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ORIGINAL PAPER

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Sequences affecting the regulation of solvent production in *Clostridium acetobutylicum*

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Abstract The high solvent phenotype of *Clostridium acetobutylicum* mutants B and H was complemented by the introduction of a plasmid that contains either an intact or partially-deleted copy of *solR*, restoring acetone and butanol production to wild-type levels. This demonstrates that the *solR* open reading frame on pSOLThi is not required to restore solvent levels. The promoter region upstream of alcohol dehydrogense E (*adhE*) was examined in efforts to identify sites that play major roles in the control of expression. A series of *adhE* promoter fragments was constructed and the expression of each in acid- and solvent-phases of growth was analyzed using a chloramphenicol acetyl-transferase reporter system. Our results show that a region beyond the 0A box is needed for full induction of the promoter. Additionally, we show that the presence of sequences around a possible processing site designated S2 may have a negative role in the regulation of *adhE* expression.

Keywords Clostridium · Solvent · *Sol* operon · CAT reporter · Alcohol dehydrogenase E

Introduction

The acetone- and butanol-producing capabilities of several strains of *Clostridium* have been known for over 80 years. Until the 1950s, solventogenic clostridia were used as the major industrial producers of acetone and butanol [13]. However the boom in the petrochemical industry during the 1950s and 1960s caused a massive reduction in the usage of clostridial fermentation for solvent production in most countries, as crude oil-derived acetone and butanol could be produced more cheaply and efficiently [21].

Current concerns regarding the depletion of the world's crude oil reserves, as well as an increasing desire for more environmentally friendly industrial practices, have led to the suggestion that solventogenic clostridia could be reinstated as industrial-scale solvent producers. If strains of clostridia could be genetically manipulated to increase solvent production from inexpensive carbon sources then clostridia may become a realistic, economic means of solvent production.

In the past 20 years, the molecular biology and biochemistry behind solvent production in *Clostridium acetobutylicum* has been investigated and partially elucidated. The principal genes involved in solventogenesis (*adc*, *adhE*, *ctfA*, *ctfB*) reside on the 192-kb pSOL1 megaplasmid, downstream of the *solR* open reading frame [5, 23]. Loss of pSOL1, forming the strain designated M5, causes a decrease in solvent production [5, 22]. Butyrate kinase (*buk*) and phosphotransacetylase (*pta*) knockout mutants both produce significantly increased amounts of butanol during exponential growth [8].

The *solR* gene has also been a subject of study over the past 3 years. Mutants B and H were generated using the plasmid pO1X, bearing a segment of the *solR* gene that has recombined with the *solR* locus, found upstream of the solvent operon, forming a disrupted, nonfunctional *solR* derivative. The resulting increase in acetone and butanol production has been documented [10, 24]. *SolR* overexpression on plasmid pCO1 in ATCC 824 causes a decrease in solvent levels, and a reduction in alcohol dehydrogense E (*adhE*) mRNA, as detected by primer extension. On the basis of these results, and reported amino acid homology to helix-turn-helix DNA binding proteins, it was suggested that SolR

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is a transcription factor, having a negative regulatory effect on solvent gene (specifically *adhE*) expression [24].

However, SolR also exhibits considerable homology to *O*-glycosylating enzymes [30]. When over-expressed on plasmid pORF5H in *C. acetobutylicum* strain DSM792, the glycosylation state of several exoproteins was significantly altered, yet little effect on solvent production was observed [30]. Furthermore, it was shown that SolR can be located to the extracellular membrane of the cell. It was concluded that SolR plays a role in protein glycosylation/deglycosylation. It was also suggested that during the construction of the pCO1 vector, a region downstream of the *solR* open reading frame was incorporated that contains genetic elements to which factors essential to the transcription of *adhE* may bind. The solvent-reducing effect of pCO1 may therefore be attributable to a titration of these factors, rather than to a direct effect due to *solR* overexpression [30, 31]. Our experiments involving the complementation of mutants B and H by pSOLThi or the modified versions of pSOLThi, and the sequencing of mutant H at the site of *solR* deletion, address this question.

The SKO1 mutant does not express *spo0A*, and is defective in both solvent production and sporulation [11]. It has been shown in *Clostridium beijerinckii* that Spo0A binds to a specific DNA sequence, the 0A box, near the promoters of the phosphotransbutyrylase (*ptb*) and acetoacetate decarboxylase (*adc*) genes [26].

