

Review

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

Tinuola Olorunsogbon ¹, Christopher Chukwudi Okonkwo ^{1,2} and Thaddeus Chukwuemeka Ezeji ^{1,3,*} 

¹ Department of Animal Science, College of Food Agricultural and Environmental Sciences, The Ohio State University, Wooster, OH 44691, USA; olorunsogbon.1@buckeyemail.osu.edu (T.O.); c.okonkwo@northeastern.edu (C.C.O.)

² Biotechnology Program, College of Science, The Roux Institute, Northeastern University, 100 Fore Street, Portland, ME 04103, USA

³ Department of Food Agricultural and Biological Engineering, College of Food Agricultural and Environmental Sciences, The Ohio State University, 1680 Madison Avenue, Wooster, OH 44691, USA

* Correspondence: ezeji.1@osu.edu; Tel.: +1-330-263-3796; Fax: +1-330-263-3949

Abstract: Solventogenic *Clostridium* species are important for establishing the sustainable industrial bioproduction of fuels and important chemicals such as acetone and butanol. 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 the production of fuels and chemicals. The functional qualities of these microbes have thus been identified to be related to complex regulatory networks that play essential roles in modulating the metabolism of this group of bacteria. Yet, solventogenic *Clostridium* species still struggle to consistently achieve butanol concentrations exceeding 20 g/L in batch fermentation, primarily due to the toxic effects of butanol on the culture. Genomes of solventogenic *Clostridium* species have a 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 for substrate utilization, solvent production, and resistance/tolerance to lignocellulose-derived microbial inhibitory compounds. Taken together, this review is anticipated to highlight the challenges impeding the re-industrialization of ABE fermentation, and inspire researchers to generate innovative strategies for overcoming these obstacles.

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



Citation: Olorunsogbon, T.; Okonkwo, C.C.; Ezeji, T.C. Molecular Markers and Regulatory Networks in Solventogenic *Clostridium* Species: Metabolic Engineering Conundrum. *Fermentation* **2024**, *10*, 297. <https://doi.org/10.3390/fermentation10060297>

Academic Editor: Christian Kennes

Received: 15 March 2024

Revised: 28 May 2024

Accepted: 29 May 2024

Published: 4 June 2024



Copyright: © 2024 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

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 [1–4]. 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 [5]. The LDMICs such as furan aldehydes (e.g., furfural and hydroxymethylfurfural; HMF) and phenolics

(hydroxybenzaldehyde, ferulic acid, syringic acid, etc.) affect the growth and sugar utilization capacity of fermenting microorganisms such as *Clostridium* species, resulting in reduced growth and product yields [6,7]. To overcome these limitations, considerable research endeavors have investigated both direct and indirect approaches targeting various factors within the ABE production network of molecules [8–10]. 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 terms of habitat/ecological niche, substrate utilization, primary and secondary metabolite secretions, and morphological structures. These variations are regulated mostly by a network of complex but precise 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. carboxydovorans*), which produce a range of products by fermenting a variety of carbohydrates [11,12]. A common feature of the *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 [13]. Others can be found in habitats like waterbodies and food/industrial waste dumps [13]. *Clostridium* species can be utilized to produce cosmetics and 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 [14]. Some *Clostridium* strains can selectively target cancer cells (e.g., *C. nolyvi*-NT, *C. sporogenes*, and *C. sordellii*), with the rare capacity to penetrate and/or replicate within solid tumors [15]. 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 [16,17].

In addition to the use of *Clostridium* species in cosmetics and medicine, they can convert a range of substrates into fuels and bulk chemicals such as acetone, butanol, ethanol, acetic acid, butyric acid, isopropanol and 1,3-propanediol (Table 1). In the early 19th century, solventogenic *Clostridium* species were used for the industrial production of acetone and butanol. Unfortunately, the discovery of a 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 industrial 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 [18]. 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 Mb 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.

<i>Clostridium</i> Species	Substrates	Products	References
<i>C. acetobutylicum</i>	Glucose, xylose, arabinose, cellobiose	Acetone, butanol, and ethanol	[19]
<i>C. beijerinckii</i>	Glucose, xylose, arabinose, cellobiose	Acetone, butanol, and ethanol	[19]
<i>C. thermocellum</i>	Lignocellulose, Cellulose	Ethanol	[20]
<i>C. butyricum</i>	Glycerol	1,3-propanediol	[21]
<i>C. Pasteurianum</i>	Glycerol	1,3-propanediol, butanol and ethanol	[22]
<i>C. carboxidivorans</i>	Carbon monoxide, hydrogen, and carbon dioxide	Ethanol, butanol and hexanol	[11]
<i>C. aurantibutyricum</i>	Glucose, lactose, maltose, galactose, xylose, starch	Acetone, isopropanol, and butanol	[23]
<i>C. ljundahlii</i>	Carbon monoxide and hydrogen	Ethanol	[24]
<i>C. tetanomorphum</i>	Glucose and maltose, glutamate	Butanol and ethanol	[25]
<i>C. saccharoperbutylacetonicum</i>	Glucose	Acetone, ethanol, and butanol	[26]

Solventogenic *Clostridium* species are a non-pathogenic group of microorganisms that belong to the *Clostridium* genus and 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. ljundahl*, the wood–ljungdahl pathway is triggered to produce ethanol from synthesis gas during solventogenesis [24,27]. In *C. tetanomorphum*, the methylaspartate pathway for glutamate conversion to acetate, butyrate, and carbon dioxide is activated [28]. In *C. acetobutylicum* and *C. beijerinckii*, both glycolytic (Embden–Meyerhof pathway) and acid production pathways are active for the concurrent uptake of glucose and acids production during the exponential growth phase [29]. 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 have focused on random mutagenesis and mutant selection [30], the modulation of acid and ABE production (*adh*, *pta*, *ptb*, *buk*, *ack*, *bdh*, *ald*, etc.), stress response (*groES*, *groEL*, *DnaK*, *DnaJ*), and LDMIC detoxification genes [9]. Some other indirect approaches focused on improving redox balance through NAD(P)H regeneration, calcium and acetate supplementations, and process and media optimizations to enhance butanol production [7,10,31,32]. Despite over a century of ABE fermentation by solventogenic *Clostridium* species, which are considered the most efficient butanol-producing microorganisms, neither wild-type nor engineered microorganisms have been able to consistently achieve butanol concentrations exceeding 20 g/L in batch fermentation. 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 the metabolic engineering of these microorganisms [33], 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 [34–38]. The redox potential regulator (Rex) and the carbon storage regulator A (CsrA) proteins also play a role in solvent production in *Clostridium* species [39,40].

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. After attaining a thorough understanding of the mechanisms within solventogenic *Clostridium* species, strategic approaches could be formulated to address many challenges impeding the industrialization and re-industrialization of butanol and 1,3-propanediol/isopropanol, respectively. These challenges include strain degeneration, carbon catabolite repression during growth in mixed sugars, as well as issues concerning poor product concentration, yield, and productivity [41–43]. This review specifically addresses the role of sigma factors, small non-coding RNAs (sRNAs) and secondary metabolites in the regulation of growth and ABE production by solventogenic *Clostridium* species. 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 [3]. 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 [44,45]. The sigma factor in the RNA polymerase holoenzyme controls the tight binding of the core RNA polymerase to the promoter to initiate transcription [46]. 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 [44,47]. 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 [48–50].

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* [51]. 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 [48–50]. Unlike σ^A , σ^{54} binds strongly to the conserved consensus sequence at the GG –24 and TGC –12 promoter elements [52]. 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* [48]. 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 [50,51]. 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 the isomerization of the closed RNAP- σ^{54} complex to form an open complex [53,54]. The EBP binds to upstream activator sequences (UASs) and forms higher-order oligomers that lead to the 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 [55]. The EBPs are grouped based on regulatory domains involved in sensing environmental or cellular signals (Table 2).

