Molecular characterization of pyrimidine biosynthesis genes from the thermophile *Bacillus caldolyticus*

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The genes encoding the six pyrimidine biosynthesis enzymes from the thermophile *Bacillus caldolyticus* were characterized by cloning and complementation in *Escherichia coli*, and by nucleotide sequence analysis. Nine cistrons are clustered within an 11 kb region of the chromosome, the gene order being: *orf1-pyrB-pyrC-pyrAa-pyrAb-orf2-pyrD-pyrF-pyrE*. This organization of the cluster is very similar to that of the *pyr* operon of *Bacillus subtilis*. Different parts of the *B. caldolyticus* cluster were cloned in two orientations in the expression shuttle vector pHP59. Complementation studies in *B. subtilis* established that expression of the *pyr* genes was dependent on the vector-borne promoter, suggesting that they are part of an operon, and that the native promoter of the operon had not been cloned. The deduced amino acid sequence of the individual cistrons showed 49 to 78% identity with the corresponding *B. subtilis* cistrons. Measurements of the aspartate transcarbamylase (*pyrB*), orotidine monophosphate decarboxylase (*pyrF*) and orotate phosphoribosyltransferase (*pyrE*) levels in cells grown under different conditions indicated that expression of the operon is repressed 7–9-fold by addition of uracil to the growth medium. Based on the nucleotide sequence in the intercistronic region between *orf1* and *pyrB* a regulatory mechanism involving transcriptional termination and antitermination is proposed to control expression of the operon.

Keywords: *Bacillus caldolyticus*, pyrimidine biosynthesis genes, thermophile

INTRODUCTION

The extreme thermophile *Bacillus caldolyticus* together with *Bacillus caldodenax* and *Bacillus caldovelox* were isolated as single isolates from a hot spring in Yellowstone National Park, USA (Heinen & Heinen, 1972). The optimal growth temperature for *B. caldolyticus* is about 72 °C and the maximal temperature for growth about 82 °C (Claus & Berkley, 1986). Differences in the mol% G + C in the genomes and in DNA/DNA homologies showed that *B. caldolyticus* does not belong to the same species as *B. caldodenax* and *B. caldovelox* (Sharp *et al.*, 1980). Recent taxonomic studies further showed that *B. caldolyticus* does not belong to the *Bacillus stearrowthermophilus* group either (De Bartolomeo *et al.*, 1991).

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Thermophilic micro-organisms have received increasing interest in recent years due to their potential use as sources for thermostable enzymes of industrial interest. A number of genes from thermophilic *Bacillus* strains have been characterized and the deduced amino acid sequences of the gene products compared with their mesophilic counterparts. In many instances the aim was to gain information on the structure–thermostability relationships of the encoded proteins (Eijsink *et al.*, 1992; Schlatter *et al.*, 1987; Van den Burg *et al.*, 1991; Zuber, 1988; Züllig *et al.*, 1987). However, very little is known about the genetic organization and control of expression of biosynthetic pathways in thermophilic *Bacillus* strains.

Pyrimidine biosynthesis follows the same pathway in all organisms. Six enzyme reactions catalyse the conversion of glutamine (ammonia), bicarbonate, aspartate and phosphoribosylpyrophosphate to UMP, the common precursor of all other pyrimidine nucleotides. In *Escherichia coli* and *Salmonella typhimurium* the enzymes are

Table 1. Bacterial strains and plasmids

Strains and plasmids	Relevant characteristics	Reference or source*
Strains		
<i>B. caldolyticus</i>		
DSM 405	Wild-type	DSM
<i>B. subtilis</i>		
6GM15	<i>trpC2 tyr met his ura rib r_M⁻ m_M⁺ lacZΔM15 Km^R</i>	Haima <i>et al.</i> (1990b)
ED215	<i>met pyrE305</i>	BGSC 1A322
ED216	<i>trpC2 pyrC</i>	BGSC 1A394
ED217	<i>trpC2 pyrF</i>	BGSC 1A400
ED316	<i>trpC2 pyrB</i>	BGSC 1A392
ED318	<i>trpC2 pyrD</i>	BGSC 1A396
ED364	<i>pyrA</i>	BGSC 1A391
<i>E. coli</i>		
SØ268	<i>pyrE60 cdd thi-1 argE3 his-4 proA2 thr-1 leu-6 mtl-1 xyl-5 ara-14 gal-K2 lacY1 str-31 supE44</i>	JF611, J. Friesen
SØ990	<i>pyrB59 argH1 thi-1 hisG1 purF1 mtl-2 xyl-7 malA1 ara-13 lacY1 or lacZ4 strA ton tsx supE44?</i>	CGSC 4517
SØ1209	<i>carBΔ8 thr thi lac r_K⁻ m_K⁺</i>	JL2944, J. L. Ingraham
SØ1263	<i>ΔpyrC araD139 Δ(argF-cod-lac)U169 rpsL thi</i>	K. F. Jensen
SØ3539	<i>araD139 Δ(ara-leu)7697 Δlac74 gal4 hsdR rpsL</i>	MC1061, N. P. Minton
SØ4058	<i>pyrD::Km^R araD139 Δ(ara-leu)7697 Δlac74 gal4 hsdR rpsL</i>	L. Linde
SØ5009	<i>pyrF30 araD139 Δ(ara-leu)7697 Δlac74 gal4 hsdR rpsL</i>	L. Stauning
Plasmids		
pHPS9	Cm ^R Er ^R <i>cat-86::lacZa</i> PT1060-pUC9 derivative	Haima <i>et al.</i> (1990a)
pHPSY4	4.65 kb <i>Xba</i> I fragment from pSY31 with <i>pyrAa</i> and <i>Ab</i> in <i>Sma</i> I site	This study
pHPSY5	Same insert as pHPSY4 with reversed orientation	This study
pHPSY6	3.1 kb <i>Sma</i> I fragment from pSY17 with <i>pyrB</i> and <i>C</i> in <i>Sma</i> I site	This study
pHPSY7	Same insert as pHPSY6 with reversed orientation	This study
pHPSY8	2.1 kb <i>Bam</i> HI fragment from pHPSY10 with <i>orf2</i> and <i>pyrD</i> in <i>Bam</i> HI site	This study
pHPSY9	Same insert as pHPSY8 with reversed orientation	This study
pHPSY10	2.1 kb <i>Eco</i> RI- <i>Pvu</i> II fragment from pSY2 with <i>orf2</i> and <i>pyrD</i> in <i>Eco</i> RI- <i>Sma</i> I sites	This study
pJS21	2.75 kb <i>Sph</i> I fragment from pJS5 with <i>pyrF</i> and <i>E</i> in <i>Sph</i> I site of pHPS9	This study
pJS22	Same insert as pJS21 with reversed orientation	This study
pUC18, 19	Ap ^R	
pJS5	2.8 kb <i>Nru</i> I- <i>Eco</i> RV fragment from pSY4 with <i>pyrF</i> and <i>E</i> in pUC18 <i>Hinc</i> II site	This study
pJS7	1.6 kb fragment from pJS5 with <i>pyrF</i> , by <i>Pst</i> I deletion	This study
pPM4	1.2 kb <i>Nsi</i> I- <i>Hind</i> III fragment from pJS7 with <i>pyrF</i> , in pUC18 <i>Pst</i> I- <i>Hind</i> III sites	This study
pPM15	1.7 kb fragment with <i>pyrE</i> by <i>Hinc</i> II- <i>Sma</i> I deletion of pJS5	This study
pSY1	4.7 kb <i>Cla</i> I fragment with <i>orf2</i> , <i>pyrD</i> , <i>F</i> and <i>E</i> in pUC19 <i>Acl</i> I site. From <i>Cla</i> I library	This study
pSY2	Same insert as pSY1 with reversed orientation	This study