In *C. acetobutylicum*, a 0A box upstream of the *adhE* open reading frame has been shown to be necessary for correct gene expression, in conjunction with two additional consensus sequences designated R2 and R3 [31]. Through the promoter fragment analysis experiments described in this paper, we present data in agreement with this.

Primer extension analysis of *adhE* from previous studies determined two 5′ transcript end-points [7]. These are designated S1 (the main transcription start site), and S2, (a secondary transcription start or processing site) [7] as shown on Fig. 2. Data from the promoter fragment analysis experiments suggest the presence of a possible site affecting expression around the S2 region, approximately 82 bases upstream of the start of the *adhE* open reading frame.

Materials and methods

Bacterial strains and plasmids

The bacterial strains and plasmids used are listed in Table 1.

Growth conditions

Escherichia coli was grown in Luria Bertani (LB) medium [20] aerobically at 37°C. For recombinant strains, liquid or agar-solidified medium was appropriately supplemented with ampicillin (Ap; 100 μg/ml), erythromycin (Em; 200 μg/ml), kanamycin (Km; 50 μg/ml) or chloramphenicol (Cm; 35 μg/ml). Strains were stored at −85°C in medium with 50% glycerol.

Table 1 Bacterial strains and plasmids used in this study. *mcrA*/ΔmcrBCMethylcytosine-specific restriction system abolished, *recA1* homologous recombination abolished, *Φ3 tI*, *Φ3 t* methylase, *lacZ* β-galactosidase open reading frame, *catP* chloramphenicol acetyl transferase open reading frame, *solR* functional copy of *solR*, *solR*⁻ *solR* knockout, *solR′* truncated non-functional copy of *solR*, *MLS*^{r} macrolide lincosamide and streptogramin B resistant, *Tc*^{r} tetracycline resistant, *Cm*^{r} chloramphenicol and thiamphenicol resistant, *Km*^{r} kanamycin resistant, *Ap*^{r} ampicillin resistant

Strain or plasmid	Description	Reference/Source
Clostridium acetobutylicum		
ATCC 824	Wild-type	ATCC
Mutant B	*solR*⁻, MLS^r	[24]
Mutant H	*solR*⁻, MLS^r	[24]
Escherichia coli		
DH10B	*mcrA*, ΔmcrBC, *recA1*	New England Biolabs, Beverly, Mass., [15]
Plasmids		
pO1X		
MLS^r, *solR′*, Tc^r	[24]	
pCATP	MLS^r, *catP*	this study
pAN1	Cm^r, *Φ3 tI*	[17]
pDHKM	Km^r, *Φ3 tI*	[33]
pSC12lacZ	Cm^r,*lacZ*	[33]
pCO1	MLS^r, Ap^r, *solR*	[24]
pSOLThi	Cm^r, *solR*	This study
pSOLThiA	Cm^r, *solR′*	This study
pSOLThiD	Cm^r, *solR′*	This study

C. acetobutylicum was grown in Clostridial Growth Medium (CGM [12]) anaerobically at 37°C. For recombinant strains, liquid and agar-solidified CGM was appropriately supplemented with Em (40 μg/ml) and the Cm-alternative, thiamphenicol (Thi; 25 μg/ml). Strains were stored as horse-serum-supplemented lyophilized stocks at room temperature, or at −85°C in medium with 10% glycerol.

Construction and modification of pSOLThi

Plasmids pCO1 [24] and pSC12lacZ [33] were both digested with *Eco*RI and *Xba*I. A fragment of ~1.5 kb from pCO1 (containing the *solR* promoter, open reading frame and two terminator sites, as well as the 0A box and R1, R2 and R3 domains from the *adhE* promoter region) and a fragment of ~3.5-kb of pSC12lacZ were purified and joined together to form plasmid pSOLThi (Fig. 1A).

Plasmid pSOLThi was digested with *Nde*I and *Bbs*I, thus removing almost all the *solR* open reading frame. pSOLThi was also digested using *Nco*I and *Bbs*I, removing a fragment of ~350 bp from the N-terminal region of the *solR* reading frame. The resulting sticky ends were blunted using DNA polymerase I Klenow fragment, and joined together to form plasmids pSOLThiA and pSOLThiD, respectively.

