

Transcriptional Regulation of Solventogenesis in *Clostridium acetobutylicum*

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Abstract

Solvent synthesis in *Clostridium acetobutylicum* is induced in concert with sporulation to counteract the dangerous effects of produced butyric and acetic acids and to provide the cell with sufficient time to complete endospore formation. Cardinal transcription units for butanol and acetone production are the *sol* and *adc* operons encoding butyraldehyde/butanol dehydrogenase and coenzyme A transferase as well as acetoacetate decarboxylase. Induction is achieved by a decreased level of DNA supercoiling and the transcription factor Spo0A, possibly in cooperation with other regulatory proteins. A number of other operons is also turned on during this metabolic switch, whose physiological relevance, however, is only partly understood. The recent completion of *C. acetobutylicum* genome sequencing will pave the way for transcriptional profiling and thus allow comprehension of the coherent regulatory networks of solventogenesis and sporulation.

Introduction

Regulation of acetone and butanol formation in *Clostridium acetobutylicum* is embedded in a complex network, including sporulation, pH, and redox control. This obligate anaerobic bacterium degrades sugars or starch via a typical butyrate fermentation. Since high substrate concentrations are tolerated, large amounts of acetic and mainly butyric acid are formed that endanger survival by dissipation of the proton gradient over the cytoplasmic membrane. As a consequence, the organism ceases to make acids and switches to the production of neutral solvents for which even part of the excreted acids is taken up again and converted into

butanol and acetone in a ratio of app. 2 to 1 (Figure 1). Concomitantly, the formation of endospores is started which eventually will guarantee survival, since butanol at increasing concentrations of up to 2% also exerts a toxic effect. Solventogenesis thus only serves as a means to provide the cell with enough time to complete endospore production (for reviews see Jones and Woods, 1986; Dürre and Bahl, 1996; Dürre, 1998).

Biological butanol synthesis served as a major feedstock of this solvent for industrial purposes until about 1950. Then, increasing substrate prices and cheap raw oil availability gave the petrochemical industry a massive economic advantage which led to the decline of the fermentation process. The oil crisis in the 1970s provoked a renewed interest in the underlying biological processes in order to eventually establish a competitive fermentation procedure. However, only at the end of the 1980s, the first molecular biological investigations on *C. acetobutylicum* were reported (for a review see Dürre, 1998). Since then, most of the genes required for solventogenesis have been cloned and sequenced. These data were the starting point for elucidation of signals and regulatory mechanisms, which is pursued by *in vitro* and *in vivo* studies, RNA analyses, reporter gene constructions, and gene knock outs. It is the aim of this mini review to present the current status of the picture thus emerging.

Cardinal Operons for Solvent Formation

Essential genes required for solventogenesis are arranged in the *sol* and *adc* operons. They were found to be located on an app. 210-kbp megaplasmid (Cornillot and Soucaille, 1996; Cornillot *et al.*, 1997). Meanwhile, sequencing revealed this plasmid to have a size of 192 kbp (see last paragraph). Both operons represent a contiguous stretch of DNA, but with convergent direction of transcription (Figure 2).

1. *sol* Operon

This transcription unit comprises genes encoding a small peptide (OrfL), a bifunctional butyraldehyde/butanol dehydrogenase (AdhE), and the two subunits of acetoacetyl-CoA: acetate/butyrate-coenzyme A transferase (CtfA/B) (Fischer *et al.*, 1993). Northern experiments revealed that this operon is one of the first being induced at the onset of solventogenesis (Sauer and Dürre, 1995). The essential role of the *adhE* gene (or *aad*, as it was designated by an American group) in butanol synthesis was also confirmed by successful complementation of a solvent-negative mutant (Nair and Papoutsakis, 1994) and the significant decrease of butanol production upon its insertional inactivation (Green and Bennett, 1996). In contrast to the CoA