Table 1. (continued).

Strains and plasmids	Relevant characteristics	Reference or source*
pSY4	6.5 kb <i>Bam</i> HI fragment with <i>orf2</i> , <i>pyrD</i> , <i>F</i> , and <i>E</i> in pUC19 <i>Bam</i> HI site. From <i>Bam</i> HI library	This study
pSY5	4.3 kb <i>Sph</i> I fragment with <i>pyrF</i> and <i>E</i> in pUC19 <i>Sph</i> I site. From <i>Sph</i> I library	This study
pSY6	11.3 kb <i>Hind</i> III fragment with <i>pyrC</i> , <i>Aa</i> , <i>Ab</i> , <i>orf2</i> , <i>D</i> , <i>F</i> and <i>E</i> in pUC19 <i>Hind</i> III site. From <i>Hind</i> III library	This study
pSY10	7 kb <i>Hind</i> III- <i>Cla</i> I fragment from pSY6 with <i>pyrC</i> and <i>Aa</i> in pUC18 <i>Hind</i> III- <i>Acc</i> I sites	This study
pSY15	7.1 kb fragment with <i>pyrB</i> , <i>C</i> , <i>Aa</i> and <i>Ab</i> in pUC19 <i>Bam</i> HI site. From partial <i>Sau</i> 3AI library	This study
pSY16	4.7 kb insert with <i>pyrAb</i> in pUC19 <i>Bam</i> HI site. From partial <i>Sau</i> 3AI library	This study
pSY17	3.1 kb <i>Sma</i> I fragment from pSY15 with <i>pyrB</i> and <i>C</i> in pUC19 <i>Sma</i> I site	This study
pSY18	Same insert as pSY17 with reversed orientation	This study
pSY31	4.7 kb <i>Nar</i> I- <i>Hind</i> III fragment from pSY15 with <i>pyrAa</i> and <i>Ab</i> in pUC18 <i>Acc</i> I- <i>Hind</i> III sites	This study

* DSM, Deutsche Sammlung für Mikroorganismen und Zell Kulturen, Braunschweig, Germany; BGSC, *Bacillus* Genetic Stock Center, Ohio State University, Columbus, OH, USA; CGSC, *E. coli* Genetic Stock Center, Yale University, New Haven, CT, USA. K. F. Jensen, L. Linde and L. Stauning. Dept Biol. Chem., Univ. Copenhagen, Denmark; J. Friesen, Hospital for Sick Children, Toronto, Canada; N. P. Minton, Microbial Technol. Lab., Salisbury, UK; J. L. Ingraham, Dept Bacteriol., Univ. Calif., Davis, CA, USA.

encoded by six genetically unlinked loci (Neuhard & Nygaard, 1987). Expression of these genes and small operons are non-coordinately regulated by an interplay of the intracellular nucleotide pools. The *pyrB* and *pyrE* genes are regulated through UTP-sensitive transcriptional attenuation (Poulsen *et al.*, 1984; Roland *et al.*, 1985), whereas *pyrC* and *pyrD* expression is controlled through CTP/GTP-sensitive selection of the transcriptional start site which in turn influences translation initiation due to formation of secondary structures on the RNA (Sørensen & Neuhard, 1991; Sørensen *et al.*, 1993; Wilson *et al.*, 1992). The *pyr* genes in *B. subtilis* are organized in a single operon with the gene order *pyrB-C-A-D-F-E* (Lerner *et al.*, 1987; Quinn *et al.*, 1991). Expression of the operon is coordinately repressed by a uracil compound by an unknown mechanism (Asahi *et al.*, 1989; Paulus *et al.*, 1982).

In this paper we report the cloning and characterization of the pyrimidine biosynthesis genes from *B. caldolyticus*. Our data indicate that the genes belong to a single operon with the same overall organization as found for the *pyr* operon of *B. subtilis*, and that expression of the operon is repressed in cells grown in the presence of uracil.

