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Review

Molecular Markers and Regulatory Networks in Solventogenic *Clostridium* Species: Metabolic Engineering Conundrum

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Abstract: Solventogenic *Clostridium* species are important for establishing sustainable industrial bioproduction of fuels and important chemicals. The inherent versatility of these species in substrate utilization and the range of solvents produced during acetone butanol-ethanol (ABE) fermentation make solventogenic *Clostridium* an attractive choice for biotechnological applications such as production of fuels and chemicals. The functional qualities of these microbes have thus been identified to be due to complex regulatory networks that play essential roles in modulating the metabolism of this group of bacteria. Genomes of solventogenic *Clostridium* species have relatively greater prevalence of genes that are intricately controlled by various regulatory molecules than most other species. Consequently, the use of genetic or metabolic engineering strategies that do not consider the underlying regulatory mechanisms will not be effective. Several regulatory factors involved in substrate uptake/utilization, sporulation, solvent production, and stress responses (Carbon Catabolite Protein A, Spo0A, AbrB, Rex, CsrA) have been identified and characterized. In this review, the focus is on newly identified regulatory factors in solventogenic *Clostridium* species, the interaction of these factors with previously identified molecules, and potential implications on substrate utilization, solvent production, and resistance/tolerance to lignocellulose-derived microbial inhibitory compounds.

Keywords: ABE fermentation; butanol; lignocellulosic biomass; furfural; LDMIC; riboswitch; sigma factor; carbon catabolite repression; *Clostridium beijerinckii*

1. Introduction

Biofuels are currently produced through the fermentation of food crops such as corn, sugarcane, sorghum, and others, potentially impacting food crop prices due to their dual use as food and fuel. As a result, the use of lignocellulosic biomass (including energy crops, agricultural residues, farm waste, etc.) as fermentation substrates for biofuel production is gaining traction as a viable alternative to using food crops (Palmqvist and Hahn-Hagerdal, 2000; Isar and Rangaswamy, 2011; Zhang and Ezeji, 2013; Okonkwo et al., 2016). However, the use of lignocellulosic biomass (LB) as fermentation substrate has a major limitation due to the presence of lignocellulosic-derived microbial inhibitory compounds (LDMICs) formed during the pretreatment and hydrolysis of LB to monomeric sugars (Ezeji et al., 2007). The LDMICs such as furan aldehydes (e.g., furfural and hydroxymethylfurfural_HMF) and phenolics (hydroxybenzaldehyde, ferulic acid, syringic acid, etc.)

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affect the growth and sugar utilization capacity of fermenting microorganisms such as *Clostridium* species, resulting in reduced growth and product yields (Baral and Shah, 2014; Ujor et al., 2016). To overcome these limitations, considerable research endeavors have investigated both direct and indirect approaches targeting various factors within the ABE production network of molecules (Agu et al., 2019; Okonkwo et al., 2019; Patil, 2019). The aim of these efforts is to enable the development of solvent-producing *Clostridium* strains that can be used economically and industrially.

The genus *Clostridium* is a diverse group of gram-positive, motile, rod-shaped anaerobes with tremendous diversity in habitat/ecological niche, substrate utilization, primary and secondary metabolite secretions, and morphological structures. These variations are regulated mostly by a network of complex but precisely molecular factors and mechanisms induced by environmental and physiological signals. Species in the genus *Clostridium* include several toxin-producing human pathogens (e.g., *C. difficile, C. botulinum, C. tetani, C. perfringens*) and many non-pathogenic species (e.g., *C. acetobutylicum, C. beijerinckii, C. butylicum, C. carboxydivorans)* which produce a range of products by fermenting a variety of carbohydrates (Liou et al., 2005; Ezeji et al., 2010). A common feature of *Clostridium* genus (over 200 species) is the production of oval sub-terminal endospores.

Clostridium species occur in a wide variety of habitats. Although most of them are found in the soil, some are also found in the intestines of animals and humans and are therefore excreted through feces (Maczulak, 2011). Others can be found in habitats like waterbodies and food/industrial waste dumps (Maczulak, 2011). *Clostridium* species can be utilized to produce cosmetics, medicine and/or for industrial biotechnological purposes. For example, *C. botulinum,* which is known to produce a neurotoxin that attacks the nerves in the body, causing muscle paralysis, difficult breathing, and potentially fatal consequences, is currently being used in the cosmetic and beauty industry. The toxin produced by *C. botulinum* has been modified and used to make Botox, a substance widely used in the cosmetic industry to remove or mask wrinkles (Small, 2014). Some *Clostridium* strains can selectively target cancer cells (e.g., *C. noyvi-NT, C. sporogenes, and C. sordellii*), with the rare capacity to penetrate and/or replicate within solid tumors (Staedtke et al., 2016). Hence, these *Clostridium* strains are potential candidates for delivering therapeutic proteins and tumor-killing agents into active tumors. The potential of using *Clostridium* species in cancer therapy related purposes has been established in various preclinical studies (Dang et al., 2001; Patyar et al., 2010).

In addition to the use of *Clostridium* species in cosmetics and medicine, they can convert a range of substrates into fuels and bulk chemicals (**Table 1**). In the early 19th century, solventogenic *Clostridium* species were used for ABE production. Unfortunately, the discovery of low-cost fossil fuel source led to the cessation of bacterial methods of butanol production. Fortunately, the urgent demand for a clean environment and the finite nature of fossil fuels have rekindled the need to exploit biotechnological applications of *Clostridium* species in the production of fuels and important industrial chemicals (**Table 1**). Genome wise, solventogenic *Clostridium* species have a wide range of genome sizes. For example, *C. acetobutylicum* ATCC 824 has a 3.94-Mb chromosome with 11 ribosomal operons and contains a large plasmid, pSOL1 (Nölling et al., 2001). The pSOL1 is about 192 kb in size and harbors the ABE production genes. Conversely, *C. beijerinckii* NCIMB 8052 possesses the largest chromosome of the *Clostridium* genus with a 6.1 Mbp genome. In addition, *C. beijerinckii* contains the largest number of ribosomal operons (13 plus) of the *Clostridium* species. A considerable portion of genes in *C. beijerinckii* have unknown functions, making it a promising candidate for exploratory research.

Table 1. Substrates for and products produced by solventogenic *Clostridium* species.

***Clostridium* species**	**Substrates**	**Products**	**References**
C. acetobutylicum	Glucose, xylose, arabinose, cellobiose	Acetone, butanol, and ethanol	Jones and Woods, 1986
C. beijerinckii	Glucose, xylose, arabinose, cellobiose	Acetone, butanol, and ethanol	Jones and Woods, 1986
C. thermocellum	Lignocellulose, Cellulose	Ethanol	Dror et al., 2003

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C. butyricum	Glycerol	1,3-propanediol	Saint-Amans et al., 1994
C. Pasteurianum	Glycerol	1,3-propanediol, butanol and ethanol	Biebl, 2001
C. carboxidivorans	Carbon monoxide, hydrogen, and carbon dioxide	Ethanol, butanol and hexanol	Liou et al., 2005
C. aurantibutyricum	Glucose, lactose, maltose, galactose, xylose, starch	Acetone, isopropanol, and butanol	(George et al., 1983)
C. ljundahlii	Carbon monoxide and hydrogen	Ethanol	Younesi et al., 2005
C. tetanomorphum	Glucose and maltose, glutamate	Butanol and ethanol	Wilde et al., 1989
C. saccharoperbutylacetonicum	Glucose	Acetone, ethanol, and butanol	Tashiro et al., 2004

Most solvent-producing *Clostridium* species exhibit a biphasic fermentation profile regardless of the type of substrate used. An initial acidogenic phase characterized by the production of hydrogen, carbon dioxide, acetate, and butyrate, leads to a decrease in the pH of the fermentation medium. The solventogenic phase of the fermentation process is marked by a metabolic shift from producing hydrogen, carbon dioxide, and acids to generating solvents or ABE (stationary phase) as the primary products. During solventogenesis, substrate uptake takes place concurrently with acid re-assimilation, resulting in a rise in the culture pH. Depending on the *Clostridium* species and the carbon source, a wide range of metabolic pathways are activated for solvent formation. For instance, in *C. carboxidivorans* and *C. ljungdahl*, the wood-ljungdahl pathway is triggered to produce ethanol from synthesis gas during solventogenesis (Younesi et al., 2005; Han et al., 2020). In *C. tetanomorphum*, the methylaspartate pathway for glutamate conversion to acetate, butyrate, and carbon dioxide is activated (Buckel and Barker, 1974). In *C. acetobutylicum* and *C. beijerinckii*, both glycolytic (Embden-Meyerhof pathway) and acid production pathways are active for concurrent uptake of glucose and acids production during the exponential growth phase (Aristilde et al., 2015). Subsequently, the solventogenic pathway is activated for the reassimilation and conversion of acids to ABE during the late exponential and stationary phases.