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
<i>C. acetobutylicum</i> ATCC 824	3	PRD-EIIA-PRD (1), PrpR-N-PAS (1), PTS_Hpr-PAS-PAS (1)	2
<i>C. autoethanogenum</i> DSM 10061	12	PAS-PAS (1), GAF-PAS (6), PAS (3) 0 (2)	-
<i>C. beijerinckii</i> NCIMB 8052	19	PRD-EIIA-PRD (12), CBS-PAS (1), PTS_Hpr-PAS-PAS (1), PAS-PAS (2), GAF-PAS (2), RR (1)	26
<i>C. carboxydovorans</i> 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)	-
<i>C. ljungdahlii</i>	19	PrpR-N-PAS (1), PAS-PAS (1), GAF-PAS (10), PAS (5), 0 (2)	-
<i>C. saccharobutylicum</i> DSM 13864	5	PRD-EIIA-PRD (3), GAF-PAS (2)	-
<i>C. saccharoperbutylacetonicum</i> 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, cystathionine β -synthase [51].

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. saccharoperbutylacetonicum*, *C. carboxydovorans*, and *C. ljungdahlii*. This suggests that σ^{54} plays an important role in the regulation of sugar metabolism and ABE production in solventogenic *Clostridium* species [48,51]. 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, these being PTS proteins, via phosphorylation/dephosphorylation [56]. 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 [49,50]. In contrast to *sigL* overexpression, *AdhR* overexpression resulted in poor cell growth and ABE production [50]. 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 the 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 the 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* the 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. The presence of EBPs and the diverse regulatory domains suggest some important functions of EBPs in signal transduction and the regulation of sugar metabolism and ABE production (Figure 1).

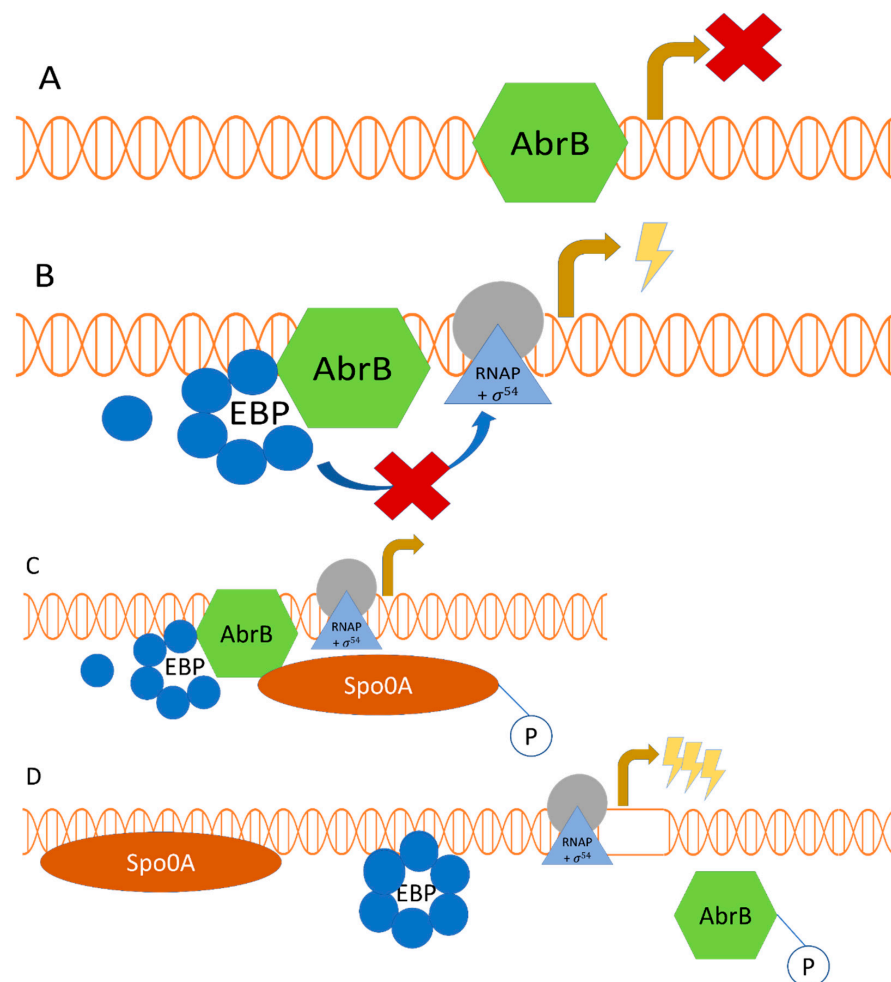


Figure 1. Proposed mechanism of regulation of ABE production by Spo0A, AbrB and EBP/sigma 54. (A) Acid re-assimilation (*ctfAB*) and acetoacetate decarboxylase (*adc*) genes with promoters that are not sigma 54-dependent are completely inhibited by AbrB and no transcription occurs in the absence of Spo0A. (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,D) Phosphorylated Spo0A inactivates AbrB through phosphorylation. Phosphorylated AbrB is released from bound DNA, and 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 the 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 the simultaneous utilization of hexose and pentose sugars because these sugars are significant components of the lignocellulosic biomass (LB) hydrolysates (LBH). CCR occurs when the presence of a preferred carbon source (usually glucose) in the growth medium induces the transcriptional repression of genes encoding proteins required for the uptake and metabolism of non-preferred sugars (e.g., xylose, arabinose, galactose, cellobiose, sucrose etc.) [37]. 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 the transcription of *sol* operon, *adhA1*, *adhA2*, *crt*, *thl*, *bcd*, *etfAB*, and *hbd*, which are genes involved in ABE formation [57,58]. 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) [48]. While no conventional *cre* site has been identified in genes regulating ABE production (e.g., *sol* operon, *adhA1*, *adhA2*, *crt*, *thl*, *bcd*, *etfAB*, *hbd*), their expression is downregulated in CcpA null mutants [33,57]. This finding indicates that *cre* is regulated by CcpA. However, sugar uptake and genes involved in ABE production are regulated to some extent by the σ^{54} regulon with EBPs in *C. beijerinckii* [48,51]. 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?

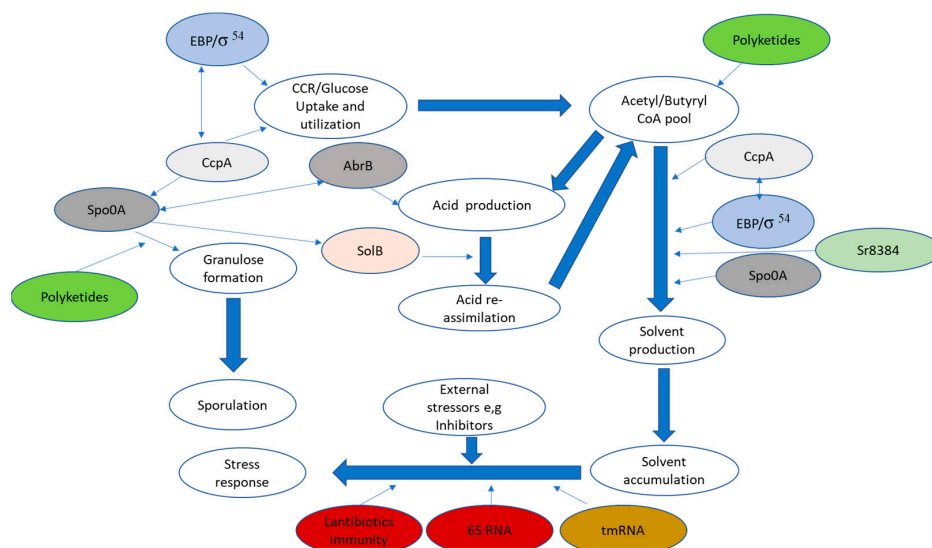


Figure 2. Reconstructed pathway of ABE production in solventogenic *Clostridium* species showing regulatory points. Colored shapes indicate regulatory molecules, white shapes indicate metabolic processes.