METHODS

Bacterial strains, plasmids and growth conditions. The bacterial strains and plasmids used are described in Table 1. *B. caldolyticus* DSM405 was grown at 60 °C in mineral salts medium

162 (Degryse *et al.*, 1978) supplemented with either yeast extract (2.5 g l⁻¹) and tryptone (2.5 g l⁻¹), or with ammonium chloride (10 mM), 0.5 % glucose and 0.2 % vitamin-free Casamino acids. *E. coli* was grown at 37 °C in LB medium (Sambrook *et al.*, 1989) or in AB medium with 0.2 % glucose and 0.2 % vitamin-free Casamino acids (Clark & Maaløe, 1967). *B. subtilis* was grown at 37 °C in LB medium or in Spizizen mineral salts medium (Spizizen, 1958) with 0.5 % glucose as carbon source. As solid medium for *B. subtilis* Spizizen mineral salts medium supplemented with 0.2 % glycerol and 0.2 % glutamate was employed. The following nutrients were added when required: uracil (20 µg ml⁻¹), hypoxanthine (15 µg ml⁻¹), individual amino acids (50 µg ml⁻¹). Antibiotics were used at the following concentrations: ampicillin (100 µg ml⁻¹), chloramphenicol (5 µg ml⁻¹), tetracycline (10 µg ml⁻¹). X-Gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside) was used at a final concentration of 40 µg ml⁻¹.

Molecular cloning and DNA manipulations. Chromosomal, plasmid and M13 DNA was prepared as described by Sambrook *et al.* (1989). Restriction endonucleases, mung bean nuclease, the Klenow fragment of DNA polymerase I and T4 DNA ligase were purchased from Boehringer or Promega and used in accordance with the specifications given by the manufacturers. *E. coli* cells were made competent for transformation by the CaCl₂ method (Sambrook *et al.*, 1989). Competence in *B. subtilis* was induced by the method of Boylan *et al.* (1972). DNA sequencing was accomplished by the chain-termination method (Sanger *et al.*, 1977) using M13mp18/19 or pUC18/19 as vectors. ssDNA was sequenced with the Sequenase kit (US Biochemical Corp.) with 7-deaza-dGTP to alleviate the problem of compression of the G+C-rich regions frequently encountered in *B. caldolyticus* DNA. For sequencing dsDNA the template

was prepared by the CTAB-DNA precipitation method (Del Sal *et al.*, 1989), and a TaqTrack sequencing system (Promega). Computer-assisted sequence analysis was done with University of Wisconsin GCG software packages (Devereux *et al.*, 1984) on a VAX computer. The deduced amino acid sequences were compared with the SwissProt databank and the NBRF protein database by using the FASTA program (Pearson & Lipman, 1988). RNA secondary structure analysis was performed with the FOLD program using the algorithm of Zuker & Stiegler (1981).

Polymerase chain reaction (PCR). The intercistronic region between *pyrF* and *pyrE* was amplified using total chromosomal *B. caldolyticus* DNA and two synthetic primers: the *pyrF* primer, 5' GCAACACGAATGGAACGGAGG 3', corresponding to nucleotides 9221 to 9241 (Fig. 2); and the *pyrE* primer 5' ATTTCAAGCCCGAGGACCAGG 3', complementary to nucleotides 9724 to 9744 (Fig. 2). cDNA was synthesized using the *pyrE* primer, *B. caldolyticus* total RNA as template, and reverse transcriptase. The resulting cDNA was phenol extracted and ethanol precipitated prior to amplification with the same two primers as above.

Enzyme assays. Exponentially growing cultures were harvested, washed and sonicated. Enzyme activities were measured in the crude sonicated extracts by published methods: aspartate transcarbamylase (Prescott & Jones, 1969), orotidine monophosphate (OMP) decarboxylase (Jensen *et al.*, 1982), orotate phosphoribosyltransferase (Paulus *et al.*, 1982; Poulsen *et al.*, 1983). The reaction temperature was 60 °C and 37 °C with *B. caldolyticus* and *B. subtilis* extracts, respectively. Protein concentrations were determined by the Lowry method with bovine serum albumin as a standard.

RESULTS

Cloning of the *pyr* genes

Genomic libraries were prepared by digesting *B. caldolyticus* DNA with various restriction endonucleases, and ligating the fragments into plasmid pUC19 digested with the same or a compatible endonuclease. The libraries were used to transform pyrimidine-requiring mutants of *E. coli* (*carB*, *pyrB*, -C, -D, -E and -F) to pyrimidine prototrophy at 42 °C. Plasmids capable of complementing a specific pyrimidine mutation were subsequently tested for their ability to complement the remaining five *E. coli* pyrimidine biosynthesis mutations. The following five plasmids were obtained from such screenings (Fig. 1a): pSY4 (*Bam*HI library), pSY5 (*Sph*I library), pSY6 (*Hind*III library), and pSY15 and -16 (partial *Sau*3AI library). The complementation pattern and the restriction map of these plasmids and of the four subclones pPM4, pPM15, pSY10 and pSY18 (Fig. 1a) showed that the six pyrimidine biosynthesis genes of *B. caldolyticus* are clustered on the chromosome, and that they are organized in the following order: *pyrB*-*pyrC*-*pyrA*-*pyrD*-*pyrF*-*pyrE*.

Expression of the *pyr* genes in *B. subtilis*

To test for the presence of Gram-positive promoters within the region, segments containing *pyrB*-C, *pyrA*, *pyrD* and *pyrF*-E, respectively, were cloned in both orientations in the *E. coli*/*B. subtilis* shuttle vector pHPS9,

and the ability of the resulting plasmids to complement individual *B. subtilis pyr* mutations was determined (Fig. 1b). Plasmid pHPS9 contains a strong *Lactococcus lactis* promoter, P59, reading into the multiple cloning region. The copy number of pHPS9 in *E. coli* is very high, whereas it is only five to six in *B. subtilis* (Haima *et al.*, 1990a). Since complementation was only observed with the fragments in one orientation, it was concluded that expression was dependent on transcription from the P59 promoter present on the vector. This further suggested that the genes are organized in an operon transcribed in the direction from *pyrB* towards *pyrE*.

