pyrG* promoter fragment in different genetic backgrounds, it has been shown that expression of *pyrG* is increased during limitation of cytidine nucleotides (25). However, no direct determinations of nucleotide pool sizes were performed. A terminator was identified in the *pyrG* mRNA leader, and deletion of this terminator abolished *pyrG* regulation, resulting in high expression both during pyrimidine starvation and during growth in the presence of pyrimidines. Further mutational analysis of the *pyrG* leader has identified two segments that are required for normal regulation of *pyrG*, and these nucleotides are conserved in the mRNA *pyrG* leaders from gram-positive bacteria (26). The conserved nucleotides are the first four nucleotides in the transcript (GGGC) and six nucleotides in the terminator stem (GCUCCC). No antiterminator structure was identified in the *B. subtilis pyrG* leader, which led to the proposal that an as-yet-unidentified protein senses the CTP concentration in the cell and during CTP starvation the protein binds to the *pyrG* leader, thus preventing termination and allowing transcription of *pyrG*.

In this study, regulation of the *pyrG* gene from *L. lactis*, including a correlation between *pyrG* expression and the CTP pool, was investigated. Expression from a *pyrG*::*lacLM* fusion

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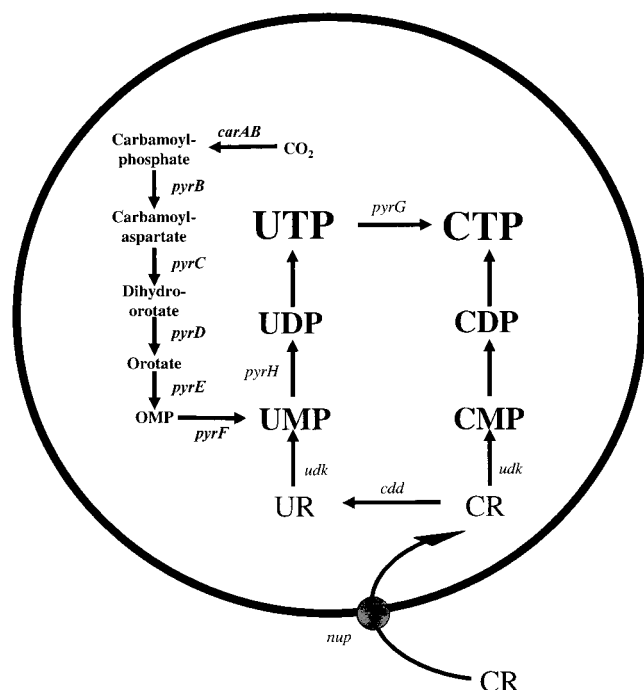
FIG. 1. Pathways for the synthesis of CTP and the salvage of cytidine in *L. lactis*. UR, uridine; CR, cytidine; *carAB*, carbamoylphosphate synthase gene; *pyrB*, aspartate transcarbamylase gene; *pyrC*, dihydroorotase gene; *pyrD*, dihydroorotate dehydrogenase gene; *pyrE*, orotate phosphoribosyltransferase gene; *pyrF*, OMP decarboxylase gene; *pyrH*, UMP kinase gene; *pyrG*, CTP synthase gene; *udk*, uridine kinase gene; *cdd*, cytidine deaminase gene; *nup*, cytidine/purine nucleoside transporter gene.

during CTP limitation was determined, and it was found that a decreased CTP pool size results in increased *pyrG* expression. To gain insight into the mechanism behind *pyrG* regulation, the rate of synthesis from the *pyrG* gene after a sudden drop in the CTP pool was studied, and it was found that *pyrG* expression is not linear during the first almost 2.5 h of CTP limitation. Profound alterations in macromolecular biosynthesis affecting both RNA and protein synthesis were observed during CTP limitation. A model for the regulation of *pyrG* is presented, in which a terminator-antiterminator mechanism independent of additional protein factors is responsible for the regulation. A similar mechanism can be proposed for *pyrG* regulation in a number of gram-positive organisms.

MATERIALS AND METHODS

Growth conditions. *Escherichia coli* cells were grown in Luria-Bertani medium at 37°C with vigorous shaking. *L. lactis* cultures were grown in SA defined medium with 1% glucose (GSA medium) (12), 1% galactose, or 1% maltose at 30°C. Erythromycin was added to a concentration of 2 or 150 μg/ml for *L. lactis* and *E. coli*, respectively. Unless noted otherwise, cytidine was added to 20 μg/ml. 5-Bromo-4-chloro-3-indolyl-β-D-galactoside was added to a final concentration of 90 μg/ml.

Bacterial strains and plasmids. The bacterial strains and plasmids used in this work are described in Table 1. Plasmid pCJ24 carries an internal fragment of *pyrG* and was constructed by digesting pSH105 (35) with *Hin*dIII and *Bam*HI and subsequently transferring a 1.1-kb fragment to the integration vector pSMA500 (19) cleaved with *Hin*dIII and *Bam*HI. The ligated plasmid was transformed to competent ABLE K cells (Stratagene, La Jolla, Calif.) and plated on Luria-Bertani plates containing 150 μg of erythromycin per ml.

The promoter region upstream of *pyrG*, including the 5′ end of *pyrG*, was amplified by PCR with primers pyrGupF2 (5′-CCCAAGCTTGGAAAATAGA CAAAGCCC-3′) and pyrG9b1-Bam (5′-CGGGATCCTCAGTAACAAAGAC TTCACC-3′). The PCR product was cloned by using the pMOS *Blue* blunt-ended cloning kit from Amersham Biosciences. The cloned fragment was

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or description	Reference or source
Strains		
E. coli ABLE K		Stratagene
L. lactis		
MG1363	Wild type	8
MB109	MG1363 *cdd*	21
MB420	MG1363 *pyrB*::IS*S1*	23
CJ217	MB109 *pyrG*::pCJ24	This study
CJ233	MB109/pAK80	This study
CJ238	MB109/pCJ29	This study
CJ240A	MB109 *pyrG*::pCJ30	This study
CJ295	MB109 *pyrG*::IS*S1*	This study
CJ298	CJ295/pCJ29	This study
CJ300	MB420/pCJ29	This study
Plasmids		
pGh9:IS*S1*	Used for IS*S1* mutagenesis	20
pAK80	Contains promoterless *lacLM* genes	11
pSMA500	Contains promoterless *lacLM* genes and no origin for replication in *L. lactis*	19
pMOSBlue	Plasmid vector	Amersham Biosciences
pCR2.1-TOPO	Plasmid vector	Invitrogen
pSH105	Contains 1.4-kb internal fragment of *pyrG*	35
pCJ24	Internal *pyrG* fragment from pSH105 in pSMA500	This study
pCJ29	*pyrG* promoter fragment (−667 to +297) in pAK80	This study
pCJ30	*pyrG* promoter fragment (−667 to +297) in pSMA500	This study
pCJ34	*pyrG* promoter fragment (−667 to +82) in pAK80	This study
pCJ35	*pyrG* promoter fragment (−667 to +69) in pAK80	This study
pCJ36	*pyrG* promoter fragment (−667 to +45) in pAK80	This study

transferred as a *Hin*dIII-*Bam*HI fragment to the promoter-probe vector pAK80 as well as pSMA500, resulting in pCJ29 and pCJ30, respectively.

Plasmids containing parts of the *pyrG* leader were cloned in the TOPO TA cloning kit from Invitrogen. The cloned fragments were made by PCR with primers pyrGupF2 and either pyrGdel_1 (5′-TCTCGGATCCAAAGAAATAA CTGGGAAA-3′), pyrGdel_2 (5′-CCTCGGATCCAAAAACAAAAACAGCT CCCC-3′), or pyrGdel_3 (5′-TCTCGGATCCGGGGAGCCTACCGTTACTG-3′). *Hin*dIII-*Bam*HI fragments were transferred to the vector pAK80, resulting in pCJ34, pCJ35, and pCJ36, respectively.

DNA isolation. Chromosomal DNA from *L. lactis* was isolated as previously described (13). Plasmid DNA from *E. coli* cells was purified by using the Qiagen plasmid midi kit.

Transformation. *E. coli* cells were made competent with $CaCl_2$ and transformed as described previously (30). *L. lactis* cells were transformed by electroporation as described previously (10).

