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Citrate Stimulates Oligosaccharide Synthesis in Metabolically Engineered *Agrobacterium* sp.

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Abstract *Agrobacterium* sp. ATCC 31749 was previously shown to be an advantageous host for oligosaccharide production. Unexpectedly, the addition of citrate to the oligosaccharide synthesis reaction resulted in up to a sixfold improvement in the production *N*-aceytl-lactosamine, a disaccharide. The possible mechanisms for this citrate-induced stimulation of oligosaccharide production were investigated, including the consumption of citrate as a carbon and energy source, enhanced metal ion solubility from citrate chelation, and the ability of citrate to act as a buffer. The main mechanisms for the effect of citrate on oligosaccharide production were determined to be carbon and energy provision from citrate consumption and pH maintenance. ATCC 31749 was shown to co-metabolize citrate along with sucrose, a preferred carbon source, indicating the lack of a catabolite repression system in this *Agrobacterium*. Metabolic flux analysis suggested an increase in flux through TCA cycle for the citrate-containing reaction, which may provide additional energy supply to support enhanced oligosaccharide production. The citrate stimulation of oligosaccharide synthesis was shown to be unique to the *Agrobacterium* strain, as a similarly engineered *Escherichia coli* strain did not show significant improvement in oligosaccharide production with citrate addition. This work provides insight into the metabolism of *Agrobacterium* sp. ATCC 31749 and highlights important factors in whole-cell oligosaccharide synthesis.

Keywords Oligosaccharide synthesis · *Agrobacterium* · ATCC 31749 · Whole-cell synthesis · Metabolic flux analysis · Citrate · Co-metabolism of citrate · Catabolite repression

Abbreviations

ACoA	Acetyl coenzyme A
ADP	Adenosine diphosphate
aKG	Alpha-ketoglutarate
ATP	Adenosine triphosphate

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CIT	Citrate
Crd	Curdlan
ED	Entner–Doudoroff
F6P	Fructose-6-phosphate
FAD	Flavin adenine dinucleotide (oxidized)
$FADH_2$	Flavin adenine dinucleotide (reduced)
Fru	Fructose
G1P	Glucose-1-phosphate
G6P	Glucose-6-phosphate
GAP	Glyceraldehyde-3-phosphate
Glc	Glucose
Glycerol-3-P	Glycerol-3-phosphate
ICT	Isocitrate
LacNAc	*N*-Aceytl-lactosamine
MAL	Malate
NAD	Nicotinamide adenine dinucleotide (oxidized)
NADH	Nicotinamide adenine dinucleotide (reduced)
OAA	Oxaloacetate
P3	Product 3 (galactose-β1,4-mannose)
PPP	Pentose phosphate pathway
PTS	Phosphotransferase system
PYR	Pyruvate
SUCC	Succinate
SUCC-CoA	Succinyl coenzyme A
TCA	Tricarboxylic acid
UDP-Gal	Uridine diphosphate galactose
UDP-Glc	Uridine diphosphate glucose
UTP	Uridine triphosphate

Introduction

Oligosaccharides are important biomolecules which participate in various cellular processes, yet until recently, the significance of oligosaccharides in disease and other medical conditions has been largely overlooked. Currently, oligosaccharides are key components of potential vaccines and treatments for diseases including cancer, HIV, malaria, and anthrax [1, 2]. While initial investigation of these treatments may show promise, they are often limited by the cost and difficulty of synthesizing the oligosaccharide component. Recombinant DNA technology has been used to metabolically engineer bacteria for oligosaccharide synthesis [3–7]. This whole-cell synthesis strategy can produce the medically relevant oligosaccharide from a cheap carbon source like glucose or sucrose, thereby reducing the cost of oligosaccharide synthesis compared to other available chemical and enzymatic methods. However, whole-cell oligosaccharide synthesis efforts often produce only small quantities of the desired oligosaccharide. We have recently demonstrated that using an unconventional bacterium, *Agrobacterium* sp. ATCC 31749, oligosaccharides can be synthesized at concentrations as high as 20 mM without the use of expensive cofactors or nutrients [8].

In our previous work, *Agrobacterium* sp. ATCC 31749 was engineered to produce *N*-acetyl-lactosamine (LacNAc), a disaccharide basal structure found in antigens overexpressed on the surface of cancerous cells. Initial synthesis experiments produced only

1.5 mM of LacNAc, yet with the addition of 5 g/L citrate, LacNAc synthesis improved sixfold. This work investigates the possible mechanisms for the observed citrate-induced stimulation of oligosaccharide synthesis in the engineered *Agrobacterium*.

Agrobacterium sp. ATCC 31749 is primarily studied for its production of curdlan, a β1,3-glucose polymer. Preliminary investigation of ATCC 31749 indicates the presence of the Entner–Doudoroff and pentose phosphate pathways as well as the tricarboxylic acid (TCA) cycle, yet little else is known about the bacterium's metabolism [9]. ATCC 31749 has been shown to utilize citrate as a sole carbon source, presumably through the metabolism of citrate in the TCA cycle [10]. Since oligosaccharide synthesis requires a significant amount of energy, the energy generated from citrate consumption may explain the observed increase in oligosaccharide production. However, the oligosaccharide synthesis reaction includes sucrose, a preferred carbon source of ATCC 31749, which may prevent citrate uptake. Several other possible mechanisms may explain the citrate-induced stimulation of oligosaccharide production. Citrate is a known chelating agent, and as such, it may increase the solubility of metal ions in the reaction medium. Metal ions are important cofactors for many metabolic reactions. In particular, manganese is a cofactor for the β1,4-galactosyltransferase that synthesizes LacNAc [11]. An increase in manganese availability due to citrate chelation may explain the increase in oligosaccharide production. Citrate may also act as a buffer to control pH during oligosaccharide synthesis. This study investigates the possible mechanisms for the citrate-induced stimulation of oligosaccharide production and utilizes metabolic flux analysis to gain insight into the effect of citrate on the entire metabolic network. We also investigate whether the effect of citrate on oligosaccharide production is unique to *Agrobacterium* sp.