Nucleotide sequence

The nucleotide sequence of a 10932 bp region was determined on both strands and across all cloning junctions (Fig. 2). Computer assisted search of the sequence revealed the presence of nine open reading frames (ORFs). The 5' end of the first ORF was not present on any of the clones. Based on the assumption that the 3' end of *B. caldolyticus* 16S rRNA is similar to that of *B. subtilis* and *B. stearothermophilus* 16S rRNA, i.e. 3' UCUUCCUCCACUAG (Douthwaite *et al.*, 1983; McLaughlin *et al.*, 1981), putative ribosomal binding sites were located 7–11 bp upstream of the start codons of the first seven fully sequenced ORFs. For the last ORF the only sequence resembling a ribosomal binding site was located 66 bp upstream of the putative start codon. The first two ORFs and the last two ORFs are separated by 169 bp and 384 bp, respectively. The remaining ORFs are overlapping or, in one case, separated by 2 bp (Fig. 2).

Identification of the ORFs

The organization of the pyrimidine genes (Fig. 1) and the corresponding ORFs (Fig. 2) indicated great similarities with the *pyr* operon of *B. subtilis* (Quinn *et al.*, 1991). Comparison of the deduced amino acid sequences of the eight fully sequenced ORFs from *B. caldolyticus* with those of the known Pyr proteins of *B. subtilis* revealed between 49 and 78 % identity. This identified seven of the ORFs as subunits of the pyrimidine biosynthesis enzymes (Table 2). One major organizational difference between the *pyr* clusters of the two organisms was that the coding regions of *B. caldolyticus pyrF* and *pyrE* appeared to be separated by a 384 bp non-translated region whereas the two cistrons overlap by 4 bp in *B. subtilis*. To ascertain that this difference was not the result of a cloning artefact this region of the *B. caldolyticus* chromosome was amplified by PCR using a primer late in the *pyrF* gene and one early in the *pyrE* gene. The reaction yielded a 523 bp fragment with a restriction endonuclease digestion pattern in accordance with the nucleotide sequence of the intercistronic region (Fig. 2). An identical 523 bp fragment was obtained by PCR amplification of *B. caldolyticus* cDNA using the same primers. The cDNA was derived from total *B. caldolyticus* RNA by reverse transcription using the early *pyrE* primer. This further indicated that the *B. caldolyticus pyrF/pyrE* intercistronic region is transcribed.

Fig. 1. Organization of the *pyr* gene-cluster of *B. caldolyticus*. Complementation by different DNA fragments of individual *pyr* mutations in *E. coli* (a) and *B. subtilis* (b). Filled bars indicate fragments obtained from the various libraries by complementation; hatched bars indicate fragments obtained by subcloning as described in Table 1. The vectors used were pUC18/19 for the pSY and pPM plasmids, and pHPS9 for the pHPSY plasmids. In all the pUC-derived constructs the *lac* promoter of the vector promotes transcription of the fragments from left to right. The orientation of the vector-borne P59 promoter is indicated by arrows. *carB* is the gene encoding the large subunit of carbamoylphosphate synthase of *E. coli*. It corresponds to the *pyrAb* gene of *B. caldolyticus*. The construction of the plasmids in Fig. 1(b) is described in Table 1. Restriction endonuclease sites are abbreviated as follows: B, *Bam*HI; C, *Cl*al; E, *Eco*RI; EV, *Eco*RV; H, *Hind*III; Hc, *Hinc*II; N, *Nsi*I; Na, *Nar*I; P, *Pst*I; Pv, *Pvu*II; S, *Sph*I; Sm, *Sma*I.

Codon usage

A mol% G+C of 59 was calculated for the protein coding regions of the *B. caldolyticus* *pyr* cluster (3181 codons). This is considerably higher than found for the homologous regions of *B. subtilis* (Table 3). In particular the third codon position showed a strong bias for G or C (73%), as has been observed for protein coding regions of other organisms with high G+C content (Kagawa *et al.*, 1984; Kimsey & Kaiser, 1992). This strong bias is particularly pronounced with the Val, Ser, Pro, Thr, Ala, Cys, Arg and Gly codons where 84 to 91% of them end with G or C (Table 4).

Transcription signals

Two regions of partial dyad symmetry were located within the untranslated region between *orf1* and *pyrB*. A transcript of this region may form two competing secondary structures. One, located at positions 335–360, resembled a Rho-independent transcriptional terminator consisting of a GC-rich stem-loop structure followed by 8

U-residues (Fig. 3a). The stability of this hairpin was calculated to be -90 kJ mol^{-1} . The other structure is located between positions 268 and 341. This large and more stable structure ($\Delta G = -215 \text{ kJ mol}^{-1}$) includes most of the left stem of the terminator hairpin (Fig. 3a). In addition, a region of partial dyad symmetry showing some resemblance to a Rho-independent transcriptional terminator was located 15 bp downstream of the last gene of the cluster (*pyrE*).

Pyrimidine regulation of *pyr* gene expression

The specific activity of aspartate transcarbamylase (*pyrB*), orotate phosphoribosyltransferase (*pyrE*), and OMP decarboxylase (*pyrF*) was determined in crude sonicated extracts of *B. caldolyticus* grown exponentially at 60 °C in minimal medium with or without uracil ($20 \mu\text{g ml}^{-1}$). For comparison, the activity of two of the enzymes was also determined in extracts of *B. subtilis* grown in similar conditions at 37 °C. As shown in Table 5, addition of uracil resulted in 7–9-fold repression of *pyrB*, *pyrE* and *pyrF* expression in *B. caldolyticus*. Regulation of the two *B. subtilis* enzymes assayed occurred to the same extent.

Fig. 2. For legend see p. 487.