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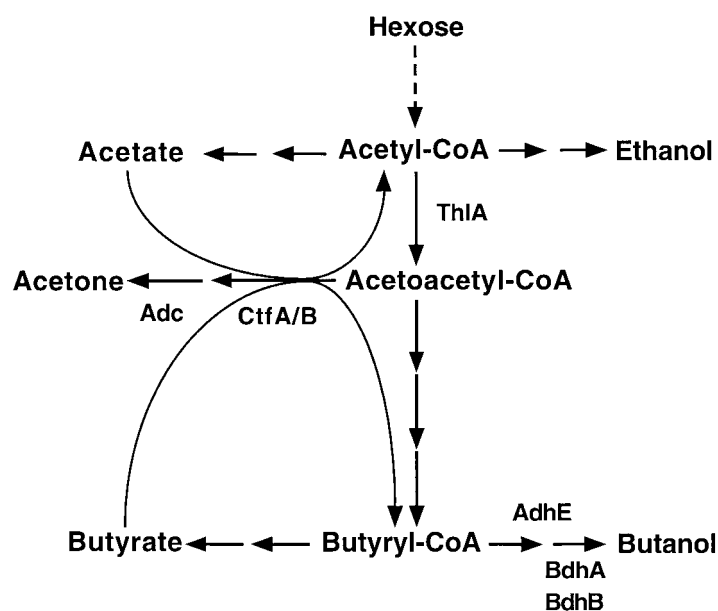


Figure 1. Catabolic pathways of acid and solvent formation in *Clostridium acetobutylicum*. Gene products discussed in the text are listed above, aside, and below the arrows indicating the catalyzed reaction(s). Adc, acetoacetate decarboxylase; AdhE, bifunctional butyraldehyde/butanol dehydrogenase; BdhA, butanol dehydrogenase; BdhB, butanol dehydrogenase; CtfA/B, acetoacetyl-CoA: acetate/butyrate-coenzyme A transferase; ThIA, thiolase.

transferase (Wiesenborn *et al.*, 1989), the AdhE enzyme has not been characterized. Using the his-tag technology, the protein could be purified, but did no longer show enzymatic activity. Two translation products are obtained from the *adhE* gene in both *C. acetobutylicum* and *Escherichia coli*: the bifunctional enzyme and separately the butanol dehydrogenase domain (Thormann *et al.*, 2002). The physiological relevance of this phenomenon is still unknown. Based on primer extension experiments, the *sol* operon was proposed to be controlled by two promoters, the distal P_1 (or S_2) and the proximal P_2 (or S_1) (Fischer *et al.*, 1993; Nair *et al.*, 1994). Signal intensities indicated that most transcripts originated from P_2 (Fischer *et al.*, 1993). However, this putative promoter structure showed 5 mismatches to the consensus in the -10 and -35 regions as well as an unusual spacing of these two boxes (Fischer *et al.*, 1993; Nair *et al.*, 1994; Dürre *et al.*, 1995). For further investigations, a reporter gene technology has been developed (Dürre *et al.*, 1995; Tummala *et al.*, 1999) that is based on the *lacZ* gene from *Thermoanaerobacterium thermosulfurigenes* (Burchhardt and Bahl, 1991). Recent experiments revealed that indeed only one promoter exists, which is P_1 . The second primer extension signal (P_2) is not due to a promoter structure, but rather represents an mRNA processing site (Thormann *et al.*, 2002).

DNA topology was found to play an important role in induction of the *sol* operon. Relaxation (i.e. less negative supercoiling), induced by inhibition of the DNA gyrase, caused an immediate increase in transcription (Ullmann *et al.*, 1996). This is in perfect accordance with the finding that DNA from *C. acetobutylicum* becomes less negatively supercoiled

at the onset of solventogenesis (Wong and Bennett, 1996). Since the environmental conditions at this time point (still excess of substrate, pH below 4.5, high concentrations of acetate and butyrate) are all known to affect DNA topology directly, it is assumed that the change in DNA supercoiling serves as a signal to induce solventogenesis (Dürre *et al.*, 1995; Dürre, 1998; Ullmann and Dürre, 1998).