Materials and Methods

Materials

The chemicals used in this study were obtained from Sigma-Aldrich (lactose, LacNAc, GlcNAc, ATP, methyl blue), Mallinckrodt (glucose), Wako Chemicals USA (curdlan), and Fisher Scientific (all other chemicals).

Bacterial Strains

The *Agrobacterium* sp. and *Escherichia coli* strains used in this study, ATCC 31749/pKEL and AD202/pMUEL, were previously constructed [5, 8].

LacNAc and Lactose Synthesis

The ATCC 31749/pKEL inoculums were prepared by overnight incubation at 30 °C with agitation of 250 rpm in 3 mL of LB containing 100 μg/mL ampicillin and 100 μg/mL kanamycin. The cells were diluted 100 times and grown in freshly prepared LB medium supplemented with antibiotics until OD_{600} reached 0.3–0.4, upon which, IPTG was added to a final concentration of 0.75 mM, and cells were incubated for another 6 h. The cells were harvested by centrifugation at 3,000×*g* and 4 °C for 10 min. Cells were washed once with 10% glycerol prior to addition of the reaction buffer. The 10 mL reaction buffer contained 50 mM Tris–HCl (pH 7.5), 1 g/L $K_2HPO_4{\cdot}3H_2O$, 5 g/L $MgSO_4{\cdot}7H_2O$, 5 mM $MnCl_2$, 50 g/L sucrose (or glucose for *E. coli* strain), 10 g/L GlcNAc, and varying

concentrations of sodium citrate. The cell concentration was 10% (wet weight), and the reaction vessel was a 50 mL Erlenmeyer flask. The reaction was carried out at 30 °C (or 25 °C for *E. coli*) and 250 rpm in a biological shaker. For reactions with constant pH, 1 M HCl or NaOH were added every 2 h to adjust the pH to the target level.

Analytical Techniques

Carbohydrate Analysis

Samples were heated in boiling water for 10 min. The supernatant was obtained by centrifugation and analyzed using a Dionex BioLC system with a CarboPac PA20 analytical column. The Dionex ED50 electrochemical detector measured carbohydrate concentrations through pulsed amperometry (waveform: t=0.41 s, p=−2.00 V; t=0.42 s, p=−2.00 V; t=0.43 s, p=0.60 V; t=0.44 s, p=−0.10 V; t=0.50 s, p=−0.10 V). Sucrose, glucose, fructose, lactose, and LacNAc concentrations were determined using calibration curves prepared from standards. The mobile phase consisted of degassed 200 mM sodium hydroxide (A) and 18 MΩ-cm water (B), pressurized with inert gas (He) at a flow rate of 0.5 mL/min. The following linear gradient was used for LacNAc and lactose detection: t=0 min, 5:95 (A:B); t=5 min, 5:95; t=10 min, 20:80; t=25 min, 20:80; t=25 min, 100:0; t=40 min, 100:0; t=40 min, 5:95; t=55 min, 5:95. The following linear gradient was used for sucrose, glucose, and fructose detection: t=0 min, 16:84 (A:B); t=40 min, 16:84; t=40 min, 100:0; t=55 min, 100:0; t=55 min, 16:84; t=70 min, 16:84.

Citrate Measurement

Samples (300 μL) were centrifuged at 13,200 rpm for 3 min. The supernatant was appropriately diluted, and citrate concentrations were measured using the pyridine–acetic anhydride method outlined by Marier and Boulet [12]. A Beckman Coulter DU 530 Life Sciences UV/Visible spectrophotometer was used for measurement.

Acetate and Glycerol Measurement

Samples (100 μL) were centrifuged at 13,200 rpm and 4 °C for 10 min. A 10% solution of trichloroacetic acid (100 μL) was added to 50 μL of the supernatant. The sample was placed on ice to allow for protein precipitation. After 10 min, the sample was centrifuged at 13,200 rpm and 4 °C for 10 min. The supernatant was analyzed using an Agilent 1100 series HPLC with a 59346 Supelcogel H HPLC column. The mobile phase was a 0.1% (*w*/*v*) phosphoric acid solution. Analysis was performed using an isocratic program with a flowrate of 0.17 mL/min and a duration of 60 min.

Curdlan Measurement

Samples for curdlan measurement were prepared using a standard method of curdlan isolation and dissolving the precipitated curdlan in 1 M NaOH [13]. Because curdlan concentrations were below the level detectable by the common dry cell weight measurement, a more sensitive aniline blue assay for detecting β1,3-glucan was used [14, 15]. Since aniline blue is very light-sensitive, this assay was performed in the dark to prevent photobleaching. The microplate reader of a Molecular Devices SpectraMax M5 spectrophotometer was used for measurement, and for this instrument, the optimum

excitation and emission wavelengths were determined to be 400 and 498 nm, respectively, with a cutoff of 475 nm.