PCR amplification of promoter fragments, and CATP plasmid construction

Eight primers were used in the amplification of fragments of the *adhE* promoter from wild-type *C. acetobutylicum*:

p1F. CCGGAATTCGACCCTGGGGTGTAACTATAG ·
p2F. CCGGAATTCTATTTATGCTCCTATAAAATT ·
p3F. CCGGAATTCTACGCCAAAATATTAGATACG ·
p4F. CCGGAATTCTTATTTACTTCATAAATTGATG ·
p5R. CGCGGATCCTCTAAAATATTTATTATATTT ·
p6R. CGCGGATCCTAGTATAGATATTATTCTTGAAAG ·
p7R. CGCGGATCCAGCAGATTTGGAGATAGATATTG ·
p8R. CGCGGATCCAATATCTATGCTTTTATTATAG

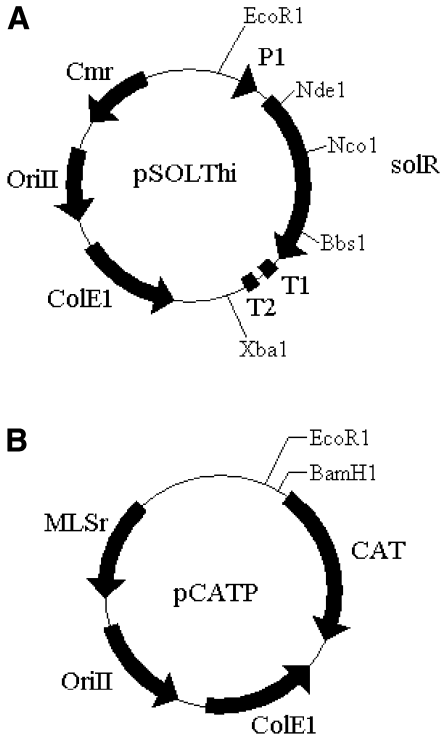


Fig. 1A, B Plasmid maps. **A** *pSOLThi* Thiamphenicol-resistant *solR* over-expression vector, detailing the *solR* promoter *P1* and two transcription terminators, *T1* and *T2*. **B** *pCATP* Reporter vector harboring a promoterless chloramphenicol acetyl transferase (*CAT*) gene. *Cmr* Chloramphenicol resistance cassette, *OriII* Gram-positive origin of replication from pIM13, *ColE1 Escherichia coli* origin of replication, *MLSr* macrolide lincosamide and streptogramin A resistance

Underlined regions correspond to restriction enzyme sites *Bam*HI (GGATCC) and *Eco*RI (GAATCC) to allow incorporation into plasmid pCATP. Figure 2 shows the location of primers p1F through p8R within the *adhE* promoter, and shows the number (1–15) allocated to the fragments amplified. All fragments were ligated into the appropriate restriction sites on pCATP (Fig. 1B) to yield plasmids pCATP1 through pCATP15.

Experimental techniques

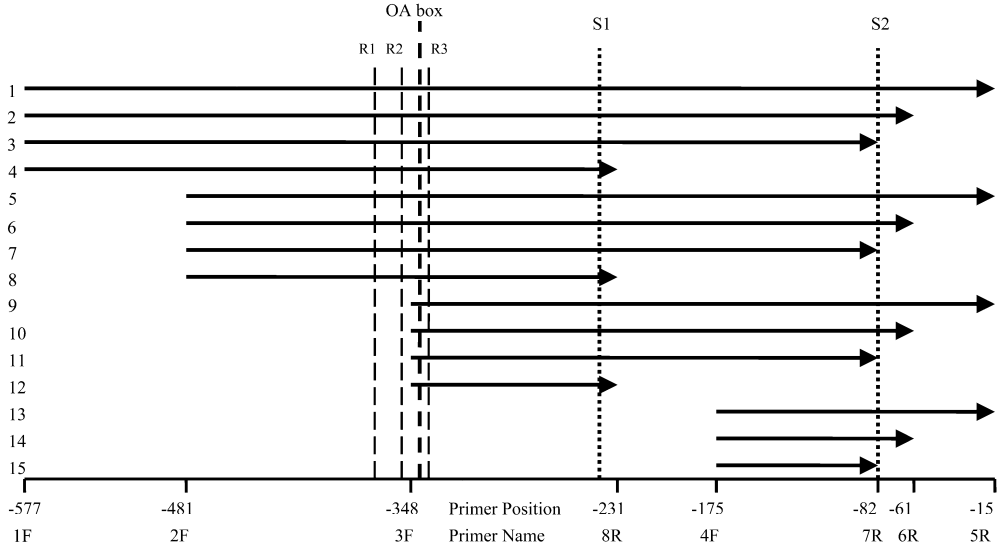
All plasmids for transformation into *C. acetobutylicum* were methylated in *E. coli* DH10α harboring plasmids pAN1 or pDHKM, as previously described [17]. *C. acetobutylicum* was electrotransformed using previously documented methods [18].

