Differential turnover of the multiple processed transcripts of the *Escherichia coli* *focA-pflB* operon

R. Gary Sawerst†

Department of Molecular Microbiology, John Innes Centre, Norwich, UK

Correspondence

R. Gary Sawers
sawers@staff.uni-marburg.de

Expression of the anaerobically inducible *focA-pflB* operon of *Escherichia coli* is subject to complex transcriptional and post-transcriptional control, which generates eight transcripts whose 5' ends span ~1.2 kb. All eight transcripts have the same 3' end. The 5' ends of three of the transcripts, termed 6, 6a and 7, are located upstream of the operon. The promoters generating transcripts 6 and 7 are anaerobically regulated by FNR and ArcA ~P, while promoter 6a is constitutively active. The 5' ends of the other five transcripts are all located within the operon. Most of the 5' ends of these operon-internal transcripts result from RNA polymerase-dependent processing of the three longer primary transcripts, 6, 6a and 7. Here, it is demonstrated that subsequent to, and distinct from, these processing events, post-transcriptional modification of these transcripts also occurs through the action of the endoribonuclease RNase E. Transcripts 6 and 7 exhibit differential stability with half-lives of 1 and 5 min, respectively. Transcript 7, which has the longer half-life, is the longest transcript of the operon and has a ~340 base untranslated leader. Two of the operon-internal transcripts, 4 and 5, also have comparatively short half-lives in the wild-type, which are significantly increased in a mutant with impaired RNase E activity. A precursor-product relationship is observed between the longer transcripts 3–7 and transcripts 1 and 2. The 5' ends of transcripts 1 and 2 are closest to the *pflB* gene and have half-lives of approximately 7–8 min. The consequence of this regulation is an accumulation of full-length *pflB* transcript and comparably low levels of dicistronic transcript. This ensures different levels of synthesis of the formate transporter FocA and pyruvate formate-lyase during anaerobic growth, while maintaining coordinate regulation. Transcript analysis throughout the growth phase revealed that maximal anaerobic expression of the *focA-pflB* operon was restricted to exponentially growing cells. Expression of transcript 7 peaked in early to mid-exponential phase, while the levels of transcript 6 steadily accumulated toward the late-exponential phase of growth. Taken together, these findings indicate that although subject to common positive control by ArcA ~P and FNR, the transcripts generated by promoters 6 and 7 are subject to differential temporal and post-transcriptional regulation.

Received 28 February 2006

Revised 9 April 2006

Accepted 11 April 2006

INTRODUCTION

In prokaryotes there are a number of levels at which gene expression is regulated. Transcriptional control is the primary means of regulation; however, post-transcriptional events such as RNA turnover can also exert regulatory control (Apirion & Gegenheimer, 1984; Coburn & Mackie, 1999). Since the mean half-life of mRNA species in bacteria like *E. coli* lies between 2 and 4 min, control of mRNA turnover and degradation play key roles in influencing gene expression (Kennell, 2002; Kushner, 2002; Li & Altman, 2004; Ow *et al.*, 2002). In particular, differential

endoribonucleolytic processing and stability of polycistronic transcripts allow modulation of the cellular concentrations of gene products while maintaining coordination of product synthesis (Coburn & Mackie, 1999; Higgins & Smith, 1986; Kennell, 2002; Kushner, 2002; Newbury *et al.*, 1987). Usually these processing reactions form part of the general turnover cycle of mRNAs and regulation is a consequence of whether particular portions of transcripts are more or less susceptible to further degradation by exoribonucleases.

There are three major characterized cytoplasmic endoribonucleases in *Escherichia coli*, and these include the enzymes RNase III, RNase E and RNase G. While RNase III plays a major role in processing specific transcripts, its role in mRNA turnover is limited (Aristarkhov *et al.*, 1996; Kushner, 2002). On the other hand, RNase E and its

†Present address: Max-Planck-Institut für Terrestrische Mikrobiologie, Karl-von-Frisch-Straße, D-35043 Marburg, Germany

Abbreviations: PFL, pyruvate formate-lyase; URS, upstream regulatory sequence(s).

paralogue RNase G have major roles in mRNA turnover (Li *et al.*, 1999; Tock *et al.*, 2000; Umitsuki *et al.*, 2001; Wachi *et al.*, 1997) and both enzymes are conserved in a large number of prokaryotes (Kushner, 2002). The activity of RNases E and G is markedly affected by the presence of a 5'-end monophosphate on transcripts, and these transcripts are cleaved at a distant site (Bouvet & Belasco, 1992; Coburn & Mackie, 1999; Mackie, 1998; Tock *et al.*, 2000). The recent structural determination of the catalytic domain of RNase E (Callaghan *et al.*, 2005) has provided important insights into how the enzyme catalyses cleavage at a distance. RNase E is also associated through its C-terminal domain with a large protein complex called the 'RNA degradosome' (Carpousis, 2002); however, there is still considerable debate as to what role the 'degradosome' plays in mRNA turnover (Ow *et al.*, 2000; Kennell, 2002; Kushner, 2002). RNase E initiates turnover of many mRNA species through selective processing at specific sites. The findings of this study show that RNase E is involved in initial events during the turnover of the anaerobically inducible *focA-pflB* transcript.