Previous research efforts focused on random mutagenesis and mutant selection (Gottumukkala et al., 2017), modulation of acid and ABE production (*adh, pta, ptb, buk, ack, bdh, ald,* etc.), stress response (*groES, groEL, DnaK, DnaJ*), and LDMIC detoxification genes (Okonkwo et al., 2019). Some other indirect approaches focused on improving redox balance through NAD(P)H regeneration, calcium and acetate supplementations, and process and media optimizations (Han et al., 2013; Ujor et al., 2016, Xue et al., 2017; Patil, 2019). The use of metabolic engineering techniques to increase butanol production by solventogenic *Clostridium* species has yielded mixed results. It is therefore imperative to focus on the regulatory factors that play roles in butanol production in the future. Understanding the functions of these factors will be helpful in identifying specific targets for metabolic engineering of these microorganisms (Yang et al., 2018) considering that *Clostridium* species contain common sets of genes that encode proteins that modulate basic processes such as ABE production, stress response and sporulation. Most *Clostridium* species share common regulatory protein networks that play crucial roles in ABE production. These networks are responsible for the regulation of various processes, including sporulation, sugar uptake/utilization, carbon catabolite repression (CCR), and the transition from acidogenesis to solventogenesis. The key proteins involved in these regulations are Spo0A, the phosphoenolpyruvate:carbohydrate phosphotransferase system (PTS), carbon catabolite protein A (CcpA), and the transition phase regulator (AbrB; Smith, 1993; Tangney et al., 2003; Scotcher et al., 2005; Ren et al., 2010; Noguchi et al., 2013). The redox potential regulator (Rex) and the carbon storage regulator A (CsrA) proteins also play a role in solvent production in *Clostridium* species (Wietzke and Bahl, 2012; Tan et al., 2015).

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A greater understanding of sporulation, sugar uptake/utilization, ABE production, and response to stressors such as LDMICs and butanol in solventogenic *Clostridium* species is crucial for deciphering the regulatory networks involving various molecular factors. This review specifically addresses the role of sigma factors, small non-coding RNAs (sRNAs) and secondary metabolites in the regulation of ABE production. It also examines their responses to solvent toxicity and LDMICs, as well as possible interactions between these regulatory elements. Furthermore, the review highlights the importance of molecular markers within these networks that may guide strategic decisions in metabolic engineering aimed at increasing butanol production by *Clostridium* species.

2. Sigma Factor 54 Directed Regulation

The transition from exponential to stationary growth phase in solventogenic *Clostridium* species results in a wave of altered gene expression patterns (Zhang and Ezeji, 2013). Sigma factors play a central role in regulating gene transcription in bacteria as they bind to RNA polymerase to form a catalytically active RNA polymerase holoenzyme (Davis et al., 2017; Buck and Cannon, 1992). The sigma factor in the RNA polymerase holoenzyme controls the tight binding of the core RNA polymerase to the promoter to initiate for transcription (Shen, 2019). The vegetative sigma factor family (σ^{70} or σ^{A}) is well established for its role in promoting exponential growth in *Clostridium* species, while sporulation-specific sigma factors (σ^{F}, σ^{E}, σ^{G}, and σ^{K}) are responsible for initiating sporulation in these microorganisms. A review of the regulatory roles of sigma factors σ^{A}, σ^{F}, σ^{E}, σ^{G}, and σ^{K} in sporulation in *Clostridium* species has been published elsewhere (Sauer et al., 1995; Davis et al., 2017). Although often overlooked, sigma factor σ^{54} plays a critical role in regulatory functions such as sugar uptake/utilization and solventogenesis in solventogenic *Clostridium* species (Nie et al., 2016, Hocq et al., 2019; Yang et al., 2020).

Sigma factor 54 (σ^{54}), encoded by the *SigL* gene, is a transcription factor that occurs as a single copy in solventogenic *Clostridium* species such as *C. acetobutylicum, C. beijerinckii C. ljungdahlii, C. carboxidivorans, and C. autoethanogenum* (Nie et al., 2019). While σ^{54} was initially determined to be involved in nitrogen metabolism in *E. coli*, this transcription factor also regulates solvent production in solventogenic *Clostridium* species (Nie et al., 2016, Hocq et al., 2019; Yang et al., 2020). Unlike σ^{A}, σ^{54} binds strongly to conserved consensus sequence at the GG −24 and TGC −12 promoter elements (Morett and Buck, 1989). Data from recent studies suggest that σ^{54} promoter elements are located upstream of most phosphotransferase system (PTS) operons (*cel, lev, man, atl,* and *gfr* operons) involved in sugar transport in *C. acetobutylicum* and *C. beijerinckii* (Nie et al., 2016). Similarly, a putative σ^{54} promoter element is located upstream of genes involved in ABE production (*adh, crt-hbd-thl-maoC-bcd-etfAB* operon) in solventogenic *Clostridium* species (Nie et al., 2019, Yang et al., 2020). The strategic location of the σ^{54} promoter element is an indication that σ^{54} may have important functions in the regulation of sugar metabolism and ABE production. Unlike most sigma factors that bind to RNA polymerase to form an open complex that initiates transcription, σ^{54} binds to RNA polymerase and forms a stable closed RNA polymerase-σ^{54} (RNAP-σ^{54}) complex which requires an activator to initiate transcription. The enhancer binding protein (EBP) serves as a vital activator for the conversion of the closed RNAP-σ^{54} complex to its open form (**Table 2**). EBP is widespread in *Clostridium* species, and it is a modular protein consisting of an N-terminal regulatory domain, a central ATPase domain and a C-terminal DNA binding domain. The central ATPase domain utilizes the energy released from ATP hydrolysis to initiate isomerization of the closed RNAP-σ^{54} complex to form an open complex (Schumacher et al., 2004; Jones, 2009). The EBP binds to upstream activator sequences (UASs) and forms higher-order oligomers that leads to formation of a stable complex with the DNA, and consequently, induces transcription activation. The EBP requirement for transcriptional activation is a unique feature of σ^{54}-dependent promoters. A review of functions of EBPs in σ^{54} mediated transcription activation in bacteria has been published elsewhere (Bush and Dixon, 2012). The EBPs are grouped based on regulatory domains involved in sensing environmental or cellular signals (**Table 2**).

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Table 2. Number of EBPs and regulatory domain groups identified in the genome of solventogenic *Clostridium* species.

Organism	Number of EBPs predicted to be present in genome	Type of EBP regulatory domains identified	Number of σ^{54} regulated operons
C. acetobutylicum ATCC 824	3	PRD-EIIA-PRD (1), PrpR-N-PAS (1), PTS_Hpr-PAS-PAS (1)	2
C. autoethanogenum DSM 10061	12	PAS-PAS (1), GAF-PAS (6), PAS (3) 0 (2)	-
C. beijerinckii NCIMB 8052	19	PRD-EIIA-PRD (12), CBS-PAS (1), PTS_Hpr-PAS- PAS (1) PAS-PAS (2). GAF-PAS (2), RR (1)	26
C. carboxydivorans P7	11	PRD-EIIA-PRD (1), PTS_Hpr-PAS-PAS (1), PAS-PAS (1) GAF-PAS (2), PAS (4), 0 (1), PrpR-N-PAS (1)	-
C. ljungdahlii	19	PrpR-N-PAS (1), PAS-PAS (1), GAF-PAS (10), PAS (5), 0 (2)	-
C. saccharobutylicum DSM 13864	5	PRD-EIIA-PRD (3), GAF-PAS (2)	-
C. saccharoperbutylacetonicum N1-4 (HMT)	14	PRD-EIIA-PRD (5), GAF-PAS (3), PAS-PAS (1), PAS (2), PrpR-N-PAS (2), V4R (1)	-

Abbreviated term definitions: PAS, Per-Arnt-Sim domain; GAF, cyclic GMP-specific phosphodiesterases, adenylyl cyclases and FhlA; PTS-HPr, PTS system histidine phosphocarrier protein HPr-like; PrpR_N, N-terminal domain of Propionate catabolism activator; CBS, cystathinoine β-synthase (Nie et al., 2019).