Isolation of *pyrG* mutants. An *L. lactis* strain with a transposon inserted in the *pyrG* gene was isolated in a *cdd* derivative of MG1363 (MB109). The mutant was isolated from a transposon library by using pGh9:IS*S1* as previously described (15). The library was isolated on GSA defined medium containing 20 μg of cytidine per ml. From this library, 5 ml of GSA medium containing 20 μg of uracil per ml was inoculated to an optical density at 436 nm (OD_{436}) of 0.1 and grown for 5 h at 37°C. Ampicillin was added to 100 μg/ml, and the culture was incubated overnight at 37°C. Cells were harvested and washed with 0.9% NaCl, and 100 μl from this cell suspension as well as 100 μl from a 10× dilution was plated on GSA plates with 50 μg of cytidine per ml and incubated for 3 days at 37°C. Colonies were screened for growth on plates with and without cytidine or uracil. Fifteen colonies with a specific cytidine requirement were restreaked twice at 28°C to select for excision of the plasmid and twice at 37°C to cure the plasmid from the strain. An isolated strain with a cytidine requirement and an erythromycin-sensitive phenotype was kept as CJ295. The IS*S1* element was inserted in the *pyrG* open reading frame as verified by PCR on chromosomal DNA from CJ295. To isolate mutants with the reporter genes *lacLM* under control of the *pyrG* promoter, competent *L. lactis* cells carrying a *cdd* mutation (MB109) were transformed with plasmid pCJ24 or pCJ30, resulting in CJ217 and CJ240A, respectively. After transformation, erythromycin-resistant colonies were isolated and purified on GSA plates with erythromycin and cytidine. The presence of the integrated plasmid in the *pyrG* region was verified by the cytidine requirement of strain CJ217 as well as by PCR analysis of chromosomal DNA from CJ217 and CJ240A. The genetic organizations of the *pyrG* region in CJ217, CJ240A, and CJ295 are shown in Fig. 2.

Growth experiments and enzyme assay. Starting with fresh colonies from GSA plates with erythromycin and cytidine, the isolated *pyrG* mutants were grown for 6 to 8 h at 30°C in 1 ml of GSA medium with erythromycin and cytidine. Different dilutions of the growing culture were added to 10 ml of medium and grown overnight. From an exponentially growing culture, new medium was inoculated to an OD_{436} of 0.05. The growth experiments were performed in glass or plastic flasks without stirring. For β-galactosidase activity determinations, 35 ml of culture was harvested, washed with 0.9% NaCl, and resuspended in 1 ml of Z buffer.

β-Galactosidase activity was determined at 30°C as previously described (30) except that the cell density was measured at 436 nm and the specific activity was determined as $OD_{420}/(OD_{436}$ per minute per milliliter of culture).

RNA extraction. *L. lactis* RNA was harvested from strain MG1363 grown exponentially in SA glucose medium to an OD_{450} of approximately 0.8. Total RNA from 200 ml of culture was isolated by using the Fast Prep system (BIO101) with the protocols of the manufacturer.

RT-PCR. *L. lactis* RNA was used as the template in the Titan one-tube reverse transcription-PCR (RT-PCR) system from Boehringer Mannheim (product number 1 888 382) in accordance with the protocols of the manufacturer. As a control, conventional PCR was conducted on both chromosomal DNA and the RNA. The following primer pairs were used: A, SLLH17 (5′-GGCAAATTGA TATTGCACTTG-3′) and SLLH18 (5′-AAAAAGAATGTTGTCTACGGCTT GG-3′); B, SLLH19 (5′-CAACTAAGTATATTTTCGTCACTGG-3′) and pyrG9b (5′-TCAGTAACAAAAACTTCCCC-3′); C, SLLH17 and pyrGb9; and D, pyrG8a (5′-GGCAAAAAATTCTTCGTT-3′) and SLLH7 (5′-TACAAAAG ATTTTGGGC-3′).

Determination of intracellular nucleoside triphosphate concentrations. Nucleotides were extracted from [^{33}P]orthophosphate-labeled cell cultures and separated by thin-layer chromatography as previously described (23).

Determination of RNA and protein synthesis. For pulse-labeling of RNA, 600 μl of culture was mixed with 1 μl of [^{14}C]adenine (50 μCi/ml) and 3 μl of 10 mM adenine to a final adenine concentration of 55 μM. Pulse-labeling of proteins was done by adding 2.6 μl of [^{14}C]leucine (50 μCi/ml) to 220 μl of culture (the

A.

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GCAAGCACTTAAGTGAAAAAAGTAATGATAAAAAATGTAGAAGCTAAAAT
1210        Stop YeiG