Metabolic Flux Analysis

The metabolic network for metabolic flux analysis was generated from the genome sequence of ATCC 31749 (unpublished results) and from biochemical evidence found in the literature [16–18]. The specific pathways included in the metabolic network are discussed in the results section. Overall, the network consists of 32 metabolic reactions and 25 metabolites. The metabolic reactions are listed in the appendix. Ten metabolic fluxes were measured as indicated in Table 2, yielding an overdetermined system of equations. Using MATLAB, the system of equations was solved by applying the following equation:

$$\mathbf{A} = -(\mathrm{inv}(\mathbf{C} \times \mathbf{C}') \times \mathbf{C}) \times \mathbf{M}' \times \mathbf{V} \quad (1)$$

where **A** is a vector of calculated intracellular fluxes, **C** is the stoichiometric matrix of intracellular metabolic fluxes, **M** is the stoichiometric matrix of measured metabolic fluxes, and **V** is a vector of measured metabolic fluxes.

Results

Citrate Stimulation of Oligosaccharide Synthesis

The addition of citrate to the reaction medium stimulated oligosaccharide synthesis in the engineered *Agrobacterium* strain, ATCC 31749/pKEL. This recombinant strain produces two main β1,4-galactose-containing oligosaccharides: LacNAc and lactose, both requiring the sugar nucleotide UDP-galactose. To optimize the amount of citrate for oligosaccharide production, various concentrations of sodium citrate were added to the reaction buffer, ranging from 0 to 10 g/L (Fig. 1). The most dramatic improvement in oligosaccharide production was observed with the addition of 5 g/L sodium citrate. Increasing the concentration of sodium citrate from 2 to 5 g/L, a 2.5-fold increase, led to nearly an equivalent improvement in oligosaccharide production, a 2.7-fold increase for LacNAc and a 2.4-fold increase for lactose. Doubling the concentration of sodium citrate to 10 g/L did

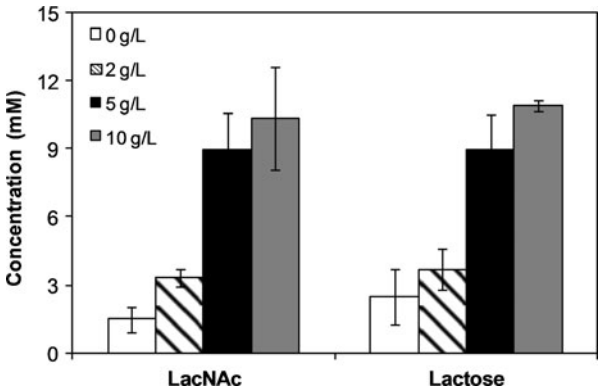

Fig. 1 Oligosaccharide production in ATCC 31749/pKEL with varying concentrations of sodium citrate

not yield a similar increase in oligosaccharide production, indicating that an additional increase in citrate concentration will result in minimal improvement. The highest oligosaccharide concentrations were achieved with addition of 10 g/L sodium citrate, representing nearly a sevenfold improvement in LacNAc and over a fourfold improvement in lactose production compared to the control with 0 g/L sodium citrate.

Mechanisms of Citrate Stimulation

From the known biological and chemical functions of citrate as a carbon and energy source, chelating agent, and buffer, there are several possible mechanisms for the observed stimulation of oligosaccharide production in ATCC 31749/pKEL. These potential mechanisms were investigated to gain insight into oligosaccharide synthesis in the engineered *Agrobacterium*.

Citrate as Carbon Source for Energy Production

Past reports have indicated that *Agrobacterium* sp. can grow using citrate as the sole carbon source [10]. As an intermediate of the TCA cycle, citrate can be directly consumed to provide both cellular energy and carbon building blocks. Citrate concentrations throughout the oligosaccharide synthesis reaction were measured to investigate citrate consumption. Nearly 40% of the initial amount of citrate was consumed by ATCC 31749/pKEL during oligosaccharide synthesis, indicating that citrate is utilized as a carbon and energy source (Fig. 2). Surprisingly, the consumption of citrate as a carbon source did not prevent the uptake of sucrose for oligosaccharide production. In fact, sucrose consumption increased by 79% with the addition of citrate. Many microorganisms possess strict catabolite repression systems, preventing the simultaneous uptake of two carbon sources. However, it appears that ATCC 31749 does not employ this type of regulation with regard to citrate consumption, allowing simultaneous uptake of both citrate and sucrose.

To confirm that the energy produced from citrate consumption leads to increased oligosaccharide synthesis, several other metabolites in the TCA cycle were investigated as potential energy sources. Using an equimolar amount of TCA metabolite, both α-ketoglutarate and succinate stimulated oligosaccharide synthesis, with approximately a threefold increase in LacNAc (Fig. 3) and twofold increase in lactose (data not shown). The

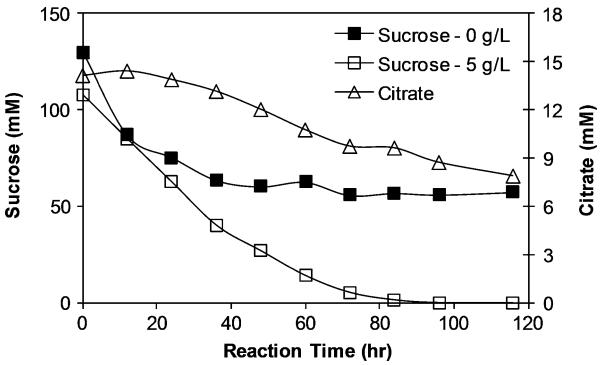

Fig. 2 Sucrose and citrate concentrations during oligosaccharide synthesis. Sucrose concentration in citrate-free reaction (*filled square*). Sucrose concentration in reaction containing 5 g/L sodium citrate (*open square*). Citrate concentration in reaction containing 5 g/L sodium citrate (*open upright triangle*)