The σ^{54}-dependent promoters and associated EBPs have been identified for various operons involved in the uptake of sugars (CelR, LevR, ManR, AtlR, and Gfr) and ABE production (AdhR, CrbR, AhcR, AorR) in *C. acetobutylicum*, *C. beijerinckii, C. saccharoperbutylicum, C. carboxydivorans,* and *C. ljungdahlii*. This suggests that σ^{54} plays important role in the regulation of sugar metabolism and ABE production in solventogenic *Clostridium* species (Nie *et al*., 2016, 2019). The EBPs associated with the PTS operons contain the PTS regulatory domain (PRD) at the N-terminals which enables the regulation of Hpr, EI and cognate EIIA/Bs, which are PTS proteins, via phosphorylation/dephosphorylation (Deutscher et al., 2014). Disruption of the *sigL* (which codes for σ^{54}) gene led to impaired cell growth and ABE production in *C. beijerinckii*, whereas the overexpression of this gene resulted in a significant increase in acetone production with no apparent change in butanol production (Hocq et al., 2019; Yang et al., 2020). In contrast to *sigL* overexpression, *AdhR* overexpression resulted in poor cell growth and ABE production (Yang et al., 2020). These data suggest that EBP mediated σ^{54} regulation may be present, effective, and concentration dependent in solventogenic *Clostridium* species. It is also plausible that a second protein/molecule may be required for transcriptional activation. The overabundance of AdhR may lead to binding exclusion of the second regulatory protein/molecule that may be required for transcriptional activation. Another possibility is the requirement of optimal AdhR concentration for UASs binding because increased abundance of AdhR may distort the concentration balance. Indeed, many proteins compete for the same UAS sites, and this competition can result in a partial or total inhibition of binding. For instance, alcohol dehydrogenase (Adh) is an important enzyme for ABE production in *Clostridium* species. This is because Adh catalyzes the conversion of precursor aldehydes (acetaldehyde and butyraldehyde) to their respective alcohols (ethanol and butanol). A precise understanding of regulatory factors involved in the expression of *Adh* gene is important to improve ABE production. Delineating the regulatory domain and activation signal within AdhR is a crucial aspect in comprehending the regulatory mechanism of AdhR and exploring possible strategies for enhancing butanol production.

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The presence of EBPs and the diverse regulatory domains suggest some important functions of EBPs in signal transduction and regulation of sugar metabolism and ABE production (**Figure 1**).

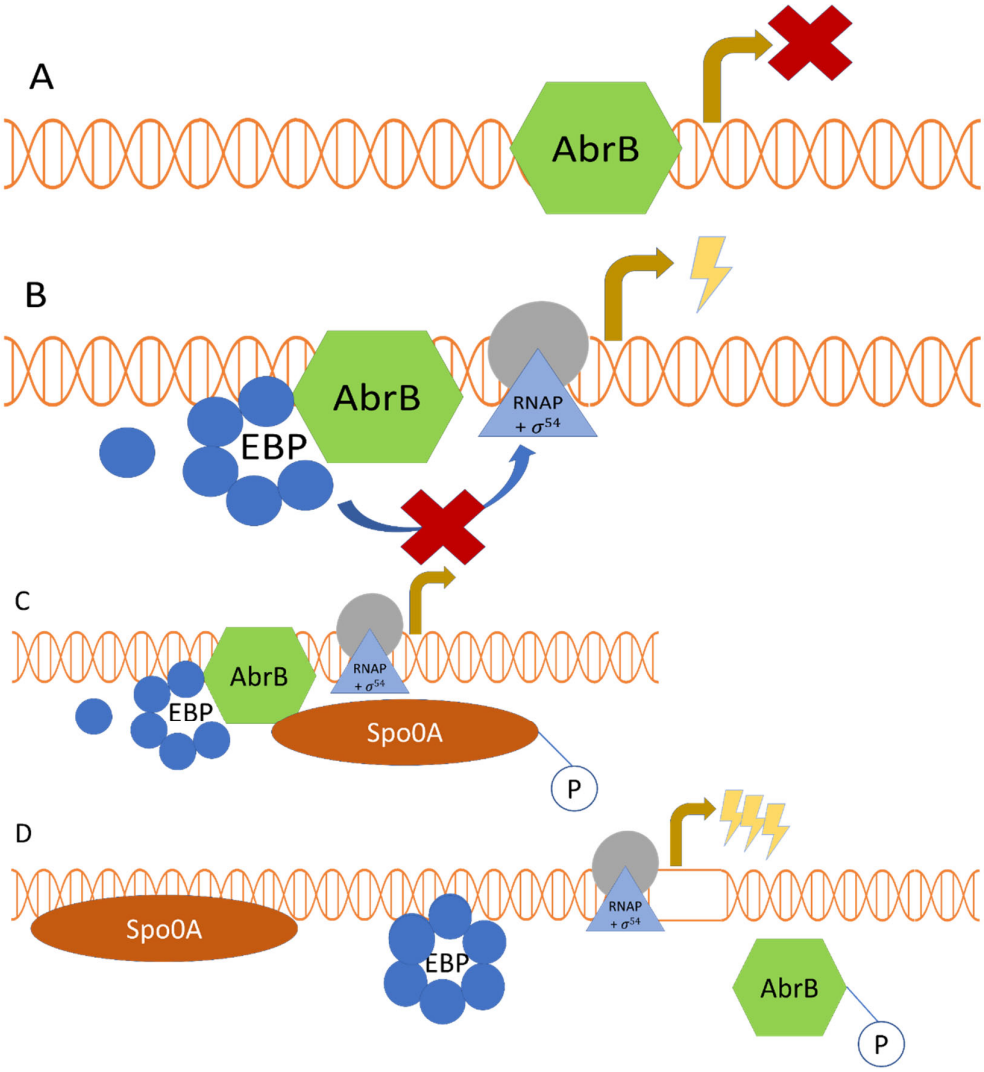

Figure 1. Proposed mechanism of regulation of ABE production by Spo0A, AbrB and EBP/sigma 54. A; Acid re-assimilation (ctfAB) and acetoacetate decarboxylase (adc) genes with promoters that are not sigma 54 dependent are completely inhibited by AbrB and no transcription occurs in the absence of Spo0A. B; Other sigma 54 dependent solvent production genes undergo significantly reduced transcription due to disrupted stability of the EBP by AbrB in the absence of Spo0A. C and D; Phosphorylated Spo0A inactivates AbrB through phosphorylation. Phosphorylated AbrB is released from bound DNA, Spo0A binds to DNA and allows the formation of higher- order EBP oligomer. EBP then induces a larger activation signal for sigma 54 dependent transcription. The golden arrow indicates the transcription start site.

2.1. Interaction between σ^{54} Mediated Regulation and Carbon Catabolite Repression/Activation

Carbon catabolite repression (CCR) is a major barrier to simultaneous utilization of hexose and pentose sugars in bacteria (**Figure 2**). Solventogenic *Clostridium* species are no exception. With ongoing efforts to utilize lignocellulose derived sugars as feedstocks for ABE production, it is important to understand and engineer solventogenic *Clostridium* species to improve simultaneous utilization of hexose and pentose sugars because these sugars are significant component of the lignocellulosic biomass (LB) hydrolysates (LBH). CCR occurs when the presence of a preferred

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carbon source (usually glucose) in the growth medium induces transcriptional repression of genes encoding proteins required for the uptake and metabolism of non-preferred sugars (e.g., xylose, arabinose, galactose, cellobiose, sucrose etc.; Ren et al., 2010). This transcriptional repression process is modulated by the HPr protein of the PTS which in turn is mediated by the catabolite control protein A (CcpA), an allosteric transcriptional regulator. The CcpA binds to the catabolite repression elements (*cre*) in the promoter or coding region of the target gene and represses transcription. Interestingly, recent research studies have shown that the CcpA of *C. acetobutylicum* and *C. beijerinckii* also activates transcription of *sol* operon, *adh*A1, *adh*A2, *crt, thl, bcd, etfAB, hbd* which are genes involved in ABE formation (Ren et al., 2012; Yang et al., 2017). In *C. acetobutylicum and C. beijerinckii,* several sugar uptake/utilization operons that contain *cre* sites (e.g., *Cel* operon, *man* operon) for CcpA mediated activation/repression possess σ^{54} dependent promoters with associated EBPs (CelR and ManR; Nie et al., 2016). While no conventional *cre* site has been identified in genes regulating ABE production (e.g., *sol* operon, *adh*A1, *adh*A2, *crt, thl, bcd, etfAB, hbd*), their expression is downregulated in CcpA null mutants (Ren et al., 2012, Yang et al., 2018). This finding indicates that *cre* is regulated by CcpA. However, sugar uptake and genes involved in ABE production, however, are regulated to some extent by the σ^{54} regulon with EBPs in *C. beijerinckii* (Nie et al., 2016, 2019). This indicates possible interactions between σ^{54} mediated and CcpA-dependent regulation processes (ccr) that modulate gene expression. Hence, the question arises: which regulatory mechanism prevails, and is there a possibility of collaborative regulation during sugar uptake and ABE production?

