R. eutropha as a bioproduction platform

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Engineering Carbon Storage Biology

Carbon Storage Biology
- Genomics/Genetics
- Biosynthesis
- Cell biology
- Degradation
- Carbon flux
- Carbon fixation
-

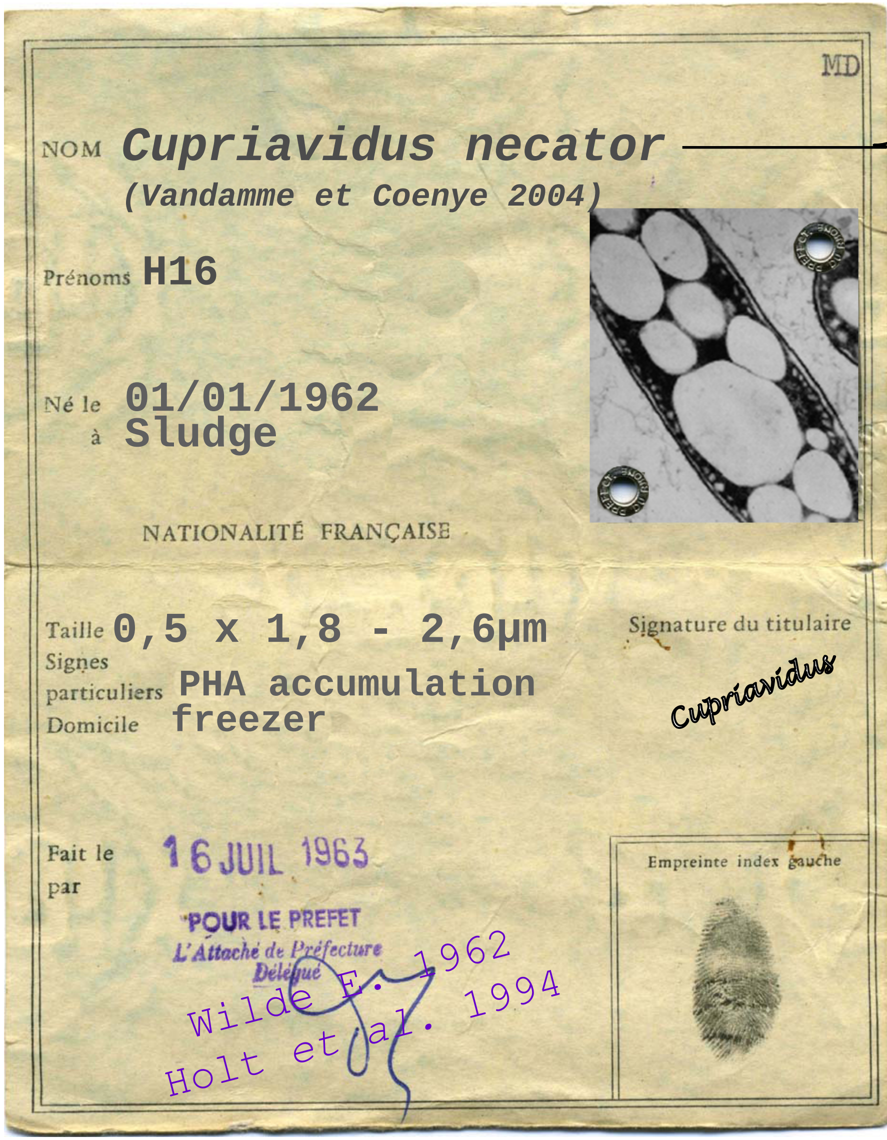
Storage/Product Molecules
- Polymers (PHAs)
- Triglycerides (TAGs)
- Isobutanol (IBT)
- Specialty chemicals

Carbon Storage Systems

Substrate Range of Biocatalyst
- Glucose, xylose
- Glycerol
- Volatile organic acids
- Oils/fatty acids
- CO_2

Bioprocess Development
- Medium composition
- Fermentation strategy
- Product recovery
- Product processing

R. eutropha as a bioproduction platform



Wautersia eutropha *(Vaneechoutte et al. 2004)*
Ralstonia eutropha *(Yabuuchi et al. 1995)*
Alcaligenes eutrophus *(Davis et al. 1969)*
Hydrogenomonas eutropha *(Wittenberger et Respaske 1958)*

Extremely versatile microorganism :