AGGTGACGCATATCGCCTTAAATACGGGACGGTGAAGGGCTCCGTCATTTCGACGTTCCGGCGTTTACGGTGCACTATCATCCGGAAGGTCGGCGGGCCCAAGATGCCAATCCGC	3600
E V T H I A L N D G T V E G L R H L D V P A F T V Q Y H P E A S P G P E D A N P	343
RBS PyrAB ---->	
TGTTTGACGAGTTTGGCGCTCATCGCGAGTTCAACAGAGGAGAGTATCATCGCTTAAGCGCCCGGACATTGAAACGATTTAGTCATCGGCTCGGGCCGATCGTCATCGGC	3720
L F D E F L A L I R E F N K K G E V I H A *	364
M P K R R D I E T I L V I G S G P I V I G	21
CAGCGGCGGAGTTTCGACTACCGGACGGACGCGTCTTAGCCCTAAAGAAGAGATACAAAGTCATTCTGGTGAATCAACCGCGGACGATCATGACCGATACGGAATCGCC	3840
Q A A E F D Y A G T Q A C L A L K E E G Y K V I L V N S N P A T I M T D T E I A	61
GATAAAGTCTATATGGAGCGCTGACGCTCGATTTCGCGCCGATCATCGCAAGAGCGCGCGGACCGGATTTCCGCGCTTGGCGGCAACCGGCTCAACTGGCGGTGAG	3960
D X V Y M E P L T L D F V A R I I R K E R P D A I L P T L G G Q T G L N L A V E	101
CTCGCAAGCGCGCGCTGTAAGATGCGCGCTGAAAATTCGCGCACGAGCTCGAGCGATCGAAAGCGCGAAGACCGCGAAGAGTTCCGCGCGCTCATGAACGAGCTTGGCGAG	4080
L A K A G V L E E C G V E I L G T K L E A I E K A E D R E Q F R A L M N E L G E	141
CCGGTCCGCAAGCGGATTTATTCACGCTCGAAGAGCGGATGCGTTTCGCAACAAATCGGCTATCGCGTCACTCGCGCGCGCGCTTACGCTCGCGCGGACCGCGCGCGGCGATT	4200
P V P E S A I I H S L E E A Y A F V E Q I G Y P V I V R P A F T L G G T G G G I	181
TGCACGAACGAAGAGCTTTGTCGAGTTTGTGCAACCGCGCTGAAGCTGATGTCGCGTGCACCAATGCTTGTGAACGAGCATCGCGGCTCAAGGAGATTGAATACGAAGTGTG	4320
C T N E E E L V E I V S T G L K L S P V H Q C L L E R S I A G Y K E I E Y E V M	221
CGCGACGCAACGAACCGGATTTGTCGTCGAACGAAACATCGATCGGCTCGGATTCATACGGGTGATCGATCGTCTCGCGCGGACGAGCATTTAAGCGACCGCGAGTAC	4440
R D A N I D N A I V L C N M E N P D F V L K G D S I V V A P S Q T L S D R E Y	261
CAGCTGCTCCGCAACCGCTGCTTAAGATCATCGCGCGCTTGGCATCGAGCGCGCTGCAACGTCGACGCTCGCGCTTGAACCGGACGCTTCCGCTATTCATGAAATGAAACCGG	4560
Q L L R N A S L K I I R A L G I E G G C N V Q L A L D P D S F R Y Y V I E V N P	301
CGCGTCAAGCGCTGCTCGCGCTTGGCGTCAAGAGCAACCGGATTCGATCGCAACTCGCGCGGCAAAATCGCGTCCGCTGACCGCTTGAATGAATGATCAACCGGTTACCGGAAA	4680
R V S R S S A L A S K A T G Y P I A K L A A K I A V G L T L D E M I N P V T G K	341
ACGTAACGCTTCGAGCGCGCTGCACTATGTCGTCGAACAAATTCGCGCTTTCGCTTGAACAAATTTGAATCGCGCAACCGCGCGCTTGGCAACGAATGAAGCGACCGCGGAA	4800
T Y A C F E P A L D Y V V T K I P R F P F T K F E S A N R R L G T Q M K A L E Y	381
GTGATGGCGATCGCGCGGAGCTTGAAGATCACTGTAAAGCGCTTGGTTCGCTCGAGATCGGGGTGATCATCTGGAATTGAACGAGCGGCAAAACCGCGCGGACGATGATGGA	4920
V M A I G R T F E E S L L K A V R S L E I G V H H L E L N E A K T A A D D V M E	421
AAACGATTCGCGCAAGCGCGCTGCAACCGCTCTTTACATCGCGAGCGCGCTCGCGCGCGGCTGACGCTGCAACGCTGCAATGCAAGCGCAATCGACCGCTTTTCCTGCAATAG	5040
K R I R K A G D E R L F Y I A E A L R R G V T V E T L H E W S Q I D R F F L H K	461
ATTCAAAACATTTAGATGCAACCGCTTGTAAACCAACCGCGCGGACCTTGAAGCTGTAAACCAACCGGAGCGGCTCGGCTTCTCGATCGCGCGGATAGCGCGCTTATGCAACAA	5160
I Q N I I E M A I T V L C N M E N P D L D V L K K A K G L G F S D A A I A G L W N K	501
ACGGAACGCGCAATTTACCGCTGCGCGCGCAACGAGGATCATCGCGGTGTAACAAATGTCGATACGTCGCGGCAAGTTCAGTTCGGAACCGCGTACTACTACGACGATCGAG	5280
T E R D I Y A V R R Q R G I M P V Y K M V D T C A A E F T S E T P Y Y Y S T Y E	541