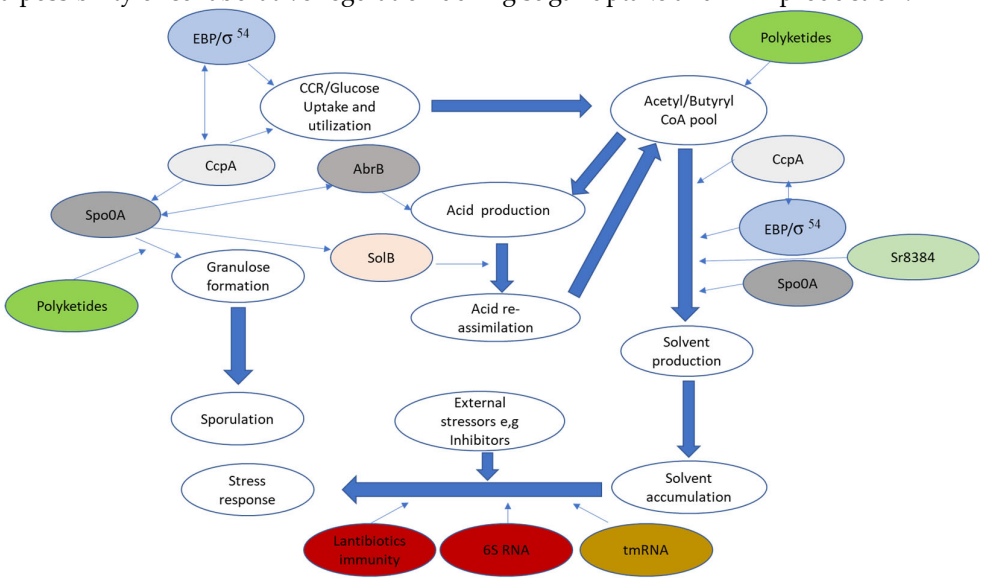

Figure 2. Reconstructed pathway for ABE production in solventogenic Clostridium species showing regulatory points. Colored shapes indicate regulatory molecules, white shapes indicate metabolic processes.

The genes of the *Cel* operon and most sugar uptake/utilization operons contain typical *cre* site (WTGWAAACGWTWWCAW: W is A or T) in the coding region. The CcpA dependent CCR of genes encoding proteins that modulate uptake/utilization of non-preferred sugars (*cel, xyl, ara* operons) is regulated by the HPr-Ser-46. The CcpA-HPr-Ser-46 complex binds to *cre* sites on the promoter or protein coding sequence of the target operon/gene to repress gene expression (Deutscher et al., 2006, 2014). This mechanism of regulation is clearly CcpA/Hpr mediated, indicating that there is no interaction with the σ^{54} promoter. The CcpA regulation of genes modulating ABE production in *C. beijerinckii* (*adh*A1, *adh*A2, *crt, thl, bcd, etfAB, hbd*), however, is not straightforward. The absence of typical *cre* sites in the promoter or coding region, even though the genes for ABE production are being regulated, is indicative that the mechanism for this type of regulation is yet to be elucidated. Data from a recent study indicate that CcpA binds to three atypical cre_{var} sites (1. AACTGCTAAATGTAAA-TTATACG-TTTACATTTAGCAGTTT, 2:

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TGTAAAAGTTGCTATTTACA. 3: TATTGTAAACCTTGTTTTGTTTTGCAGTTTACAA TA) upstream (within -350 to -1 base pairs proximal to transcription start site) of the *sol* operon promoter in *C. acetobutylicum* in a regulatory manner (Yang et al., 2017). These atypical binding sites involving CcpA are predominantly present in genes associated with sporulation and ABE formation, and the binding of CcpA to cre_{var} was thought to be associated with gene activation. Additionally, transcriptomic analysis show that CcpA is constitutively expressed in *C. acetobutylicum* and *C. beijerinckii* during ABE fermentation (Alsaker et al., 2004; Wang et al., 2012). This suggests that at least one gene or protein molecule may be required to mediate regulation. Because EBPs consist of a N-terminal regulatory domain through which various cellular signals and proteins are regulated, EBPs could possibly serve as a direct control molecule of the CcpA. The EBPs possibly interact with CcpA to induce a greater activation signal for the σ^{54}-dependent transcription in the presence of activating cellular signals. The cre_{var} binding motif has been identified to consist of a core palindromic sequence **TGT**AAA/TTT**ACA** with intervening sequence of variable length (Yang et al., 2017). Similarly, EBPs bind to three UASs (-80 to -150 bp upstream of the promoter sequence) in a cooperative manner to mediate σ^{54}-dependent transcription (Nie et al., 2016; Yang et al., 2020). Specifically, UASs for EBPs genes (AdhR, AhcR, CrbR) modulating ABE production genes (*adh, atoE-hbd-cotX, crt-hbd-thl-maoC-bcd-etfAB*) are of variable length with flanking TGT/ACA palindromic sequences (Nie et al., 2019). The matching flanking palindromic sequences and similarly flexible binding sequence lengths recognized by both EBPs and CcpA at locations upstream of the promoter indicate the occurrence of either competitive or cooperative binding to induce repression or activation of gene transcription. Binding of EBPs to the three UASs may be required for activation and modulation of target gene transcription (Nie et al., 2016, Yang et al., 2020). Competitive binding of CcpA to one or more of these sites could either distort or enhance (possibly depending on position) EBP oligomeric structure stability required for ATPase activity necessary for the formation of the open RNA polymerase- σ^{54} complex. Another potential scenario involves the activation or repression facilitated through direct interaction of the CcpA and EBP regulatory domains, specifically through protein-protein interaction. EBPs can be activated or repressed through interaction with other proteins.

2.2. Regulatory Interplay between σ54-Mediated Regulation and the Transition Phase Regulator AbrB, along with the Sporulation Master Regulator Spo0A

The metabolic shift from acid to solvent production may be regulated by the transition phase regulator AbrB (Scotcher et al., 2005). AbrB was identified to play a role in activating transcription for the phosphotransferase acetylase (*pta*), acetate kinase (*ack*), phosphotransferase butyrylase (*ptb*), and butyrate kinase (*buk*) genes. The proteins encoded by these genes are accountable for acid production in *C. acetobutylicum*. Proteins associated with ABE production (*sol* operon: *adhE1-ctfa-ctfb*, *adc*) are negatively regulated by the AbrB. Three homologues of the AbrB gene (*AbrB1941*, *AbrB0310* and *AbrB3647*) are active in regulating different genes in *C. acetobutylicum* (Scotcher et al., 2005; Xue et al., 2016). Data from an *AbrB3647* gene knockout experiment in which transcriptomic analysis was conducted in *C. acetobutylicum* mutants indicated a marked upregulation of the *sol* operon and other genes that encode proteins involved in ABE production (*bdhA, bdhB, edh,* and *adc*; Xue et al., 2016). Although there are σ^{54} dependent promoters and associated EBPs that modulate genes of *C. acetobutylicum* involved in ABE production, orthologs of these genes (*adh*A1, *adh*A2, *crt, thl, bcd, etfAB, hbd*) have been predicted in *C. beijerinckii, C. carboxidivorans* and *C. ljungdahlii* to encode the σ^{54} dependent promoters of associated EBPs (AdhR, AhcR, CrbR; Nie et al., 2019). Albeit the function of AbrBs has not been fully elucidated in solventogenic *Clostridium* species, orthologs of these genes are present in their genomes, suggesting there may be AbrB mediated regulation (Yang et al., 2018). Data from enzyme mobility shift assays in *C. acetobutylicum* show that AbrB binds upstream of solvent production genes (*sol* operon, *adhE2, edh, bdhA, bdhB*) promoters (Xue et al., 2016) in the same region as the putative UAS for EBP binding. This suggests that AbrBs can repress the expression of genes that modulate ABE production by either inhibiting EBPs or preventing interaction of EBP with the RNA polymerase holoenzyme. The AbrB possibly competes for one or more UASs thereby

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preventing the formation of the higher order oligomer of EBPs required for stabilization in the DNA and induction of the ATPase activity of the central domain.