The genes of the *Cel* operon and most sugar uptake/utilization operons contain the typical *cre* site (WTGWAAACGWTWWCAW: W is A or T) in the coding region. The CcpA-dependent CCR of genes encoding proteins that modulate the 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 [56,59]. 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* (*adhA1*, *adhA2*, *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—TGTAAGTTGCTATTTACA, 3—TATTGTAAACCTTGTTTTGTTTTGCAGTTTACAATA) upstream (within −350 to −1 base pairs proximal to transcription start site) of the *sol* operon promoter in *C. acetobutylicum* in a regulatory manner [58]. 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 shows that CcpA is constitutively expressed in *C. acetobutylicum* and *C. beijerinckii* during ABE fermentation [60,61]. 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 **TGTAAG/TTTACA** with an intervening sequence of variable length [58]. 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 [48,50]. 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 [51]. 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 the repression or activation of gene transcription. The binding of EBPs to the three UASs may be required for the activation and modulation of target gene transcription [48,50]. Competitive binding of CcpA to one or more of these sites could either distort or enhance (possibly depending on position) the 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 the 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 σ^{54} -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* [36]. *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 homologs of the *AbrB* gene (*AbrB1941*, *AbrB0310* and *AbrB3647*) are active in regulating different genes in *C. acetobutylicum* [36,62]. 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*) [62]. Although there are σ^{54} -dependent promoters and associated EBPs that modulate genes of *C. acetobutylicum* involved in ABE production, orthologs of these genes (*adhA1*, *adhA2*, *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) [51]. Although 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 [33]. 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 [62] 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 the interaction of EBP with the RNA polymerase holoenzyme. The AbrB possibly competes for one or more UASs, thereby 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) [63–66]. The activation is facilitated by Spo0A binding to the 0A boxes upstream of the promoters of genes involved in ABE production. Data from functional analyses of Spo0A provide insights that Spo0A is a negative regulator of AbrB [34]. Because the onset of ABE production coincides with the onset of sporulation in solventogenic *Clostridium* species, the deletion of *Spo0A* in *C. beijerinckii* resulted in a strain with lesser capacity to produce butanol and ethanol with no ability for acetone production relative to the wild type [63,67]. 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 an impaired ability to produce acetone since the acetoacetate decarboxylase (*adc*) gene's expression is regulated by Spo0A [68]. The Adc protein is recognized for facilitating the transformation of acetoacetate into acetone and CO₂. Nevertheless, research has revealed that the inactivation of the *adc* gene in *C. beijerinckii* and *C. acetobutylicum* does not completely abolish acetone production, owing to an operational non-enzymatic acetoacetate decarboxylation route for acetone production in these organisms [69,70]. 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 [51]. The production of butanol and ethanol (although in low concentrations) when there was Spo0A inactivation could be due to the 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 [71]. 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 modulates 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 the formation of the higher-order oligomeric structure, which promotes transcription activation (Figure 1). The transient binding of Spo0A may also result in the 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 the 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 [72]. 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) [73]. The sRNAs are usually located at intergenic regions, which are independently transcribed or are nucleolytically cleaved from the 3' untranslated region of mRNAs [74,75]. 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 a catalysis for the associated proteins, or inhibit their normal functions [76,77]. 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 [78,79]. 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 [73] 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 [80] (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* 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 [81]. It is worth noting that only a small proportion of the validated sRNAs have been subjected to experimental characterization [50,81–85].

Table 3. Number of sRNAs identified from different *Clostridium* species adapted from [80].

<i>Clostridium</i> Species	Number of sRNA Identified in Genome	Genome Size (mb)	GC Content (%)	Metabolism Type
<i>Clostridium acetobutylicum</i> ATCC 824	113	4.13	30.90	Saccharolytic
<i>C. beijerinckii</i> NCIMB 8052	366	6.00	29.90	Saccharolytic
<i>C. cellulolyticum</i> H10	45	4.07	37.40	Cellulolytic
<i>C. kluyveri</i> DSM 555	126	4.02	32.02	-
<i>C. kluyveri</i> NBRC 12016	136	3.96	32.02	-
<i>C. phytofermentans</i> ISDg	42	4.85	35.30	Cellulolytic
<i>C. thermocellum</i>	15	3.84	39.00	Cellulolytic
<i>C. carboxidivorans</i> p7	NA	5.75	29.89	-

NA; not available.

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* [84]. The *solB* coding region lies upstream of the *adhE2* gene on the *sol* operon as a 196 bp sequence [86]. Data from transcriptomic analysis indicated that *solB* was upregulated specifically during butanol stress [87]. The overexpression of *solB* in solventogenic *Clostridium* species induces downregulation at the *sol*-locus leading to modulation in the rate of gene transcription [88]. While the repression of the *sol* operon by *solB* results in longer cell viability, it also leads to reduced ABE production [88]. Interestingly, coenzyme A transferase (CoAT) activity *was* abolished in *solB* knockout mutants, resulting in the complete loss of acetone production with a marked decrease in butanol production [88]. 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 the *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 shown that *solB* transcripts are present in low quantities in *C. acetobutylicum* wildtype during the acidogenic phase, followed by a remarkable increase in expression as fermentation progresses [87,88]. The 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 [88]. Conversely, the deletion of *solB* impacted the expression of *ctfAB* and *adhE1*, but did not affect *adc* expression [88]. 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. This 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 via 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 the butanol titer reaches a toxic threshold. Wang et al. [87] noted that *solB* expression increased following the 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* [89]. In response to sulfur concentration in the cell, the activation of the S-box riboswitch induces the production of antisense RNA to regulate the transcription of sulfur utilization genes [89]. 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 [89]. It is possible that *solB* is transcribed because of the 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 the 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 [63,67]. 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 *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 the inhibition of exponential-phase RNA polymerase holoenzyme, while enabling the accumulation and activity of the stationary-phase RNA polymerase holoenzyme [90–92]. 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 the transcription of genes associated with the exponential growth phase in *Clostridium* species. Interestingly, 6S RNA is associated with stress response and survival in *E. coli* and *B. subtilis* [93]. While the regulatory functions of 6S RNA in *E. coli* and *B. subtilis* have been extensively studied [85], there is 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 [94]. 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 [95]. 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 [82,83].

Meanwhile, there are three homologs of the 6S RNA gene in the genome of *C. acetobutylicum*. The overexpression of a homolog (6S RNA) in *C. acetobutylicum* resulted in increased ABE production, growth, and cell survival under butanol stress [96]. 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 the stationary phase. The relatively larger abundance of 6S RNA in the stationary phase relative to the 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* [97]. Similarly, in Gram-positive bacteria such as *B. subtilis* and *L. monocytogenes*, the 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 is known to modulate the stress response machinery (e.g., *GrpE*, *DnaK* *DnaJ*) in the microorganisms [96,98]. Additionally, the vegetative sigma factor (σ^A) in *Clostridium* species regulates the initiation of sporulation during the stationary phase (see [99] for a review on *Clostridia* sporulation). The sporulation-specific σ^E and SpoIIGA genes required for the initiation of sporulation contain the σ^A promoter-specific sequence along with O_A boxes consistent with the regulation of transcriptional activation by both σ^A and Spo0A [100]. The constitutive overexpression of 6S RNA, which inhibits σ^A -dependent promoters, could delay or inhibit the onset of sporulation. Meanwhile, the constitutive overexpression of tmRNA only results in the increased butanol tolerance and cell survival of *C. acetobutylicum* without increasing ABE production [96]. 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 [83,101,102]. Additionally, tmRNAs might play a specific role in the degradation of mRNAs that encode proteins required for substrate uptake and acid production prior to the onset of solventogenesis [83]. Taken together, while elevated levels of tmRNAs in *C. acetobutylicum* improved microbial tolerance of ABE, they 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 been shown to control both growth and ABE production in *C. acetobutylicum*. The 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 [50]. The 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 a ~1.6-fold reduction in cell population and a ~1.4-fold decrease in butanol titer relative to the wildtype strain with intact sr8384 [50]. 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 this, sr8889 shares a 51% nucleotide homology with sr8384, and is predicted to be a critical regulator of growth and ABE production in *C. beijerinckii* [50]. 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 a competitive advantage against competitive microbes, and occasionally play a role in cell-to-cell signaling [103]. 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 [103]. Polyketides (PKs) and non-ribosomal peptides (NRPs) are the most widely 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. [104]. Solventogenic *Clostridium* species produce secondary metabolites in addition to ABE. For example, *C. beijerinckii* produces antibiotic clostrubin, which is active against methicillin- and vancomycin-resistant bacteria [105].