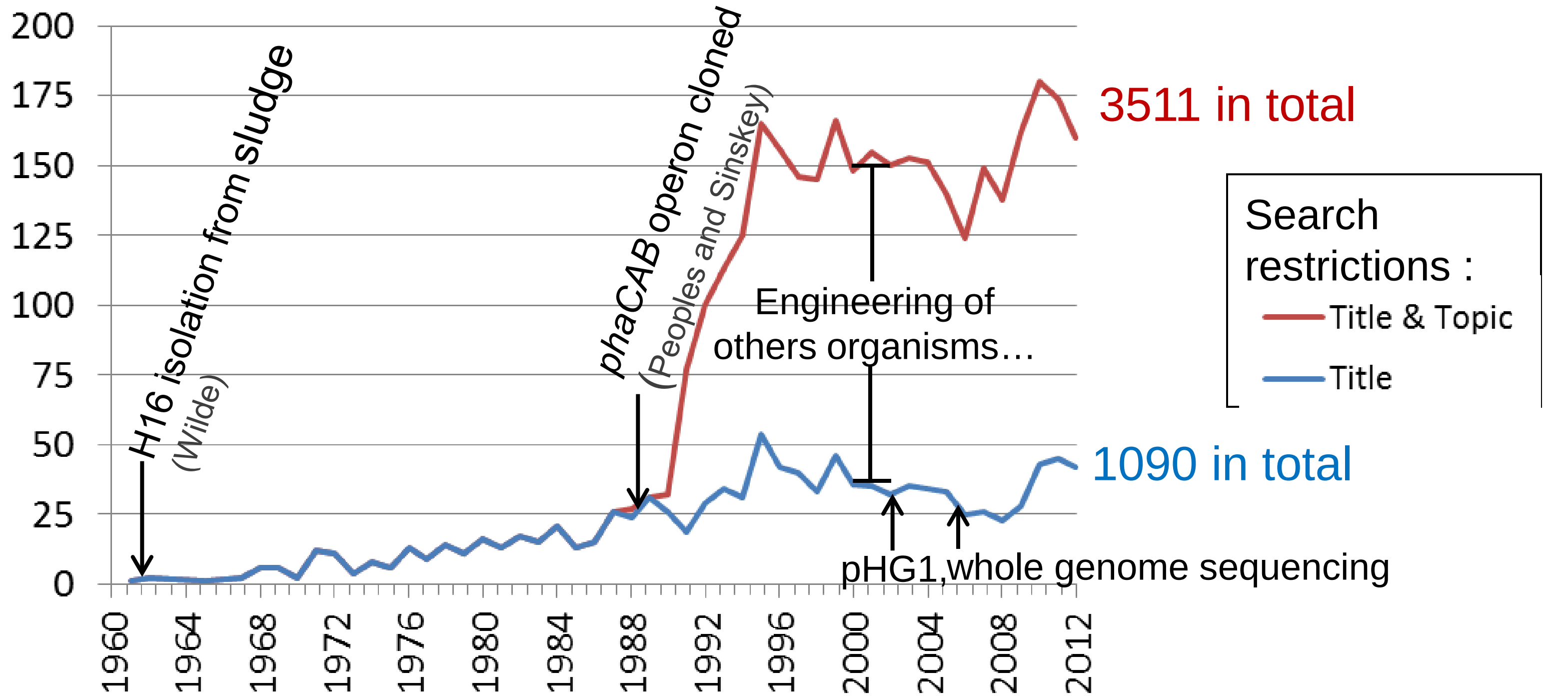
- Autotroph : CO_2-H_2, formic acid
- Heterotroph : fructose, xylose, acetic, propionic, butyric, lactic, succinic, malic, valeric, palmitic, oleic, linoleic… acids, benzoate, phenol, glycerol…
- 2 final electron acceptors: O_2, nitrate

Natural producer of PHA (Poly-Hydroxy-Alkanoates)

Intracellular content of PHA ≥ 80% (mass)

R. eutropha research history

Publications per year concerning *R. eutropha*

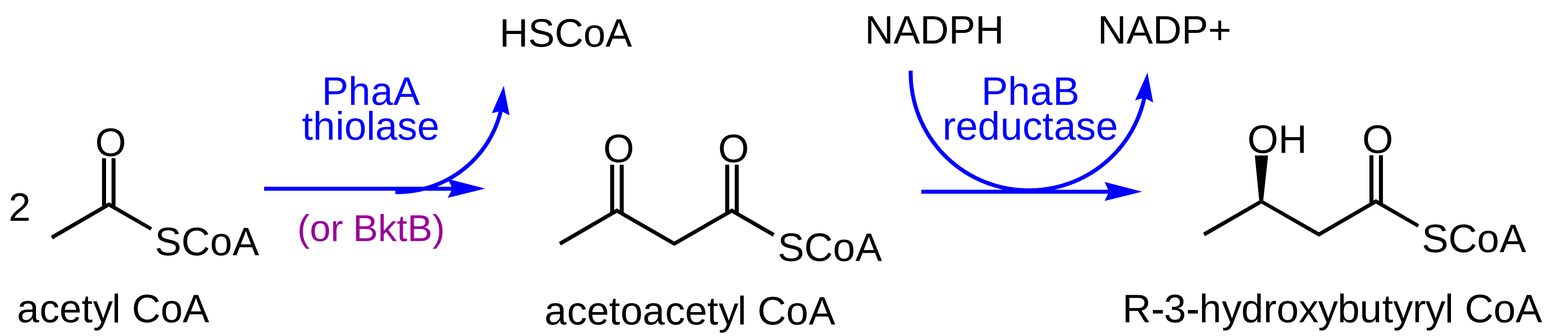


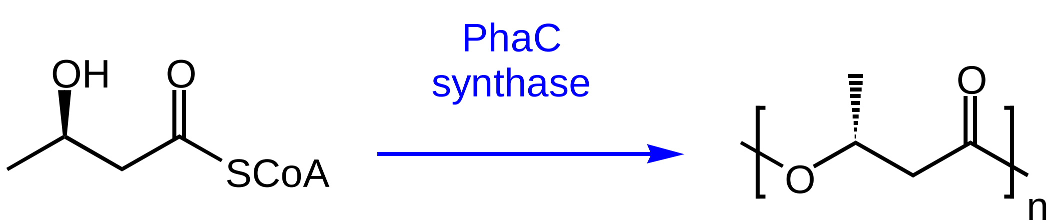
Search : ((ralstonia eutropha)or(alcaligenes eutrophus)or(hydrogenomonas eutropha)or(cupriavidus necator)or(wautersia eutropha))
In Web of Knowledge, Thomson Reuters, 2013/03/04

- 60's : Physiology studies : autotrophic/mixotrophic/heterotrophic metabolism, PHB biosynthesis…
- 70's : Applied research started with SCP (Single Cell Protein) production (first PHB$^-$ mutants)
- 80's : Operon *phaCAB* for PHB synthesis cloned
- 90's : Expression of *phaCAB* in many other organisms, copolymers exploration

Pathway for synthesis of PHB

The *phaA*/*bktB*, *phaB*, and *phaC* genes are required for conversion of acetyl CoA to PHB.