Activated sporulation transcriptional regulator protein, Spo0A, inactivates AbrBs via phosphorylation. The Spo0A protein regulates sporulation and solventogenesis by activating genes (*ctfA-ctfB*, *adc*, *ptb*, *adhE*) that modulate acid and ABE production in *Clostridium* species (**Figures 1 and 2;** Ravagnani et al., 2000; Thormann et al., 2002; Dürre and Hollergschwandner, 2004; Hollergschwandner and Dürre, 2004). The activation is facilitated by Spo0A binding to the 0A boxes upstream of the promoters of genes involved in ABE production. Data from functional analysis of Spo0A provided insights that Spo0A is a negative regulator of AbrB (Smith, 1993). Because the onset of ABE production coincides with the onset of sporulation in solventogenic *Clostridium* species, deletion of *Sp0A* in *C. beijerinckii* resulted in a strain with lesser capacity to produce butanol and ethanol with no ability for acetone production relative to the wildtype (Harris et al., 2002; Ravagnani et al., 2000). This indicates that in the absence of the Spo0A protein, the genes involved in ABE production may not be optimally expressed in solventogenic *Clostridium* species. It is also plausible that the production of lesser quantities of butanol and ethanol may be due to the continued AbrB repression of *ctfAB* (CoA-transferase) transcription. The CoA-transferase mediates acid re-assimilation via Coenzyme A transfer to acetic and butyric acids, thus activating these acids for subsequent conversion to solvents during solventogenesis. The *C. beijerinckii* Spo0A null mutant has impaired ability to produce acetone since the acetoacetate decarboxylase (*adc*) gene expression is regulated by Spo0A (Sullivan and Bennett, 2006). The Adc protein is recognized for facilitating the transformation of acetoacetate into acetone and CO_2. Nevertheless, research has revealed that inactivation of *adc* gene in *C. beijerinckii* and *C. acetobutylicum* do not completely abolish acetone production, owing to an operational non-enzymatic acetoacetate decarboxylation route for acetone production in these organisms (Jiang et al., 2009; Han et al., 2011). The analysis of the σ^{54} regulon in *C. beijerinckii* demonstrated the presence of σ^{54}-dependent promoters in nearly all genes involved in regulating ABE production, except for the aldehyde dehydrogenase (*ald*), *ctfAB*, and *adc* genes (Nie et al., 2019). The production of butanol and ethanol (although in low concentration) when there was Spo0A inactivation could be due to EBP-σ^{54} mediated transcriptional activation of other genes such as *adhE* that modulate ABE production. The dependence of the *adc* and *ctfAB* genes on Spo0A expression (AbrB inhibition and binding to the 0A box to allow a conformation favorable for transcription activation) could be attributed to the suppression of acetone production in the *C. beijerinckii* Spo0A null mutant. The results of DNAse I foot-printing assays validated the precise binding of Spo0A to the 0A boxes at the promoter region of the *abrB* gene (Ravagnani et al., 2002). The binding of Spo0A, however, did not protect 0A boxes from restriction digest, which suggests that Spo0A does not bind tightly to 0A boxes. It is possible that Spo0A binds transiently to the 0A boxes of genes and modulate ABE production, while simultaneously regulating transcription via AbrB inactivation. A plausible mechanism for Spo0A regulation of ABE production is that Spo0A phosphorylates and inactivates AbrB which dissociates from the 0A boxes on the DNA allowing the binding of Spo0A. The change in DNA conformation following Spo0A binding possibly favors the binding of EBP and formation of the higher order oligomeric structure, which promotes transcription activation (**Figure 1**). The transient binding of Spo0A may also result in modulation of the conformation of DNA leading to stabilization of the EBP, improved contact of EBP with σ^{54} for ATPase activity, and ultimately, transcriptional activation.

3. Regulation by Small Non-Coding RNAs

Bacterial small non-coding RNAs, also referred to as small RNAs (sRNAs; 50 to 400 nucleotides), regulate expression of diverse genes post-transcriptionally. The sRNAs modulate the expression of genes that control important cellular functions such as virulence, quorum sensing, iron homeostasis and stress responses (Vogel and Sharma, 2005). The sRNAs are involved in a wide range of biological functions such as translation (transfer messenger RNA) and RNA processing by RNase P (ribozyme moiety and protein secretion, 4.5S RNA; **Table 3**; Brosse and Guillier, 2018). The sRNAs are usually located at intergenic regions which are independently transcribed or are nucleolytically cleaved from

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the 3′ untranslated region of mRNAs (Chao et al., 2012; Argaman et al., 2001). Generally, sRNA synthesis is induced in response to environmental and cellular signals, and many regulate gene expression post transcriptionally. The sRNAs imperfectly base pair with target mRNAs to inhibit protein synthesis or serve as markers for mRNAs degradation. Furthermore, sRNAs can bind to proteins to form ribonucleoproteins (RNPs). These complexes can either stabilize and facilitate specific functions or catalysis for the associated proteins or inhibit their normal functions (Richards and Vanderpool, 2011; Storz et al., 2011). Conventionally, sRNAs are encoded by the sense strand of DNA. Nevertheless, recent research findings indicate an increasing number of sRNAs are encoded on the anti-sense strand of coding nucleotide sequences. These are known as antisense RNAs (asRNAs) and they possess the ability to influence the expression of the corresponding coding gene (Thomason and Storz, 2010; Georg and Hess, 2011). The sRNA-mediated regulation is often closely associated with transcriptional regulatory networks that modulate growth, virulence, biofilm formation, etc., in different bacteria. While Brosse and Guillier (2018) published a review on the regulatory roles of sRNAs in diverse bacterial species, the review did not delve into the regulatory functions of sRNAs in solventogenic *Clostridium* species or elucidate how these functions impact ABE production and stress response. The need to understand the regulatory functions of sRNAs in solventogenic *Clostridium* species is beginning to emerge. Data from predictive comparative genome analysis suggest that seven solventogenic *Clostridium* species harbor sRNA genes (Chen et al., 2011; **Table 3**). The data also highlight the prevalence of metabolism-dependent sRNA genes in solventogenic *Clostridium* species, revealing that saccharolytic *Clostridium* species possess a higher number of sRNAs genes compared to their cellulolytic counterparts (**Table 3**). Furthermore, the presence of several predicted sRNAs has been validated using RT-qPCR and Northern analysis of *C. acetobutylicum* of mRNA. An abundance of sRNAs that respond to cellular stressors through either transcriptional regulation (6S, S-box and solB) or translational (tmRNA and SRP-RNA) processes have also been predicted (Venkataramanan *et al*., 2013). It is worth noting that only a small proportion of the validated sRNAs have been subjected to experimental characterization (Keiler, 2008, Venkataramanan *et al*., 2013; Craig *et al*., 2014, Wach *et al*., 2014; Wassarman, 2018, Yang *et al*., 2020).

Table 3. Number of sRNAs identified from different *Clostridium* species adapted from Chen et al., 2011.