Expression of the *focA-pflB* operon is remarkably complex. The operon encodes the central enzyme of fermentative metabolism, pyruvate formate-lyase (PFL), and a membrane protein, FocA, thought to transport formate (Suppmann & Sawers, 1994; Sawers & Clark, 2004). Expression of the operon is subject to redox-dependent transcriptional activation by FNR and ArcA ~ P (Sawers & Suppmann, 1992; Sawers, 1993; Drapal & Sawers, 1995; Kaiser & Sawers, 1995). Transcription of the operon is also controlled from multiple promoters. Three of these promoters are located in the regulatory region 5' of the operon, and two of these (promoters 6 and 7) are anaerobically regulated by FNR and ArcA ~ P, while the third is a weakly transcribed constitutive promoter termed 6a (Fig. 1; Sawers & Böck, 1989). The 5' ends of five further transcripts map within the operon. The

3' ends of all eight transcripts map to the end of the *pflB* gene (Sawers & Böck, 1989). Although the transcripts generated from promoters 6, 6a and 7 are the result of primary transcription events (Sawers, 1993; Kaiser & Sawers, 1997), recent studies have demonstrated that the majority of the five operon-internal transcripts result from specific processing of the longer primary transcripts (Sawers, 2005b). Surprisingly, these processing events appear to be RNA-polymerase-dependent and although it is currently uncertain what factor(s) is responsible for this processing, it is clear that it is not RNase E, RNase G or RNase III. Nevertheless, it is possible that one or more of these endonucleases is involved in the general turnover of these transcripts subsequent to their generation. Since nothing is known regarding the stage in the growth phase that *focA-pflB* operon transcription occurs and what controls the initiation of transcript turnover and degradation, this study aimed to shed light on these events. As well as identifying a role for RNase E in transcript turnover, this work also shows that transcription of the operon is restricted to the exponential phase of growth, that the anaerobically inducible promoters 6 and 7 are expressed at different stages of the growth phase and that their transcripts have different stabilities.

METHODS

Bacterial strains, plasmids and culture conditions. Bacterial strains and plasmids used in this study are listed in Table 1. Bacteria were grown routinely at 37 °C in the buffered rich medium TGYEP, pH 6.5, which included 20 mM glucose (Begg *et al.*, 1977). Where indicated, pyruvate was added to 10 mM final concentration. Aerobic cultures were grown in flasks filled maximally to one-tenth of their volume, while anaerobic cultures were grown in stoppered bottles filled with medium to the top.

Cells for RNA analysis were generally grown to mid-exponential phase ($OD_{600} = 0.4-0.5$) prior to harvest. In the case where RNA was isolated from late-stationary-phase cells, cultures were grown for 16 h prior to harvest.

Antibiotics were added to the following final concentrations ($\mu\text{g ml}^{-1}$): ampicillin, 75; chloramphenicol, 15; tetracycline, 15. Media were solidified by the inclusion of 1.5% (w/v) agar.

Analysis of RNA transcripts. Two methods were used to isolate total RNA from cells. The standard method involved use of a Qiagen RNeasy kit, according to the manufacturer's instructions. In experiments where the half-life of the *focA-pflB* transcripts was analysed, cells were grown anaerobically to an OD_{600} of 0.5 and rifampicin was added to the cultures to a final concentration of 0.2 mg ml^{-1} . To obtain the 0 min time-point, immediately prior to addition of rifampicin, a 5 ml aliquot of the culture was removed and rapidly added to 5 ml hot (65 °C) phenol (Aiba *et al.*, 1981). At appropriate time-points after rifampicin addition, a 5 ml aliquot of the culture was removed and immediately added to 5 ml hot phenol for RNA preparation. In experiments where RNA was isolated from CH1826 (*rne-1*), cells were grown to an OD_{600} of approximately 0.5 at 30 °C and then cultures were shifted to 42 °C for 30 min prior to harvesting of cells for RNA preparation or addition of rifampicin to determine the chemical half-life of transcripts. The chemical half-life determinations were performed at least twice for each strain and representative gels are shown for each experiment.

Fig. 1. Transcriptional organization of the *focA-pflB* operon. A schematic representation of the *focA-pflB* operon is shown along with an indication of the location of the 5' ends of the transcripts, shown as angled arrows, generated. Transcripts 6, 6a and 7 result from primary transcription events, while transcripts 1–5 mainly result from processing (see Sawers, 2005b). The locations of DNA probes used for S1 nuclease protection experiments and Northern blotting are shown. The probe for Northern blotting extended from the *Bam*HI site at nucleotide position 390 within the *pflB* gene to position 2380 at the end of the gene. The scale bar is representative for the whole operon.