CTGGCAATTTCGTCATTAAAATCTACTGATTACAAAAAAATAAAAATAGA


AATGCCCGATATTACTGGGCTTCATAAAAAAATTAAAAACAGACTTTTGAC
1310                                            - 35

AAAAAAGGAAAAATCAGATATAATTATTTTCGGGCACTGGTTTTTTATTA
        - 10

TAAAAAATCAGTAACGGTAGGCTCCCCTCTGGGGAGCTGTTTTTGTTTTT
1410

CCCAGTTATTTCTGTTTTGTAAAAAGATAAGAGGAGATTTAGATGTCAAC
                                            Start PyrG
```
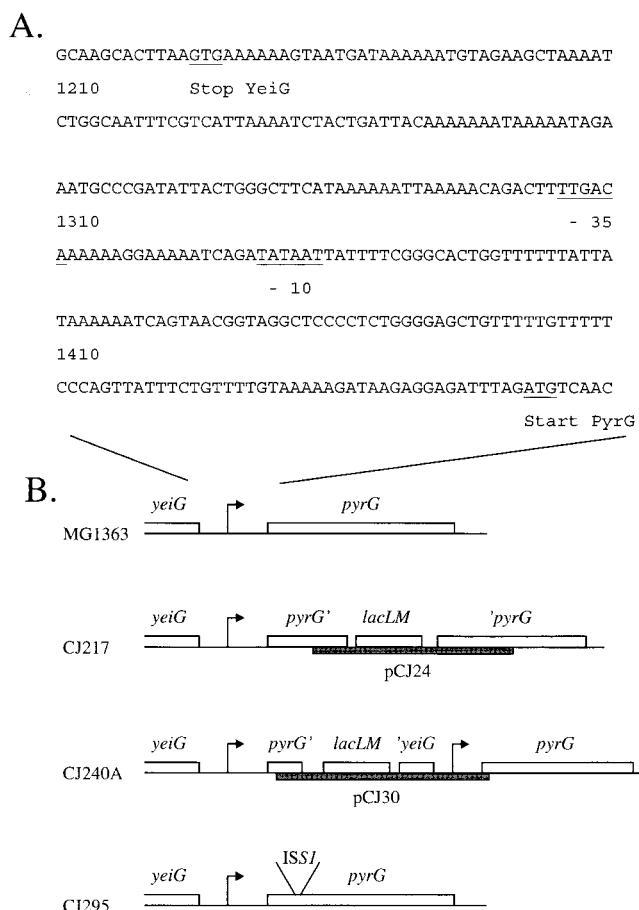
B.

FIG. 2. (A) Nucleotide sequence of the noncoding region in front of *pyrG*. The numbers refer to the sequence found in the EMBL data library (accession number AJ010153). The −10 and −35 sequences of the putative promoter are shown. (B) Genetic maps of the *pyrG* regions in the strains used in this study. Grey boxes indicate plasmids with the reporter genes *lacLM* integrated in the chromosome. Arrows indicate the position of the putative *pyrG* promoter. The maps are not drawn to scale.

concentration of leucine in SA medium is 0.8 mM [12]). After 10 min of labeling with either [^{14}C]adenine or [^{14}C]leucine, 200 μl of culture was transferred to a tube with 3 ml of cold 5% trichloroacetic acid (TCA) and put on ice for 0.5 to 1.5 h. The precipitated macromolecules were collected on a membrane filter (0.45-μm pore size; Schleicher & Schuell, Dassel/Reliehausen, Germany), washed twice with cold 5% TCA and once with boiling water, and left to air dry (5). The radioactivity on the filters was counted in an Instant Imager.

mRNA half-life determinations. Determination of mRNA half-life was based on a method described previously (36). A 1.65-ml portion of culture was mixed with 11 μl of [^{14}C]adenine (50 μCi/ml) and 8 μl of 10 mM adenine and pulse-labeled for 4 min. At time zero, actinomycin D was added to a final concentration of 4 μg/ml and nalidixic acid was added to a final concentration of 20 μg/ml in order to stop transcription. At different time intervals between 0 and 10 min and at 60 min, 200 μl of labeled culture was mixed with 3 ml of cold 5% TCA and left on ice for 30 to 60 min. Precipitated RNA was collected on a membrane and treated as described above. The mean of the values obtained at 8, 10, and 60 min after inhibition of transcription was taken to represent stable RNA, a value which was subtracted from the values obtained at 0, 1, 2, 4, and 6 min.

RESULTS

Identification of the *pyrG* transcript. The region upstream of *pyrG* was sequenced and found to contain a potential pro-

TABLE 2. Regulation of *pyrG::lacLM* fusion in *L. lactis*

Strain	Genotype	Plasmid	Addition(s) to medium[a] (μg/ml)	T_d (min)[b]	β-Galactosidase activity (U/OD$_{436}$ unit)[c]	Relative expression
CJ217	*cdd pyrG::lacLM*	None	CR (20)	56 ± 3	3.7 ± 1.4	1
			CR (20), UR (500)	109 ± 7	55 ± 8	15
			CR (20), AR (100)	110 ± 14	24 ± 5	6
			CR (20), CdR (500)	151 ± 10	57 ± 1	15
CJ298	*cdd pyrG::ISS1*	pCJ29	CR (20)	59 ± 4	71 ± 19	1
			CR (20), UR (500)	119 ± 8	1,275 ± 98	18
			CR (20), AR (100)	114 ± 10	380 ± 93	5
			CR (20), CdR (500)	166 ± 8	1,150 ± 44	16
CJ238	*cdd*	pCJ29	None	60 ± 2	76 ± 8	1
			CR (20)	58 ± 1	47 ± 6	0.62
			CR (50)	59 ± 2	46 ± 5	0.60
			CR (500)	59 ± 2	48 ± 5	0.63
			UR (50)	59 ± 3	78 ± 9	1
CJ233	*cdd*	pAK80	None	57 ± 1	<0.02	

[a] Abbreviations: CR, cytidine; UR, uridine; AR, adenosine; CdR, deoxycytidine. The nucleosides were added to exponentially growing cells at an OD$_{436}$ of 0.2.
[b] T_d, doubling time GSA in defined medium. The data are averages and standard deviations from three to five independent experiments.
[c] The cells were harvested at an OD$_{436}$ of 0.8. The data are averages and standard deviations from three to five independent experiments.

moter sequence with consensus −10 and −35 regions separated by 17 nucleotides. A 950-bp DNA fragment covering bp −667 to +297 with respect to +1, thus including the promoter region and the 5′ end of the *pyrG* open reading frame, was cloned in the pAK80 promoter-probe vector (pCJ29). The cloned fragment has promoter activity, as it results in expression of the *lacLM* reporter genes (compare CJ238 with CJ233 in Table 2).

No putative terminator can be identified after the gene upstream of *pyrG*, and it cannot be ruled out that upstream transcription may contribute to the overall expression of *pyrG*. To test whether transcription initiated upstream of the *pyrG* promoter may contribute to the overall expression of *pyrG*, an RT-PCR experiment was conducted. Total RNA isolated from the wild-type strain *L. lactis* MG1363 was analyzed by RT-PCR as described in Materials and Methods, and the results are presented in Fig. 3. It can be seen that it is possible to obtain RT-PCR products spanning the intergenic region of *pyrG* and *yeiG* (Fig. 3, PCR product c.). It is not possible to obtain an RT-PCR product including both *pyrG* and the downstream open reading frame (Fig. 3, PCR product d.). This indicates that in vivo upstream-initiated transcription does contribute to the overall expression of *pyrG* and that all *pyrG* transcription is terminated immediately after the *pyrG* open reading frame.

The 950-bp fragment with the *pyrG* promoter was also transferred to a plasmid that is unable to replicate in *L. lactis*. Integration of this plasmid in the chromosome resulted in a strain with a functional *pyrG* open reading frame expressed only from the promoter present on the cloned fragment (CJ240A) (Fig. 2). This strain grows at a normal rate in the absence of exogenous cytidine, showing that the promoter immediately in front of *pyrG* has adequate strength to ensure sufficient production of CTP.

The expression of *pyrG* is repressed by cytidine. To investigate whether *pyrG* expression is affected by addition of cytidine, strain CJ238, containing the plasmid with the *pyrG* promoter controlling expression of *lacLM*, was grown in the presence of cytidine (Table 2). Expression from the reporter

genes was reduced by 40% compared to growth in the absence of cytidine. Uridine does not repress *pyrG* expression. These results suggest that expression of *pyrG* is regulated at the level of transcription by the availability of cytidine compounds, as proposed for *B. subtilis* (25).