***Clostridium* species**	**Number of sRNA identified in genome**	**Genome size (mb)**	**GC content (%)**	**Metabolism type**
Clostridium acetobutylicum ATCC 824	113	4.13	30.90	Saccharolytic
C. beijerinckii NCIMB 8052	366	6.00	29.90	Saccharolytic
C. cellulolyticum H10	*45*	*4.07*	*37.40*	*Cellulolytic*
C. kluyveri DSM 555	126	4.02	32.02	-
C. kluyveri NBRC 12016	136	3.96	32.02	-
C. phytofermentans ISDg	*42*	*4.85*	*35.30*	*Cellulolytic*
C. thermocellum	*15*	*3.84*	*39.00*	*Cellulolytic*
C. carboxidivorans p7	*??*	*5.75*	*29.89*	-

3.1. SolB Mediated Regulation

SolB is an sRNA that was identified by Peter Durre. This sRNA is associated with response to butanol stress in *C. acetobutylicum* (Wach et al., 2014). The *SolB* coding region lies upstream of the *adhE2* gene on the sol operon as a 196 bp sequence (Zimmerman, 2013). Data from transcriptomic analysis indicated that *SolB* was upregulated specifically during butanol stress (Wang et al., 2013). Overexpression of *SolB* in solventogenic *Clostridium* species induces downregulation at the sol-locus leading to modulation in rate of gene transcription (Jones et al., 2018). While the repression of *sol* operon by *SolB* results in a longer cell viability, it also leads to reduced ABE production (Jones et al.,

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2018). Interestingly, coenzyme A transferase (CoAT) activity *was* abolished in *solB knockout mutants*, resulting in complete loss of acetone production with a marked decrease in butanol production (Jones et al.,2018). This suggests that the regulatory activity of *solB* may be concentration dependent. In addition, a small abundance of *SolB* transcripts is probably necessary for the stabilization of CoAT for optimal activity. The lack of acetone production in *solB knockout mutant* could be primarily due to the lack of *ctfAB* transcription. Transcriptomic analysis and ABE production pattern in the *SolB* overexpressing mutant show that the plausible mechanism of regulation of ABE production is via *SolB* in solventogenic *Clostridium* species. Northern blotting, q-RT-PCR and transcriptomics analysis have showed that *solB* transcripts are present in low quantities in *C. acetobutylicum* wildtype during the acidogenic phase followed by remarkable increase in expression as fermentation progresses (Wang et al., 2013; Jones et al., 2018). Overexpression of *SolB* in *C. acetobutylicum* led to a notable decrease in transcript levels of solvent producing sol operon and *adc* gene in the mutant (Jones et al., 2018). Conversely, deletion of *solB* impacted the expression of *ctfAB* and *adhE1*, but did not affect *adc* expression (Jones et al., 2018). Therefore, a plausible regulatory mechanism for solvent production in *C. acetobutylicum* arises when considering the upregulation of *SolB* during butanol stress and its correlation with the progression of fermentation, leading to the accumulation of primarily butanol. This is further influenced by the impact of ctfAB and adhE1 deletion on coenzyme A expression and solvent production. It suggests that initial low levels of *solB* are necessary for the expression of sol operon genes. However, as butanol builds up in the fermentation medium during the process, the transcription of *SolB* intensifies, targeting mRNAs produced from the sol operon for degradation. This, in turn, leads to the regulation of ABE production. This could help elucidate the decrease in ABE production rate once a certain butanol concentration threshold is reached in the fermentation medium. Given these observations, it is conceivable that *SolB* acts as a regulatory inhibitor of butanol biosynthesis, potentially exerting its influence even before butanol titer reaches a toxic threshold. Wang et al. (2013) noted that *solB* expression increased following supplementation of the fermentation medium with 2 g/L butanol. Nevertheless, the precise threshold and mechanism by which butanol triggers the accumulation of *SolB* transcripts remain unclear. Given that the coding region of *SolB* is located upstream of the *adhE2* gene, it is conceivable that the *solB* sRNA transcript acts in a cis-like manner and promptly triggers the degradation of mRNA transcripts of adjacent genes.

Furthermore, riboswitches can mediate gene control by modulating ligand binding and influencing transcriptional regulation. For example, an S-box riboswitch is involved in the regulation of sulfur metabolism in *C. acetobutylicum* (André et al., 2008). In response to sulfur concentration in the cell, activation of the S-box riboswitch induces the production of antisense RNA to regulate the transcription of sulfur utilization genes (André et al., 2008). The abundance of synthesized antisense RNAs corresponds to changes in sulfur concentrations in the cells, leading to adjustments in gene transcript levels and the activities of proteins involved in sulfur utilization (André et al., 2008). It is possible that *SolB* is transcribed because of activation of a riboswitch located at the 5′ untranslated region of the sol operon. While a direct interaction between butanol and a regulatory element on DNA is unlikely, there is a possibility that one or more intermediate regulatory protein(s) bind to the riboswitch or directly to the operator sequence on the solB promoter. Moreover, the regulatory protein(s) may either detach or bind in the presence of butanol, facilitating transcription of the *solB* gene. It is also plausible that the riboswitch/*solB* gene senses butanol concentration through the intermediary regulatory protein, leading to the production of *SolB* transcripts in response to increasing butanol levels, providing an explanation for the observed phenotype. Notably, Spo0A null mutants did not produce acetone, but rather butanol and ethanol in relatively small amounts relative to the *C. beijerinckii* wildtype (Ravagnani et al., 2002, Harris et al., 2002). These findings are consistent with the ABE production pattern in *SolB* null mutants indicating an association between *SolB* and Spo0A functions. Although, Spo0A is the master regulator of ABE production, *SolB* may serve as the regulation effector for ABE production to maintain cellular homeostasis. A comprehensive investigation of the regulation of solvent production by *solB* requires in-depth studies on the impact of butanol on the upregulation of *solB* expression. Understanding the interaction between *SolB* and

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sol locus genes during transcription and translation processes and their role in modulating CoAT activity, is vital. It may be crucial to uncover the relationships between the accumulation of butanol in the fermentation medium and the expression of *SolB* and Spo0A. This knowledge may be instrumental in applying metabolic engineering techniques to enhance the ABE production capacity of solventogenic *Clostridium* species. Additionally, insight into the influence of *SolB* on genes regulating ABE production (*hbd, bcd, bdhB, bdhA, thl*) would deepen our understanding of the regulatory mechanisms of *SolB*.

3.2. The 6S RNA and tmRNA Mediated Regulation

The 6S RNA in *E. coli* and *Bacillus subtilis* activates the transcription of stationary phase genes via inhibition of exponential phase RNA polymerase holoenzyme, while enabling the accumulation and activity of the stationary phase RNA polymerase holoenzyme (Barrick et al., 2005; Gildehaus et al., 2007; Neusser et al., 2010). Specifically, 6S RNA forms a binding interaction with σ^A (σ^{70}) RNA polymerase, thereby impeding DNA binding and repressing transcription initiated by σ^A-dependent promoters during the stationary phase. Sigma A (σ^A), commonly known as vegetative sigma factor, initiates transcription of genes associated with exponential growth phase in *Clostridium* species. Interestingly, 6S RNA is associated with stress response and survival in *E. coli* and *B. subtilis* (Trotochaud and Wassarman, 2006). While the regulatory functions of 6S RNA in *E. coli* and *B. subtilis* have been extensively studied (Wassarman, 2018), there are limited information on its role in solventogenic *Clostridium* species. Given that ABE production in solventogenic *Clostridium* species occurs predominantly during the stationary growth phase, it is crucial to investigate the regulatory functions of 6S RNA in this group of microorganisms at this phase. Likewise, further studies are needed to understand the contribution of transfer-messenger RNA (tmRNA) in restoring suppressed ribosomal functions, which play a role in cell survival under stress conditions (Shin and Price, 2007). In cases where protein synthesis is suppressed due to damaged mRNA, the tmRNA complex, consisting of tmRNA together with small protein B, ribosomal protein S1, and elongation factor EF-Tu, triggers the activation of complex factors. This activation leads to an improvement in ribosomal functions and the recycling of ribosomes, allowing ongoing protein synthesis to continue. At the same time, tmRNA serves as a marker for damaged proteins that are destined for intracellular degradation (Withey and Friedman, 2003). The multifunctional nature of tmRNA, encompassing both tRNA and mRNA functions, facilitates the recycling of ribosomes for continuous repair and protein synthesis, thereby promoting cell survival (Keiler, 2008; Craig et al., 2014).