Table 1. Strains and plasmids used in this study

Strain/plasmid	Genotype*	Reference
MC4100	F ⁻ <i>araD139</i> Δ (<i>argF-lac</i>) <i>U169 ptsF25 deoC1 relA1 flbB530 rpsL150 λ⁻</i>	Casadaban & Cohen (1979)
RM4100	Like MC4100 but with wild-type <i>fnr</i> gene regulatory region	Sawers (2005a)
CH1826	Like CH1827 but <i>rne-1</i> (Ts)	Mudd <i>et al.</i> (1990)
CH1827	<i>araD139</i> Δ (<i>araABC-leu</i>)7697 Δ (<i>lac</i>) <i>X74 galU galK hsdR rpsL150 thi zce-726::Tn10</i>	Mudd <i>et al.</i> (1990)
RM6521	Like MC4100 but <i>rnc14::Tn10</i>	Sawers (2005b)
MC4100rng	Like MC4100 but <i>rng::cat</i>	Sawers (2005b)
Plasmids		
p29	Cm ^R <i>focA</i> ⁺ <i>pflB</i> ⁺ <i>act</i> ⁺	Christiansen & Pedersen (1981)
pBR322	Ap ^R Tc ^R	Bolivar <i>et al.</i> (1977)

*Ap, Ampicillin; Cm, chloramphenicol; Tc, tetracycline.

S1 nuclease mapping of *focA-pflB* transcripts was performed according to either Sawers & Böck (1989) or Sawers (2005b) using 50 µg total RNA. The *Bam*HI DNA fragment used for hybridization was derived from plasmid p29 (Christiansen & Pedersen, 1981). Labelling of this fragment with [γ -³²P]ATP, as well as treatment of the fragment prior to S1 analysis, was carried out exactly as described previously (Sawers, 2005b).

Northern blotting of total RNA was performed according to Sawers & Böck (1989). A total of 10 µg total RNA from each sample was applied to a 1% (w/v) agarose gel containing formaldehyde. The ~2 kbp DNA probe used to detect the *pflB* transcripts is shown in Fig. 1 and was labelled by nick-translation (Sambrook *et al.*, 1989) using [α -³²P]dATP.

Other methods. Protein concentration was determined by the Lowry method. SDS-PAGE was performed using 7.5% (w/v) polyacrylamide gels according to the method of Laemmli (1970). Western blotting was carried out according to Towbin *et al.* (1979). Anti-PFL antiserum was diluted 1:10 000 and the antibody-antigen reaction was visualized using the ECL chemiluminescent method (Amersham Bioscience), exactly as recommended by the manufacturer. These experiments were repeated at least twice and representative gels are shown. Densitometric analyses were performed using SynGene Gene Tools analysis software.

RESULTS

High *focA-pflB* transcript levels in exponential-phase cells

Multiple transcripts control expression of the *focA-pflB* operon (Fig. 1). Five of these transcripts result from processing of three longer transcripts, which originate in the upstream regulatory sequences (URS) (Fig. 1). These processing events are catalysed by a currently unknown factor(s), but they appear to be distinct from RNA turnover and degradation events (Sawers, 2005b). To determine precisely when during the growth phase the *focA-pflB* operon is expressed, total RNA from cells grown aerobically and anaerobically in the exponential and stationary phase was isolated and analysed by S1 nuclease protection (Fig. 2). Analysis of RNA from aerobic cells (Fig. 2, lane 1) grown to mid-exponential phase revealed low levels of transcripts 1–5, with barely detectable levels of transcript 6A (anaerobically induced, FNR-dependent transcript 6 is not present in

aerobically grown cells: Sawers, 1993; Reyes-Ramírez & Sawers, 2006). Transcript 7 is never observed in aerobically grown cells (Sawers, 1993; Reyes-Ramírez & Sawers, 2006). Supplementation of the growth medium with pyruvate, which was shown previously to activate *pflB-lacZ* expression (Sawers & Böck, 1989), had only a marginal effect in slightly increasing the levels of transcript 6A (Fig. 2, lane 2). RNA isolated from cells grown aerobically with glucose into late stationary phase had a slightly altered transcript pattern where the level of transcript 6 or 6a (this gel system does not distinguish these transcripts) increased approximately threefold relative to the level in exponential-phase cells (Fig. 2, lane 3). This is probably due to increased transcription from promoter 6, as no such increase in transcript level was observed when glucose was omitted from the growth medium (see lane 4) and transcript 6a has been shown to be constitutively transcribed (Kaiser & Sawers, 1997).

Notably, the level of transcript 5 in aerobic stationary-phase cells grown on glucose also increased relative to the level in exponentially growing cells, while the levels of transcripts 1 and 2 were clearly reduced. This might suggest that the processing activity responsible for cleavage of the transcripts originating in the URS is maximal in cells growing in the exponential phase (see also below).

Anaerobic growth to exponential phase showed substantial induction of transcript levels (Fig. 2, lanes 5 and 6), which is consistent with previous observations (Sawers & Böck, 1989). While the levels of all transcripts increased coordinately relative to levels during aerobic growth, transcript 7 was only detectable under anaerobic conditions. Pyruvate had no discernible effect on the overall transcript pattern (Fig. 2, lane 6).