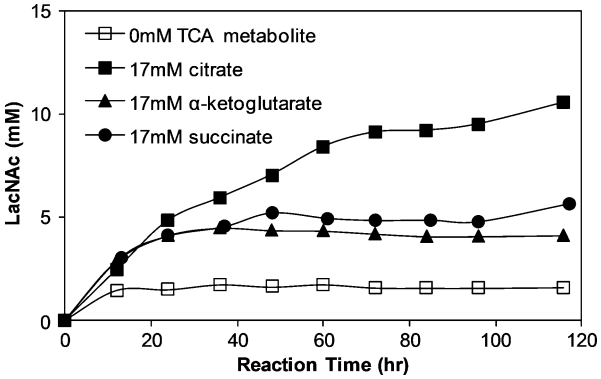

Fig. 3 LacNAc synthesis in ATCC 31749/pKEL with addition of TCA cycle metabolites. Control with 0 mM TCA cycle metabolites (*open square*). Addition of 17 mM citrate (*filled square*). Addition of 17 mM α-ketoglutarate (*filled upright triangle*). Addition of 17 mM succinate (*filled circle*)

effect of α-ketoglutarate and succinate on oligosaccharide synthesis is not as dramatic as the effect of citrate, yet this is to be expected since the energy yield from α-ketoglutarate (9 ATP equivalents) and succinate (5 ATP equivalents) is less than that from citrate (12 ATP equivalents). However, both α-ketoglutarate and succinate, like citrate, can serve as chelating agents and buffers. These mechanisms must also be investigated.

Citrate as Chelating Agent

Like many cellular enzymes, the recombinant β1,4-galactosyltransferase (LgtB) from *Neisseria meningitidis* used for production of the β1,4-galactose-containing disaccharides (LacNAc and lactose) in ATCC 31749/pKEL requires metal ions as cofactors. In particular, manganese (Mn^{2+}) is an essential cofactor for LgtB activity, with magnesium (Mg^{2+}) and calcium (Ca^{2+}) substitutions yielding only half the activity observed with Mn^{2+} [19]. As a chelating agent, citrate may enhance the solubility of Mn^{2+} in the reaction medium, thereby increasing the intracellular concentration of Mn^{2+} available as cofactor for LgtB. Subsequently, the enhanced activity of LgtB may lead to higher production of the disaccharides LacNAc and lactose. To test this theory, oligosaccharide production was measured using a reaction buffer containing an alternative chelating agent, phytic acid.

Similar to citrate, phytic acid is a well-known chelating agent, yet unlike citrate, it is not a known metabolite of *Agrobacterium* sp. Furthermore, phytic acid has been used in previous fermentations for oligosaccharide synthesis, presumably due to its chelating ability [20, 21]. Substituting phytic acid for citrate in the reaction medium yields oligosaccharide levels comparable to the control reaction, containing neither citrate nor phytic acid (Fig. 4). These results indicate that citrate chelation does not contribute significantly to the mechanism for citrate-induced stimulation of oligosaccharide synthesis.

Citrate as a Buffer

As a weak acid, citrate may act as a buffer to maintain the pH at an optimal level for oligosaccharide synthesis. In fact, citrate is a common buffer for enzymatic studies [22]. Citrate may either control the extracellular pH of the reaction, or if transported into the cell, citrate may regulate intracellular pH. During the course of oligosaccharide synthesis, the pH

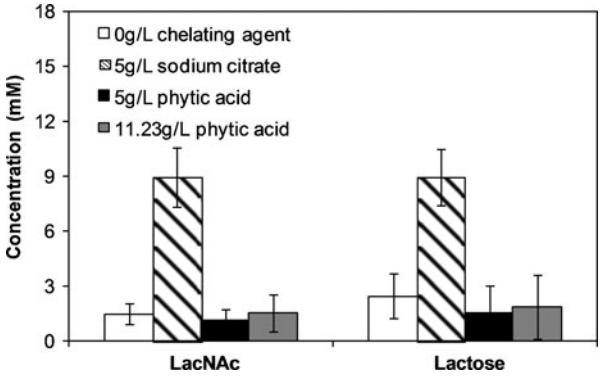

Fig. 4 Oligosaccharide synthesis with the addition of a chelating agent

of the reaction becomes more acidic with time. In an effort to maintain constant pH, a Tris–HCl buffer (pH 7.5) was added to the reaction medium, yet with a buffer concentration of 50 mM, the buffering capacity may be limited. Therefore, the influence of citrate's buffering capacity was investigated by analyzing the effect of citrate under constant pH conditions. A pH of 6.0 was selected as this was the optimum pH determined by experimental testing of constant pH conditions ranging from 5.5 to 7.5. The results under constant pH conditions were compared to the previous results with no pH adjustment.

The buffering capacity of citrate had a significant influence on oligosaccharide production (Fig. 5b). Over 60% of the increase in LacNAc production with the addition of citrate can be accounted for by the buffering mechanism. During oligosaccharide production, the pH of the reaction drops precipitously at the beginning of the reaction, reaching a minimum after 16 h (Fig. 5a). Without the addition of citrate, the pH falls to a minimum of 4.8, while with citrate, the pH minimum of the reaction is 5.4. This small difference in pH clearly has a dramatic effect on enzyme activity and oligosaccharide production. The increase in sucrose consumption with the addition of citrate can also be attributed to the buffering effect. With pH adjustment, the rate of sucrose consumption is similar for both reactions, regardless of the presence of citrate (data not shown). While the buffering mechanism is a significant contribution to citrate stimulation of oligosaccharide production, it does not fully account for the stimulation as a considerable increase in oligosaccharide production remains even with pH adjustment. This additional improvement may be due to the use of citrate as a carbon and energy source as discussed above. This is consistent with the significant consumption of citrate during synthesis, yet this does not rule out other mechanisms such as an unidentified regulatory mechanism of citrate.