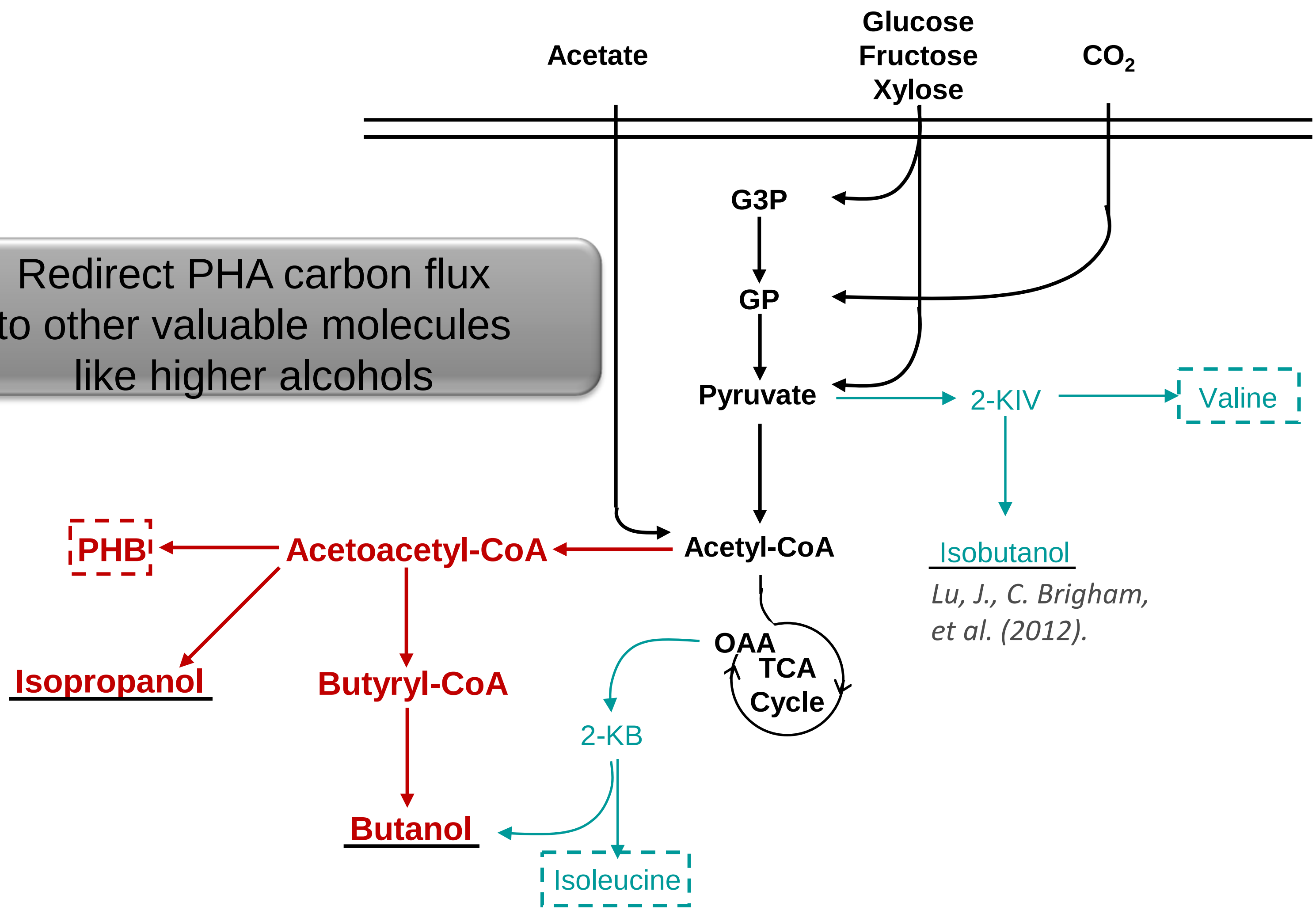




R. eutropha as a bioproduction platform

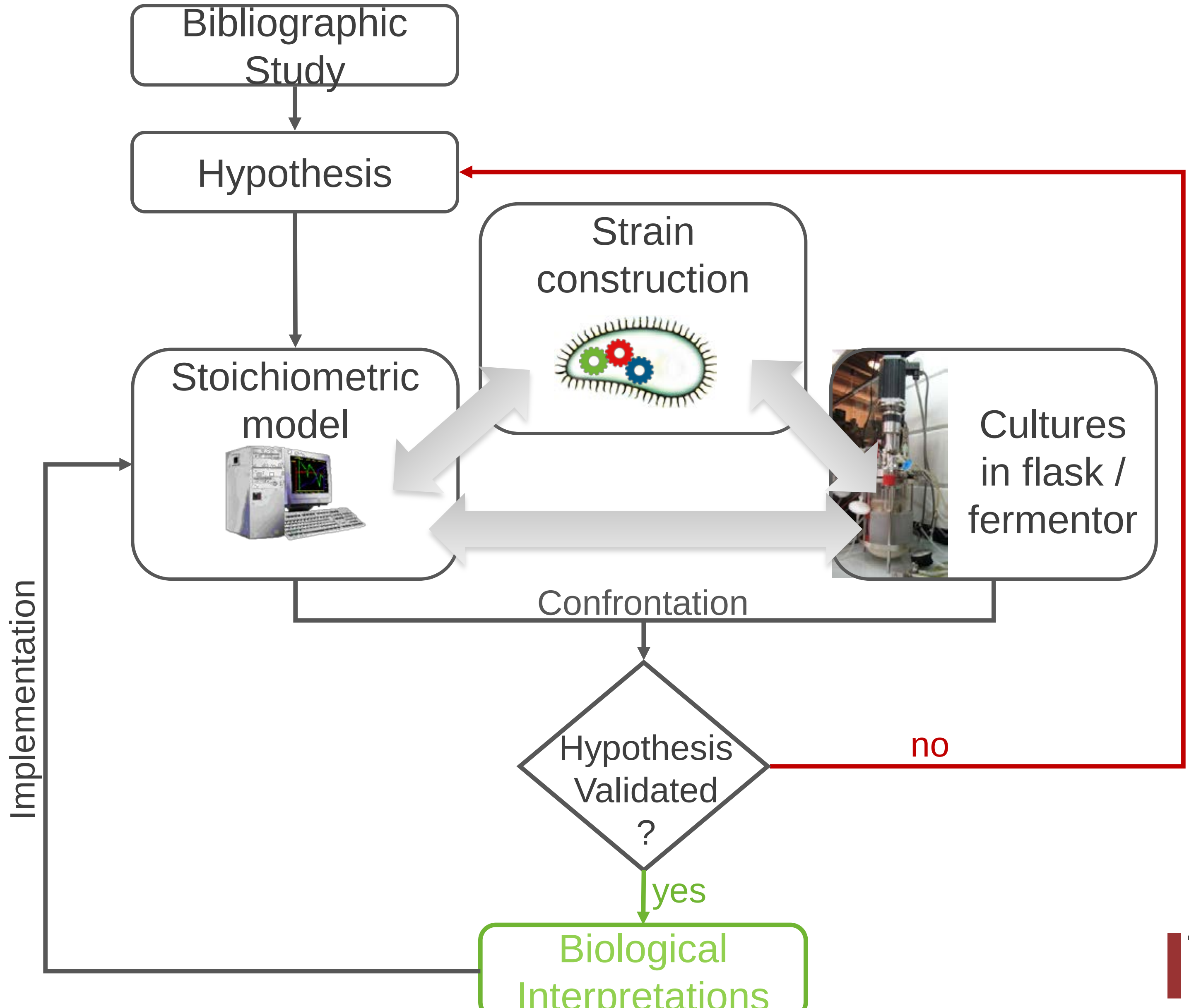
Exploits high potential of this versatile microorganism to build a flexible production platform with special interest in cheap substrates

Redirect PHA carbon flux