In cells grown anaerobically to late stationary phase, transcripts 4, 5, 6 and 7 were not detectable and transcripts 1, 2 and 3 were dramatically reduced in level compared with exponentially grown cells. This finding indicates that *focA-pflB* operon transcription is virtually non-existent in late stationary phase. This result contrasts with what was

Fig. 2. S1 nuclease protection analysis of *focA-pflB* transcripts generated after growth of wild-type *E. coli* to mid-exponential and late stationary phase in the presence and absence of oxygen. Total RNA was isolated from strains grown in TGYEP medium and hybridized with radioactively labelled DNA as described in Methods. Lanes: S, radioactively labelled DNA size standards generated by digestion of pBR322 with *Hin*III; 1, S1 nuclease analysis of total RNA isolated from cells grown aerobically to mid-exponential phase ($OD_{600}=0.4-0.5$) of growth; 2, as lane 1 but the growth medium was supplemented with 10 mM pyruvate; 3, as lane 1 but RNA was isolated from cells grown to late stationary phase (16 h growth); 4, as lane 3 but without addition of glucose or pyruvate; 5, RNA isolated from cells grown anaerobically in TGYEP medium to mid-exponential phase of growth; 6, as lane 5 but the growth medium was supplemented with 10 mM pyruvate; 7, as lane 5 but RNA was isolated from cells grown to late stationary phase (16 h growth). The location of the 5' ends of the transcripts is indicated on the right. Samples were separated in a denaturing 4% (w/v) polyacrylamide gel. Note that the transcripts generated from promoters 6 and 6A are not separated in this gel system and are thus collectively referred to as transcript 6.

observed in aerobically grown cells and suggests that transcription initiation was reduced under these conditions in anaerobic cells and/or that the transcripts were degraded.

To determine more precisely in the growth phase when the *focA-pflB* transcript levels started to decrease during anaerobiosis, RNA was prepared from cells throughout growth, from early exponential to early stationary phase (Fig. 3). S1 nuclease protection analysis revealed that even in cells that

were emerging from the lag phase (Fig. 3b, lane 1), anaerobic *focA-pflB* transcription was activated. Moreover, the pattern of transcripts 1–6 was similar until late-exponential phase (Fig. 3b, lane 5), indicating that there was no difference in the level of co-transcriptional processing. Interestingly, expression of transcript 7 peaked at early to mid-exponential phase (Fig. 3b, lanes 2 and 3). On the other hand, the intensity of transcript 6, as determined by densitometric analysis, peaked at late exponential phase (Fig. 3b, lane 5). As soon as cells entered stationary phase, transcript 7 was no longer detectable and the levels of transcripts 4, 5 and 6 were substantially reduced (Fig. 3b, lane 6). This suggested either that transcription of the operon was reduced or that turnover of the transcripts was significantly increased, or that both of these events contributed to reduced levels of the transcripts.

Since the S1 nuclease protection analyses undertaken here only provide information about the 5' ends of transcripts, it was important to examine the levels of full-length *pflB* transcripts during the growth phase. The same RNA samples were analysed in a Northern blot experiment (Fig. 3c). The level of the full-length *pflB* transcript was clearly reduced in stationary phase in accord with what was observed in the S1 nuclease protection experiment.

A Western blot using anti-PFL antiserum revealed that the level of PFL polypeptide was approximately fivefold lower in the lag phase (Fig. 3d, lane 1) compared with the maximal level observed in late exponential phase (Fig. 3d, lane 5). The level of PFL polypeptide began to decrease in early stationary phase, but was still present at significant levels, suggesting that the half-life of the protein is long. Notably, the Western blot revealed a protein doublet, the lower band of which, termed PFL', is characteristic of radical-bearing enzyme that has undergone oxygenolytic cleavage (Knappe & Sawers, 1990; Wagner *et al.*, 1992) and indicates that the polypeptide in early stationary phase was biologically active.

Differential half-lives of *focA-pflB* transcript 5' ends

The half-lives of the 5' ends of seven of the eight *focA-pflB* transcripts were determined using rifampicin to inhibit DNA-dependent RNA polymerase transcription initiation (see Methods) and S1 nuclease protection to analyse the complete set of transcripts (Fig. 4). RNA was analysed from cultures grown to the mid-exponential phase, equivalent to an OD_{600} of ~ 0.5 (see sample 2 in Fig. 3a). The transcripts exhibited different turnover profiles in response to rifampicin treatment. Whilst transcript 6 was rapidly degraded with a half-life of 1 min (see Fig. 4a, b), the half-life of transcript 7 was shown reproducibly to be between 5 and 6 min. This finding further emphasizes the different regulation of these transcripts. Transcript 5 also had a similar turnover profile to that of transcript 6 and thus had a similar half-life of approximately 1 min. The remaining transcripts exhibited a precursor-product relationship, in particular transcript 1, the level of which increased during the course of