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 [60,106,107]. Genome mining efforts have revealed that *Clostridium* species possess unique potential for polyketide synthesis [106]. 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 *pks* gene during the early stationary growth phase [60]. In vitro studies revealed that PKS is responsible for the production of three polyketide compounds (clostrienic acid, unidentified polyketide and clostrienose) from the condensation of malonyl coenzyme A [107]. The *C. acetobutylicum* *pks* null mutant showed a significantly delayed sporulation pattern, reduced granulose accumulation, and produced more butanol than the wildtype strain, although both strains had similar growth profiles [107]. Remarkably, the addition of clostrienose to the medium resulted in a resurgence of granulose accumulation, overcoming its previous suppression [107]. Polyketide compounds may have a broader presence and play a 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.

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 [108]. Additionally, bioinformatics investigation revealed the presence of seven biosynthesis gene clusters (*nrps1-4* and *hyb1-3*) [108]. In addition, transcriptomics analysis confirmed the expression of five of these gene clusters in *C. saccharoperbutylacetonicum* N1-4 during fermentation [108]. The *nrps3* gene has been reported to be widely expressed and conserved in other solventogenic *Clostridium* species [108]. This gene codes for an N-acylated dipeptidyl alcohol thought to be involved in microbial tolerance of ABE [108]. Indeed, the *C. saccharoperbutylacetonicum* N1-4 *nrps3* null mutant showed a defective exponential growth pattern in medium supplemented with butanol. Transcriptomics analysis of the *nrps3* null mutant revealed a downregulation of the glycerol metabolism operon. Despite the inability of *C. saccharoperbutylacetonicum* N1-4 to use glycerol as the sole carbon source, it has been suggested that the glycerol metabolism operon contributes to ABE tolerance [108].

Typically, bacteria synthesize glycerol to facilitate the production of glycerophospholipids, which are essential for cell membrane formation. Consequently, the gene activation of glycerol metabolism is essential during the bacterial growth process. The glycerol metabolism operon *DhaKLM* in *C. beijerinckii* is predicted to be regulated by a σ^{54} -dependent promoter activated by the *dhaQ* EBP [51]. Thus, *nrp3* could serve as an EBP activator signal for the 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 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. The 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 EIIB/B) at the PTS regulatory domains (PRD). According to Nie et al. [48], 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, the binding of CcpA–Hpr-Ser46 to the *Cre* site within the coding region results in the 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 the phosphorylation of Hpr's ser46 or targeting its CcpA interaction domain, thereby preserving the structural integrity of CcpA for the regulation of processes that are not connected to CCR (Figure 1).

Second, the need for P~His-HPr phosphorylation on His-551 that triggers 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 the 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-transport and -utilization genes and ABE production. Delineating the relationship between EBPs and CcpA, and how this association affects σ^{54} -dependent transcription, 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 the knockout and overexpression of *solB* in solventogenic *Clostridium* species typically result in the production of strains that produce relatively reduced amounts of ABE, the metabolic manipulation of *solB* through interference with butanol sensors will likely be 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 the maintenance of expression of ABE formation genes. The metabolic engineering of *solB* can be performed in conjunction with the overexpression of tmRNA. Accumulated ABE can influence mRNA structures, leading to inhibited ribosomal functions and the misfolding of proteins, and ultimately impacting the viability of solventogenic *Clostridium* species. The 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 [104,108–110]. 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 attention 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, the regulation of processes

in fermenting microorganisms such as solventogenic *Clostridium* species during growth and ABE fermentation is essential. Although much has been achieved in gaining 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. The 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 effective metabolic engineering approaches in solventogenic *Clostridium* species to sustainably produce fuels and chemicals.

Funding: This work was funded by the National Science Foundation Cellular & Biochemical Engineering program (Award Number: 1803022), USDA NIFA Hatch grant (Project No. OHO01333), CFAES SEED and U.S. Department of Agriculture under the award number 2014-38502-22598 through South Central Sun Grant Program USDA-NIFA Project number OKL03163.

Acknowledgments: The authors express their gratitude to James Kinder from the Department of Animal Sciences at The Ohio State University for reviewing the manuscript and offering invaluable feedback.

Conflicts of Interest: The authors declare no conflict of interest.

References

- Ezeji, T.C.; Atiyeh, H.; Mariano, A.P.; Rakshit, S.K. Innovative bioconversion of non-food substrates to fuels. *Front. Bioeng. Biotechnol.* **2023**, *11*, 1163513. [\[CrossRef\]](#) [\[PubMed\]](#)
- Panahi, S.H.K.; Dehghani, M.; Guillemain, G.J.; Okonkwo, C.C.; Kinder, J.E.; Ezeji, T.C. Biobutanol Production: Scope, Significance, and Applications. In *Advances in Pollution Research, Advances and Developments in Biobutanol Production*; Segovia-Hernandez, J.G., Behera, S., Sanchez-Ramirez, E., Eds.; Woodhead Publishing: Sawston, UK, 2023; pp. 1–45. ISBN 9780323911788. [\[CrossRef\]](#)
- Zhang, Y.; Ezeji, T.C. Transcriptional analysis of *Clostridium beijerinckii* NCIMB 8052 to elucidate role of furfural stress during acetone butanol ethanol fermentation. *Biotechnol. Biofuels* **2013**, *6*, 66. [\[CrossRef\]](#) [\[PubMed\]](#)
- Okonkwo, C.C.; Azam, M.M.; Ezeji, T.C.; Qureshi, N. Enhancing ethanol production from cellulosic sugars using *Scheffersomyces (Pichia) stipitis*. *Bioprocess Biosyst. Eng.* **2016**, *39*, 1023–1032. [\[CrossRef\]](#) [\[PubMed\]](#)
- Ezeji, T.; Qureshi, N.; Blaschek, H.P. Butanol production from agricultural residues: Impact of degradation products on *Clostridium beijerinckii* growth and butanol fermentation. *Biotechnol. Bioeng.* **2007**, *97*, 1460–1469. [\[CrossRef\]](#) [\[PubMed\]](#)
- Baral, N.R.; Shah, A. Microbial inhibitors: Formation and effects on acetone-butanol-ethanol fermentation of lignocellulosic biomass. *Appl. Microbiol. Biotechnol.* **2014**, *98*, 9151–9172. [\[CrossRef\]](#) [\[PubMed\]](#)
- Ujor, V.; Okonkwo, C.; Ezeji, T.C. Unorthodox methods for enhancing solvent production in solventogenic *Clostridium* species. *Appl. Microbiol. Biotechnol.* **2016**, *100*, 1089–1099. [\[CrossRef\]](#) [\[PubMed\]](#)
- Agu, C.V.; Ujor, V.; Ezeji, T.C. Metabolic engineering of *Clostridium beijerinckii* to improve glycerol metabolism and furfural tolerance. *Biotechnol. Biofuels* **2019**, *12*, 50. [\[CrossRef\]](#) [\[PubMed\]](#)
- Okonkwo, C.C.; Ujor, V.; Ezeji, T.C. Chromosomal integration of aldo-keto-reductase and short-chain dehydrogenase/reductase genes in *Clostridium beijerinckii* NCIMB 8052 enhanced tolerance to lignocellulose-derived microbial inhibitory compounds. *Sci. Rep.* **2019**, *9*, 7634. [\[CrossRef\]](#) [\[PubMed\]](#)
- Patil, R.C.; Suryawanshi, P.G.; Kataki, R.; Goud, V.V. Current challenges and advances in butanol production. In *Sustainable Bioenergy*; Elsevier: Amsterdam, The Netherlands, 2019; pp. 225–256. [\[CrossRef\]](#)
- Thunuguntla, R.; Atiyeh, H.K.; Huhnke, R.L.; Tanner, R.S. CO₂-based production of C₂–C₆ acids and alcohols: The potential of novel Clostridia. *Bioresour. Technol. Rep.* **2024**, *25*, 101713. [\[CrossRef\]](#)
- Ezeji, T.; Milne, C.; Price, N.D.; Blaschek, H.P. Achievements and perspectives to overcome the poor solvent resistance in acetone and butanol-producing microorganisms. *Appl. Microbiol. Biotechnol.* **2010**, *85*, 1697–1712. [\[CrossRef\]](#)
- Li, C.J.; Zhang, Z.; Zhan, P.C.; Lv, A.P.; Li, P.P.; Liu, L.; Li, W.J.; Yang, L.L.; Zhi, X.Y. Comparative Genomic Analysis and Proposal of *Clostridium yunnanense* sp. nov., *Clostridium rhizosphaerae* sp. nov., and *Clostridium paridis* sp. nov., Three Novel *Clostridium sensu stricto* Endophytes with Diverse Capabilities of Acetic Acid and Ethanol production. *Anaerobe* **2023**, *79*, 102686. [\[PubMed\]](#)
- Mitra, R.; Raja, V.B.K.K.; Panneerselvam, E. Comparison of Wrinkle Patterns Generated by Intradermal and Intramuscular Botulinum Toxin Injections by Clinical Evaluation. *J. Maxillofac. Oral Surg.* **2024**. [\[CrossRef\]](#)
- Staedtke, V.; Roberts, N.J.; Bai, R.Y.; Zhou, S. *Clostridium novyi*-NT in cancer therapy. *Genes Dis.* **2016**, *3*, 144–152. [\[CrossRef\]](#) [\[PubMed\]](#)
- Dang, L.H.; Bettegowda, C.; Huso, D.L.; Kinzler, K.W.; Vogelstein, B. Combination bacteriolytic therapy for the treatment of experimental tumors. *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 15155–15160. [\[CrossRef\]](#) [\[PubMed\]](#)