Limitation of cytidine availability results in increased *pyrG* expression. In order to test whether *pyrG* expression in *L. lactis* is subject to changes during CTP depletion, a chromosomal fusion which carries the *lacLM* reporter genes under control of the *pyrG* promoter was constructed. Strain CJ217 (Fig. 2) was constructed by a chromosomal integration of plasmid pCJ24, which carries an internal fragment of *pyrG* in front of the reporter genes. This strain contains a truncated *pyrG* gene and has a cytidine requirement for growth. Moreover, cytidine degradation is prevented, since the strain has no cytidine deaminase activity due to an inactive *cdd* gene. As shown previously, an *L. lactis cdd pyrG* mutant grown with cytidine has a growth rate and intracellular nucleotide concentrations similar to those of the wild-type strain (23). The *pyrG::lacLM* fusion strain was grown with a limited concentration of cytidine in the growth medium, resulting in a decline in growth rate when the supplied cytidine had been utilized (Fig. 4A). Determination of the intracellular concentrations of nucleoside triphosphates revealed that the cells were indeed starved for CTP, since the concentration of CTP was below the level of detection at the onset of the growth arrest (Fig. 4B). The β-galactosidase activity encoded by the *lacLM* reporter genes was assayed in order to monitor expression of the *pyrG* gene. No increase in expression was detected, even after 24 h of CTP starvation (specific activity, 1.3 U/OD unit), compared to cells grown without cytidine limitation (specific activity, 3.7 U/OD unit), showing that expression from the *pyrG* gene is not induced when the cell is completely starved for cytidine.

In a previous study, the *pyrG* gene of *B. subtilis* was shown to be induced in growing cells during pyrimidine limitation (25). Addition of high concentrations of nucleosides to a *pyrG* mutant grown with cytidine has been identified as a way to reduce
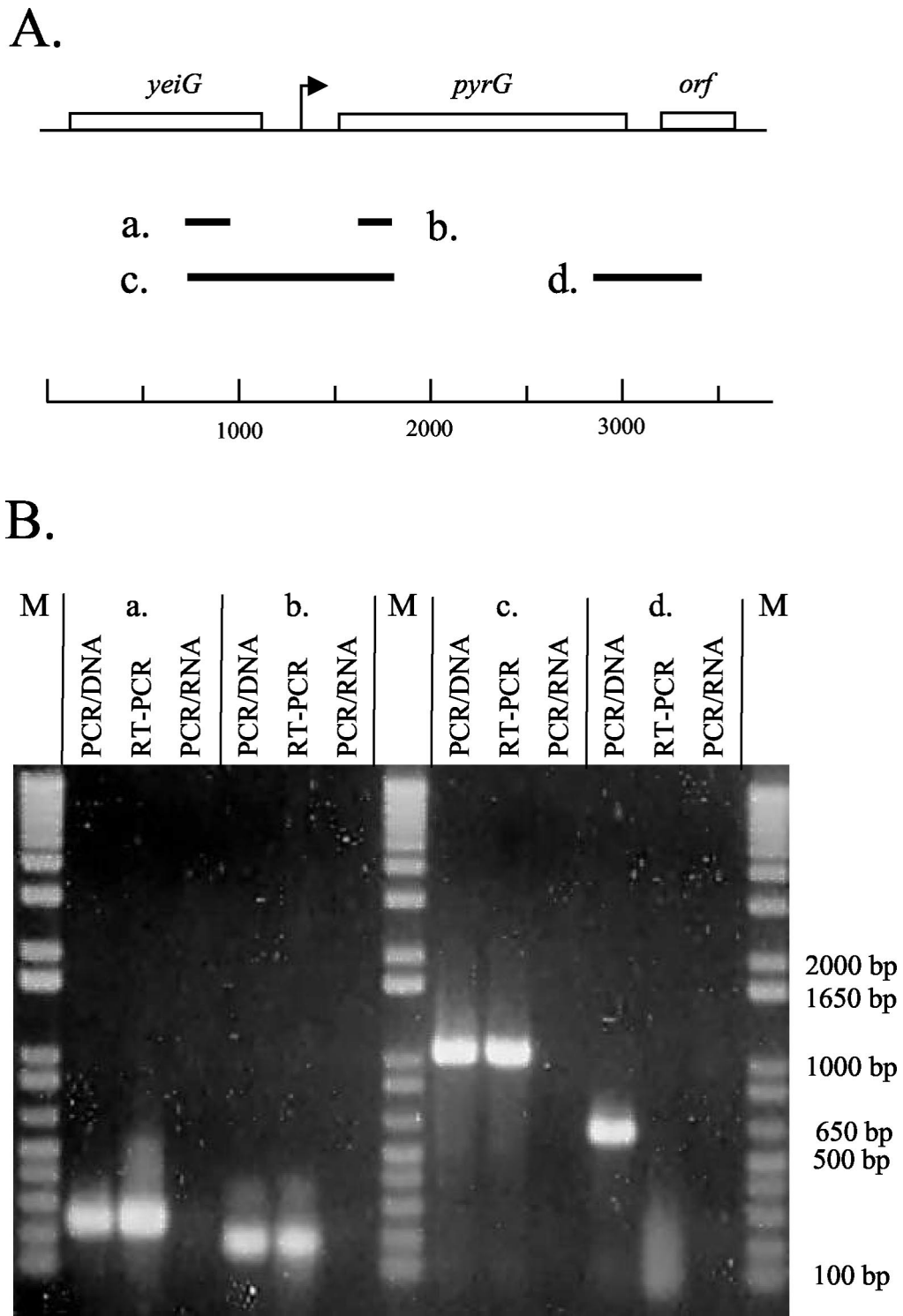
FIG. 3. RT-PCR analysis of transcription of *yeiG*, *pyrG*, and *orf* in *L. lactis*. (A) Physical map of the *pyrG* region from *L. lactis*. Numbering refers to the DNA sequence submitted to the EMBL data library and assigned accession number AJ010153. The theoretical PCR products are shown. a, PCR fragment obtainable with oligonucleotides SLLH17 and SLLH18; b, PCR fragment obtainable with oligonucleotides SLLH19 and pyrG9b; c, PCR fragment obtainable with oligonucleotides SLLH17 and pyrG9b; d, PCR fragment obtainable with oligonucleotides pyrG8a and SLLH7. (B) Agarose gel electrophoresis of the RT-PCR products. Lanes PCR/DNA, ordinary PCR with DNA as the template; lanes RT-PCR, RT-PCR with RNA as the template; PCR/RNA, PCR with RNA as the template. a, b, c, and d refer to the theoretical PCR fragments shown in panel A. Lanes M, 1-kb Plus DNA ladder from GibcoBRL.
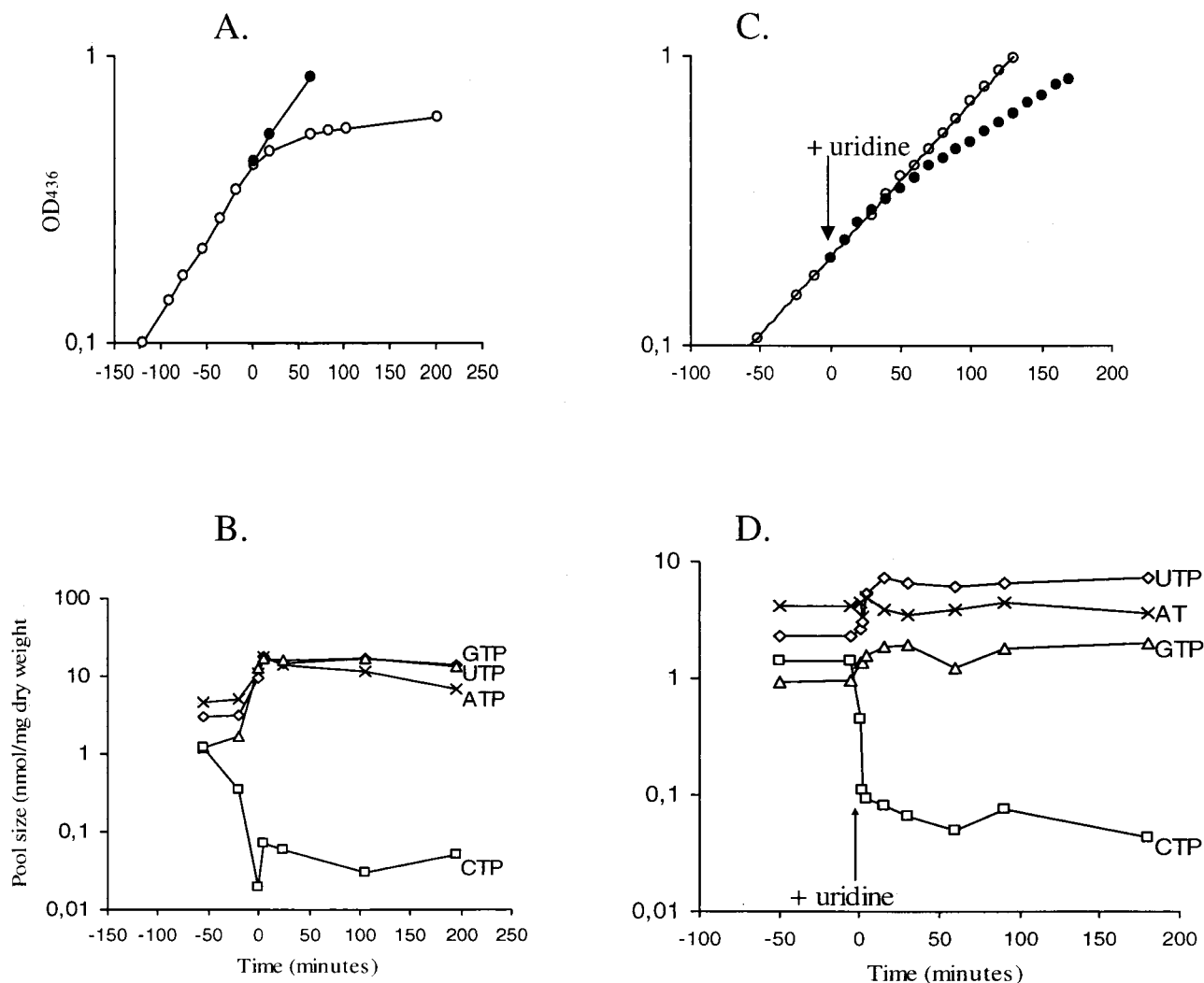
FIG. 4. Growth curves (A and C) and nucleotide pool size changes (B and D) during starvation or limitation for cytidine. A *cdd pyrG::lacLM* mutant was grown in GSA defined medium. (A and B) Cytidine starvation. Cells were grown either with 20 μg of cytidine per ml (●) or with a limited amount of cytidine (○). Time zero indicates the point of reduced growth rate at an OD_{436} of 0.4. (C and D) Cytidine limitation. Cells were grown with cytidine (○) or with cytidine plus uridine (●). Uridine (500 μg/ml) was added at time zero at an OD_{436} of 0.2.

the CTP concentration in *L. lactis* (23). The only nucleosides that do not inhibit growth of a cytidine-requiring strain are thymidine and deoxyuridine. The expression of the *pyrG* gene during growth with high concentrations of purine or pyrimidine nucleosides and consequently a low CTP pool size was therefore tested. On plates with cytidine and large amounts of (deoxy)pyrimidine or (deoxy)purine nucleosides, growth of the *pyrG* mutant CJ217 (*pyrG-lacLM*) is inhibited, as it takes several days for the mutant to show visible growth. Moreover, expression of the *pyrG-lacLM* fusion was clearly induced as judged by 5-bromo-4-chloro-3-indolyl-β-D-galactoside indicator plates, since the colonies turned blue when growth was inhibited by nucleosides compared to the same strain growing in the presence of only cytidine.

The effect on *pyrG* expression in a *L. lactis pyrG* mutant during CTP limitation was also investigated in liquid medium with cytidine. As shown in Table 2, the addition of uridine, deoxycytidine, or adenosine to strain CJ217 resulted in a de-

crease in growth rate and increased expression from the *pyrG-lacLM* chromosomal fusion when measured after two generations of growth with CTP limitation. Uridine and deoxycytidine resulted in a 15-fold induction, whereas adenosine increased expression only sixfold. In order to test whether all regulatory signals needed for *pyrG* regulation are present on the sequence immediately in front of the *pyrG* open reading frame, a *pyrG* mutant with an IS*S1* element inserted in the gene (CJ295) (Fig. 2) was transformed with the promoter fusion clone pCJ29. The cloned promoter fragment is responsible for the observed regulation of *pyrG*, since CTP limitation by uridine, adenosine, or deoxycytidine addition resulted in induction ratios of 18-, 5-, and 16-fold, respectively. These ratios are identical to those observed for the chromosomal *pyrG::lacLM* fusion in CJ217. These results show that all required regulatory elements are present immediately upstream of *pyrG* and furthermore that the regulatory mechanism is fully functional even when multiple copies of the elements are present in the cell.