Fig. 3. The *focA-pflB* transcript levels are maximal in exponentially growing anaerobic cells of *E. coli*. (a) Graphical representation of anaerobic growth of *E. coli* in TGYEP medium. The vertical arrows indicate times when samples of cells were removed from the culture and total RNA isolated for S1 nuclease analysis. (b) S1 nuclease analysis of *focA-pflB* transcripts generated throughout the anaerobic growth phase. Lanes: S, DNA size markers (see legend to Fig. 2); P, radioactively labelled DNA probe used for the S1 analysis; 1-6, RNA samples isolated from cells harvested at points 1-6, respectively, in (a). The numbers 1-7 on the right indicate the locations of transcripts 1-7, respectively. (c). Northern analysis of full-length *pflB* transcripts. Total RNA from samples of the culture shown in (a) were separated in a denaturing agarose gel, transferred to nylon membrane and hybridized with radioactively labelled DNA from the *pflB* gene as described in Methods (see Fig. 1). Lanes: 1-6, RNA samples isolated from the culture at positions 1-6 in (a). The location and size range of the full-length *pflB* transcript are indicated. An image of the 16S and 23S rRNA species from the same gel and visualized by staining with acridine orange is shown below the Northern blot. The order of the lanes is identical to that in the upper panel. The weak bands above the main *pflB* signals represent 3.2-3.6 kb *focA-pflB* transcripts. (d) A western blot of the six culture samples used for RNA preparation and for visualizing the PFL antigen is shown. Whole-cell lysates (100 µg protein) were subjected to 7.5% (w/v) SDS-PAGE. PFL and PFL' represent full-length and oxygenolytically cleaved species of the protein, respectively. For details of the Western blotting procedure, see Methods.

Fig. 4. Determination of the half-life of the 5' ends of the *focA-pflB* transcripts. (a) S1 nuclease analysis of *focA-pflB* transcripts after the addition of rifampicin to inhibit further transcription initiation. Lanes: S, radioactive DNA size markers (see legend to Fig. 2); P, radioactively labelled DNA probe used in the S1 nuclease analyses; 0–7, labelled to indicate the time (min) after addition of rifampicin. (b) The results of densitometric analysis of selected transcripts are shown graphically. The intensity level of each transcript at time point 0 min was taken as 100% and the intensity of each transcript at subsequent time points was determined. In the interests of clarity, the data for only transcripts 3, 5, 6 and 7 are shown and are labelled T3, T5, T6 and T7, respectively. (c) Northern analysis of full-length *pflB* transcripts. Total RNA from samples of the culture shown in (a) was separated in a denaturing agarose gel, transferred to a nylon membrane and hybridized with radioactively labelled DNA from the *pflB* gene as described in Methods. Lanes are labelled with respect to the time points of RNA sample isolation from the culture at 0, 1, 2, 3, 5 and 7 min after rifampicin addition. The location and size range of the full-length *pflB* transcript are indicated. An image of the 16S and 23S rRNA species from the same gel and visualized by staining with acridine orange is shown below the Northern blot. The order of the lanes is identical to that in the upper panel. The weak bands above the main *pflB* signals represent 3.2–3.6 kb *focA-pflB* transcripts.

the experiment (Fig. 4a). These findings indicated that transcript 1 was a degradation-intermediate for at least some of the longer transcripts.

The accumulation of transcript 1 suggested that the half-life of the full-length *pflB* transcript was considerably longer than that of the full-length *focA-pflB* transcript. A Northern blot analysis of the *pflB* transcript revealed that the half-life was approximately 7–8 min, which is in agreement with the S1 nuclease protection analysis of transcripts 1, 2 and 3 (Fig. 4c).

RNase E is responsible for *focA-pflB* transcript turnover

While the RNases III, E and G have been shown not to be involved in the initial co-transcriptional processing of the *focA-pflB* transcripts (Sawers, 2005b), it was important to determine whether any of them had a role in turnover of the transcripts. The half-lives of the 5' ends of the transcripts were therefore analysed in various RNase mutant

backgrounds (Fig. 5a). Transcripts 4, 5 and 6 were no longer detectable in the wild-type, and the *rng* and *rnc* mutants, after 10 min treatment with rifampicin. Indeed, the transcript profiles were essentially identical (Fig. 5a). In contrast, in a mutant with a temperature-sensitive RNase E enzyme, transcripts 4, 5 and 6 could readily be detected after 10 min treatment with rifampicin. Densitometric analysis of the transcripts indicated that the half-lives of transcripts 5 and 6 had increased from 1 min in the wild-type to approximately 5 min in the *rne-1* mutant (data not shown). Moreover, transcripts 1 and 2 had neither diminished in level nor accumulated intermediates on the degradation pathway. This finding indicates that RNase E has an important role minimally in the turnover of the 5' ends of the *focA-pflB* transcripts.