Simultaneous Uptake of Multiple Carbon Sources

With the ubiquitous evolution of catabolite repression mechanisms in bacteria, the simultaneous consumption of sucrose and citrate is an unexpected occurrence. To determine if this phenomenon is due to the specific combination of sucrose and citrate, glucose, an alternative carbon source, was tested with citrate for simultaneous consumption. ATCC 31749/pKEL consumed both glucose and citrate throughout the reaction, and similarly, oligosaccharide synthesis and glucose consumption increased with the addition of citrate (Table 1). However, the increase in oligosaccharide production is much higher when sucrose is used as the carbon source (4.5-fold increase) than when glucose is employed

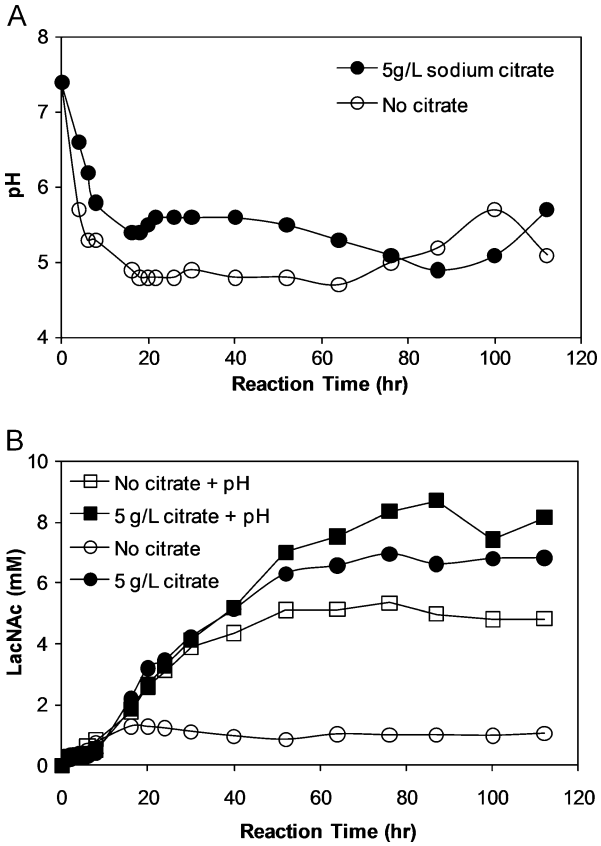

Fig. 5 **a** pH measurement in LacNAc synthesis reactions with and without the addition of citrate, **b** LacNAc synthesis by ATCC 31749/pKEL with and without citrate and pH adjustment. Conditions: 0 g/L sodium citrate, no pH adjustment (*open circle*); 5 g/L sodium citrate, no pH adjustment (*filled circle*); 0 g/L sodium citrate with pH adjustment (*open square*); 5 g/L sodium citrate with pH adjustment (*filled square*)

(1.8-fold increase). This may be due to the fact that sucrose is a preferred carbon source of ATCC 31749. As evidence, the increase in sucrose consumption with the addition of citrate, a 79% increase, is greater than the increase in glucose consumption, a 55% increase. Overall, these results indicate that the simultaneous consumption of multiple carbon sources is not restricted to the use of sucrose, but instead, appears to be independent of the carbon source.

Table 1 Oligosaccharide synthesis with different carbon sources

Carbon source	Sodium citrate	LacNAc (mM)	Lactose (mM)	Carbon source consumed (mM)	Citrate consumed (mM)
50 g/L sucrose	0 g/L	1.48±0.58	2.47±1.22	80.1±9.62	N/A
	5 g/L	8.93±1.64	8.97±1.56	143±2.03	6.25±2.04
50 g/L glucose	0 g/L	0.74±0.18	5.30±0.08	58.4±19.6	N/A
	5 g/L	1.92±0.33	8.93±0.93	90.5±18.1	4.64±1.84

Metabolic Flux Analysis

From the results presented above, it is apparent that the addition of citrate influences multiple pathways including oligosaccharide synthesis, energy production, and carbon uptake. Metabolic flux analysis was performed to gain further insight into the effect of citrate on the entire metabolic network of the cell.