GAAAGAAACGAATCGATCGTGCGGAAACCGAGCGTCACTGCTGCTTGGCTCGCGCGGATCGCGATCGCGCAAGGATCGAATTTGACTATGCGACCGTCCATTGCGTCTGGCGATT	5400
E E N E S I V T E K P S V I V L G S G P I R I G Q G I E F D Y A T G V C W A I	581
AAGCAACCGCGCTTACGCGGATCATCAACCAACCGGAAACGCTCGACCGGATTCAGCACTCGCGCAAACTGATTTGAGCGCGCTGATCGCGCAAGCGGATGATGATGTC	5520
K Q A G Y E A I I I N N P E A T I V S T D F S T D K L Y F E P L T A E D V M H V	621
ATCGACCTCGACGAGCGATCGCGCTCATCGTCCAAATTCGCGGCTCAGACGGGCTCAACTTGGCGCGGAACTCGAGCGCGCGGCTCGCGTCTGCTTGGGCAACCGCTTGAAGATTG	5640
I D L E Q P I G V I V Q F G G Q T A I N L A A E L E A R G V R L L G T T L E D L	661
GAACGCGCGGAGACCGCGCAAAATTTGAACAGCGCTCTCGGAACTCGGATTCGCAACCGCGCGGCAAAACCGCGCTTTCGCTGCAAGAGCGGTGCGCATCGCTGAAGAAATCGG	5760
D R A E D K F E Q A L S E L G I P K T A V S V E E A V A I A G L W N K	701
TACCGGCTGCTCGTCTGCTTCTGTAAGTCTGCTCGCGCGGCGGAGTGAATTCGTGTAACATCGCGCGGAGCTGCTTCAATACATGGAACACCGCGTGGCGCTCAATCAACGACCCA	5880
Y P V L V R P S Y V L G G R A M E I V Y N R G E L L H Y M E N A V R V N P Q H P	741
GTGCTTGTGACCGCTACATCACTGGCAAGAGTGAAGTCGATGCCATCGCGGACGGGAGAGCGGTGTCATCCCGGATCATGGAACATATCGAGCGCGCGCGCTCCATTCCGCGC	6000
V L V D R Y I T G K E V E V D A I A D G E T V V I P G I M E H I E R A G V H S G	781
GATTGATTCGCGCTTACCGCGGCAACATTAAGCGCGGAACTGATCGCAAGATTCGCGGATTCACGATCGCGACTCGCGCGCGGCTGCAATTTGCGGCTGTTGAACATTGAGTTT	6120
D S I A I V P P O T L S A E I D I A E I T I R L A R G L H I V L N I Q F	821
GTGCTCTCGGCAACGATGTCTACGCTTGGAGTGAACCGCGCTCAAGCGGCAACGTCGCGCTTTTAAACAAATTCACCGCGTGGCGATGGCGAATCTCGCGCAAGAGCCATTTTA	6240
V V S G S D V Y V L E V N P R S S R T V P F L S K I T G V P M A N L A T K A I L	861
GGGACGAAGCTCGCGGAGTGGGCTATGAGCGCGGCTCGCGCGTGGCGGCTGACGTAAGATCGCGGTGTTCTCGTTTGGCAATTCGCGCAAGTGCACATTCGCTCGGC	6360
G T K L A E M G Y E T G V C P V R P G V Y V K V P V F S F A K L R N V D I S L G	901
CCGGAATGAATCGACCGCGAAGTATCGCGCAAGAGCTGACGTTTGAAGAGCGCTTGAAGAGCGGCTTGTGCGCTCGGCAATCCATATCCAGCGCATGGCGCGGCTGCTGTTGACG	6480
P E M K S T G E V I G K D V T F E K A L Y K G L V A S G I H I Q P H G A V L L T	941
GTGGCGCAAAAGCAAGAGAGCGGTGAGCTGCGCGCGGCTTTCGCGCAACGCGGATTCACGCTACGAGCTTGGCGCAAGCGGCAAGCGGATTTGAAGCGCGCGGCTTCCGCTG	6600
V A D K D K E E A V E L A R R F A D I G Y Q L L A T N G T A E T L K A A G I P V	981
ACGGTCTCAATAAATTCGCTCGCGCTACCGCAACATTTGATGTCATCGCGCAAGGAGCGGCAAGTCTGATCAACGCTGACGAAGGAAAGCAGCGCGGAAAGCGGCGGCTT	6720
T V V N K I H S A S P N I L D V I R O G K A Q V V I N T L T K G K Q P E S D G F	1021
RBS	
CCGATCCGCGCGGAGCGGTCGAAAAACGCGATCCCGTCTTGAAGTCTCGCTCGATACCGCGCGGCGATGCTGCAAGTCTCGAATCGATGACGTTTCAACGACCGCGGATGACGAGCGG	6840
R I R R E A V E N G I P C L T S L D T A R A M L Q V L E S M T F S T T A M T E G	1061
Orf2 ---->	
CTGGTGGGTCATGATCGCGCGGAGCGATGACGGTGCAGCGGACGCGGTCATCGCGAGCGGACGATGAATGACGCTTTCAGGCGCGCTGTCGACAGATCGCGCAACCGGCGC	6960
L V R S *	1065
M I G R E R M T V A S Q R L I A E R T Y E L T L S G R L V Q E M R Q P G	36
AGTTGCTTCAATGAAGTGGCGGATCGCGGATCCGCTCTGCGCGCGCGGCTCAGCGTTTGCATATCGACCAAGAGCGGCAATGCAAGTATTTACCGCGCAAGAGGAGG	7080
Q F V H V K V A A S A D P L L R R P L S L C H I D H K Q G Q C T I I Y R Q E G K	76