For the *solR* complementation assays, cultures were grown for 100 h in 15 ml CGM supplemented with the appropriate antibiotic. Samples (1 ml) were centrifuged at 16,000 *g* for 15 min at room temperature in a Sorvall Biofuge PICO, the supernatant fluid was collected and acidified (20 µl 50% H_2SO_4/ml supernatant). Samples (5 µl) were injected into a Hewlett Packard 5890 Series II gas chromatograph for solvent content analysis. All measurements were repeated in triplicate.

For the strains harboring the pCATP-variant plasmids, cultures were grown in 10 ml CGM supplemented with the appropriate antibiotic. Aliquots of samples were plated on antibiotic-containing and antibiotic-free media to monitor for plasmid loss. Samples were taken at mid-acidogenic and in mid-solventogenic phase, as confirmed by OD_{600} readings and solvent analysis. Aliquots (10 ml) of culture were centrifuged at 2,700 *g* for 10–12 min at 4°C. The cell pellet was resuspended in 0.5 ml MOPS buffer (50 mM MOPS, 500 mM $(NH_4)_2SO_4$, 20% glycerol v/v, pH 7.0) and centrifuged at 10,000 *g* for 1 min at room temperature. Cell pellets from mid-acidogenic stage cultures were resuspended in 0.5 ml MOPS buffer and cell pellets from mid-solventogenic stage cultures were resuspended in 1 ml MOPS buffer. All samples were sonicated for 18 min at 6°C in a W225-R sonicator (Heat systems; Ultrasonics, Farmingdale, N.Y.). Cell debris was pelleted at 10,000 *g* for 20 min at 4°C. The supernatant was extracted for chloramphenicol acetyltransferase (CAT) activity assay.

Each sample was assayed using a Cary118 spectrophotometer at 37°C, according to the method of Shaw [28]. Each sample was assayed with and without chloramphenicol to enable subtraction of background activity. CAT activity was calculated using the following formula:

Fig. 2 Scale diagram of promoter region upstream of the alcohol dehydrogense E (*adhE*) gene (GenBank accession no. L14817, positions 1,484–2,046), indicating the location of all 15 promoter fragments within this region. Primer position is numbered relative to the start codon of the *adhE* open reading frame, designated +1. Key regions within the *adhE* promoter include the 0A box with the *R1*, *R2* and *R3* flanking regions [31], the primary start site of transcription designated *S1*, and a secondary putative transcription start or processing site designated *S2* as identified by primer extension experiments [6]



$$\text{Units of CAT} = \frac{(\Delta \text{ absorbance/min} \times \text{total reaction volume } (\mu l)/13.6)}{\text{Volume of sample used } (\mu l)} \quad (1)$$

Protein content of the crude samples was assayed according to Bradford [3]. CAT expression was quantified as units of CAT per milligram protein. For each sample, five cultures were grown and analyzed.

Results

Complementation of mutants B and H with *solR*

Using plasmid pSOLThi, a functional copy of *solR* was introduced back into mutants B and H, and solvent production in these recombinant strains was analyzed by gas chromatography.

Figure 3 shows the acetone, butanol and butyrate levels after growth for 100 h in all strains. As expected, acetone and butanol levels were reduced to near zero in wild-type cells complemented with the *solR*-bearing plasmid. These results are consistent with those reported using the pCO1 vector for *solR* expression [24], but solvent levels were also reduced in wild-type strains bearing the modified pSOLThiA and pSOLThiD plasmids.