Meanwhile, there are three homologues of the 6S RNA gene in the genome of *C. acetobutylicum* contains. Over-expression of a homolog (6S RNA) in *C. acetobutylicum* resulted in increased ABE production, growth, and cell survival under butanol stress (Jones et al., 2016). Other homologs are probably inactive in the cells. Although 6S RNA is constitutively expressed in cells, it is present in small abundances during the exponential growth phase and its accumulation is characteristic of the transition to stationary phase. The relatively larger abundance of 6S RNA in the stationary phase relative to exponential growth phase results in the repression of σ^A dependent gene transcription and other genes activated by the alternative sigma factor B (also called σ^{38} in *E. coli*). The σ^{38} is a stationary phase sigma factor that is associated with the regulation of stress response genes, which are activated during conditions such as osmotic shock, oxidative stress, and nutrient starvation of Gram-negative microbes such as *E. coli* (Sharma and Chatterji, 2010). Similarly, in Gram positive bacteria such as *B. subtillis* and *L. monocytogenes*, transcription of stress-related genes is regulated by sigma factor B **(**σ^B**),** which is more abundant when there are stressors such as heat, acids, and solvent accumulations. The σ^B protein has been detected in *C. acetobutylicum* and it is known to modulate the stress response machinery (e.g., *GrpE, DnaK DnaJ*) in the microorganisms (Venkataramanan et al., 2015; Jones et al., 2016). Additionally, the vegetative sigma factor (σ^A) in *Clostridium* species regulates initiation of sporulation during the stationary phase (see Durré, 2016 for a review on *Clostridia* sporulation). The sporulation specific σ^E and SpoIIGA genes required for initiation of sporulation contain the σ^A promoter-specific sequence along with 0A boxes consistent with regulation of transcriptional activation by both σ^A and Spo0A (Santangelo et al., 1998). Constitutive overexpression of 6S RNA,

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which inhibits σ^A-dependent promoters, could delay or inhibit onset of sporulation. Meanwhile, constitutive overexpression of tmRNA only results in increased butanol tolerance and cell survival of *C. acetobutylicum* without increasing ABE production (Jones et al., 2016). Excess tmRNAs likely trigger a stress response and subsequent protein degradation driven by the action of the σ^E (stress and extracellular protein sigma factor) proteins which lead to the activation of genes encoding proteases. These proteases target repressors of membrane proteins and ultimately cause the bacterial membrane to thicken (Craig et al., 2014; Rhodius et al., 2005; Konovalova et al., 2018). Additionally, tmRNAs might play a specific role in degradation of mRNAs that encode proteins required for substrate uptake and acid production prior to the onset of solventogenesis (Craig et al., 2014). Taken together, while elevated levels of tmRNAs in *C. acetobutylicum* improved microbial tolerance to ABE, it did not result in enhanced ABE production. The use of inductive or regulatable promoters to control tmRNA abundance may address this limitation and potentially increase ABE production.

3.3. Sr8384 Mediated Regulation of Growth and ABE Production

A recently identified type of small non-coding RNA has shown to control both growth and ABE production in *C. acetobutylicum*. Phenotypic screening of a transposon-mediated mutant library of *C. acetobutylicum* revealed the existence of a coding sequence for a novel sncRNA (sr8384) located in the intergenic region between CAC2383 and CAC2384. (Yang et al., 2020). Knockdown of *sr8384* in *C. acetobutylicum* resulted in a strain with impaired growth and poor ABE production. In fact, a 50% knockdown of sr8384 mRNA transcripts resulted in ~1.6-fold reduction in cell population and ~1.4-fold decrease in butanol titer relative to the wildtype strain with intact sr*8384* (Yang et al., 2020). Furthermore, when *sr8384* was overexpressed in *C. acetobutylicum*, the growth and ABE production capacity of the generated strain improved ~1.38- and ~1.35-fold, respectively, relative to the wildtype strain. Consequently, sr8384 was predicted to exert a regulatory influence directly or indirectly on both growth and ABE production in *C. acetobutylicum*. In addition, a homolog of *sr8384* was discovered in the genome of *C. beijerinckii* (*sr8889*) in the intergenic region between *Cbei*_1788 and *Cbei*_1789. Interestingly, the genes *Cbei*_1788 and *Cbei*_1789 in *C. beijerinckii* show homology with the CAC2385 and CAC2383 genes, respectively, in *C. acetobutylicum*. The genes code for a hypothetical protein and a polysaccharide/xylanase/chitin deacetylase, respectively. Besides, *sr8889* share a 51% nucleotide homology with *sr8384* and is predicted to be a critical regulator of growth and ABE production in *C. beijerinckii* (Yang et al., 2020). For a comprehensive understanding of the regulatory functions of sr8384 in solventogenic *Clostridium* species, it is imperative to identify sr8384 proteins/genes, target markers, transcription inducers, and their expression pattern throughout the fermentation period.

4. Bacteria Secondary Metabolites and Their Potential Role in Regulation

Secondary metabolites, often referred to as biologically active compounds, are generated by microorganisms. While these secondary metabolites typically do not have a direct impact on the growth or survival of microorganisms, they can provide protection and competitive advantage against competitive microbes, and occasionally play a role in cell-to-cell signaling (Gokulan et al., 2014). Secondary metabolites are mostly species specific, and their production can occur in response to environmental stress such as extreme pH, temperature, nutrient deficiency, etc. Secondary metabolites in bacteria are synthesized via the β-lactam, oligosaccharide, shikimate, polyketide, and non-ribosomal pathways (Gokulan et al., 2014). Polyketides (PKs) and non-ribosomal peptides (NRPs) are the most studied chemically diverse families of secondary metabolites in bacteria. While polyketides are synthesized by polyketide synthases (PKSs), NRPs are produced when non-ribosomal peptide synthetases (NRPSs) are activated. Antimicrobial agents such as endo- and exo-toxins are examples of secondary metabolites, and some bacteria including the genus, *Clostridium*, produce secondary metabolites. Most prominent is the botulinum toxin synthesized by *Clostridium botulinum*, which is of clinical and cosmetic importance. The production of bioactive compounds, including antibiotics and lantibiotics, by *Clostridium* species has been reviewed by Pahalagedara et al. (2020). Solventogenic *Clostridium* species produce secondary metabolites in addition to ABE. For

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example, *C. beijerinckii* produce antibiotic clostrubin, which is active against methicillin- and vancomycin-resistant bacteria (Yang et al., 2015).

Previous research suggests the existence of secondary metabolites that serve as signal transduction molecules that may trigger important processes such as sporulation and ABE formation in solventogenic *Clostridium* species (Li et al., 2019 Alsaker et al., 2004; Herman et al., 2017). Genome mining efforts have revealed that *Clostridium* species possess unique potential for polyketide synthesis (Li et al., 2019). Furthermore, transcriptome analysis of *C. acetobutylicum* revealed the presence and expression of the type I modular polyketide synthase (pks) gene. There was a notable surge in the transcription of type I modular polyketide synthase (pks) gene during the early stationary growth phase (Alsaker et al., 2004). In vitro studies revealed that pks is responsible for the production of three polyketide compounds (clostrienoic acid, unidentified polyketide and clostrienose) from the condensation of malonyl coenzyme A (Herman et al., 2017). The *C. acetobutylicum pks* null mutant had a significantly delayed sporulation pattern, reduced granulose accumulation, and produced more butanol than the wildtype strain, although both strains had similar growth profiles (Herman et al., 2017). Remarkably, the addition of clostrinose to the medium resulted in a resurgence of granulose accumulation, overcoming its previous suppression (Herman et al., 2017). Polyketide compounds may have a broader presence and play more important role in regulating processes within solventogenic *Clostridium* species than previously thought. Hence, they represent promising targets for metabolic engineering aimed at delaying sporulation and increasing the duration of ABE production in these species.

4.1. Non-Ribosomal Peptides (NRPs) Mediated Regulation

The genome analysis of *C. saccharoperbutylacetonicum* N1-4 revealed its capacity to produce polyketides and non-ribosomal peptide secondary metabolites (Li et al., 2020). Additionally, bioinformatics investigation revealed the presence of seven biosynthesis gene clusters (nrps1-4 and hyb1-3; Li et al., 2020). In addition, transcriptomics analysis confirmed the expression of five of these gene clusters in *C. saccharoperbutylacetonicum* N1-4 during fermentation (Li et al., 2020). The *nrps*3 gene has been reported to be widely expressed and conserved in other solventogenic *Clostridium* species (Li et al., 2020). This gene codes for an N-acylated dipeptidyl alcohol thought to be involved in microbial tolerance to ABE (Li et al., 2020). Indeed, the *C. saccharoperbutylacetonicum* N1-4 *nrps*3 null mutant had a defective exponential growth pattern in medium supplemented with butanol. Transcriptomics analysis of the *nrps*3 null mutant revealed a downregulation of the glycerol metabolism operon. Despite the inability of *C. saccharoperbutylacetonicum* N1-4 to use glycerol as sole carbon source, it has been suggested that the glycerol metabolism operon contributes to ABE tolerance (Li et al., 2020).

Typically, bacteria synthesize glycerol to facilitate the production of glycerophospholipids, which are essential for cell membrane formation. Consequently, gene activation of glycerol metabolism is essential during the bacterial growth process. The glycerol metabolism operon *Dha*KLM in *C. beijerinckii* is predicted to be regulated by a σ^{54} dependent promoter activated by the dhaQ EBP (Nie et al., 2019). Thus, *nrp*3 could serve as an EBP activator signal for transcriptional activation of genes that regulate glycerol metabolism.