Recently, it has been proposed that the *sol* operon is regulated by a transcriptional repressor (Orf5), which is encoded by the monocistronically organized gene directly upstream of *adhE* (Figure 2) (Nair *et al.*, 1999). The conclusion of the authors was based on experiments indicating that i) insertional inactivation of the *orf5* gene (then designated *solR*) yielded strains with significantly increased solvent production, ii) overexpression of this gene yielded strains with a solvent-negative phenotype, and iii) a potential helix-turn-helix (HTH) DNA-binding motif is present in the *orf5* gene product (Nair *et al.*, 1999). However, this suggestion was disproved by data showing that Orf5 i) is localized at the extracellular side of the cytoplasmic membrane, ii) is involved in glycosylation/deglycosylation reactions, and iii) contains a tetratricopeptide repeat motif for protein-protein interactions rather than a HTH-motif (Thormann and Dürre, 2001). The dominant glycosylated protein detected in the course of these experiments represents most probably flagellin, which recently was found to be post-translationally modified by a terminal sialyl residue (Lyristis *et al.*, 2000). Most strikingly, the claim that overexpression of *orf5* leads to a solvent-negative phenotype could not be reproduced. Respective constructions even produced 15% more butanol than the wild type (Thormann and Dürre, 2001).

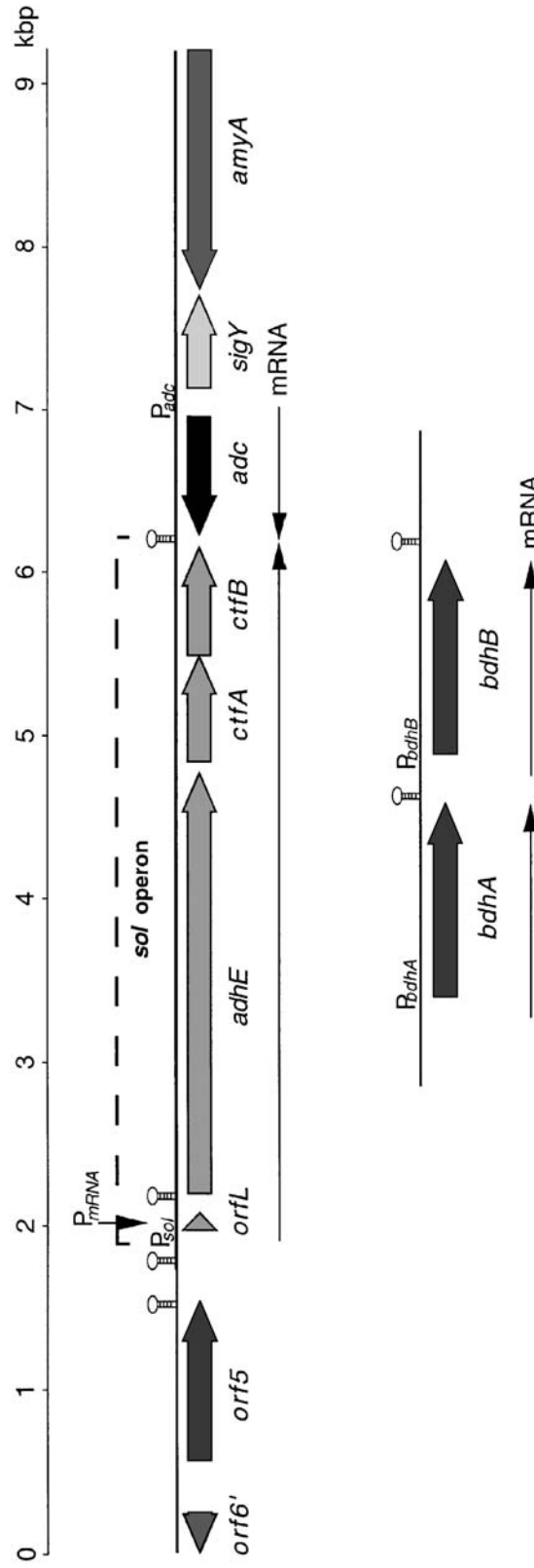


Figure 2. Schematic map of *C. acetobutylicum* DNA regions, encoding essential enzymes for solventogenesis. Arrows and arrowheads represent lengths, locations, and orientation of genes; lines with arrows indicate primary mRNA transcripts. A prime at the end of a gene indicates truncation of the respective open reading frame. Promoter positions of operons directly involved in solventogenesis are marked by P_{sol} , P_{adc} , P_{bdthA} , P_{bdthB} . P_{mRNA} indicates a putative mRNA processing site; possible stem-loop structures are indicated by hairpin symbols. Functionally characterized proteins from indicated genes: *adhE*, bifunctional butyraldehyde/butanol dehydrogenase; *ctfA/B*, acetoacetyl-CoA: acetate/butyrate-coenzyme A transferase; *adc*, acetoacetate decarboxylase; *sigY*, alternative sigma factor; *amyA*, α -amylase; *bdthA*, butanol dehydrogenase A; *bdthB*, butanol dehydrogenase B.