When complemented with pSOLThi, pSOLThiA or pSOLThiD, production of acetone in both mutants B and H is significantly reduced, from approximately 25 mM to 5–10 mM in both mutant strains. Similarly, butanol levels decrease from 55–60 mM to approximately 20–30 mM in mutant B and 30–40 mM in mutant H. In mutants B and H, butyrate levels decrease as elevated solvent production draws carbon from the system, but when complemented with pSOLThi, pSOLThiA or pSOLThiD, the decrease in solvent levels is reflected in the increase of butyrate due to the alteration of carbon flux through the system. The OD_{600}, and the levels of acetate and ethanol were not significantly altered between the 12 strains (data not shown).

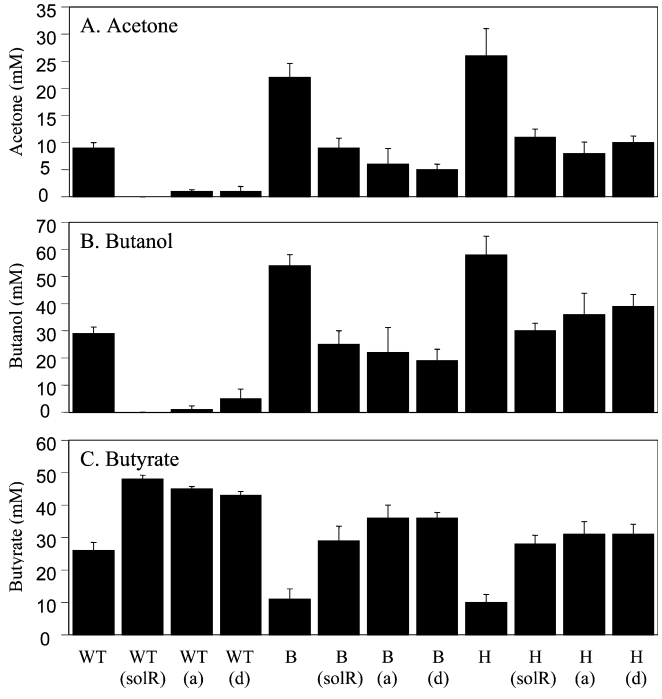


Fig. 3 Butyrate, butanol and acetone concentrations from cultures of *Clostridium acetobutylicum* strains ATCC 824, mutant B and mutant H harboring the control plasmid pSC12lacZ, the pSOLThi plasmid, or the modified pSOLThi plasmids. *WT* Wild-type *C. acetobutylicum* ATCC 824/pSC12lacZ, *WT(solR)* wild-type/pSOLThi, *WT(a)* wild-type/pSOLThiA, *WT(d)* wild-type/pSOLThiD, *B* mutant B/pSC12lacZ, *B(solR)* mutant B/pSOLThi, *B(a)* mutant B/pSOLThiA, *B(d)* mutant B/pSOLThiD, *H* mutant H/pSC12lacZ, *H(solR)* mutant H/pSOLThi, *H(a)* mutant H/pSOLThiA, *H(d)* mutant H/pSOLThiD. *Bars* +1SE

DNA sequence analysis of mutant H

Figure 4 shows the arrangement of the open reading frames within the *sol* operon on mutant H as compared to the wild-type sequence (GenBank accession no. L14817, positions 441–4,649) [25]. A region 2,891 bases upstream of the *adhE* start codon in mutant H was sequenced and analyzed to identify any potential element that may contribute to the phenotype of elevated solvent production.

As shown, far upstream of *adhE* is the divergently transcribed erythromycin-resistance cassette introduced on pO1X. The *solR* gene is lacking its promoter region, and the first 53 bases of the open reading frame. From position 494 up to the start of the *adhE* open reading frame, the sequence in mutant H is identical to that in the wild type.

These results do not reveal the mechanism for the high solvent phenotype of mutant H, but apparently rule out the inadvertent introduction of a new strong promoter element into this region. In both mutants B and H, *adhE* is significantly upregulated during solventogenesis [24]. We therefore proceeded to locate and identify the sequence essential for solvent-phase induction of the promoter.