The first challenge in applying metabolic flux analysis is to determine which pathways are active during the oligosaccharide synthesis reaction. Previous experimental results indicate the Entner–Doudoroff (ED) pathway is utilized for sugar metabolism in *Agrobacterium* sp. [9]; therefore, this pathway was added to the metabolic network. The primary purpose of the pentose phosphate pathway is to produce reducing equivalents for anabolic reactions and to provide the cell with ribose-5-phosphate for synthesis of nucleotides. Under the conditions of the oligosaccharide synthesis reaction, both anabolic reactions and the need for nucleotides are minimal, allowing the pentose phosphate pathway to be excluded from the metabolic network. The oxygen level during oligosaccharide synthesis is a major influence on cell metabolism. Ideally, MFA should be performed with data obtained from experiments under well-defined D.O. conditions. Earlier in this research, we carried out several experiments in a bioreactor with DO controlled within aerobic regimes. This condition, however, failed to yield any product due to the assimilation of acceptor sugar (*N*-acetyl-glucosamine) as nitrogen source, which resulted in cell growth instead of oligosaccharides synthesis. Completely anaerobic conditions yielded no products either, presumably due to the low energy yield of the metabolism. We found that the shake-flask conditions employing high cell density culture (10% (*w*/*v*)) with vigorous shaking (250 rpm) gave the best product yield. This condition may fall well within the microaerobic condition, which exhibits both fermentative and respiratory characteristics. As evidence of this microaerobic conditions, acetate was found to accumulate during the synthesis and a functional TCA cycle was supported by the rapid consumption of citrate, as well as other TCA cycle metabolites. Combining the oligosaccharide synthesis, ED, and acetate production pathways with the glyoxylate and TCA cycles provides the basic metabolic network for ATCC 31749 during oligosaccharide synthesis.

Several metabolites were found to accumulate during oligosaccharide synthesis, namely glucose and fructose. Additional reactions were added to the metabolic network to account for the accumulation of these sugars. Furthermore, ATCC 31749 was found to consume the residual glycerol remaining from the wash step of cell preparation, and subsequently, the metabolic network was altered to reflect this additional carbon source (refer to the Appendix for a list of all reactions included in the network).

With the metabolic network established, metabolic flux analysis was performed using fluxes measured from oligosaccharide synthesis reactions with and without the addition of citrate. Figures 3 and 5 show three distinct phases within the LacNAc synthesis reaction. In the first phase (0–12 h), both reactions, with and without citrate, synthesize LacNAc, have a rapid decline in pH, and consume a negligible amount of citrate. In the second phase (12–60 h), the pH is stabilized, and citrate is consumed, allowing the citrate-containing reaction to continue LacNAc synthesis, while synthesis no longer occurs in the citrate-free reaction. In the third phase (60–120 h), sucrose and citrate consumption slowly decrease for the citrate-containing reaction, leading to a lower rate of oligosaccharide synthesis. Because the fluxes are very similar during the first phase and the fluxes are not constant in the third phase, only the second phase was used for metabolic flux analysis.

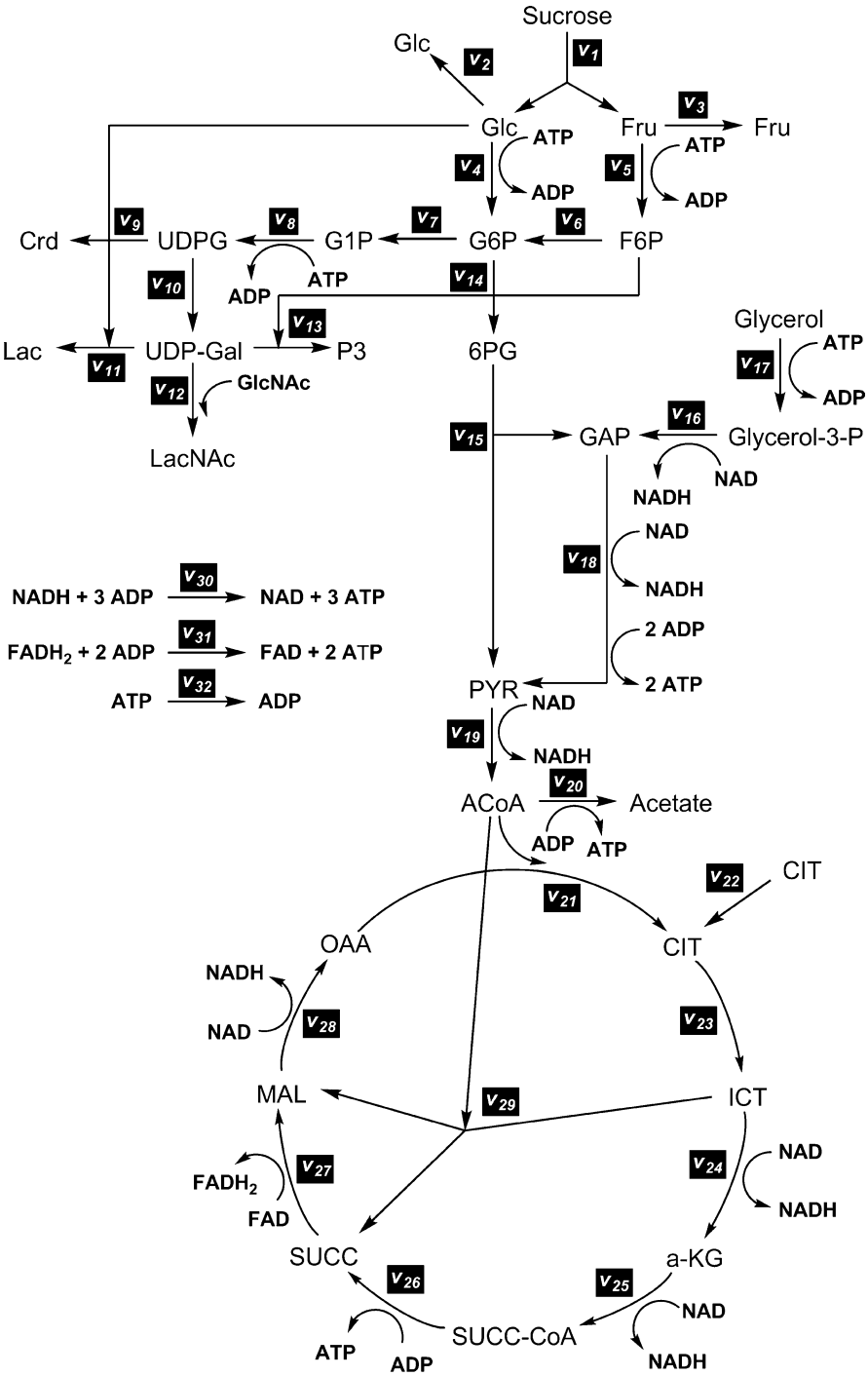

Fig. 6 Metabolic network for ATCC 31749/pKEL. Metabolic fluxes are indicated by v_i with i=1 to 32. Fluxes v_{10}–v_{13} make up the engineered pathway for oligosaccharide synthesis, while all other fluxes are native to ATCC 31749

