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How can alcohol production be improved in carboxydotrophic clostridia?

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ABSTRACT

Clostridia are well-known organisms within the scientific community, and many aspects of their life cycle have been studied. Autotrophic strains are especially interesting as microbial cell factories for industrial purposes. Carboxydotrophic clostridia can use CO_2/H_2 and/or CO as their only carbon and reducing power sources, and they produce alcohols and acids. Both products can be used as either biofuels or feedstock chemicals for many industrial processes, confirming the control of carboxydotrophic clostridia metabolism as one of the most profitable scientific ambitions to be explored. Here, we review the current state of research on alcohol production by carboxydotrophic clostridia, including a comprehensive overview of their metabolic pathways and the key experimental variables that govern alcohol production. Additionally, the genetic and genomic tools for selective and enhanced solvent production, which are currently under intense development, are discussed. In this context, this review covers the main genetic engineering methods that have been utilized to improve the capabilities of solventogenic carboxydotrophic bacteria.

1. Introduction

Clostridia are well-known organisms in the scientific community. They have been identified as an ancient lineage that likely evolved from the early oxygen-free earth [1], and serve as a model experimental target for research on organisms living in "extreme" environmental conditions (i.e., high temperatures and strict anaerobiosis). In addition, the potential infectious threat to humanity of some *Clostridium* isolates (i.e., *Clostridium botulinum*, *Clostridium tetani*, and *Clostridium perfringens*, among others) has deepened our knowledge of the genus on both physiological and genetic levels [2]. Clostridia metabolisms have been thoroughly studied and, whenever possible, harnessed to benefit humankind. As an example, we can thank clostridia for the onset of the first industrial bioproduction of organic solvents (acetone and butanol), far in the late 1920s (the Weizmann organism, *Clostridium acetobutylicum*) [3].

However, until very recently, less attention has been paid to one of the most unique features of some clostridia, their ability to grow autotrophically and fix carbon in a non-cyclic metabolism, through the acetyl-CoA pathway (described in depth in Section 3). Most, if not all, clostridia have been isolated from organic matter rich environments, such as a anaerobic sediments, animal manure and sewage sludge [4,5], but some possess a very desirable treat from the industrial perspective, the ability to grow in strict autotrophy in non-photosynthetic conditions as well as to produce added-value compounds (i.e., acids and/or alcohols) as end-metabolites. In this light, clostridia have recently raised significant industrial interest as potential platforms for the sustainable production of carbon neutral biofuels [6,7]. However, the successful production of alcohols in clostridia relies on the metabolic shift from acidogenesis (production of acids) to solventogenesis (production of alcohols). The mechanisms governing this shift have been extensively investigated, especially in acetone–butanol–ethanol (ABE) fermenting clostridia. This review aims to provide deeper insights into the underlying mechanisms controlling the solventogenic shift in carboxydotrophic bacteria, identify key factors that can potentially enhance alcohol production, and outline a road map for future research on controlling the advent of solventogenesis in carboxydotrophic clostridia.

2. The need for a change in the energy model: alternatives involving *Clostridium* spp.

One challenge of scientific research in the early 21st century is the development of new technologies that allow for the progressive transition from the current energy model, based on fossil

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Table 1
Carboxydotrophic *Clostridia* able to ferment inorganic carbon CO_2/H_2 or/and CO to alcohols.

Clostridia species	Isolation source	Alcohols produced	Reference
Acetogenium (Thermoanaerobacter) kivui	Lake Kivui	Ethanol	[20]
Alkalibaculum bacchi	Livestock-impacted soil	Ethanol	[21]
Butyribacterium methylotrophicum	Sewage digester sludge	Ethanol, butanol	[22,23]
Clostridium autoethanogenum	Rabbit feces	Ethanol	[24]
Clostridium carboxidivorans P7	Agricultural settling lagoon sediment	Ethanol, butanol, hexanol	[4,25]
Clostridium glycolicum RD-1	Sea-grass roots	Ethanol	[26]
Clostridium ljungdahlii	Chicken waste	Ethanol	[5]
Clostridium methozybenzovorans	Olive oil mill wastewater	Ethanol	[27]
Clostridium ragsdalei P11	Duck pond sediment	Ethanol, butanol	[28]
Eubacterium limosum	Sheep rumen	Ethanol, butanol	[16,29]
Morella sp. HUC22-1	Mud from underground hot water	Ethanol	[30]
Peptostreotococcus productus U-1	Anaerobic sewage digester sludge	Ethanol	[31]

fuels, to a more sustainable energy model, based on renewable and carbon-neutral fuels. To date, many alternatives have been experimentally explored to achieve this goal, i.e., generating electricity from renewable sources and catalytic or bio-catalytic transformation of renewable feedstocks into fuels. Among the latter, the use of biologically produced alcohols as substitutes for oil-based energy sources has achieved great acceptance in the transportation industry because the use of alcohols requires simple and economically feasible adaptation of existing engines. In this context, the utilization of clostridia for producing ethanol and butanol, two of the most promising alternative biofuels [8,9], has been extensively studied over the last three decades [4,10–12]. Initial efforts have focused on the production of "first generation biofuels" that are mainly obtained through biological transformations of starch, corn and molasses. Although successful, this approach relied on the utilization of food feedstocks, which resulted in a subsequent increase in the crop prices and reduced food security in developing countries. In recent years, there has been a growing research interest in the development of a "second generation of biofuels", which are aimed at alcohol production from non-food feedstocks, such as forestry, agricultural and municipal solid wastes [13,14]. However, the direct fermentation of these substrates into biofuels is critically hampered by the low efficiency of cellulose and lignin conversion processes [15].