to other valuable molecules
like higher alcohols



Lu, J., C. Brigham, et al. (2012).

Complementary approach

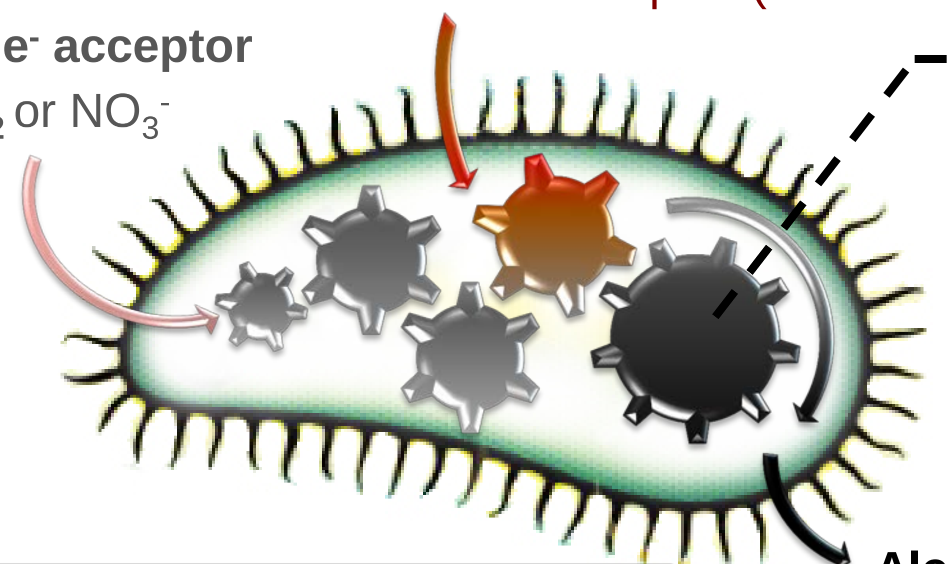


Stoichiometric modeling

- System considered for *R. eutropha*

Carbon and energy sources
autotrophic (CO_2, H_2 or HCO_2^-)
or heterotrophic (**Fructose**)