DISCUSSION

This study has highlighted several new features of *focA-pflB* operon regulation. First, operon transcription under

Fig. 5. S1 nuclease analysis of *focA-pflB* transcript stability in mutants affected in RNA turnover. (a) Total RNA was isolated from *E. coli* strains treated for different lengths of time with rifampicin and analysed by S1 nuclease protection as described in Methods. The times (min) after rifampicin treatment prior to RNA isolation are shown above each lane and the strains in which the experiments were conducted are indicated. The horizontal bar delimits the experimental series for a particular strain. The locations of the individual transcripts in the gel are indicated on the left of the figure as 1–6. Note that in this series of experiments, S1 nuclease treatment was done by a method that does not clearly identify transcript 7. The signal at the top of the gel represents undigested radioactive DNA probe. Lane S, DNA size markers generated by digesting Φ X174 with *Hinf*I. The sizes of the respective fragments are shown on the right of the figure.

anaerobic growth conditions is restricted to exponentially growing cells; second, transcripts with different 5' ends are subject to differential turnover and degradation; third, one of the enzymes that is involved in this turnover is RNase E; fourth, the transcript derived from promoter 6 is turned over rapidly, while the longer transcript derived from promoter 7 is degraded five times more slowly; and finally, the combined processing and turnover of the multiple transcripts has the consequence that although FocA and PFL synthesis remains coordinated, comparatively higher amounts of PFL polypeptide can be produced.

One of the unusual features of the *focA-pflB* operon is the long URS, which comprises minimally 416 bp with an untranslated region of 346 bases between the 5' end of transcript 7 and the GUG translation initiation codon of the *focA* gene (Sawers & Böck, 1989). Within this URS are three promoters, the weak, constitutive P6a promoter (Kaiser & Sawers, 1997), and the anaerobically inducible P6 and P7 promoters (Sawers, 1993; Drapal & Sawers, 1995). Both P6 and P7 are regulated by FNR and ArcA ~ P, but while the upstream P7 promoter is absolutely dependent on both FNR and ArcA ~ P, the strong P6 promoter is still functional in the absence of ArcA ~ P. Until this study, this was the only regulatory distinction between these promoters. The transcription start site of the P7 promoter is far upstream of the *focA* gene and the function of this promoter in governing operon expression has been an enigma, mainly because of its location, but also because the activity of the promoter appears to be constrained (Drapal & Sawers, 1995). Here it is clearly shown that the level of the P7 transcript peaks in the early exponential phase of growth, with the level becoming reduced in the mid- to late exponential phase. In contrast,

the level of P6-generated transcript slowly increased until the late exponential phase of growth, where it peaked, and then decreased rapidly upon entry into stationary phase. Based on this experiment alone, it is not possible to state unequivocally that the temporal difference in P6 and P7 transcript levels is a consequence of a difference in transcription activation, since the absolute transcript level observed is the sum of transcription plus turnover. The results of the transcription inhibition experiment, however, revealed that transcript 7 has a comparatively long half-life of approximately 5 min, while that of transcript 6 was only of the order of 1 min. Whilst it cannot be excluded that specific transcript stability may change throughout the growth phase, this finding suggests that the differences in temporal regulation observed for transcripts 6 and 7 are through transcriptional control, indicating that these promoters are responding in their activity to different signals. Since both are regulated positively by ArcA ~ P and FNR, the difference in their regulation could be due to either promoter 7 functioning only when both ArcA ~ P and FNR are maximally active, or a further factor to which this particular promoter responds.

It is intriguing that transcript 7, which has a 346 base untranslated region, has a significantly longer half-life than transcript 6, which lies downstream of it and is within 26 bases of the *focA* gene. This difference in stability might be conferred by the additional 320 bases of AU-rich sequence at the 5' end of the P7 transcript.

The rapid degradation of transcripts 4 and 5 is also slowed markedly by the temperature-sensitive *rne-1* mutation, suggesting that they are also substrates for RNase E. At least in

the case of transcripts 1 and 2 it appears clear that they are likely intermediates in the degradation of the longer *focA-pflB* transcripts (Fig. 6) and they act as a barrier to further rapid degradation of the full-length *pflB* transcript. The factor(s) determining the comparative longevity of these transcripts is also currently unclear. Northern and Western blotting experiments indicated that the *pflB* transcripts and the PFL protein are relatively long-lived species in the growing anaerobic cell. This correlates well with the key role that PFL plays in primary metabolism during both fermentative and anaerobic respiratory growth (Sawers & Clark, 2004). The formate channel FocA is required in lower amounts and presumably the amount synthesized from the weakly expressed but long-lived dicistronic *focA-pflB* transcript is sufficient to meet the requirements of the cell to export and reimport formate (Suppmann & Sawers, 1994). Further work will be required to determine the precise level of FocA in the cell.