17. Patyar, S.; Joshi, R.; Byrav, D.P.; Prakash, A.; Medhi, B.; Das, B. Bacteria in cancer therapy: A novel experimental strategy. *J. Biomed. Sci.* **2010**, *17*, 21. [\[CrossRef\]](#) [\[PubMed\]](#)
18. Nölling, J.; Breton, G.; Omelchenko, M.V.; Makarova, K.S.; Zeng, Q.; Gibson, R.; Lee, H.M.; Dubois, J.; Qiu, D.; Hitti, J.; et al. Genome Sequence and Comparative Analysis of the Solvent-Producing Bacterium *Clostridium acetobutylicum*. *J. Bacteriol.* **2001**, *183*, 4823–4838. [\[CrossRef\]](#) [\[PubMed\]](#)
19. Lin, Z.; Cong, W.; Zhang, J. Biobutanol Production from Acetone–Butanol–Ethanol Fermentation: Developments and Prospects. *Fermentation* **2023**, *9*, 847. [\[CrossRef\]](#)
20. Choua, K.J.; Croft, T.; Hebdon, S.D.; Magnusson, L.R.; Xiong, W.; Reyes, L.H.; Chen, X.; Miller, E.J.; Riley, D.M.; Dupuis, S.; et al. Engineering the cellulolytic bacterium, *Clostridium thermocellum*, to co-utilize hemicellulose. *Metab. Eng.* **2024**, *83*, 193–205. [\[CrossRef\]](#) [\[PubMed\]](#)
21. Wang, X.L.; Sun, Y.Q.; Pan, D.T.; Xiu, Z.-L. Kinetics-based development of two-stage continuous fermentation of 1,3-propanediol from crude glycerol by *Clostridium butyricum*. *Biotechnol. Biofuels Bioprod.* **2024**, *17*, 38. [\[CrossRef\]](#) [\[PubMed\]](#)
22. Jones, D.T.; Schulz, F.; Roux, S.; Brown, S.D. Solvent-Producing Clostridia Revisited. *Microorganisms* **2023**, *11*, 2253. [\[CrossRef\]](#)
23. Höfele, F.; Schoch, T.; Oberlies, C.; Dürre, P. Heterologous Production of Isopropanol Using Metabolically Engineered *Acetobacterium woodii* Strains. *Bioengineering* **2023**, *10*, 1381. [\[CrossRef\]](#) [\[PubMed\]](#)
24. Schulz, S.; Molitor, B.; Angenent, L.T. Acetate augmentation boosts the ethanol production rate and specificity by *Clostridium ljungdahlii* during gas fermentation with pure carbon monoxide. *Bioresour. Technol.* **2023**, *369*, 128387. [\[CrossRef\]](#) [\[PubMed\]](#)
25. Gedam, P.S.; Raut, A.N.; Dhamole, P.B.; Gole, V.L. Challenges in Biobutanol Fermentation and Separation. In *Biorefinery: A Sustainable Approach for the Production of Biomaterials, Biochemicals and Biofuels*; Pathak, P.D., Mandavgane, S.A., Eds.; Springer: Singapore, 2023. [\[CrossRef\]](#)
26. Thang Ma, Y.; Guo, N.; Wang, S.; Wang, Y.; Jiang, Z.; Guo, L.; Wei Luo, W.; Wang, Y. Metabolically engineer *Clostridium saccharoperbutylacetonicum* for comprehensive conversion of acid whey into valuable biofuels and biochemicals. *Bioresour. Technol.* **2024**, *400*, 130640. [\[CrossRef\]](#) [\[PubMed\]](#)
27. Han, Y.F.; Xie, B.T.; Wu, G.X.; Guo, Y.Q.; Li, D.M.; Huang, Z.Y. Combination of trace metal to improve solventogenesis of *Clostridium carboxidivorans* P7 in syngas fermentation. *Front. Microbiol.* **2020**, *11*, 577266. [\[CrossRef\]](#) [\[PubMed\]](#)
28. Buckel, W.; Barker, H.A. Two Pathways of Glutamate Fermentation by Anaerobic Bacteria. *J. Bacteriol.* **1974**, *117*, 1248–1260. [\[CrossRef\]](#) [\[PubMed\]](#)
29. Aristilde, L.; Lewis, I.A.; Park, J.O.; Rabinowitz, J.D. Hierarchy in Pentose Sugar Metabolism in *Clostridium acetobutylicum*. *Appl. Environ. Microbiol.* **2015**, *81*, 1452–1462. [\[CrossRef\]](#) [\[PubMed\]](#)
30. Gottumukkala, L.D.; Haigh, K.; Görgens, J. Trends and advances in conversion of lignocellulosic biomass to biobutanol: Microbes, bioprocesses and industrial viability. *Renew. Sustain. Energy Rev.* **2017**, *76*, 963–973. [\[CrossRef\]](#)
31. Han, B.; Ujor, V.; Lai, L.B.; Gopalan, V.; Ezeji, T.C. Use of proteomic analysis to elucidate the role of calcium on acetone-butanol-ethanol (ABE) fermentation in *Clostridium beijerinckii* NCIMB 8052. *Appl. Environ. Microbiol.* **2013**, *79*, 282–293. [\[CrossRef\]](#) [\[PubMed\]](#)
32. Xue, C.; Zhao, J.; Chen, L.; Yang, S.-T.; Bai, F. Recent advances and state-of-the-art strategies in strain and process engineering for biobutanol production by *Clostridium acetobutylicum*. *Biotechnol. Adv.* **2017**, *35*, 310–322. [\[CrossRef\]](#) [\[PubMed\]](#)
33. Yang, Y.; Nie, X.; Jiang, Y.; Yang, C.; Gu, Y.; Jiang, W. Metabolic regulation in solventogenic *Clostridia*: Regulators, mechanisms and engineering. *Biotechnol. Adv.* **2018**, *36*, 905–914. [\[CrossRef\]](#)
34. Smith, I. Regulatory Proteins That Control Late-Growth Development. In *Bacillus subtilis and Other Gram-Positive Bacteria*; John Wiley & Sons, Ltd.: Hoboken, NJ, USA, 1993; pp. 785–800. [\[CrossRef\]](#)
35. Tangney, M.; Galinier, A.; Deutscher, J.; Mitchell, W. Analysis of the Elements of Catabolite Repression in *Clostridium acetobutylicum* ATCC 824. *J. Mol. Microbiol. Biotechnol.* **2003**, *6*, 6–11. [\[CrossRef\]](#)
36. Scotcher, M.C.; Rudolph, F.B.; Bennett, G.N. Expression of *abrB310* and *sinR*, and Effects of Decreased *abrB310* Expression on the Transition from Acidogenesis to Solventogenesis, in *Clostridium acetobutylicum* ATCC 824. *Appl. Environ. Microbiol.* **2005**, *71*, 1987–1995. [\[CrossRef\]](#)
37. Ren, C.; Gu, Y.; Hu, S.; Wu, Y.; Wang, P.; Yang, Y.; Yang, C.; Yang, S.; Jiang, W. Identification and inactivation of pleiotropic regulator CcpA to eliminate glucose repression of xylose utilization in *Clostridium acetobutylicum*. *Metab. Eng.* **2010**, *12*, 446–454. [\[CrossRef\]](#)
38. Noguchi, T.; Tashiro, Y.; Yoshida, T.; Zheng, J.; Sakai, K.; Sonomoto, K. Efficient butanol production without carbon catabolite repression from mixed sugars with *Clostridium saccharoperbutylacetonicum* N1-4. *J. Biosci. Bioeng.* **2013**, *116*, 716–721. [\[CrossRef\]](#)
39. Wietzke, M.; Bahl, H. The redox-sensing protein Rex, a transcriptional regulator of solventogenesis in *Clostridium acetobutylicum*. *Appl. Microbiol. Biotechnol.* **2012**, *96*, 749–761. [\[CrossRef\]](#)
40. Tan, Y.; Liu, Z.-Y.; Liu, Z.; Zheng, H.-J.; Li, F.-L. Comparative transcriptome analysis between *csrA*-disruption *Clostridium acetobutylicum* and its parent strain. *Mol. BioSystems* **2015**, *11*, 1434–1442. [\[CrossRef\]](#)
41. Qureshi, N.; Lin, X.; Liu, S.; Saha, B.C.; Mariano, A.P.; Polaina, J.; Ezeji, T.C.; Friedl, A.; Maddox, I.S.; Klasson, K.T.; et al. Global View of Biofuel Butanol and Economics of Its Production by Fermentation from Sweet Sorghum Bagasse, Food Waste, and Yellow Top Presscake: Application of Novel Technologies. *Fermentation* **2020**, *6*, 58. [\[CrossRef\]](#)