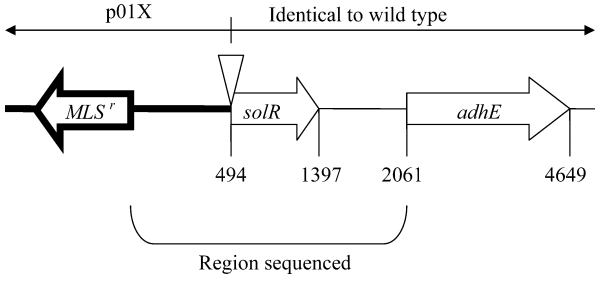


Fig. 4 Schematic representation of the region upstream of *adhE* in mutant H, illustrating the position of the insertion of plasmid p01X in *solR*. Numbers indicating base position correspond to the wild-type sequence, GenBank accession no. L14817. *MLS*^{r} Macrolide lincosamide and streptogramin B resistance

Effects of the 0A box on CAT expression

To investigate different parts of the *solR* promoter, PCR primers were designed to amplify various segments with and without the S1 and S2 sites, the 0A box and long upstream segments (Fig. 2).

To analyze the effects of the various promoter fragments on CAT expression, results from the CAT assays were pooled: fragments 1–4, 5–8, 9–12 and 13–15. The results are shown in Table 2.

There was no detectable CAT expression from constructs 13–15, suggesting that the region of the promoter from bases −175 to −15 is insufficient to allow transcription.

Constructs 9–12 showed some CAT expression during the acidogenic phase, and an increase in expression of approximately 2-fold during the solventogenic phase (Table 2). Although these constructs include the 0A box and R3 region, CAT expression was very low, less than 1 U/mg protein.

Constructs 1–4 and 5–8 exhibited similar CAT expression during acid phase (approximately 1 U/mg protein), and a 4- to 5-fold induction of CAT expression during solvent phase (Table 2). These constructs contain the 0A box and R3 region, but they also include the R1 and R2 regions, implying that all these regions are necessary for maximum gene transcription in response to Spo0A binding. These results also suggest that there are no elements between bases −577 and −481 that affect transcription, as there is little difference in CAT expression between constructs 1–4 and 5–8.

Effects of S2 on CAT expression

To investigate the effects of the promoter S2 site on gene expression, results were pooled as follows: fragments 1 and 5, 2 and 6, 3 and 7, 4 and 8 (see Fig. 2). The results for constructs 9–12 were disregarded due to low CAT expression, and 13–15 were discarded since they exhibited very little CAT expression. The results are shown in Table 2.

Table 2 CAT expression from pCATP reporter vector constructs in acidogenic and solventogenic phases. Data from constructs were combined as described in the first column, and are shown ±1SE. *CAT* Chloramphenicol acetyl transferase

Plasmid constructs	CAT expression (Units/mg protein)	
	Acidogenic phase	Solventogenic phase
pCATP control	0.01 ± 0.01	0.01 ± 0.01
1, 2, 3, 4	0.84 ± 0.20	4.96 ± 1.15
5, 6, 7, 8	1.07 ± 0.24	4.64 ± 1.03
9, 10, 11, 12	0.42 ± 0.12	0.73 ± 0.09
13, 14, 15	0.03 ± 0.01	0.01 ± 0.01
1, 5	1.37 ± 0.36	2.24 ± 0.42
2, 6	0.21 ± 0.07	2.45 ± 0.40
3, 7	1.40 ± 0.24	6.96 ± 1.48
4, 8	0.83 ± 0.1	7.56 ± 1.33

Constructs that possess the promoter S2 site (1 and 5, 2 and 6) exhibited low CAT expression in acid and solvent phase (maximum expression = 2.5 U/mg protein). Constructs that do not possess S2 (3 and 7, 4 and 8) exhibited similar CAT expression during acid phase to constructs possessing S2, but exhibited considerable induction of CAT expression during solvent phase (maximum expression = 7.56 U/mg protein). These results imply that the S2 region may have a negative effect on gene expression.

Discussion

Plasmid pSOLThi bearing either a complete or partially-deleted copy of the solR open reading frame complements mutants B and H, resulting in solvent levels returning to approximately wild-type levels. These results suggest that the data obtained using pCO1 to overexpress *solR* in wild-type *C. acetobutylicum* are not correlated with *solR* overexpression [24], and that any effect that *solR* overexpression may be having is masked by an unknown phenomenon, most likely the titering effect described by Thormann et al. [31].