Determination of nucleotide pool sizes and *pyrG* transcriptional rate during CTP limitation. Expression from the *pyrG-lacLM* chromosomal fusion during growth inhibition by uridine was analyzed in detail. Ribonucleoside triphosphate pool sizes during growth inhibition by uridine were determined, and the results clearly show that addition of uridine results in an instantaneous lowering of the CTP pool by more than one order of magnitude (Fig. 4C and D). Simultaneously, a threefold increase in UTP concentration and a twofold increase in GTP concentration were observed. No significant change in the ATP pool size was observed.

Figure 5A shows the rate of synthesis from the reporter genes *lacLM* under control of the *pyrG* promoter during CTP limitation. The slope of the curve for uninduced cells is 3.1, which, as expected, is similar to the specific activity found in Table 2 for CJ217 (3.7 ± 1.4 U/OD unit). After uridine was added and the CTP concentration was lowered, *pyrG* expression immediately increased ninefold to 28 U/OD unit. Expression from the *pyrG-lacLM* gene fusion did not, however, attain a new steady-state level but gradually increased until 140 min of CTP limitation, when a final induction of 37-fold was reached. Growth of the *pyrG* mutant for more than two generations after this point resulted in no further increase in expression; i.e., full induction of the *pyrG-lacLM* fusion is first observed after more than 1.5 generations of growth during CTP limitation.

The expression of *pyrG* responds primarily to CTP and not UTP. Transformation of the promoter fusion clone pCJ29 to a pyrimidine de novo mutant made it possible to determine *pyrG* expression during UTP limitation. A *pyrB* mutant grown with cytidine as a pyrimidine source will be subject to cytidine limitation in the presence of inosine, since cytidine and inosine transport is facilitated by the same transport system. As a result, the strain will have a decreased UTP pool and an increased CTP pool (23). As shown in Table 3, a fourfold increase in the CTP pool was followed by a sevenfold decrease in expression of the reporter genes, again suggesting that *pyrG* expression responds to the CTP concentration in the cell.

Reduced nucleotide pool sizes, including CTP, during growth on galactose and maltose induce *pyrG* expression. When grown on galactose or maltose as a carbon source, *L. lactis* shifts from homolactic fermentation to mixed acid fermentation with a reduction in growth rate. Growth of the *pyrG::lacLM* fusion strain in defined medium with cytidine and galactose or maltose as a carbon source increased the doubling time to 157 and 111 min, respectively, compared to 56 min when grown on glucose (Table 3). These doubling times are similar to previous observations of growth of MG1363 on defined medium (1, 24). The nucleoside triphosphate pools, and thus the CTP pool, are lower when the strain is grown on these carbon sources than when it is grown on glucose. The reduced pool sizes are followed by an increase in *pyrG* expression, as the *lacLM* reporter genes are induced almost 10-fold, suggesting that induction of *pyrG* expression is triggered by a lowered CTP pool size.

A correlation between CTP concentration and *pyrG* expression in *L. lactis*. Distorted nucleotide pool sizes were also achieved by the use of decoyinine, an inhibitor of GMP synthase and hence of GTP synthesis. Addition of decoyinine to strain CJ238, which harbors plasmid pCJ29, reduced the
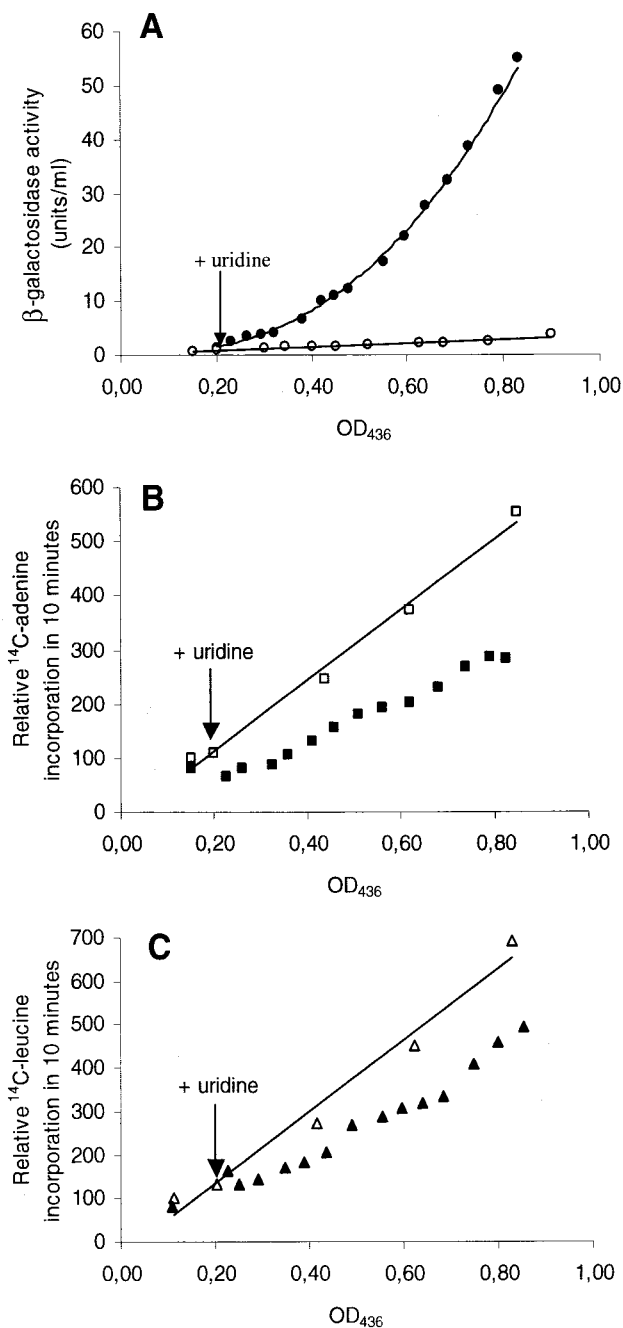

FIG. 5. Synthesis of *pyrG*, RNA, and protein during CTP limitation. (A) Rate of synthesis from a *pyrG::lacLM* chromosomal fusion mutant (CJ217) grown in GSA defined medium with cytidine (○) or with cytidine and uridine (●). (B and C) Synthesis of RNA (squares) and protein (triangles), respectively, either during growth with cytidine (open symbols) or during growth with cytidine and uridine (CTP limitation) (closed symbols). Uridine (500 μg/ml) was added at an OD_{436} of 0.2. The amount of incorporated labeled adenine or leucine in a 10-min pulse-labeling is set to 100 for the first determination for cells grown only with cytidine in the medium and is plotted against the OD value after 5 min of labeling.

growth rate threefold and not only reduced the GTP pool but also caused a general reduction in pool sizes (Table 3). These reduced pool sizes were followed by an increased *pyrG* expression from 76 to 126 U/OD unit. Next, the cells were inhibited

TABLE 3. Effects of varying nucleotide pool sizes on expression from a *pyrG*::*lacLM* fusion.

Strain	Genotype	Plasmid	Carbon source[a]	Cytidine (μg/ml)	Added inhibitor[b]	T_d (min)[c]	β-Galactosidase activity (U/OD$_{436}$ unit)[d]	Ribonucleotide concn (nmol/mg [dry wt])[e]			
								GTP	ATP	CTP	UTP
CJ217	*cdd pyrG*::*lacLM*	None	Glucose	20	None	56 ± 3	3.7 ± 1.4	1.41 ± 0.30	5.91 ± 0.63	1.73 ± 0.48	3.56 ± 0.28
			Galactose	20		157 ± 11	32 ± 2	0.64 ± 0.05	1.92 ± 0.12	0.65 ± 0.10	1.14 ± 0.19
			Maltose	20		111 ± 17	38 ± 6	0.74 ± 0.18	3.24 ± 0.60	0.51 ± 0.14	2.21 ± 0.29
CJ238	*cdd*	pCJ29	Glucose	0	None	61 ± 2	76 ± 8	1.28 ± 0.11	8.02 ± 1.02	1.52 ± 0.32	4.22 ± 0.50
					Decoyinine	181 ± 6	126 ± 1	0.95 ± 0.21	4.59 ± 0.16	0.88 ± 0.07	2.79 ± 0.43
				50	None	60 ± 2	46 ± 5	1.09 ± 0.10	6.50 ± 0.23	1.96 ± 0.26	3.37 ± 0.22
					Decoyinine	159 ± 7	44 ± 4	0.74 ± 0.12	6.47 ± 0.75	3.05 ± 0.17	2.86 ± 0.38
CJ300	*pyrB*::IS*S1*	pCJ29	Glucose	20	None	59 ± 3	96 ± 5	1.36 ± 0.46	5.78 ± 1.00	0.78 ± 0.30	1.27 ± 0.83
					Inosine	215 ± 21	13 ± 1	2.43 ± 0.14	6.07 ± 2.47	4.91 ± 0.11	0.22 ± 0.06

[a] All strains were grown in defined medium with the indicated carbon source at 1%.
[b] Decoyinine and inosine were added at an OD$_{436}$ of 0.2. Decoyinine was added to a concentration of 5 μg/ml, and inosine was added at 100 μg/ml.
[c] T_d, doubling time. The data are averages and standard deviations from three to five independent experiments.
[d] The cells were harvested at an OD$_{436}$ of 0.8. The data are averages and standard deviations from three to five independent experiments.
[e] The data are averages and standard deviations from three to five independent experiments.