Final e^- acceptor
O_2 or NO_3^-



Alcohols
isopropanol

Modeling 37-54 reactions, up to 55 metabolites

Takes into account carbon, energy and coenzymes

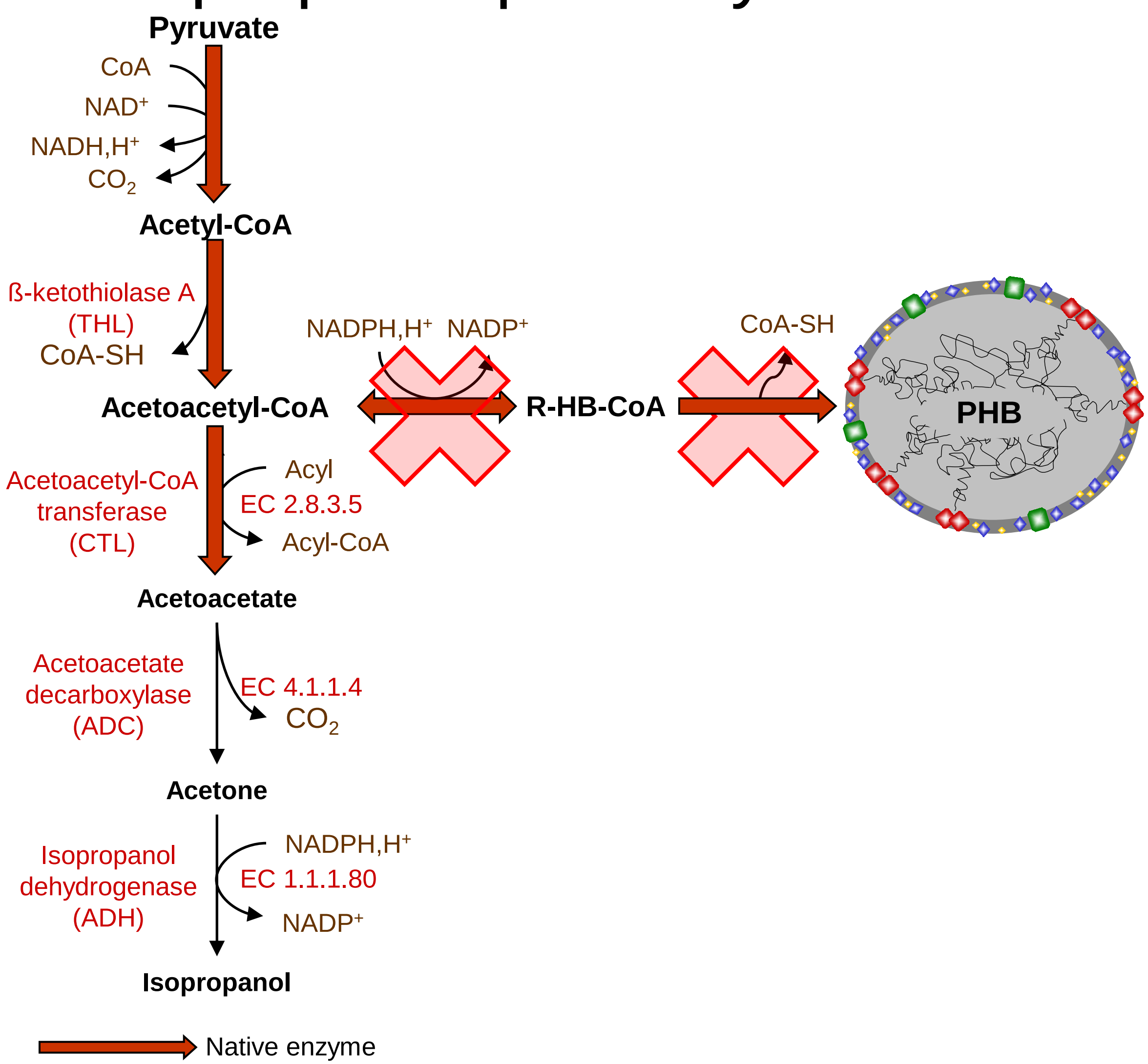
System solved with steady state assumption, with null production of internal metabolites

	THF	Myl	Met	Mer	F-TI	S	NH3	ATF	NAD	NAD	AcC	eryl	fruF	glul	aKg	oaa	pen
r THF-FTHF	-1	0	0	0	1	0	0	0	0	-1	0	0	0	0	0	0	0
r myleneTHF-Methyl-THF	0	-1	1	0	0	0	0	0	0	-1	0	0	0	0	0	0	0
r myleneTHF-Methenyl-THF	0	-1	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0
r myleneTHF-THF	1	-1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
r gluP	0	0	0	0	0	0	0	-1	0	0	0	0	0	1	0	0	0
r F6P	0	0	0	0	0	0	0	0	0	0	0	0	1	-1	0	0	0
r gluconateP	0	0	0	0	0	0	0	0	0	1	0	0	0	-1	0	0	0
r Fruct-diP	0	0	0	0	0	0	0	-1	0	0	0	0	-1	0	0	0	0
r FrcutdiP rev	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
r trioseP	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
r GlyatediP	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
r Pga	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
r PEP	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
r pyr	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
r AcCoA	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0
r acté-AcCoa	0	0	0	0	0	0	0	-1	0	0	1	0	0	0	0	0	0
r isocitrate	0	0	0	0	0	0	0	0	0	0	-1	0	0	0	0	-1	0
r a-kg	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0
r SucCoA	0	0	0	0	0	0	0	0	1	0	0	0	0	0	-1	0	0
r Succina	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
r fumarate	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
r malate	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
r OAA	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0
r pep-oaa	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
r anapl pyr-oaa	0	0	0	0	0	0	0	-1	0	0	0	0	0	0	0	1	0
r oaa pep	0	0	0	0	0	0	0	-1	0	0	0	0	0	0	0	-1	0
r ribulP HMP	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1
r riboseP HMP	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	-1
r xyluloseP	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	-1
r hep HPM	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
r TrioseFruc	0	0	0	0	0	0	0	0	0	0	0	1	-1	0	0	0	0
r erythrP HMP	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0
r trioseP HMP	0	0	0	0	0	0	0	0	0	0	0	-1	1	0	0	0	0
r GlycerolP	0	0	0	0	0	0	0	0	-1	0	0	0	0	0	0	0	0

In collaboration with LISBP (Toulouse, France)