There remains the question of what is occurring in mutants B and H to cause the solvent overproduction phenotype. One possibility is that during the homologous recombination event that generated the *solR* knockout, a mutation or some element was introduced into the region upstream of *adhE* causing its overexpression. DNA sequence analysis revealed no such element. The upstream region of *adhE* in mutant H is identical to that of the wild type for 1,577 bases prior to the *adhE* start codon (GenBank accession no. L14817, positions 484–2,061), and it is highly unlikely that any element further upstream would have an effect on the expression of the sol operon. Whilst an explanation for the high solvent phenotype is puzzling, it could relate to the sensitivity of expression to the conformation of the DNA in this region in vivo, which may influence the correct assembly or function of the transcriptional complex.

Results obtained from the pCATP analysis of promoter fragments from *adhE* are consistent with other studies, and also imply new ways in which the *adhE* promoter may function [6, 7, 11, 31]. These results also confirm reports that the S2 site is insufficient to initiate transcription of *adhE* [31]. These data suggest that there is no element within the 175 bases directly upstream of the *adhE* start codon that can initiate transcription.

It has been reported that for correct *adhE* expression in response to Spo0A, the 0A box in conjunction with the R1 and R3 domains is required [31]. Our results support this conclusion, as in the absence of R1 and R2, R3 and the 0A box are insufficient to elicit maximal *cat* expression. It is unlikely that there are any promoter elements more than 481 bases upstream of the *adhE* start codon, as there is no significant difference in CAT expression between constructs 1–4 and 5–8.

The results shown in Table 2 suggest that there may be a negative element within the *adhE* leader mRNA sequence, most likely associated with the S2 region. Constructs extending beyond the S2 point (1, 2, 5, 6) exhibit severely decreased CAT expression compared with those that halt at S2 (3, 7) or halt just after S1 (4, 8). These data suggest that the region between −61 and −82 plays a key role in eliciting the observed decrease in CAT expression. The similarity in CAT expression between 1 and 5, and 2 and 6, suggests that the region between bases −15 and −61 does not contribute to this effect. The same can be said for the region between bases −82 and −231. It cannot be determined whether the decrease in CAT expression is due solely to the −61 to −82 base region, or to this region in conjunction with a preceding sequence, or the junction with the reporter element of the plasmid.

Extensive secondary structure has been predicted for the untranslated 246 bases of the *adhE* mRNA [31], and it is possible that the decrease in expression is due to effects on the secondary structure of the mRNA.

The nature of S2 remains unknown. One possibility is that a terminator/antiterminator site is near S2, such as is found in the *sac*PA operon in *C. acetobutylicum* [29]. Antiterminator sequences tend to form an imperfect palindromic stem-loop structure designated the ribonucleic antiterminator (RAT), to which the antiterminator protein binds, partially overlapping a terminator region [2, 9]. Sequence analysis of the S2 region reveals considerable nucleotide homology to the *sac*PA terminator and sucrose operon RAT site from *C. acetobutylicum*. Internet-based RNA-fold analysis also predicts a stem-loop structure for this region. However, there is no known suitable candidate in *C. acetobutylicum* that may act as the antiterminator protein to bind to this region. Additionally, the *adhE* gene in other systems such as *E. coli* does not appear to be under the control of an antiterminator [1, 4, 14, 16, 19, 32].

Alternatively, the S2 segment may be required for RNA processing, and may involve the action of a secondary protein. In *E. coli*, *adhE* expression may be regulated at the transcription initiation level by Cra, and post-transcriptionally by RNase G and RNase III [1, 14, 32,]. In *E. coli*, Cra represses transcription by binding to the Cra-box, located 250 bases upstream of the start codon of *adhE* [14, 27]. RNase III and RNase G are required for cleavage of the *adhE* transcript, and are important for optimal expression of *adhE* [1, 32]. The Cra-box from *E. coli* exhibits limited homology to the S2 region although no protein has been designated as Cra in *C. acetobutylicum* [25]. Open reading frames found in *C. acetobutylicum* have been designated as RNase G/E and a double-stranded RNA-specific ribonuclease III [25], exhibiting ∼30% identity, and 55% amino acid similarity to those in *E. coli*.

It is possible that one or several of these proteins may be important in the regulation of *adhE* expression and therefore that they may affect the production of solvents within *C. acetobutylicum*. Presently they represent targets for investigation and future work directed at understanding their possible roles and potential for adjusting the genetic profile of *C. acetobutylicum* to maximize solvent production.

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