Further investigations revealed that the results obtained by Nair *et al.*, stemmed from erroneous subcloning of a part of the regulatory region of the *sol* operon together with the *orf5* gene. This fragment, when subcloned, yielded a solvent-negative phenotype (thus explaining the result obtained by Nair *et al.*) and was found to be the control region of the P₁ promoter of the *sol* operon (Thormann *et al.*, 2002). It contained an imperfect three times repeat and a potential binding site for the transcription factor Spo0A. Targeted mutagenesis of this box revealed that Spo0A is indeed involved in the control of the *sol* operon (Thormann *et al.*, 2002). This is in perfect agreement with a recent report that the other cardinal operon of solventogenesis (*adc*) is also regulated by Spo0A (Ravagnani *et al.*, 2000). Spo0A is not only a key regulator of sporulation, but directly or indirectly controls competence, motility, and production of peptide antibiotics and extracellular hydrolases in *Bacillus* (Strauch and Hoch, 1993). The findings that in *C. acetobutylicum* this transcription factor is involved in regulation of *sol*, *adc*, and *ptb-buk* operons (the latter encoding enzymes for butyrate formation) (Ravagnani *et al.*, 2000; Thormann *et al.*, 2002) provides the long sought-for link between solventogenesis and sporulation. It must be added, however, that the reported data do not rule out the participation of additional proteins in the transcriptional regulation.

The genome sequencing project (see last paragraph) revealed that a second *adhE* gene is present on the megaplasmid. It is monocistronically organized and does not play a role in the usual acetone/butanol formation. Induction takes place under conditions that lead to a so-called 'alcohologenic' fermentation, when only butanol and ethanol, but no acetone, are formed (Girbal and Soucaille, 1998). This happens during simultaneous feeding of glucose and glycerol (Vasconcelos *et al.*, 1994). Artificial induction can be achieved by adding methyl viologen to an actively growing culture (Rao and Mutharasan, 1986, 1987). RNA analyses had shown that none of the at that time known genes of solventogenic enzymes was involved in this type of fermentation. A weak hybridization obtained with an *adhE* probe had led to the suggestion that a homologous gene might be present (Dürre *et al.*, 1995). This prediction has now been substantiated by the sequencing project and investigations on the second *adhE* gene (Fontaine, Meynial-Salles, and Soucaille, poster presentations at Clostridium 2000, the 6th Int. Workshop on Regulation of Metabolism, Genetics, and Development of the Solvent- and Acid-forming Clostridia, Urbana-Champaign, Ill., May 26–27, 2000). However, no data are yet available on the regulation of this operon.