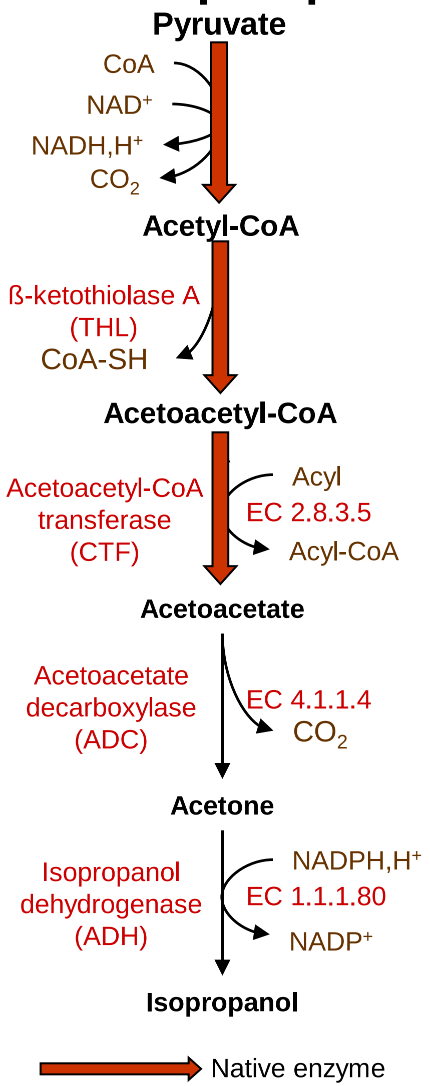
Stoichiometric modeling

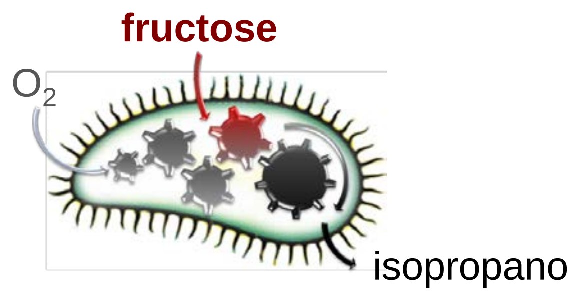
- Isopropanol pathway overview



Stoichiometric modeling

- Isopropanol production from fructose





1 Fructose + $Y_{S,O2}$ O_2 ➔ $Y_{S,ISOPROP}$ Isopropanol + $Y_{S,CO2}$ CO_2 + $Y_{S,H2O}$ H_2O + Energy

$$Y_{S,ISOPROP} = \frac{\text{N\# of Carbon molecule of ISOPROPANOL produced}}{\text{N\# of Carbon molecule of FRUCTOSE consumed}} < 1$$

$Y_{S,ISOPROP\ THEO} = 0.50\ Cmole.Cmole^{-1}$ ➔ 100% of the maximum

The chosen pathway is validated

Strain constructions

- 1- How to overcome the poor expression of heterologous genes due to codon usage differences ?

R. eutropha genes

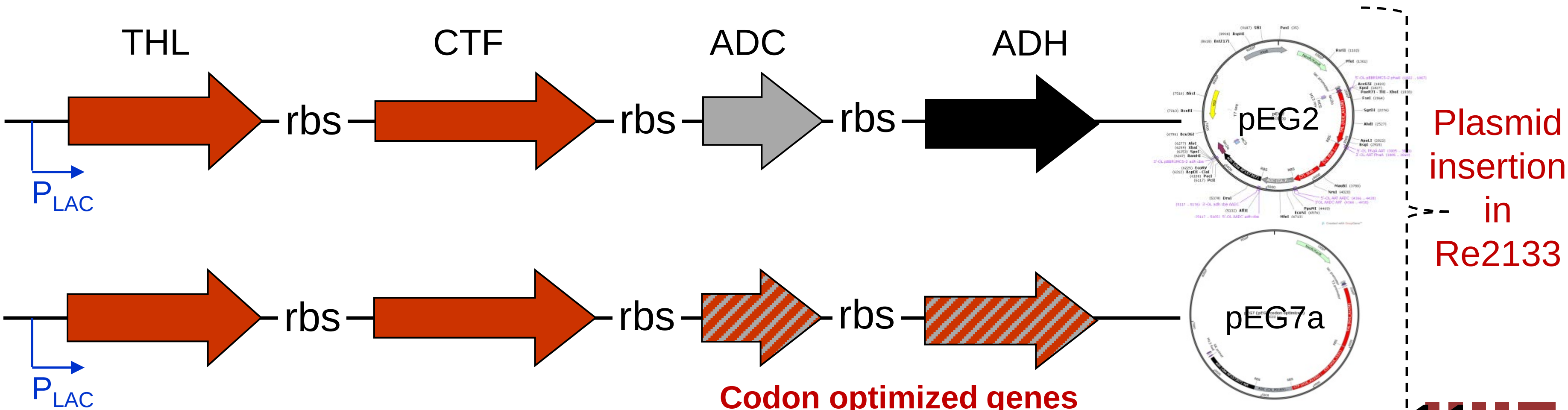
Heterologous genes from *Clostridium acetobutylicum and beijerinckii*

phaA H16_A1438 (1182 bp)

H16_A1331 H16_A1332 (1352 bp)

CA_P0165 (735 bp)