An alternative, to circumvent the low biological degradation efficiencies of cellulose- and lignin-enriched wastes, is the so-called syngas platform, which relies on an initial gasification step of the wastes into synthesis gas or syngas (a mixture of primarily CO, CO_2, and H_2), and its subsequent transformation into organic acids and alcohols by carboxydotrophic acetogenic bacteria [16]. As of today, several international companies (i.e., Lanzatech, INEOS Bio, and Coskata, among others) are commercializing and licensing the syngas fermentation process for the production of ethanol and other added-value chemicals from various feedstocks [6,17].

Acetogenic bacteria rely on the acetyl-CoA pathway or Wood–Ljungdahl pathway (WLP) for the reduction of CO_2 to acetyl-CoA, energy conservation and assimilation of CO_2 into cell carbon. Acetic acid is the main (and sometimes only) product of their fermentative metabolism (first described in *Clostridium aceticum* in 1936 [18]), although, under certain conditions, some bacteria can also produce significant concentrations of alcohols. To date, over 100 bacterial isolates, belonging to distantly related phylogenetic groups, are known to be acetogenic. Among them, only a few are able to reduce organic acids into alcohols and these mostly belong to the clostridia class [19] (Table 1).

3. Transformation of syngas into alcohols by clostridia

The carbon fixation process through the WLP has acetyl-CoA as an end-product. Acetyl-CoA is a key metabolite in many anabolic and catabolic reactions in bacteria. Mainly, it can further be transformed into cell building blocks through successive, additional carbon incorporations, or converted to fermentation end products that are excreted from the cell. The latter are of interest for the industrial production of alternative biofuels because, among the product spectra, ethanol, butanol and longer carbon-chain alcohols can be produced by some carboxydotrophic bacteria.

3.1. Carbon fixation in carboxydotrophic clostridia

The WLP is an example of an irreversible, non-cyclic carbon fixation pathway in which no intermediate metabolites are furnished for additional carbon assimilation. The WLP is dispersed among phylogenetically distant groups and can function in both the oxidative and reductive directions. However, it only takes place under strictly anaerobic conditions [16]. In acetogens, the pathway is a terminal electron accepting process used in the reductive direction for energy conservation as well as for autotrophic carbon assimilation into biomass and cell components [32,33].

Two separate branches, the Eastern or methyl branch and Western or carbonyl branch, are present in the WLP (Fig. 1). In the methyl branch, one molecule of CO_2 is stepwise reduced to a methyl group using six electrons. The much shorter carbonyl branch catalyzes the incorporation of a carbon monoxide, which is condensed with the methyl group formed in the methyl branch and coenzyme A (CoA) into acetyl-CoA [7,34] (Eq. (1)).

$$[CH_3\text{--}CFeSP] + CO + HS\text{-}CoA \rightarrow CH_3COS\text{–}CoA + [Co\text{-}E]. \quad (1)$$

According to the currently available information on bacterial genomes, the genes encoding the methyl branch of the WLP are present in many anaerobic bacteria (e.g., strict anaerobes, methanogens and autotrophic sulfate reducers), although those genes do not form a contiguous operon in all strains, revealing the complex evolutionary history of this metabolic pathway. Contrarily, the genes coding for enzymes of the carbonyl branch, acetyl-CoA synthase (ACS), are exclusively found in carboxydotrophic clostridia [35]. Acetyl-CoA synthase is a multifunctional enzyme that is also responsible for the reversible conversion of CO into CO_2, showing carbon monoxide dehydrogenase activity [33]. Therefore, the enzyme complex is often referred to as ACS/CODH. CO oxidation is coupled to the transfer of electrons to ferredoxin (Fd) and constitutes the unique source of reducing equivalents in the absence of H_2 [36,37] (Eq. (2)).

$$CO + H_2O \leftrightarrow CO_2 + 2H^+ + 2e^- \quad (2)$$

Acetyl-CoA is further oxidized to acetate in a two-step reaction involving phosphotransacetylase and acetate kinase enzymes. The latter is the main reaction from which carboxydotrophic clostridia derive energy in a substrate level phosphorylation reaction. However, no net ATP gain is obtained in the WLP because one molecule of ATP is consumed to activate formate. As the synthesis of acetate from two moles of CO_2 or CO enables the growth of acetogens, the