2. *adc* Operon

The *adc* operon encoding acetoacetate decarboxylase, which catalyzes the last step of acetone formation, was the first cloned gene involved in solventogenesis (Gerischer and Dürre, 1990; Petersen and Bennett, 1990). It is located on the megaplasmid and shares the transcription terminator (which is working in both directions) with the *sol* operon (Figure 2). These two operons are the first to be induced at the onset of

solventogenesis (Sauer and Dürre, 1995). The *adc* gene is controlled by a single promoter (Gerischer and Dürre, 1992), which recently was shown to be induced by Spo0A (Ravagnani *et al.*, 2000). However, destruction of the Spo0A binding sites did lower, but not completely abolish the promoter activity (determined by the *gusA* reporter gene system) (Ravagnani *et al.*, 2000), which might indicate the involvement of additional transcription factors. Two-dimensional protein gels loaded with extracts from acidogenic and solventogenic cells, respectively, and subsequent N-terminal amino acid sequencing revealed two different spots for acetoacetate decarboxylase (Schaffer *et al.*, 2002). Since according to the genome sequence only one *adc* gene is present, this finding indicates a post-translational modification of Adc. Both, nature and physiological relevance of this modification are still unknown.

As for the *sol* operon, relaxation of DNA was found to have an inducing effect on the *adc* gene (Ullmann *et al.*, 1996). Adjacent to *adc*, but with divergent direction of transcription, is the gene for an alternative sigma factor (*sigY*) located, whose target operons are so far unknown (Behrens *et al.*, 2000). Interestingly, at the late solventogenic phase a protein was induced that was found to bind to the *sigY* promoter region by gel retardation studies (Nakotte, 1998). It is thus tempting to speculate that globally changed DNA supercoiling in combination with Spo0A (and possibly other transcription factors) leads to the induction of *adc*, which, however, is only transient (Sauer and Dürre, 1995). Termination could then be achieved by starting transcription at the *sigY* promoter leading to a deactivation of the adjacent *adc* promoter by local reverse DNA topology changes according to the 'twin transcriptional-loop model' (Liu and Wang, 1987). According to this model, transcription from a divergent promoter (in this case P_{*sigY*}) causes an increased negative supercoiling in an area of app. 250 bp behind the RNA polymerase (i.e. at the *adc* promoter). This would exactly match the regulatory pattern of the *adc* promoter, i.e. inactive at a high degree of negative supercoiling, induction after DNA relaxation due to global DNA topology changes by altered environmental conditions, shut-down late in the solventogenic phase due to a locally increased degree of negative supercoiling. Future experiments will have to prove or disprove this hypothesis.

At first glance, it might appear strange that the genes encoding the acetone-forming enzymes (Ctf and Adc) are arranged in different operons. However, at the onset of solventogenesis a high concentration of acids is converted by the CoA transferase to mainly butyryl-CoA, which is in turn transformed into butanol by AdhE. The respective genes are arranged in the *sol* operon. Adc is only needed to remove the end product of the CoA transferase reaction (acetoacetate), since this conversion is thermodynamically less favorable. Thus, Adc is not required if, for example, plenty of reducing equivalents are available (Dürre *et al.*, 1995). Indeed, depending on the substrates used, the ratio of butanol to acetone was found to increase up to 50fold (Bahl *et al.*, 1986).

Other Genes Related to Solventogenesis

1. *bdhA* and *bdhB* Operons

These two operons are consecutively located on the chromosome and are arranged in monocistronic transcription units each (Walter *et al.*, 1992). RNA analyses indicated that *bdhB* plays a major role in butanol formation and is induced later than the *adc* and *sol* operons. On the other hand, the function of the *bdhA* gene product seems to be a sink for excess reducing equivalents by low level butanol formation (Sauer and Dürre, 1995; Dürre *et al.*, 1995). Upstream of both promoters, one and two Spo0A-binding sites, respectively, can be deduced from the sequence, suggesting an involvement of this transcription factor in *bdhB* and *bdhA* induction as well (Ravagnani *et al.*, 2000).

2. *thlA* Operon

Two thiolase-encoding genes were identified in *C. acetobutylicum* (Stim-Herndon *et al.*, 1995; Winzer *et al.*, 2000). *thlB* forms an operon together with *thlR* (a putative regulator gene) and *thlC* (no significant homology to known genes or their deduced products). The physiological function of ThlB has yet to be elucidated (Winzer *et al.*, 2000). *thlA* is monocistronically organized and codes for the essential metabolic enzyme involved in both, acido- and solventogenesis. Interestingly, the gene is actively transcribed during the exponential, acidogenic growth, repressed in the transition phase, and induced again together with the *sol* and *adc* operons at the onset of solventogenesis (Winzer *et al.*, 2000). No other known gene of *C. acetobutylicum* shows such a regulatory pattern. Since no putative Spo0A-binding boxes could be found upstream of the *thlA* promoter, its control mechanism still needs to be resolved.