5. Potential Target Points for Metabolic Engineering

Metabolic network proteins in solventogenic *Clostridium* species are tightly regulated as each regulatory molecule is associated with a different process and ultimately influences ABE production directly or indirectly (**Figure 2**). Throughout the diverse stages of growth in solventogenic *Clostridium* species and ABE production, there appear to be regulatory controls and equilibria in place. These mechanisms likely evolved to counteract potential adverse effects in the event of regulatory perturbations during different phases of ABE fermentation and ultimately ensure cell survival. The toxicity of butanol to solventogenic *Clostridium* species is probably the main reason for the tight regulation of ABE production. Hence, the intricately interactive regulatory proteins and RNA

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networks involved in ABE production make metabolic engineering of solventogenic *Clostridium* species challenging.

Research efforts are currently underway to identify new regulatory molecules and to gain an in-depth understanding of the functions of existing regulatory molecules in solventogenic *Clostridium* species during ABE fermentation. Findings from overexpression and knockout experiments involving genes responsible for regulatory molecules such as Spo0A, AbrB, CcpA, Rex, and CsrA suggest a trade-off scenario. This trade-off entails a balance between production-oriented phenotypes (e.g., increased population growth and ABE production) and survival-oriented phenotypes (e.g., ABE tolerance, stress response adaptation). Indeed, investigations into protein/RNA regulatory networks are starting to offer insights into important molecular markers and crucial molecular interactions that influence ABE production. These discoveries are significant for identifying promising targets for metabolic engineering, particularly when integrated into a comprehensive approach. A thorough understanding of the regulatory networks involving EBPs/sigma factor 54, sRNAs and secondary metabolites can be used to metabolically manipulate solventogenic *Clostridium* species to generate industrial strains with desirable phenotypes. Some potentially beneficial strategies are described in this review.

5.1. EBP/Sigma Factor 54 as Potential Targets for Metabolic Engineering

Sigma factors play a crucial and precise role in the regulation of gene transcription due to their ability to influence a series of cascading processes that can lead to either desirable or undesirable microbial phenotypes. The functional integrity of sigma factors depends on their sequence integrity. Direct alteration of the *sigL* gene that encodes sigma factor 54 (σ^{54}), could therefore be counterproductive. Thus, altering enhancer binding proteins that control σ^{54}-dependent transcription could be an effective target for modulation. The EBP regulatory domains can be modified so that they are non-responsive to gene repressors. For example, EBPs of non-preferred sugar PTS operons are regulated by proteins of the PTS (Hpr, EI or EIIA/B) at the PTS regulatory domains (PRD). According to Nie et al. (2016), activated CelR and σ^{54} directly regulate the transcription of the *Cel* operon which is involved in cellobiose utilization. The P~His-HPr (Hpr protein phosphorylated on histidine residue) phosphorylation of His-551 activates CelR, while P~EIIB^{Cel} (phosphorylated cellobiose-specific EIIB protein) phosphorylation on His-829 in PRD2 inactivates CelR. In addition to this regulation, binding of CcpA-Hpr-Ser46 to the *Cre* site within the coding region results in repression of target genes. This results in non-preferred sugars either remaining unused, being utilized only after preferred sugars are exhausted, or utilized to a limited degree—a phenomenon known as Carbon Catabolite Repression (CCR). Two possible approaches are necessary to counteract the repression of genes responsible for encoding proteins/enzymes involved in non-preferred sugar utilization and abolish CCR without interfering with other processes regulated by CcpA (**Figure 2**). To alleviate the repression of genes responsible for non-preferred sugar utilization and eliminate CCR without impacting other CcpA-regulated processes, two potential mechanisms are considered. First, the interaction of CcpA with the typical *Cre* site hinges on its interaction with Hpr-ser46. This interaction can be disrupted by either suppressing phosphorylation of Hpr's ser46 or targeting its CcpA interaction domain, thereby preserving the structural integrity of CcpA for regulation of processes that are not connected to CCR (**Figure 1**). Second, the need for P~His-HPr phosphorylation on His-551 that trigger CelR activation can be bypassed by modifying CelR's His-551, rendering it constitutively active. Even in the presence of glucose, CelR remains active and ensures the transcription of the *Cel* operon genes. A process akin to desensitization of CelR to P~His-HPr phosphorylation can be extended to other PTS/non-PTS sugars or non-sugars. This will help in the simultaneous utilization of diverse sugars (e.g., hydrolysates from lignocellulosic biomass) to increase ABE production. Moreover, identifying activators/repressors of EBPs is crucial for understanding how they can be modified to resist repression, thereby promoting a well-regulated expression of sugar transports and utilization genes and ABE production. Delineating the relationship between EBPs and CcpA and how this association affects σ^{54}-dependent transcription

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can be a useful strategy in developing effective metabolic engineering approaches for enhanced ABE production (**Figure 1**, **Table 2**).

5.2. sRNAs as Potential Targets for Metabolic Engineering

Given that knockout and overexpression of *SolB* in solventogenic *Clostridium* species typically result in the production of strains that produce relatively reduced amounts of ABE, metabolic manipulation of *SolB* through interference with butanol sensors will be likely more feasible. If *SolB* is an sRNA produced as part of a riboswitch-mediated regulation, then the intermediary regulatory protein(s) can be engineered to reduce sensitivity to butanol. Decreased sensitivity leads to a decrease in *SolB* RNA transcripts, resulting in maintenance of expression of ABE formation genes. Metabolic engineering of *SolB* can be performed in conjunction with overexpression of tmRNA. Accumulated ABE can influence mRNA structures, leading to inhibited ribosomal functions and misfolding of proteins, and ultimately impacting the viability of solventogenic *Clostridium* species. Overexpression of tmRNA could potentially boost the ability of solventogenic *Clostridium* species to revive ribosomal functionality and improve cellular responses to disrupted functions. This metabolic engineering strategy can lead to the development of strains with enhanced ABE production. Understanding the scope of SolB regulation necessitates a thorough investigation into its effects on non-sol operon ABE formation genes. Furthermore, the recently identified sr8384 represents a promising target marker molecule that warrants further investigation. Examining the direct or indirect target(s) of sr8384 and elucidating its induction mechanism would offer valuable insights into the role of sr8384 in regulating ABE production in solventogenic *Clostridium* species.

5.3. Secondary Metabolites as Potential Targets for Metabolic Engineering

Recent evidence has shown that solventogenic *Clostridium* species produce metabolites that are of medicinal importance (Li et al., 2020; Pahalagedara et al., 2020; Pahalagedara et al., 2021; Lincke et al., 2010). The understanding that solventogenic *Clostridium* species possess the capability to produce secondary metabolites with potential medical significance is emerging. The potential role of these secondary metabolites or compounds in regulating ABE production could spark a new avenue of exploration in the study of solventogenic *Clostridium* species and ABE production. Apart from metabolic engineering for enhanced ABE production, conferring cellular resistance to LDMICs present in LBH may be of great importance for the development of industrially applicable strains. For example, the synthesis of lantibiotics and immunity proteins from solventogenic *Clostridium* species can be exploited for metabolic engineering to generate ABE- and LDMICs-tolerant strains. Polyketide synthesis factors in solventogenic *Clostridium* species can also be engineered to delay sporulation and prolong the duration of ABE production.

6. Conclusions

Solventogenic *Clostridium* species are gaining increasing interest in biotechnology due to their inherent ability to metabolize a wide variety of substrates to produce fuels and chemicals. To ensure sustainability in the energy, transportation, and chemical sectors, it is essential to develop sustainable raw materials with environmentally compatible technologies to produce fuels and chemicals. To achieve this goal, regulation of processes in fermenting microorganisms such as solventogenic *Clostridium* species during growth and ABE fermentation is essential. Although much has been achieved to gain a better understanding of the complex regulatory networks in this group of microorganisms, there are still knowledge gaps that need to be filled to piece together the physiological solventogenic functions of *Clostridium* species. Targeted metabolic engineering and development of solventogenic *Clostridium* species, therefore, needs to be a priority if there is to be efficacious production of fuels and chemicals, which may not be possible without a comprehensive understanding of the physiology of this group of microorganisms. Clearly, understanding the regulatory molecules and networks highlighted in this review can lead to the development of

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effective metabolic engineering approaches in solventogenic *Clostridium* species to sustainably produce fuels and chemicals.

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