Metabolic flux analysis for the metabolic network shown in Fig. 6 was performed with ATP balance. The results from this analysis are shown in Table 2, with the fluxes corresponding to the metabolic network in Fig. 6. The citrate-containing reaction has higher rates of consumption for both sucrose and glycerol. In fact, no glycerol is consumed in the citrate-free reaction. The high rate of carbon consumption leads to a higher flux through the ED pathway, nearly a 400% increase compared to the citrate-free reaction, but surprisingly, the production of acetate only increased by 47%. The majority

Table 2 Metabolic flux analysis

Flux	Enzyme(s)	Citrate-free reaction	5 g/L sodium citrate reaction
v_1[a]	Sucrose hydrolase	19	100
v_2[a]	Glucose accumulation	3.3	24
v_3[a]	Fructose accumulation	4.3	8.8
v_4	Glucokinase	16	73
v_5	Fructokinase	15	91
v_6	Phosphoglucose isomerase	15	90
v_7	Phosphoglucomutase	−0.08	9.0
v_8	UTP-Glucose-1-phosphate uridylyltransferase	−0.08	9.0
v_9[a]	Curdlan synthase	0.39	0.44
v_{10}	UDP-Galactose 4′-epimerase	−0.47	8.6
v_{11}[a]	β1,4-Galactosyltransferase (glucose as substrate)	−0.23	3.6
v_{12}[a]	β1,4-Galactosyltransferase (GlcNAc as substrate)	−0.17	3.3
v_{13}[a]	β1,4-Galactosyltransferase (mannose as substrate)	−0.07	1.6
v_{14}	Glucose-6-phosphate dehydrogenase; 6-phosphoglucolactonase	31	153
v_{15}	6-Phosphogluconate hydrolase; 2-keto-3-deoxygluconate aldolase	31	153
v_{16}	Glycerol-3-phosphate dehydrogenase	−3.5	24
v_{17}[a]	Glycerol kinase	−3.5	24
v_{18}	Glyceraldehyde-3-phosphate dehydrogenase; phosphoglycerate kinase; phosphoglycerate mutase; pyruvate kinase	27	177
v_{19}	Pyruvate dehydrogenase	58	330
v_{20}[a]	Acetyl-CoA synthetase	17	25
v_{21}	Citrate synthase	41	308
v_{22}[a]	Citrate uptake	0	2.8
v_{23}	Aconitase	41	310
v_{24}	Isocitrate dehydrogenase	41	314
v_{25}	α-Ketoglutarate dehydrogenase complex	41	314
v_{26}	Succinyl-CoA synthetase	41	314
v_{27}	Succinate dehydrogenase; fumarase	41	311
v_{28}	Malate dehydrogenase	41	308
v_{29}	Isocitrate lyase; malate synthase	0	−2.8
v_{30}	Oxidative phosphorylation	205	1466
v_{31}	Oxidative phosphorylation	41	311
v_{32}	Maintenance energy	784	5516

Values normalized based on sucrose consumption in the 5 g/L sodium citrate reaction

[a] Measured fluxes

of the increased carbon flux is predicted to be directed through the TCA cycle, leading to over a sixfold increase in ATP production. This high rate of ATP generation may be responsible for the increase in oligosaccharide synthesis in the citrate-containing reaction. As expected, the flux through the glyoxylate cycle is negligible for both reactions since the glyoxylate cycle is primarily used for acetate and fatty acid metabolism [23].

The above MFA with ATP balance based on oxidative phospholation may overestimate ATP production as the condition used for synthesis is microaerobic not aerobic. MFA without ATP balance was also performed for a network essentially the same as Fig. 6 but without the last three reactions and without cofactor balance (NADH/NAD and $FADH_2$/FAD). This alternative approach yielded exactly the same flux as the first case. While there may be some uncertainty about the extent of involvement of respiratory metabolism, MFA does point to a trend of increased flux to the TCA cycle, at least a portion of which may be used for enhanced ATP production through coupling of the respiratory chain.

Is the Citrate Effect Unique to *Agrobacterium* sp.?

To determine if the citrate stimulation of oligosaccharide synthesis is unique to *Agrobacterium* sp. ATCC 31749, an engineered *E. coli* strain, AD202/pMUEL, was also tested. Oligosaccharide synthesis in this *E. coli* strain increased slightly with the addition of citrate (Table 3), yet this 30% increase is insignificant compared to the 350% improvement observed with the engineered *Agrobacterium* strain. Furthermore, citrate consumption by AD202/pMUEL is negligible throughout the synthesis reaction, and glucose consumption is similar for both the citrate-free and citrate-containing reactions. This result is surprising as the buffering mechanism was determined to be a major influence of citrate-induced oligosaccharide synthesis in ATCC 31749. Perhaps the citrate-buffered pH is still too low for enhanced oligosaccharide synthesis in *E. coli*. Based on these results, the citrate effect appears to be unique to *Agrobacterium* sp. ATCC 31749.