3. *ser* Operon

In the proteome analysis approach mentioned above, several proteins were significantly induced at the onset of solventogenesis (among them AdcI and AdcII). N-terminal amino acid sequencing revealed that three spots represented enzymes with high homology to serine aminotransferase (SerC), 3-phosphoglycerate dehydrogenase (SerA), and a seryl-tRNA synthetase (SerS) (Schaffer *et al.*, 2002). The respective genes form a common operon together with a gene encoding a protein of unknown function (*serX*) in the sequence *serC-serA-serX-serS*, whose transcription is induced concomitantly with the *sol* and *adc* operons (Schaffer *et al.*, 2002). A promoter structure could be deduced from primer extension experiments (Schaffer *et al.*, 2002), but no Spo0A boxes were found in its vicinity. The gene products catalyze the biosynthesis of serine and its incorporation into proteins (via the tRNA synthetase). The only enzyme missing is the 3-phosphoserine phosphatase. SerX does not show homology to such an enzyme, but might catalyze such an activity. The reason, why *C. acetobutylicum* enhances serine biosynthesis and incorporates it into proteins at the onset of solventogenesis has yet to be elucidated. There are no known proteins with an

unusually high serine content that play a role in solventogenesis or sporulation.

4. *gap* Operon

The proteome analysis also showed that the protein level of glyceraldehyde-3-phosphate dehydrogenase (Gap) was app. twofold higher during solventogenesis (Schaffer *et al.*, 2002). Cloning and sequencing of the respective gene region revealed a common operon consisting of *gap* – *pgk* (encoding phosphoglycerate kinase) – *tpi* (encoding triosephosphate isomerase). Adjacent to *gap* is the *pgm(i)* gene coding for a 2,3-bisphosphoglycerate-independent phosphoglycerate mutase (Schreiber and Dürre, 1999). Transcription analysis indicated promoters upstream of the *gap* and *pgm(i)* operons, as well as an internal start point within the *gap* operon upstream of *tpi* (Schreiber and Dürre, 2000). The *gap* promoter proved to be constitutively active (although the resulting transcript was processed in different patterns according to the growth conditions (Schreiber and Dürre, 2000)), which is in accordance with the finding that although the protein level was higher in solventogenic cells, its synthesis rate determined by incorporation of labelled methionine remained unchanged (Schaffer *et al.*, 2002). On the other hand, the *tpi* promoter showed an enhanced activity during solventogenesis if compared to the *gap* control unit (Schreiber and Dürre, 2000). The physiological relevance of this phenomenon is not yet understood.

Perspective and Future Directions

Recombinant DNA methods are meanwhile well developed for *C. acetobutylicum* (e.g., Nakotte *et al.*, 1998; Young *et al.*, 1999). Thus, use of reporter genes and knock-out mutations has contributed enormously to our understanding of the regulatory mechanisms in this bacterium (and will continue to do so at an even increasing rate). Most importantly, at the end of the 1990s, the type strain has been selected for a genome sequencing project, which was performed by Genome Therapeutics Corp. (USA) as a component of the US Department of Energy (DOE) Microbial Genome Project. Sequencing is meanwhile complete and the data are available in the internet (<http://www.ncbi.nlm.nih.gov> and http://www.genomecorp.com/programs/sequence_data_clost.shtml) (Nölling *et al.*, 2001). This will enable a functional analysis by transcriptional profiling, supported by the proteome approach already mentioned, and renders other technologies such as e.g., differential RNA display obsolete. The primary goal will be comprehension of the coherent regulatory networks of solventogenesis and sporulation, thus forming the basis for new industrial and medical applications in solvent formation and cancer therapy by recombinant clostridial spores (Bahl and Dürre, 2001).

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