with decoyinine in the presence of cytidine. Cells inhibited with decoyinine had a higher CTP pool when grown with cytidine and did not have increased *pyrG* expression, again suggesting a correlation between the CTP pool size and *pyrG* expression. The latter experiment also indicates that *pyrG* expression is not triggered by a reduced growth rate as such.

Figure 6 shows a plot of the activity from the *pyrG* promoter and the corresponding concentrations of the four nucleotides from the experiments with decoyinine and the *pyrB* mutant from Table 3. There is a clear correlation between the CTP pool size and expression of *pyrG*. The *pyrG* expression is elevated at low CTP concentrations and decreased at high CTP concentrations. No correlation between the ATP, GTP, and UTP pools and the expression of *pyrG* was found.

Macromolecular biosynthesis is reduced during CTP limitation. In experiments using nucleosides as inhibitors of cytidine metabolism, we saw no decrease in the growth rate for more than half an hour after the inhibiting nucleoside was added, even though the CTP concentration in the cell was below our detection limit (Fig. 4C and D). Intuitively, one would expect that during deprivation of CTP, central cellular processes such as RNA and protein synthesis would be affected, immediately resulting in a decrease in the rate by which the OD is increased. In *L. lactis* the amount of ribosomes, representing the vast majority of RNA in a cell, may not contribute significantly to the OD, as the amount of stable RNA in *L. lactis* has been determined to be only ca. 11% of the total dry weight (3, 6). Proteins comprise more than 50% of the total dry weight (3, 27), and therefore the rate of synthesis of proteins has a greater influence on OD measurements than synthesis of rRNA. A change in cell size may also contribute significantly to the OD, but the cells do not change in size during CTP limitation as observed in the microscope (data not shown). To explain the observed phenotypes of the *pyrG* mutant during CTP limitation, we therefore decided to determine the rates of synthesis of RNA and protein by pulse-labeling for 10 min with [¹⁴C]adenine or [¹⁴C]leucine, respectively (Fig. 5B and C). Limitation for CTP had an immediate effect on RNA synthesis, as the amount of RNA synthesized decreased to about 50% of the wild-type steady-state level. Since CTP is one

of the substrates for RNA synthesis, this is not surprising. Protein synthesis was also affected during CTP limitation and was reduced to about 65% of the values obtained for cells not limited for CTP. A gradual increase in protein synthesis was seen, as after almost two generations of CTP limitation, the protein synthesis rate had increased from 65 to almost 75% of the reference level. However, protein synthesis in the time interval from 5 to 15 min after CTP limitation was at the level for the uninhibited cells, although RNA synthesis decreased to half its normal value. The delayed growth inhibition seen during uridine inhibition may therefore partly be due to unaffected protein synthesis during the first 15 min of CTP limitation. As unaffected protein synthesis could be explained by an increase in mRNA stability, we determined the mRNA half-life during CTP limitation. An mRNA half-life of 70 s was obtained both for the *pyrG* mutant after 30 and 100 min of CTP limitation and for the *pyrG* mutant with a normal CTP pool, suggesting that CTP limitation has no effect on mRNA half-life.

Identification of a terminator structure in the *pyrG* promoter region. As in *B. subtilis* (25), a terminator structure can be predicted to form in the *L. lactis pyrG* leader sequence. The existence of this terminator in vivo was supported by deletion analysis of the *pyrG* leader and subsequent starvation for CTP in a *pyrG* background. The plasmids pCJ34 and pCJ35 contain the predicted terminator, and transcription is low in these promoter constructs when cytidine is present in the medium; CTP limitation increased expression about 20-fold (Fig. 7). The fragment in pCJ36 lacks half of the terminator stem-loop structure and has high expression from the reporter genes even when cells are grown in the presence of cytidine, suggesting that this structure is required for repression during high CTP concentrations. A threefold regulation, however, was still observed for this construct. This effect has also been reported for *B. subtilis* and may be a nonspecific effect of the pyrimidine starvation condition (25). Expression from pCJ29 is severalfold higher than expression from the three plasmids in Fig. 7. This is probably due to the presence of the *pyrG* open reading frame in pCJ29. There are very few base pairs between the stop codons and the ribosome binding site for the *lacL* gene in the pAK80 vector, which may cause an increase in expression from
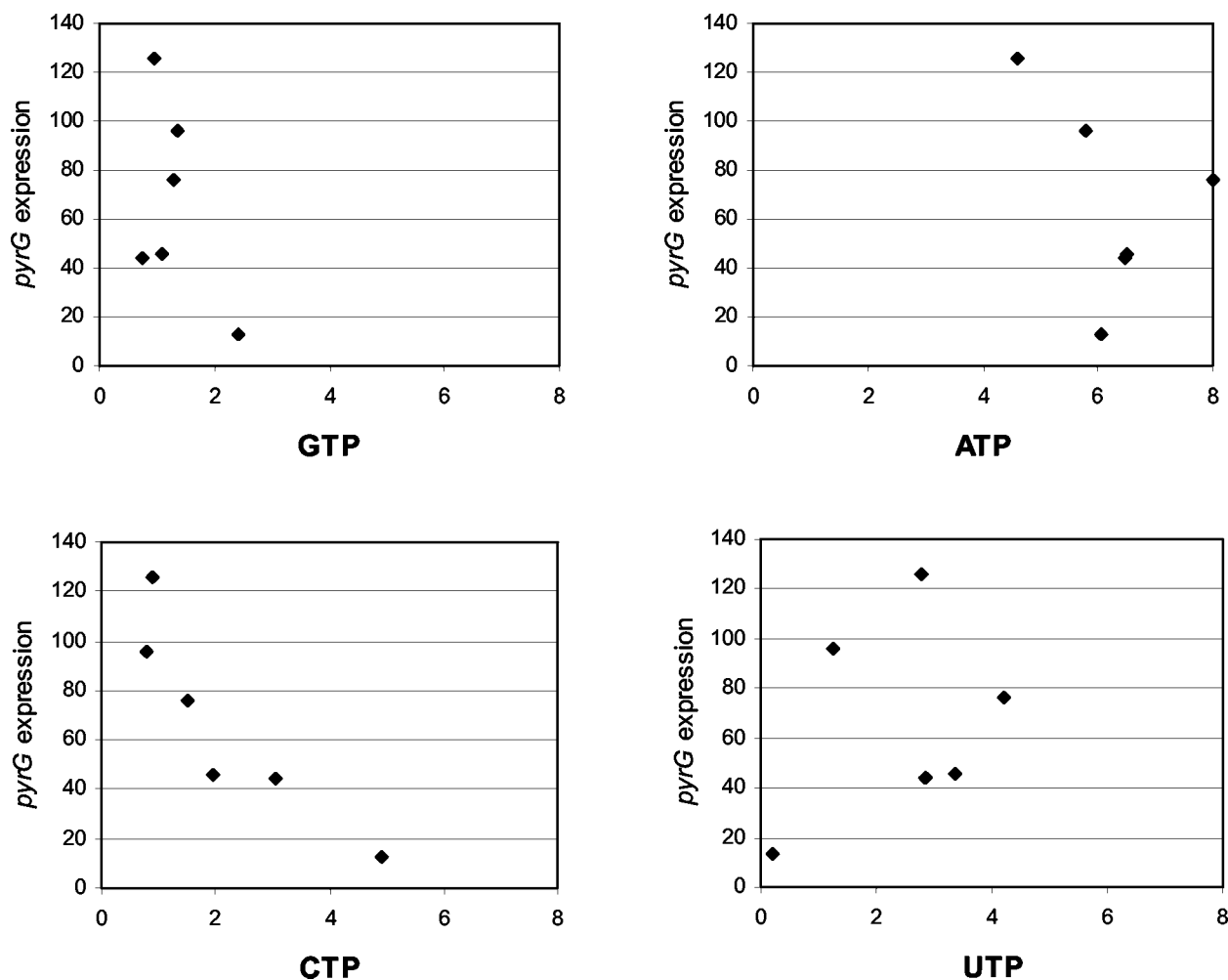
FIG. 6. Correlation between the different nucleoside triphosphate concentrations and expression of *pyrG* in *L. lactis*. Plots of β-galactosidase activity as a measure for *pyrG* expression and nucleoside triphosphate concentrations for CJ238 and CJ300 under different growth conditions (Table 3) are shown. The nucleoside triphosphate concentration is given in nanomoles per milligram (dry weight), and *pyrG* expression is presented as units/OD unit.

the reporter genes due to translational coupling to the upstream open reading frame. This is a common phenomenon with this vector (14).

DISCUSSION

The work presented here shows that a promoter immediately upstream of the *pyrG* open reading frame has sufficient strength to ensure full expression of *pyrG*. On the other hand, an RT-PCR experiment indicated that transcription arising from upstream promoters could contribute to the overall expression of *pyrG*. From Table 2 it is evident that the expression of *pyrG* is increased approximately 20-fold when the fusion is present on a plasmid compared to a chromosomal localization, as judged by the β-galactosidase expression in CJ217 and CJ298 grown under identical conditions. Since the copy number of the plasmid is reported to be lower than 30, it can be concluded that all significant expression of *pyrG* arises from a promoter present on the cloned fragment, provided that no regulatory factor is titrated by the plasmid. This is not the fact,