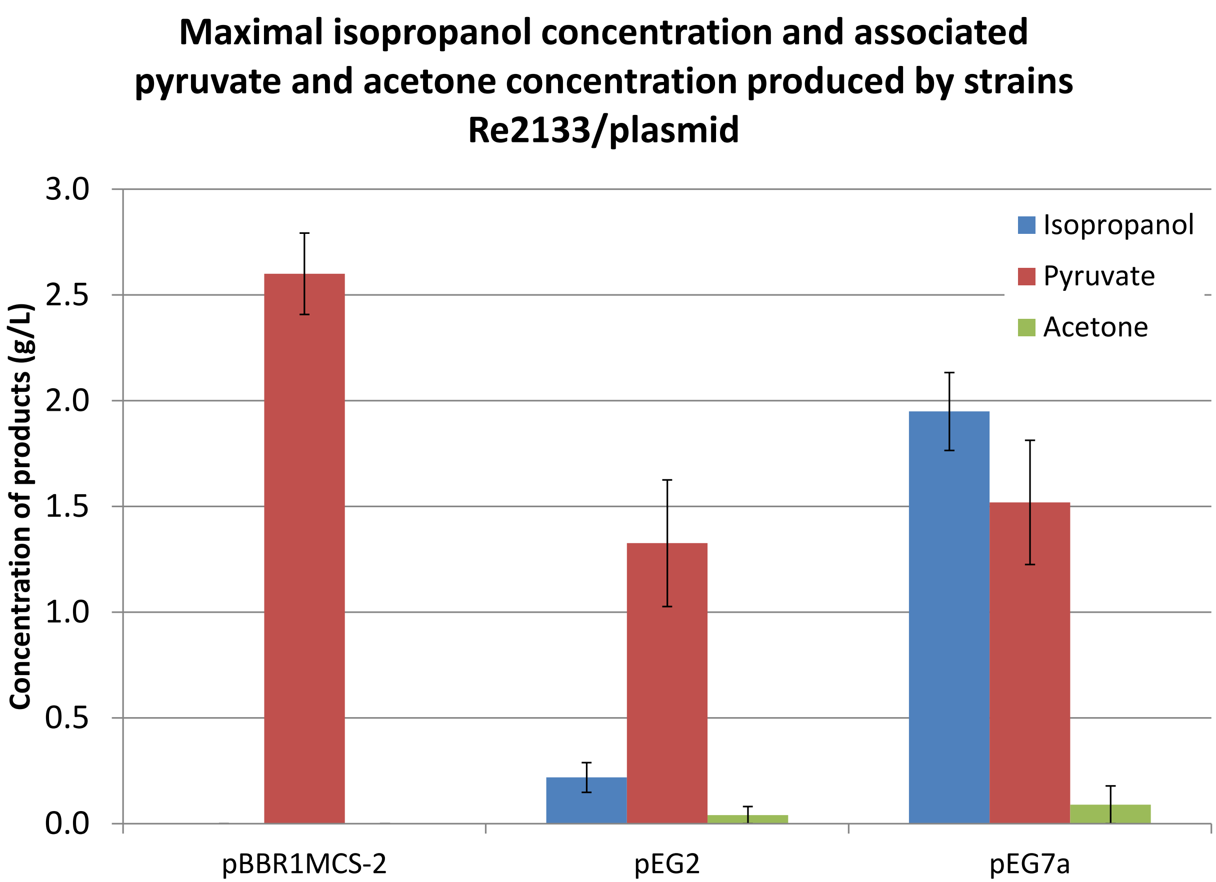
cbe AF157307 (1056 bp)



Strain Re2133: H16 ΔphaB1B2B3C1 Gen^r (*Budde, C. F., A. E. Mahan, et al. (2010)*)