It is important to emphasize that RNase E is not responsible for the initial processing of the *focA-pflB* transcript, which is a co-transcriptional event and appears to be somehow associated with RNA polymerase (Sawers, 2005b), since in an *rne-1* mutant grown at the non-permissive temperature,

Fig. 6. Schematic representation of the events underlying the transcriptional control of the *focA-pflB* operon. Only the 5' end of the operon is shown. The angled arrows represent the locations of the 5' ends of the transcripts and the thickness of the arrow gives an indication of transcript intensity. The dotted horizontal arrow in the top panel indicates transcriptional orientation and the fact that transcription from the upstream regulatory sequence is required for the appearance of the downstream, processed transcripts 1–5. The dotted, angled arrows indicate transcript ends particularly susceptible to degradation by RNase E.

the characteristic pattern of eight transcripts (including transcript 6a) is observed. Nevertheless, it appears that RNase E contributes to the production of the comparatively stable transcripts 1 and 2, since their abundance in the *rne-1* mutant was significantly reduced compared with the wild-type. It will be of interest to determine whether it functions alone or as part of the 'degradosome' in its capacity to turnover the *focA-pflB* transcript.

ACKNOWLEDGEMENTS

Dr Roger Harris is thanked for his help with the densitometric analyses. This work was supported by a competitive strategic grant to the John Innes Centre from the Biotechnology and Biological Sciences Research Council.

REFERENCES

- Aiba, H., Adhya, S. & de Crombrughe, B. (1981). Evidence for two functional *gal* promoters in intact *Escherichia coli* cells. *J Biol Chem* **256**, 11905–11910.
- Apirion, D. & Gegenheimer, P. (1984). Molecular biology of RNA processing in prokaryotic cells. In *Processing of RNA*, pp. 36–52. Edited by D. Apirion. Boca Raton, FL: CRC Press.
- Aristarkhov, A., Mikulskis, A., Belasco, J. G. & Lin, E. C. C. (1996). Translation of the *adhE* transcript to produce ethanol dehydrogenase requires RNaseIII cleavage in *Escherichia coli*. *J Bacteriol* **178**, 4327–4332.
- Begg, Y. A., Whyte, J. N. & Haddock, B. A. (1977). The identification of mutants of *Escherichia coli* deficient in formate dehydrogenase and nitrate reductase activities using dye indicator plates. *FEMS Microbiol Lett* **2**, 47–50.
- Bolivar, F., Rodriguez, R. L., Greene, P. J., Betlach, M. C., Heynecker, H. L., Boyer, H. W., Crosa, J. H. & Falkow, S. (1977). Cloning and characterisation of new cloning vehicles. II A multipurpose system. *Gene* **2**, 95–113.
- Bouvet, P. & Belasco, J. G. (1992). Control of RNase E-mediated RNA degradation by 5'-terminal base pairing in *E. coli*. *Nature* **360**, 488–492.
- Callaghan, A. J., Marcaida, M. J., Stead, J. A., McDowall, K. J., Scott, W. G. & Luisi, B. F. (2005). Structure of *Escherichia coli* RNase E catalytic domain and implications for RNA turnover. *Nature* **437**, 1187–1191.
- Carpousis, A. J. (2002). The *Escherichia coli* RNA degradosome: structure, function and relationship in other ribonucleolytic multi-enzyme complexes. *Biochem Soc Trans* **30**, 150–155.
- Casadaban, M. J. & Cohen, S. N. (1979). Lactose genes fused to exogenous promoters in one step using Mu-lac bacteriophage: *in vivo* probe for transcriptional control sequences. *Proc Natl Acad Sci U S A* **76**, 4530–4533.
- Christiansen, L. & Pedersen, S. (1981). Cloning, restriction endonuclease mapping and post-transcriptional regulation of *rpsA*, the structural gene for ribosomal protein S1. *Mol Gen Genet* **181**, 548–551.
- Coburn, G. A. & Mackie, G. A. (1999). Degradation of mRNA in *Escherichia coli*: an old problem with some new twists. *Prog Nucleic Acid Res Mol Biol* **62**, 55–108.
- Drapal, N. & Sawers, G. (1995). Promoter 7 of the *Escherichia coli pfl* operon is a major determinant in the anaerobic regulation of expression by ArcA. *J Bacteriol* **177**, 5338–5341.