since the fold of regulation is the same on both chromosomal and plasmid fusions. Consequently, only the promoter immediately in front of *pyrG* has physiological relevance.

The results presented here clearly show that expression of the *pyrG* gene from *L. lactis* is regulated by the concentration of cytidine nucleotides within the cell, as illustrated in Fig. 6. The conclusion is based on a valid determination of intracellular nucleotide pools. The method is based on labeling of cultures with radioactive phosphate for two generations, immediate quenching and extraction of the nucleotides with 2 M formic acid, and separation of the nucleotides by thin-layer chromatography. The description of the method and the validation of the different steps in the method were presented in a recent paper (23). Reducing the CTP pool either by growth of a *pyrG* mutant with uridine at a concentration 25 times higher than that of cytidine or by growth with galactose or maltose as a carbon source results in increased *pyrG* expression. Under all of these growth conditions, the strains have lower growth rates than the uninduced control strain. However, reduction in growth rate is not the mechanism triggering *pyrG* induction,
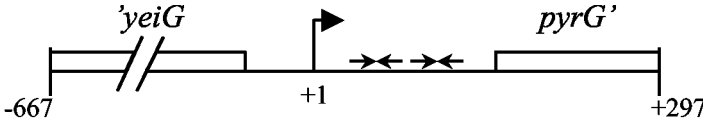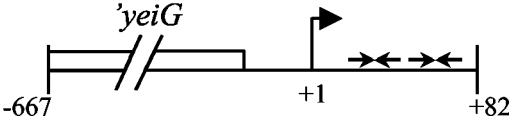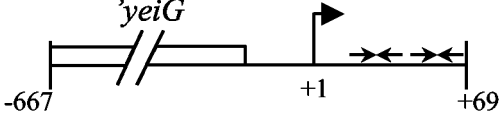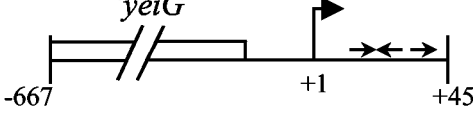
Plasmid	*pyrG* promoter region	β-galactosidase activity (units /OD$_{436}$)		Ratio
		Cytidine	Cytidine + uridine	
pCJ29		71 ± 19	1275 ± 98	18
pCJ34		7 ± 1	164 ± 8	23
pCJ35		21 ± 5	342 ± 7	16
pCJ36		115 ± 4	330 ± 47	3

FIG. 7. Deletion analysis of the *pyrG* promoter region. The indicated *pyrG* promoter regions were cloned in the promoter-probe vector pAK80. The plasmids were transformed into a *pyrG*::IS*S1* mutant and grown in defined medium either with cytidine or under CTP-limiting conditions (cytidine plus uridine) by the addition of uridine at a 25-fold excess at an OD$_{436}$ of 0.2. Cells were harvested at an OD$_{436}$ of 0.8, and the β-galactosidase activity was determined. Arrows indicate the two stem-loop structures predicted to form in the leader sequence. The activity of the negative control harboring only the vector pAK80 was determined to be less than 0.02 U/OD unit.

since decoyinine addition in the presence of cytidine inhibits growth but does not induce *pyrG* expression (Table 3). Growth inhibition is also observed when pyrimidine uptake in a *pyrB* mutant (CJ300) is inhibited by addition of inosine; however, this treatment does not reduce the CTP concentration in the cell and gives no *pyrG* induction (Table 3). The latter experiment also shows that *pyrG* expression is not induced by a decrease in the UTP pool; the regulation of *pyrG* is thus specific to cytidine nucleotides. Expression of *pyrG* in *L. lactis* is repressed by cytidine, as found in other bacteria (2, 25, 37). The CTP dependence of *pyrG* expression is similar to what has been proposed for *pyrG* from *B. subtilis* (25), but a correlation with CTP pool size was not demonstrated. In this work, nucleotide pool sizes during pyrimidine limitation have been determined and it has been shown directly that the concentration of CTP and *pyrG* expression are correlated in *L. lactis*. The fact that *pyrG* is not induced upon complete CTP starvation is most likely due to a general stop in transcription.

The rate of synthesis from *pyrG* during uridine-inhibited cytidine phosphorylation in a *pyrG* mutant was found not to be linear (Fig. 5A). Initially, *pyrG* is induced 9-fold, but the synthesis rate increases for 1.5 generations to a final induction ratio of 37-fold. This pattern of induction cannot be explained from altered macromolecular synthesis, since neither RNA nor protein synthesis changes with the same kinetics. The low CTP concentration during cytidine limitation is at the limit of detection, and the CTP concentration may therefore decrease

further during the growth experiment after uridine addition, not reaching its final lower value until 1.5 generations of growth, where *pyrG* expression reaches a new steady-state level. It is important to emphasize that the concentration of CTP does not reflect the flow through the CTP pool. The small amount of cytidine phosphorylated to CTP during uridine inhibition is rapidly used for, e.g., RNA production, causing a high turnover of CTP.

When the kinetics of the effects on macromolecular synthesis of cytidine limitation by addition of uridine was monitored (Fig. 5B and C), it was found that the rate of RNA synthesis was immediately changed to 50% of that found in uninhibited cells. In contrast, the rate of protein synthesis was unchanged during the first 15 min of cytidine limitation. The unchanged protein synthesis was not due to an increased mRNA half-life under these growth conditions (data not shown), and therefore we must conclude that the amount of mRNA synthesized during the first 15 min is unaltered compared to that in the control cells without uridine. The 50% inhibition of RNA synthesis observed is thus proposed to be primarily inhibition of stable RNA synthesis. In the final steady state obtained after 1.5 generations of growth, the growth rate is half of the uninhibited growth rate and the number of ribosomes is expected to be reduced. The immediate stop in synthesis of rRNA after uridine addition would help the cells to a fast adjustment to the new growth conditions. The rate of total RNA synthesis during the period after uridine addition was 50% of the uninhibited

Terminator   Antiterminator

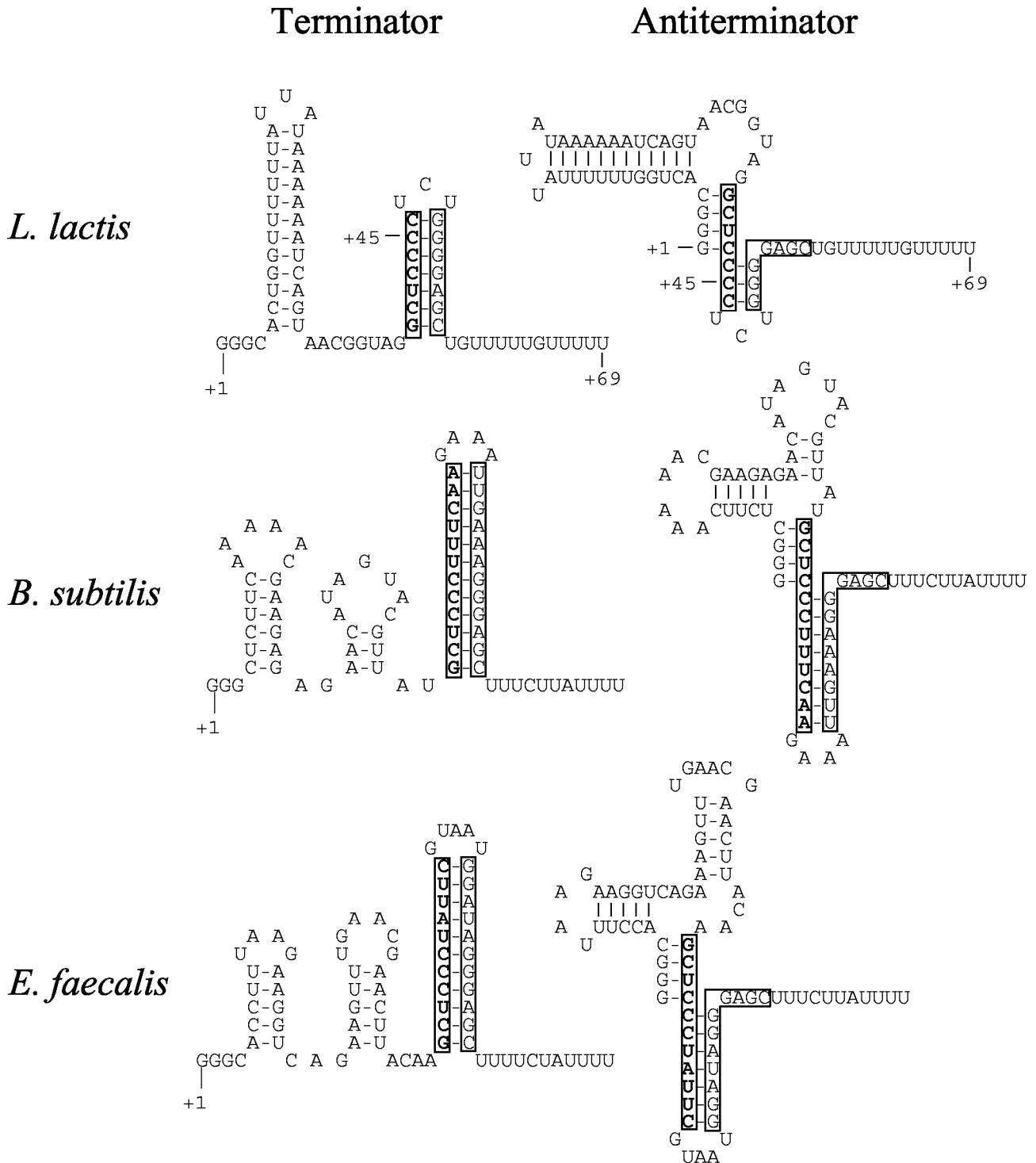*L. lactis*

B. subtilis

E. faecalis