[illegible]

AACCATTCGCATTCTCCATTTCACTCAATATTTTACTCAACCTGCGGATTTCCCTGCTGGCAATCAAGATTGGAAACCGTCCGCCGCTTTCGATTCAACCTTCCAAGCCAATTGATAAA 10680
 ATCGACATATCCGCTGTCTTCCGCGCAATTACACGGGACGGTATGCTTTGACGCGATCCGGCTCATGGAGGAACCGTGTGACGAAJAAJCAJATAAGATGCCAGCGGAAATGATGTC 10800
 ACGGAGAACTGGATGAAGAGCTATGTGCGGAAJAAJATTATGATGCCGTAGAACTTGTAAAGCGAGTACGATAAGAAJAAJCAAGAAAGATGACACCTATGATTGAAGAAJGA 10920
 TGACTCTAGAT 10932

Fig. 2. Nucleotide sequence of the *B. caldolyticus* pyrimidine biosynthesis gene-cluster. Below the sequence the deduced amino acid sequence is given in the one-letter code. Start and stop codons of the coding regions as well as putative ribosomal binding sites are underlined. Broken arrows above the sequence indicate regions of dyad symmetry (SS1 to SS6). The positions of the PCR primers employed are indicated by dotted underlines.

Table 2. *B. caldolyticus* *pyr* gene-enzyme correlations

Genes are listed in their order on the chromosome in the direction of transcription. All data are based on amino acid sequences deduced from the DNA sequences.

Gene	Enzyme	Amino acids	M_r	Amino acid identity (%) with corresponding gene products from:*	
				<i>B. subtilis</i>	<i>E. coli</i>
<i>pyrB</i>	Aspartate transcarbamylase	308	34871	61 (304)	31 (310)
<i>pyrC</i>	Dihydroorotase	427	46047	65 (428)	21 (348)
<i>pyrAa</i>	Carbamoylphosphate synthetase (glutaminase subunit)	364	40289	73 (364)	42 (380)
<i>pyrAb</i>	Carbamoylphosphate synthetase (catalytic subunit)	1065	116482	78 (1071)	49 (1072)
<i>orf2</i>	Unknown function	257	27641	57 (256)	
<i>pyrD</i>	Dihydroorotate dehydrogenase	313	32857	76 (311)	17 (336)
<i>pyrF</i>	OMP decarboxylase	244	26547	49 (239)	31 (245)
<i>pyrE</i>	Orotate phosphoribosyltransferase	206	22401	58 (210)	16 (238)

* Numbers in parentheses indicate number of amino acids in the polypeptide.

Table 3. DNA base composition of pyrimidine biosynthesis genes from *B. caldolyticus* and *B. subtilis*

Organism	Mol % G + C at each codon position				Mol % G + C of whole genome
	1	2	3	Mean	
<i>B. caldolyticus</i>	62	42	73	59	53.4†
<i>B. subtilis</i> *	59	38	45	47	42.9§

* From Quinn *et al.* (1991).

† From De Bartolomeo *et al.* (1991).

§ From Fahmy *et al.* (1985).

DISCUSSION

The present paper describes the cloning and structural characterization of the *pyr* genes, encoding the six enzymes of pyrimidine biosynthesis, from the thermophile *B. caldolyticus*. Complementation studies in *E. coli* indicate

that the genes are clustered within an 11 kb region. The nucleotide sequence of the cluster reveals the presence of nine cistrons of which eight are fully sequenced. The 5' end of the first, ORF1, is not present on any of the cloned fragments. Comparison of the deduced amino acid sequences with the known Pyr proteins of *B. subtilis* and *E. coli* identifies seven of the ORFs as subunits of the six pyrimidine biosynthesis enzymes (Table 2). Furthermore, it shows that the first enzyme of the pathway, carbamoylphosphate synthetase (*pyrA*), is composed of two non-identical subunits, like the *E. coli* and *B. subtilis* enzymes. So far no function has been assigned to ORF2, and a search in the protein databases revealed that the only protein with significant similarity was ORF2 of the *B. subtilis* *pyr* operon. The overall genetic organization of the *B. caldolyticus* *pyr* region, *orf1-pyrB-pyrC-pyrAa-pyrAb-orf2-pyrD-pyrF-pyrE* (Fig. 1), is the same as in *B. subtilis* (Quinn *et al.*, 1991). The major difference is that the *pyrF* and *pyrE* cistrons are separated by 384 bp in *B. caldolyticus*, whereas they overlap with 4 bp in *B. subtilis*.

The *B. subtilis* *pyr* cluster is organized as a single

Table 4. Codon usage of pyrimidine biosynthesis genes from *B. caldolyticus* and *B. subtilis*

Bc, *B. caldolyticus*. Bs, *B. subtilis* (Quinn *et al.*, 1991).

	2nd position															
1st position	U				C				A				G			
		Bc	Bs			Bc	Bs			Bc	Bs			Bc	Bs	
U	Phe	UUU	60	61	Ser	UCU	2	32	Tyr	UAU	29	54	Cys	UGU	6	21
	Phe	UUC	44	43	Ser	UCC	20	24	Tyr	UAC	54	36	Cys	UGC	39	25
	Leu	UUA	25	57	Ser	UCA	12	48	END	UAA	4	3	END	UGA	3	4
	Leu	UUG	60	41	Ser	UCG	48	15	END	UAG	1	1	Trp	UGG	23	15
C	Leu	CUU	57	83	Pro	CCU	8	44	His	CAU	53	47	Arg	CGU	10	13
	Leu	CUC	71	37	Pro	CCC	10	7	His	CAC	32	21	Arg	CGC	114	26
	Leu	CUA	5	20	Pro	CCA	18	23	Gln	CAA	42	64	Arg	CGA	8	10
	Leu	CUG	70	60	Pro	CCG	126	72	Gln	CAG	40	47	Arg	CGG	48	21
A	Ile	AUU	58	102	Thr	ACU	4	17	Asn	AAU	20	60	Ser	AGU	2	13
	Ile	AUC	148	105	Thr	ACC	38	21	Asn	AAC	70	64	Ser	AGC	45	46
	Ile	AUA	2	9	Thr	ACA	20	100	Lys	AAA	113	146	Arg	AGA	3	49
	Met	AUG	77	78	Thr	ACG	126	52	Lys	AAG	33	54	Arg	AGG	4	7
G	Val	GUU	19	54	Ala	GCU	14	62	Asp	GAU	45	87	Gly	GGU	12	41
	Val	GUC	144	75	Ala	GCC	126	37	Asp	GAC	92	65	Gly	GGC	174	77
	Val	GUA	6	61	Ala	GCA	19	86	Glu	GAA	159	197	Gly	GGA	25	89
	Val	GUG	101	62	Ala	GCG	175	86	Glu	GAG	99	75	Gly	GGG	66	42

Fig. 3. Potential secondary structures of the intercistronic transcripts. (a) Mutually exclusive antiterminator and terminator structures of the *orf1-pyrB* intercistronic region. Bold letters represent bases overlapping in the two structures. (b) Putative ribosomal binding site for *pyrE* in the *pyrF-pyrE* intercistronic region.