- Higgins, C. F. & Smith, N. H. (1986). Messenger RNA processing, degradation and the control of gene expression. In Regulation of Gene Expression – 25 Years On (Society for General Microbiology Symposium no. 39), pp. 179–198. Edited by I. R. Booth & C. F. Higgins. Cambridge: Cambridge University Press.
- Kaiser, M. & Sowers, G. (1995). FNR activates transcription from the *P*₆ promoter of the *pfl* operon *in vitro*. *Mol Microbiol* **18**, 331–342.
- Kaiser, M. & Sowers, G. (1997). Overlapping promoters modulate Fnr- and ArcA-dependent anaerobic transcriptional activation of the *focApfl* operon in *Escherichia coli*. *Microbiology* **143**, 775–783.
- Kennell, D. (2002). Processing endoribonucleases and mRNA degradation in bacteria. *J Bacteriol* **184**, 4645–4657.
- Knappe, J. & Sowers, G. (1990). A radical route to acetyl-CoA: the anaerobically induced pyruvate formate-lyase system of *Escherichia coli*. *FEMS Microbiol Rev* **75**, 383–398.
- Kushner, S. R. (2002). mRNA decay in *Escherichia coli* comes of age. *J Bacteriol* **184**, 4658–4665.
- Laemmli, U. K. (1970). Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
- Li, Y. & Altman, S. (2004). Polarity effects in the lactose operon of *Escherichia coli*. *J Mol Biol* **339**, 31–39.
- Li, Z., Pandit, S. & Deutscher, M. P. (1999). RNase G (CafA protein) and RNase E are both required for the 5' maturation of 16S ribosomal RNA. *EMBO J* **18**, 2878–2885.
- Mackie, G. A. (1998). Ribonuclease E is a 5'-end-dependent endonuclease. *Nature* **395**, 720–723.
- Mudd, E. A., Krisch, H. M. & Higgins, C. F. (1990). RNase E, an endoribonuclease, has a general role in the chemical decay of *Escherichia coli* mRNA: evidence that *rne* and *ams* are the same genetic locus. *Mol Microbiol* **4**, 2127–2135.
- Newbury, S. F., Smith, N. H. & Higgins, C. F. (1987). Differential mRNA stability controls relative gene expression within a polycistronic operon. *Cell* **51**, 1131–1143.
- Ow, M. C., Liu, Q. & Kushner, S. R. (2000). Analysis of mRNA decay and rRNA processing in *Escherichia coli* in the absence of RNase E-based degradosome assembly. *Mol Microbiol* **38**, 854–866.
- Ow, M. C., Liu, Q., Mohanty, B. K., Andrew, M. E., Maples, V. F. & Kushner, S. R. (2002). RNase E levels in *Escherichia coli* are controlled by a complex regulatory system that involves transcription of the *rne* gene from three promoters. *Mol Microbiol* **43**, 159–171.
- Reyes-Ramírez, F. & Sowers, R. G. (2006). Aerobic activation of the anaerobically inducible *Escherichia coli* *focA-pfl* operon transcription by FNR. *FEMS Microbiol Lett* **255**, 262–267.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Sawers, G. (1993). Specific transcriptional requirements for positive regulation of the anaerobically inducible *pfl* operon by ArcA and FNR. *Mol Microbiol* **10**, 737–747.
- Sawers, R. G. (2005a). Expression of *fnr* is constrained by an upstream IS5 insertion in certain *Escherichia coli* K-12 strains. *J Bacteriol* **187**, 2609–2617.
- Sawers, R. G. (2005b). Evidence for novel processing of the anaerobically inducible dicistronic *focA-pfl* mRNA transcript in *Escherichia coli*. *Mol Microbiol* **58**, 1441–1453.
- Sawers, G. & Böck, A. (1989). Novel transcriptional control of the pyruvate formate-lyase gene: upstream regulatory sequences and multiple promoters regulate anaerobic expression. *J Bacteriol* **171**, 2485–2498.
- Sawers, G. & Suppmann, B. (1992). Anaerobic induction of pyruvate formate-lyase gene expression is mediated by the ArcA and FNR proteins. *J Bacteriol* **174**, 3474–3478.
- Sawers, R. G. & Clark, D. P. (2004). Fermentative pyruvate and acetyl CoA metabolism. In *EcoSal – Escherichia coli and Salmonella Cellular and Molecular Biology*, chapter 3.5.3; www.ecosal.org (posted July 2004). Editor-in-Chief, R. Curtiss, III. Washington, DC: American Society for Microbiology.
- Suppmann, B. & Sawers, G. (1994). Isolation and characterisation of hypophosphite-resistant mutants of *Escherichia coli*: identification of the FocA protein, encoded by the *pfl* operon, as a putative formate transporter. *Mol Microbiol* **11**, 965–982.
- Tock, M. R., Walsh, A. P., Carroll, G. & McDowall, K. J. (2000). The CafA protein required for the 5'-maturation of 16S rRNA is a 5'-end-dependent ribonuclease that has context-dependent broad sequence specificity. *J Biol Chem* **275**, 8726–8732.
- Towbin, H., Staehelin, T. & Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci U S A* **76**, 4350–4354.
- Umitsuki, G., Wachi, M., Takada, A., Hikichi, T. & Nagai, K. (2001). Involvement of RNase G in *in vivo* mRNA metabolism in *Escherichia coli*. *Genes Cells* **6**, 403–410.
- Wachi, M., Umitsuki, G. & Nagai, K. (1997). Functional relationship between *Escherichia coli* RNase E and the CafA protein. *Mol Gen Genet* **253**, 515–519.
- Wagner, A. F. V., Frey, M., Neugebauer, F. A., Schäfer, W. & Knappe, J. (1992). The free-radical in pyruvate formate-lyase is located on glycine-734. *Proc Natl Acad Sci U S A* **89**, 996–1000.