42. Ujor, V.C.; Lai, L.B.; Okonkwo, C.C.; Gopalan, V.; Ezeji, T.C. Ribozyme-Mediated Downregulation Uncovers DNA Integrity Scanning Protein A (DisA) as a Solventogenesis Determinant in *Clostridium beijerinckii*. *Front. Bioeng. Biotechnol.* **2021**, *9*, 669462. [\[CrossRef\]](#)
43. Olorunsogbon, T.; Adesanya, Y.; Atiyeh, H.K.; Okonkwo, C.C.; Ujor, V.C.; Ezeji, T.C. Effects of *Clostridium beijerinckii* and Medium Modifications on Acetone–Butanol–Ethanol Production From Switchgrass. *Front. Bioeng. Biotechnol.* **2022**, *10*, 942701. [\[CrossRef\]](#)
44. Davis, M.C.; Kesthely, C.A.; Franklin, E.A.; MacLellan, S.R. The essential activities of the bacterial sigma factor. *Can. J. Microbiol.* **2017**, *63*, 89–99. [\[CrossRef\]](#)
45. Buck, M.; Cannon, W. Activator-independent formation of a closed complex between $\sigma 54$ -holoenzyme and nifH and nifU promoters of *Klebsiella pneumoniae*. *Mol. Microbiol.* **1992**, *6*, 1625–1630. [\[CrossRef\]](#)
46. Shen, C.-H. Gene expression: Transcription of the genetic code. In *Diagnostic Molecular Biology*; Academic Press: Cambridge, MA, USA, 2019; pp. 59–86. [\[CrossRef\]](#)
47. Sauer, U.; Santangelo, J.D.; Treuner, A.; Buchholz, M.; Dürre, P. Sigma factor and sporulation genes in *Clostridium*. *FEMS Microbiol. Rev.* **1995**, *17*, 331–340. [\[CrossRef\]](#)
48. Nie, X.; Yang, B.; Zhang, L.; Gu, Y.; Yang, S.; Jiang, W.; Yang, C. PTS regulation domain-containing transcriptional activator CelR and sigma factor $\sigma 54$ control cellobiose utilization in *Clostridium acetobutylicum*. *Mol. Microbiol.* **2016**, *100*, 289–302. [\[CrossRef\]](#)
49. Hocq, R.; Bouilloux-Lafont, M.; Lopes Ferreira, N.; Wasels, F. $\Sigma 54$ (σL) plays a central role in carbon metabolism in the industrially relevant *Clostridium beijerinckii*. *Sci. Rep.* **2019**, *9*, 7228. [\[CrossRef\]](#)
50. Yang, B.; Nie, X.; Gu, Y.; Jiang, W.; Yang, C. Control of solvent production by sigma-54 factor and the transcriptional activator AdhR in *Clostridium beijerinckii*. *Microb. Biotechnol.* **2020**, *13*, 328–338. [\[CrossRef\]](#)
51. Nie, X.; Dong, W.; Yang, C. Genomic reconstruction of $\sigma 54$ regulons in *Clostridiales*. *BMC Genom.* **2019**, *20*, 565. [\[CrossRef\]](#)
52. Morett, E.; Buck, M. In Viva Studies on the Interaction of RNA Polymerase- $\sigma 54$ with the *Klebsiella pneumoniae* and *Rhizobium meliloti* *nzfw* Promoters. *J. Mol. Biol.* **1989**, *210*, 65–77. [\[CrossRef\]](#)
53. Danson, A.E.; Jovanovic, M.; Buck, M.; Zhang, X. Mechanisms of $\sigma 54$ -dependent transcription initiation and regulation. *J. Mol. Biol.* **2019**, *431*, 3960–3974. [\[CrossRef\]](#)
54. Jones, S. Sigma 54 minds the gap. *Nat. Rev. Microbiol.* **2009**, *7*, 3. [\[CrossRef\]](#)
55. Bush, M.; Dixon, R. The Role of Bacterial Enhancer Binding Proteins as Specialized Activators of 54-Dependent Transcription. *Microbiol. Mol. Biol. Rev.* **2012**, *76*, 497–529. [\[CrossRef\]](#)
56. Deutscher, J.; Aké, F.M.D.; Derkaoui, M.; Zébré, A.C.; Cao, T.N.; Bouraoui, H.; Kentache, T.; Mokhtari, A.; Milohanic, E.; Joyet, P. The Bacterial Phosphoenolpyruvate: Carbohydrate Phosphotransferase System: Regulation by Protein Phosphorylation and Phosphorylation-Dependent Protein-Protein Interactions. *Microbiol. Mol. Biol. Rev.* **2014**, *78*, 231–256. [\[CrossRef\]](#) [\[PubMed\]](#)
57. Ren, C.; Gu, Y.; Wu, Y.; Zhang, W.; Yang, C.; Yang, S.; Jiang, W. Pleiotropic functions of catabolite control protein CcpA in Butanol-producing *Clostridium acetobutylicum*. *BMC Genom.* **2012**, *13*, 349. [\[CrossRef\]](#) [\[PubMed\]](#)
58. Yang, Y.; Zhang, L.; Huang, H.; Yang, C.; Yang, S.; Gu, Y.; Jiang, W. A Flexible Binding Site Architecture Provides New Insights into CcpA Global Regulation in Gram-Positive Bacteria. *MBio* **2017**, *8*, e02004-16. [\[CrossRef\]](#) [\[PubMed\]](#)
59. Deutscher, J.; Francke, C.; Postma, P.W. How Phosphotransferase System-Related Protein Phosphorylation Regulates Carbohydrate Metabolism in Bacteria. *Microbiol. Mol. Biol. Rev.* **2006**, *70*, 939–1031. [\[CrossRef\]](#) [\[PubMed\]](#)
60. Alsaker, K.V.; Spitzer, T.R.; Papoutsakis, E.T. Transcriptional Analysis of spo0A Overexpression in *Clostridium acetobutylicum* and Its Effect on the Cell's Response to Butanol Stress. *J. Bacteriol.* **2004**, *186*, 1959–1971. [\[CrossRef\]](#) [\[PubMed\]](#)
61. Wang, Y.; Li, X.; Mao, Y.; Blaschek, H.P. Genome-wide dynamic transcriptional profiling in *Clostridium beijerinckii* NCIMB 8052 using single-nucleotide resolution RNA-Seq. *BMC Genom.* **2012**, *13*, 102. [\[CrossRef\]](#) [\[PubMed\]](#)
62. Xue, Q.; Yang, Y.; Chen, J.; Chen, L.; Yang, S.; Jiang, W.; Gu, Y. Roles of three AbrBs in regulating two-phase *Clostridium acetobutylicum* fermentation. *Appl. Microbiol. Biotechnol.* **2016**, *100*, 9081–9089. [\[CrossRef\]](#)
63. Ravagnani, A.; Jennert, K.C.; Steiner, E.; Grünberg, R.; Jefferies, J.R.; Wilkinson, S.R.; Young, D.I.; Tidwell, E.C.; Brown, D.P.; Youngman, P.; et al. Spo0A directly controls the switch from acid to solvent production in solvent-forming clostridia. *Mol. Microbiol.* **2000**, *37*, 1172–1185. [\[CrossRef\]](#)
64. Thormann, K.; Feustel, L.; Lorenz, K.; Nakotte, S.; Dürre, P. Control of butanol formation in *Clostridium acetobutylicum* by transcriptional activation. *J. Bacteriol.* **2002**, *184*, 1966–1973. [\[CrossRef\]](#)
65. Dürre, P.; Hollergschwandner, C. Initiation of endospore formation in *Clostridium acetobutylicum*. *Anaerobe* **2004**, *10*, 69–74. [\[CrossRef\]](#)
66. Bengelsdorf, F.R.; Poehlein, A.; Flitsch, S.K.; Linder, S.; Schiel-Bengelsdorf, B.; Stegmann, B.A.; Krabben, P.; Green, E.; Zhang, Y.; Minton, N. Host organisms: *Clostridium acetobutylicum*/*Clostridium beijerinckii* and related organisms. *Ind. Biotechnol. Microorg.* **2017**, *1*, 327–364.
67. Harris, L.M.; Welker, N.E.; Papoutsakis, E.T. Northern, Morphological, and Fermentation Analysis of spo0A Inactivation and Overexpression in *Clostridium acetobutylicum* ATCC 824. *J. Bacteriol.* **2002**, *184*, 3586–3597. [\[CrossRef\]](#) [\[PubMed\]](#)
68. Sullivan, L.; Bennett, G.N. Proteome analysis and comparison of *lostridium acetobutylicum* ATCC 824 and Spo0A strain variants. *J. Ind. Microbiol. Biotechnol.* **2006**, *33*, 298–308. [\[CrossRef\]](#) [\[PubMed\]](#)
69. Jiang, Y.; Xu, C.; Dong, F.; Yang, Y.; Jiang, W.; Yang, S. Disruption of the acetoacetate decarboxylase gene in solvent-producing *Clostridium acetobutylicum* increases the butanol ratio. *Metab. Eng.* **2009**, *11*, 284–291. [\[CrossRef\]](#) [\[PubMed\]](#)