Discussion

From the results presented in this study, it appears that the citrate-induced stimulation of oligosaccharide synthesis in ATCC 31749/pKEL is due to two primary mechanisms: (1) the consumption of citrate as a carbon and energy source and (2) pH maintenance due to the buffering capacity of citrate. While metal ions are important cofactors for oligosaccharide synthesis, citrate chelation does not appear to be significant.

Table 3 Oligosaccharide synthesis and carbon consumption in AD202/pKEL with and without citrate addition

	Citrate-free reaction buffer	5 g/L sodium citrate
LacNAc (mM)	0.30±0.02	0.40±0.01
Lactose (mM)	0.09±0.05	0.12±0.06
Glucose consumed (mM)	165±15	180±8.7
Citrate consumed (mM)	N/A	2.89±0.33

A major finding of this work is the simultaneous uptake and utilization of multiple carbon sources by the engineered *Agrobacterium*. In many bacteria, catabolite repression prevents the simultaneous uptake of multiple carbon sources, making the co-metabolism of sucrose and citrate in ATCC 31749 a unique property. Only a few other bacterial species have been found to co-metabolize citrate and another carbon source. Microorganisms from the *Enterococcus*, *Leuconostoc*, *Lactobacillus*, and *Lactococcus* genera have been identified which can simultaneously utilize citrate and another carbon source such as glucose or lactose [24–29]. Most of these bacteria are grown under anaerobic or microaerophilic conditions, similar to the microaerobic conditions used for oligosaccharide synthesis with ATCC 31749/pKEL. Unlike ATCC 31749, these other citrate co-metabolizing organisms are all members of the Lactobacillales order. Several *Pseudomonas* species also co-metabolize citrate along with other carbon sources [30, 31], and the results of this work demonstrate that members of the *Rhizobiaceae* family may also possess this metabolic feature.

The synthesis of oligosaccharides in ATCC 31749/pKEL is an energy-intensive process, requiring two high-energy compounds, ATP and UTP, for each glycosidic linkage. The utilization of citrate by the TCA cycle provides additional energy for oligosaccharide production, with each citrate molecule generating 12 molecules of ATP for each turn through the TCA cycle. Assuming each citrate molecule goes through one complete turn of the TCA cycle, the consumption of 65 μmol of citrate would produce 780 μmol of ATP. The measured increase in oligosaccharide production due to citrate stimulation is 140 μmol, requiring 280 μmol of ATP for synthesis. Thus, the energy required for increased oligosaccharide production can be completely accounted for by the ATP generated from citrate consumption.

This work highlights several important aspects of whole-cell oligosaccharide synthesis. First, cellular energy provision is essential for oligosaccharide synthesis. The additional energy from the consumption of citrate accounted for up to 40% of the sixfold increase in LacNAc synthesis observed with citrate addition (Fig. 5b). Second, pH maintenance via the buffering capacity of citrate was essential for optimal metabolic activity. The addition of buffering agents like citrate can maintain the pH at a level for optimum oligosaccharide production, relieving the requirement for *in situ* pH measurement and adjustment. Lastly, judicious selection of the host organism is an important consideration for whole-cell biocatalysis. By selecting *Agrobacterium* sp. ATCC 31749 as opposed to other common hosts like *E. coli*, the ability of the *Agrobacterium* host to co-metabolize multiple carbon sources can be exploited for enhanced oligosaccharide production. The *Agrobacterium* host also has a low pH optimum for polysaccharide and oligosaccharide synthesis, allowing for increased oligosaccharide production with citrate buffering. Investigation of the citrate-induced stimulation of oligosaccharide synthesis in the engineered *Agrobacterium* has revealed important insights into metabolism of *Agrobacterium* sp. ATCC 31749 as well as key factors in whole-cell oligosaccharide synthesis.

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Appendix

(1) Sucrose → Glc + Fru
(2) Glc → Glc (extracellular)

(3) Fru → Fru (extracellular)
(4) Glc + ATP → G6P + ADP
(5) Fru + ATP → F6P + ADP
(6) F6P → G6P
(7) G6P → G1P
(8) G1P + ATP → UDP-Glc + ADP
(9) UDP-Glc → Crd
(10) UDP-Glc → UDP-Gal
(11) UDP-Gal + Glc → Lactose
(12) UDP-Gal + GlcNAc → LacNAc
(13) UDP-Gal + F6P → P3
(14) G6P → 6PG
(15) 6PG → GAP + PYR
(16) Glycerol-3-P + NAD → GAP + NADH
(17) Glycerol + ATP → Glycerol-3-P + ADP
(18) GAP + 2 ADP + NAD → PYR + 2 ATP + NADH
(19) PYR + NAD → ACoA + NADH
(20) ACoA + ADP → Acetate + ATP
(21) ACoA + OAA → CIT
(22) CIT (extracellular) → CIT
(23) CIT → ICT
(24) ICT + NAD → aKG + NADH
(25) aKG + NAD → SUCC-CoA + NADH
(26) SUCC-CoA + ADP → SUCC + ATP
(27) SUCC + FAD → MAL + $FADH_2$
(28) MAL + NAD → OAA + NADH
(29) ICT + ACoA → SUCC + MAL
(30) NADH + 3 ADP → NAD + 3 ATP
(31) $FADH_2$ + 2 ADP → FAD + 2 ATP
(32) ATP → ADP

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