Table 5. Pyrimidine biosynthesis enzyme activities in *B. caldolyticus* and *B. subtilis*

Cells were grown exponentially in minimal medium with uracil ($20 \mu\text{g ml}^{-1}$) or without uracil at 60°C for *B. caldolyticus* and at 37°C for *B. subtilis*.

Organism	Growth condition	Specific activity*		
		ATCase (<i>pyrB</i>)	OPRTase (<i>pyrE</i>)	OMP decase (<i>pyrF</i>)
<i>B. caldolyticus</i>	– Uracil	9.9	30.7	13.2
	+ Uracil	1.5	3.5	1.4
<i>B. subtilis</i>	– Uracil	86	ND	17.1
	+ Uracil	7.0	ND	2.3

* Specific activities are $\text{nmol min}^{-1} (\text{mg protein})^{-1}$. ATCase, aspartate transcarbamylase; OPRTase, orotate phosphoribosyltransferase; OMP decase, OMP decarboxylase. *B. caldolyticus* extracts were assayed at 60°C and *B. subtilis* extracts at 37°C . Assays were done in duplicate and the values represent the mean of two assays having less than 20% variance. ND, Not determined.

transcriptional unit with the promoter located upstream of the *orf1* gene (Quinn *et al.*, 1991). Two lines of evidence suggest that this is also the case in *B. caldolyticus*. Firstly, expression of the cloned *B. caldolyticus pyr* genes in *B. subtilis* is dependent on the presence of the P59 promoter located on the vector (Fig. 1b). Secondly, most of the cistrons within the cluster are overlapping. It should be noted that we have not obtained clones containing the entire *orf1* gene and the putative promoter region from any of our pUC-based libraries. This may be related to the observation that plasmids containing the *pyr* promoter and *orf1* of *B. subtilis* are unstable in *E. coli* unless the plasmid copy number is reduced (Quinn *et al.*, 1991).

Shortly downstream of the last gene in the cluster (*pyrE*) the nucleotide sequence indicates a region of hyphenated dyad symmetry followed by a series of T-residues. This may represent the transcriptional terminator of the operon.

A common feature of several *B. subtilis* biosynthetic operons, including the *trp*, *pur* and *pyr* operons, is that they contain overlapping cistrons (Ebbole & Zalkin, 1987; Henner *et al.*, 1984; Quinn *et al.*, 1991). A similar organization is observed for most of the cistrons in the *B. caldolyticus pyr* cluster. Overlapping reading frames may indicate that the genes in question are translationally coupled, i.e. the translational efficiency of one gene in an operon is determined by the translational efficiency of the adjacent upstream gene (Gold, 1988; Oppenheim & Yanofsky, 1980). The observation that transcripts of the junctions between the five overlapping gene-pairs, *pyrB/pyrC*, *pyrC/pyrAa*, *pyrAb/orf2*, *orf2/pyrD* and *pyrD/pyrF*, have the potential to form stable secondary structures sequestering the ribosomal binding regions of

the downstream genes (indicated by SS1 to SS5 on Fig. 2) suggests that translational coupling may play a role in expression of the *B. caldolyticus pyr* genes. The stability of the secondary structures (in kJ mol^{-1}) is calculated to be -228 , -163 , -128 , -85 and -208 respectively. Thus, translational initiation may be inefficient unless formation of the secondary structure on the transcript in the junction region is precluded by ribosomes translating the 3' end of the upstream gene.

The nucleotide sequence of the *pyrF-pyrE* intercistronic region does not indicate a ribosomal binding site immediately 5' of the *pyrE* start codon. However, a good Shine–Dalgarno sequence is found 66 bp upstream of the *pyrE* start codon at positions 9576 to 9585. The transcript between this putative strong ribosomal binding site and the start codon has the potential to form a stable hairpin with a ΔG of -133 kJ mol^{-1} (SS6 on Fig. 2 and Fig. 3b), which may permit the upstream Shine–Dalgarno sequence to function as an efficient ribosomal binding site for *pyrE*. A ribosomal binding site including a secondary structure has previously been found in gene 38 of the *E. coli* bacteriophage T4 (Gold, 1988; Tuerk *et al.*, 1988). It was proposed that the mRNA is positioned on the 30S subunit such that the hairpin protrudes into the solvent, and hence does not interfere with binding of the 50S subunit (Gold, 1988).

The specific activities of aspartate transcarbamylase (*pyrB*), orotate phosphoribosyltransferase (*pyrE*) and OMP decarboxylase (*pyrF*) in extracts of *B. caldolyticus* are decreased in cells grown with uracil in the medium. This indicates that expression of the *pyr* operon is negatively regulated by pyrimidines in much the same way as the *pyr* operon of *B. subtilis* (Paulus *et al.*, 1982; Table 5). Inspection of the nucleotide sequence in the intercistronic region upstream of *pyrB* shows that the transcript of that region may form two mutually exclusive stem and loop structures of which the most distal resembles a factor-independent transcriptional terminator. The proximal structure precludes the terminator hairpin from forming and may therefore act as an antiterminator (Fig. 3a). Since the homologous region of the *B. subtilis pyr* operon encodes a transcript with the potential to form similar secondary structures, we propose that these structures are involved in pyrimidine control of the *pyr* operon, and that regulation involves transcriptional termination and anti-termination by a mechanism similar to the one shown to be involved in the control of *trp* operon and *pur* operon expression in *B. subtilis* (Babitzke & Yanofsky, 1993; Kuroda *et al.*, 1988; Zalkin & Ebbole, 1988).

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