70. Han, B.; Gopalan, V.; Ezeji, T.C. Acetone production in solventogenic *Clostridium* species: New insights from non-enzymatic decarboxylation of acetoacetate. *Appl. Microbiol. Biotechnol.* **2011**, *91*, 565–576. [[CrossRef](#)] [[PubMed](#)]
71. Chen, G.; Kumar, A.; Wyman, T.H.; Moran, C.P., Jr. Spo0A-dependent activation of an extended—10 region promoter in *Bacillus subtilis*. *J. Bacteriol.* **2006**, *188*, 1411–1418. [[CrossRef](#)] [[PubMed](#)]
72. Vogel, J.; Sharma, C.M. How to find small non-coding RNAs in bacteria. *Biol. Chem.* **2005**, *386*, 1219–1238. [[CrossRef](#)]
73. Brosse, A.; Guillier, M. Bacterial Small RNAs in Mixed Regulatory Networks. In *Regulating with RNA in Bacteria and Archaea*; John Wiley & Sons, Ltd.: Hoboken, NJ, USA, 2018; pp. 453–469. [[CrossRef](#)]
74. Lalaouna, D.; Simoneau-Roy, M.; Lafontaine, D.; Massé, E. Regulatory RNAs and target mRNA decay in prokaryotes. *Biochim. Biophys. Acta (BBA)-Gene Regul. Mech.* **2013**, *1829*, 742–747. [[CrossRef](#)]
75. Argaman, L.; Hershberg, R.; Vogel, J.; Bejerano, G.; Wagner, E.G.H.; Margalit, H.; Altuvia, S. Novel small RNA-encoding genes in the intergenic regions of *Escherichia coli*. *Curr. Biol.* **2001**, *11*, 941–950.
76. Richards, G.R.; Vanderpool, C.K. Molecular call and response: The physiology of bacterial small RNAs. *Biochim. Biophys. Acta (BBA) Gene Regul. Mech.* **2011**, *1809*, 525–531. [[CrossRef](#)]
77. Storz, G.; Vogel, J.; Wassarman, K.M. Regulation by Small RNAs in Bacteria: Expanding Frontiers. *Mol. Cell* **2011**, *43*, 880–891. [[CrossRef](#)] [[PubMed](#)]
78. Thomason, M.K.; Storz, G. Bacterial Antisense RNAs: How Many Are There, and What Are They Doing? *Annu. Rev. Genet.* **2010**, *44*, 167–188. [[CrossRef](#)] [[PubMed](#)]
79. Georg, J.; Hess, W.R. Cis-Antisense RNA, Another Level of Gene Regulation in Bacteria. *Microbiol. Mol. Biol. Rev.* **2011**, *75*, 286–300. [[CrossRef](#)] [[PubMed](#)]
80. Chen, Y.; Indurthi, D.C.; Jones, S.W.; Papoutsakis, E.T. Small RNAs in the Genus *Clostridium*. *MBio* **2011**, *2*, 10–1128. [[CrossRef](#)] [[PubMed](#)]
81. Venkataramanan, K.P.; Jones, S.W.; McCormick, K.P.; Kunjeti, S.G.; Ralston, M.T.; Meyers, B.C.; Papoutsakis, E.T. The *Clostridium* small RNome that responds to stress: The paradigm and importance of toxic metabolite stress in *C. acetobutylicum*. *BMC Genom.* **2013**, *14*, 1–16. [[CrossRef](#)] [[PubMed](#)]
82. Keiler, K.C. Biology of trans-Translation. *Annu. Rev. Microbiol.* **2008**, *62*, 133–151. [[CrossRef](#)] [[PubMed](#)]
83. Craig, N.L.; Cohen-Fix, O.; Storz, G. *Molecular Biology: Principles of Genome Function*; Oxford University Press: Oxford, UK, 2014.
84. Wach, W.; Harms, K.; Klingenberg, M.; Duerre, P.; Nold, N.; Schiel, B. Production of Acid and Solvent in Microorganisms. United States Patent No. US8679799B2, 25 March 2014.
85. Wassarman, K.M. 6S RNA: A regulator of transcription. *Mol. Microbiol.* **2007**, *65*, 1425–1431. [[CrossRef](#)] [[PubMed](#)]
86. Zimmermann, T. Untersuchungen zur Butanolbildung von *Hyperthermus butylicus* und *Clostridium acetobutylicum*. Ph.D. Dissertation, Universität Ulm, Ulm, Germany, 2013. [[CrossRef](#)]
87. Wang, Q.; Venkataramanan, K.; Huang, H.; Papoutsakis, E.T.; Wu, C.H. Transcription factors and genetic circuits orchestrating the complex, multilayered response of *Clostridium acetobutylicum* to butanol and butyrate stress. *BMC Syst. Biol.* **2013**, *7*, 120. [[CrossRef](#)] [[PubMed](#)]
88. Jones, A.J.; Fast, A.G.; Clupper, M.; Papoutsakis, E.T. Small and Low but Potent: The Complex Regulatory Role of the Small RNA *SolB* in Solventogenesis in *Clostridium acetobutylicum*. *Appl. Environ. Microbiol.* **2018**, *84*, e00597-18. [[CrossRef](#)]
89. André, G.; Even, S.; Putzer, H.; Burguière, P.; Croux, C.; Danchin, A.; Martin-Verstraete, I.; Soutourina, O. S-box and T-box riboswitches and antisense RNA control a sulfur metabolic operon of *Clostridium acetobutylicum*. *Nucleic Acids Res.* **2008**, *36*, 5955–5969. [[CrossRef](#)]
90. Barrick, J.E.; Sudarsan, N.; Weinberg, Z.; Ruzzo, W.L.; Breaker, R.R. 6S RNA is a widespread regulator of eubacterial RNA polymerase that resembles an open promoter. *RNA* **2005**, *11*, 774–784. [[CrossRef](#)] [[PubMed](#)]
91. Gildehaus, N.; Neußer, T.; Wurm, R.; Wagner, R. Studies on the function of the riboregulator 6S RNA from *E. coli*: RNA polymerase binding, inhibition of in vitro transcription and synthesis of RNA-directed de novo transcripts. *Nucleic Acids Res.* **2007**, *35*, 1885–1896. [[CrossRef](#)] [[PubMed](#)]
92. Mahendran, G.; Jayasinghe, O.T.; Thavakumaran, D.; Arachchilage, G.M.; Silva, G.N. Key players in regulatory RNA realm of bacteria. *Biochem. Biophys. Rep.* **2022**, *30*, 101276. [[CrossRef](#)] [[PubMed](#)]
93. Trotochaud, A.E.; Wassarman, K.M. 6S RNA Regulation of *pspF* Transcription Leads to Altered Cell Survival at High pH. *J. Bacteriol.* **2006**, *188*, 3936–3943. [[CrossRef](#)] [[PubMed](#)]
94. Shin, J.-H.; Price, C.W. The SsrA-SmpB Ribosome Rescue System Is Important for Growth of *Bacillus subtilis* at Low and High Temperatures. *J. Bacteriol.* **2007**, *189*, 3729–3737. [[CrossRef](#)] [[PubMed](#)]
95. Withey, J.H.; Friedman, D.I. A salvage pathway for protein synthesis: tmRNA and trans-translation. *Annu. Rev. Microbiol.* **2003**, *57*, 101–123. [[CrossRef](#)] [[PubMed](#)]
96. Jones, A.J.; Venkataramanan, K.P.; Papoutsakis, E.T. Overexpression of two stress-responsive, small, non-coding RNAs, 6S and tmRNA, imparts butanol tolerance in *Clostridium acetobutylicum*. *FEMS Microbiol. Lett.* **2016**, *363*, fnw063. [[CrossRef](#)] [[PubMed](#)]
97. Sharma, U.K.; Chatterji, D. Transcriptional switching in *Escherichia coli* during stress and starvation by modulation of σ^{70} activity. *FEMS Microbiol. Rev.* **2010**, *34*, 646–657. [[CrossRef](#)]
98. Venkataramanan, K.P.; Min, L.; Hou, S.; Jones, S.W.; Ralston, M.T.; Lee, K.H.; Papoutsakis, E.T. Complex and extensive post-transcriptional regulation revealed by integrative proteomic and transcriptomic analysis of metabolite stress response in *Clostridium acetobutylicum*. *Biotechnol. Biofuels* **2015**, *8*, 81. [[CrossRef](#)]