FIG. 8. Predicted structures of terminators and antiterminators in the 5' *pyrG* leader sequences from *L. lactis* (accession number AJ010153) *B. subtilis* (accession number Z49782), and *E. faecalis* (accession number AE016950). The first nucleotide in the mRNA is marked +1. The +1 positions in *E. faecalis* and *L. lactis* are not supported by experimental evidence, whereas the +1 position of *B. subtilis* was determined by Meng and Switzer (25). The translational start codons are found further downstream. The stems in the terminator structure are marked by boxes in both the terminator and the antiterminator. +45 and +69 in the structure for *L. lactis* indicate the deletion points in plasmids pCJ35 and pCJ36 (Fig. 7).

rate. This is likely to reflect different proportions of stable RNA and mRNA rates during the experiment, as discussed above. In the final steady state, the protein synthesis rate is 75% of the uninhibited rate. These numbers also indicate that a major fraction of RNA made is mRNA and hence that cells with a lower growth rate contain fewer ribosomes and more protein per cell mass. The relationship between the growth rate of a bacterial cell and the content of stable RNA was

identified for *E. coli* and *S. enterica* serovar Typhimurium by Maaløe and coworkers, and they found that synthesis of rRNA increased with the square of the growth rate (16, 31). Later this was shown to apply for many other bacteria, including *L. lactis* (4). On the other hand, there has been controversy in the literature as to whether nucleotide pools vary with the growth rate in the same way (7, 28). The different results obtained have been attributed to variations in the methods used for nucleotide pool determinations (32). The results obtained in this study for *L. lactis* suggest a correlation between growth rate and nucleoside triphosphate pools, as growth on galactose or maltose as a carbon source results in decreased pool sizes. The variance of nucleoside triphosphate pools in *L. lactis*, however, may not be due to the method used but rather may be a consequence of the relatively simple metabolism of this bacterium, in which sugar is fermented to lactate (homolactic growth, as seen with growth on glucose) or to other acids such as acetate and ethanol (mixed acid fermentation, as seen with growth on galactose or maltose). In *E. coli*, oxidative phosphorylation, a pathway not active in *L. lactis*, may have a large influence on the turnover rate of nucleoside triphosphate.

With respect to the mechanism for the CTP-regulated expression of *pyrG*, it has been shown to be due to termination and antitermination in *B. subtilis*, and a terminator structure has been identified in the untranslated leader sequence of *pyrG* (25). In *L. lactis*, deletion analysis of the *pyrG* leader also demonstrates that the terminator structure is required for regulation (Fig. 7). *pyrG* leaders from gram-positive bacteria contain several conserved segments and all have the potential to fold into transcription terminator structures, suggesting similar regulatory mechanisms for *pyrG* in these bacteria (25). Mutational analysis of the *pyrG* leader in *B. subtilis* has identified several interesting points regarding the mechanism of *pyrG* regulation. The first four nucleotides (GGGC) in the *pyrG* leader of *B. subtilis* are required for normal regulation of *pyrG*, as mutagenesis of these nucleotides results in increased termination under all growth conditions (26). Additionally, it was shown that antitermination during CTP starvation requires the nucleotides GCUCCC in the stem of the terminator structure. Deletion of the nucleotides between these two conserved segments has no effect on regulation of *pyrG*. It was proposed that a regulatory protein senses the CTP availability and binds to the conserved segments in the *pyrG* leader during CTP starvation, thereby preventing termination (25). However, the work presented here has revealed that the cloning of the *pyrG* leader on a plasmid does not lead to titration of an effector, since the fold regulation is conserved despite the copy number. Consequently, another explanation is possible, which does not include a regulatory protein but rather involves the formation of an antiterminator structure. Such a structure is presented in Fig. 8, and the structure includes base pairing between the two identified conserved segments. The predicted terminator-antiterminator structure can be identified in several gram-positive bacteria, including *L. lactis*, *B. subtilis*, *E. faecalis*, *Streptococcus pyogenes*, *Lactobacillus plantarum*, and *Listeria monocytogenes*, suggesting the presence of the structures in these organisms. In Fig. 8 the putative terminator and antiterminator structures of *L. lactis*, *B. subtilis*, and *E. faecalis* are shown. It is interesting that a mutation in the *B. subtilis pyrG* leader removing base pairs between the two conserved segments (e.g., changing one

of the first three Gs to an A) abolishes *pyrG* regulation whereas a mutation that strengthens the antiterminator (e.g., changing the U at position +5 to a G) increases *pyrG* expression (26). The region between the two conserved segments is not needed for formation of the antiterminator, explaining the lack of homology in this part of the leader between gram-positive bacteria and the fact that this part can be deleted without loss of regulation (26).

We suggest the following model for *pyrG* regulation in *L. lactis* and probably in other gram-positive bacteria as well. At normal CTP concentrations the terminator in the *pyrG* leader is preferentially formed, thereby reducing expression of CTP synthase (Fig. 8). When the CTP concentration is reduced, RNA polymerase pauses at the stretch of C residues in the *pyrG* leader, thus allowing the antiterminator to form. Since the terminator and antiterminator are mutually exclusive structures (Fig. 8), transcription will proceed, thus allowing expression of the *pyrG* gene. We will pursue the verification of the model by in vivo and in vitro experiments.

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