99. Mehdizadeh, G.I.; Edwards, A.N.; McBride, S.M.; McClane, B.A. The impact of orphan histidine kinases and phosphotransfer proteins on the regulation of clostridial sporulation initiation. *mBio* **2024**, *15*, e02248-23. [\[CrossRef\]](#)
100. Furuya, K.; Kiyoshi, K.; Punjuy, C.; Yoshida, N.; Maruyama, R.; Yasuda, T.; Watanabe, K.; Kadokura, T.; Nakayama, S. Effect of spo0A, sigE, sigG, and sigK disruption on butanol production and spore formation in *Clostridium saccharoperbutylacetonicum* strain N1-4 (ATCC13564). *J. Biosci. Bioeng.* **2023**, *136*, 198–204. [\[CrossRef\]](#) [\[PubMed\]](#)
101. Rhodius, V.A.; Suh, W.C.; Nonaka, G.; West, J.; Gross, C.A. Conserved and Variable Functions of the σ^E Stress Response in Related Genomes. *PLoS Biol.* **2005**, *4*, e2. [\[CrossRef\]](#) [\[PubMed\]](#)
102. Konovalova, A.; Grabowicz, M.; Balibar, C.J.; Malinverni, J.C.; Painter, R.E.; Riley, D.; Mann, P.A.; Wang, H.; Garlisi, C.G.; Sherborne, B.; et al. Inhibitor of intramembrane protease RseP blocks the σ^E response causing lethal accumulation of unfolded outer membrane proteins. *Proc. Natl. Acad. Sci. USA* **2018**, *115*, E6614–E6621. [\[CrossRef\]](#) [\[PubMed\]](#)
103. Iqbal, S.; Begum, F.; Rabaan, A.A.; Aljeldah, M.; Al Shammari, B.R.; Alawfi, A.; Alshengeti, A.; Sulaiman, T.; Khan, A. Classification and Multifaceted Potential of Secondary Metabolites Produced by *Bacillus subtilis* Group: A Comprehensive Review. *Molecules* **2023**, *28*, 927. [\[CrossRef\]](#) [\[PubMed\]](#)
104. Pahalagedara, A.S.N.W.; Flint, S.; Palmer, J.; Brightwell, G.; Gupta, T.B. Antimicrobial production by strictly anaerobic *Clostridium* spp. *Int. J. Antimicrob. Agents* **2020**, *55*, 105910. [\[CrossRef\]](#) [\[PubMed\]](#)
105. Yang, M.; Li, J.; Li, A. Total synthesis of clostrubin. *Nat. Commun.* **2015**, *6*, 6445. [\[CrossRef\]](#) [\[PubMed\]](#)
106. Li, J.S.; Barber, C.; Zhang, W. Natural Products from Anaerobes. *J. Ind. Microbiol. Biotechnol.* **2019**, *46*, 375–383. [\[CrossRef\]](#) [\[PubMed\]](#)
107. Herman, N.A.; Kim, S.J.; Li, J.S.; Cai, W.; Koshino, H.; Zhang, W. The industrial anaerobe *Clostridium acetobutylicum* uses polyketides to regulate cellular differentiation. *Nat. Commun.* **2017**, *8*, 1514. [\[CrossRef\]](#)
108. Li, J.; Barber, C.; Herman, N.; Cai, W.; Zafir, E.; Du, Y.; Zhu, X.; Skyrud, W.; Zhang, W. Investigation of secondary metabolism in the industrial butanol hyper-producer *Clostridium saccharoperbutylacetonicum* N1-4. *J. Ind. Microbiol. Biotechnol.* **2020**, *47*, 319–328. [\[CrossRef\]](#)
109. Pahalagedara, A.S.; Jauregui, R.; Maclean, P.; Altermann, E.; Flint, S.; Palmer, J.; Brightwell, G.; Gupta, T.B. Culture and genome-based analysis of four soil *Clostridium* isolates reveal their potential for antimicrobial production. *BMC Genom.* **2021**, *22*, 686. [\[CrossRef\]](#)
110. Lincke, T.; Behnken, S.; Ishida, K.; Roth, M.; Hertweck, C. Closthioamide: An unprecedented polythioamide antibiotic from the strictly anaerobic bacterium *Clostridium cellulolyticum*. *Angew. Chem.* **2010**, *122*, 2055–2057. [\[CrossRef\]](#)

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.