Strain constructions

- 1- Native genes vs codon-optimized genes



Empty plasmid

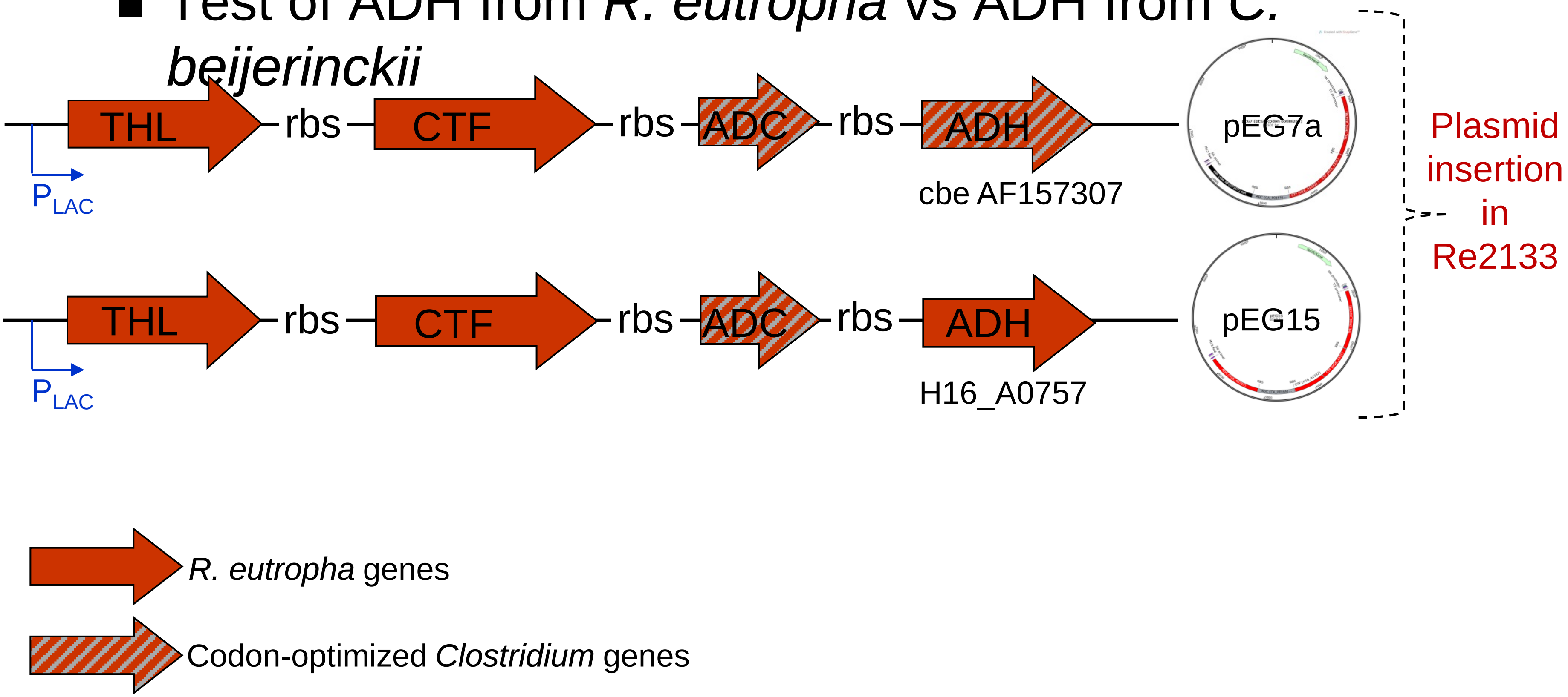
Plasmid with **native** genes

Plasmid with **codon-optimized** genes

Codon optimization of genes increases by 8.9 ± 3.0 folds the isopropanol production

Strain constructions

- 2 - Is *R. eutropha* Alcohol Dehydrogenase (ADH) able to use acetone as substrate ?
  - Test of ADH from *R. eutropha* vs ADH from *C. beijerinckii*

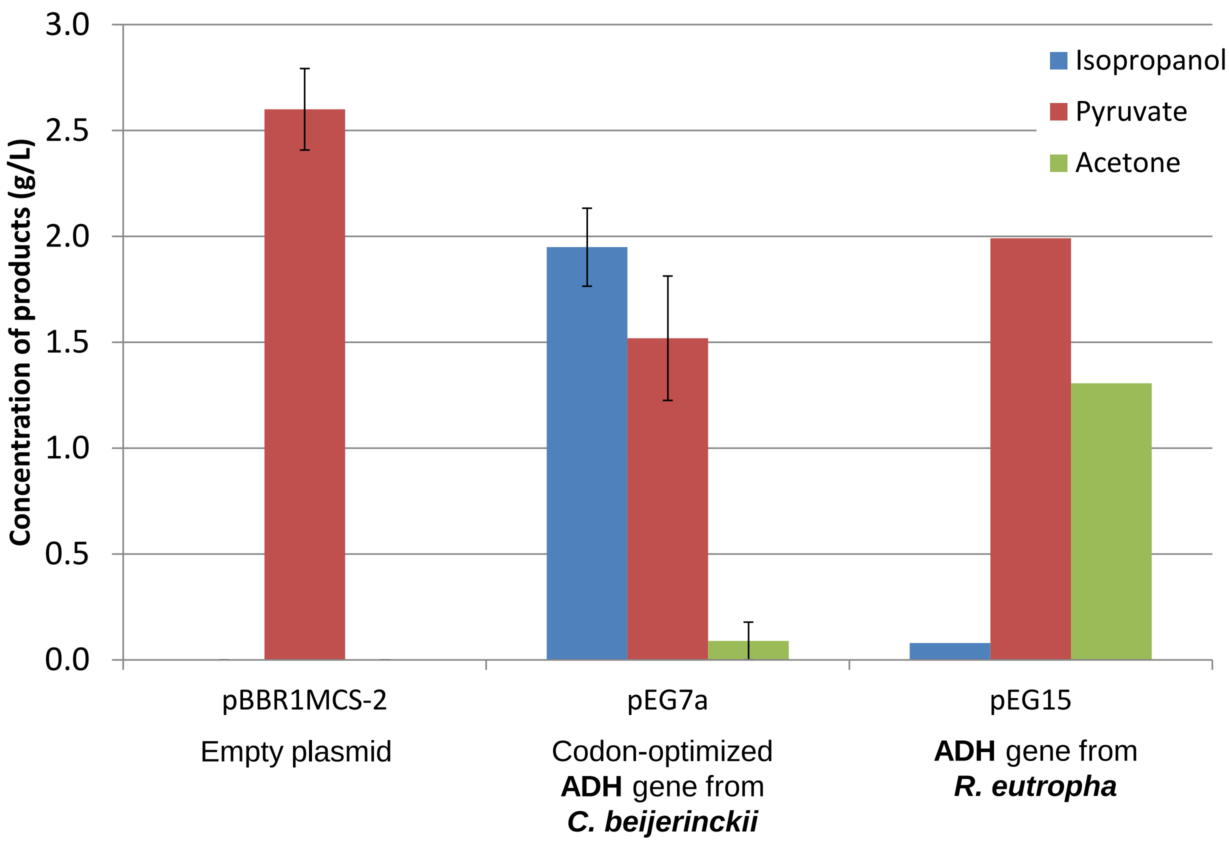


Strain Re2133: H16 ΔphaB1B2B3C1 Gen^r (*Budde, C. F., A. E. Mahan, et al. (2010)*)

Strain constructions

- 2 - ADH from *R. eutropha* vs ADH from *C. beijerinckii*

Maximal isopropanol concentration and associated pyruvate and acetone concentration produced by strains Re2133/plasmid

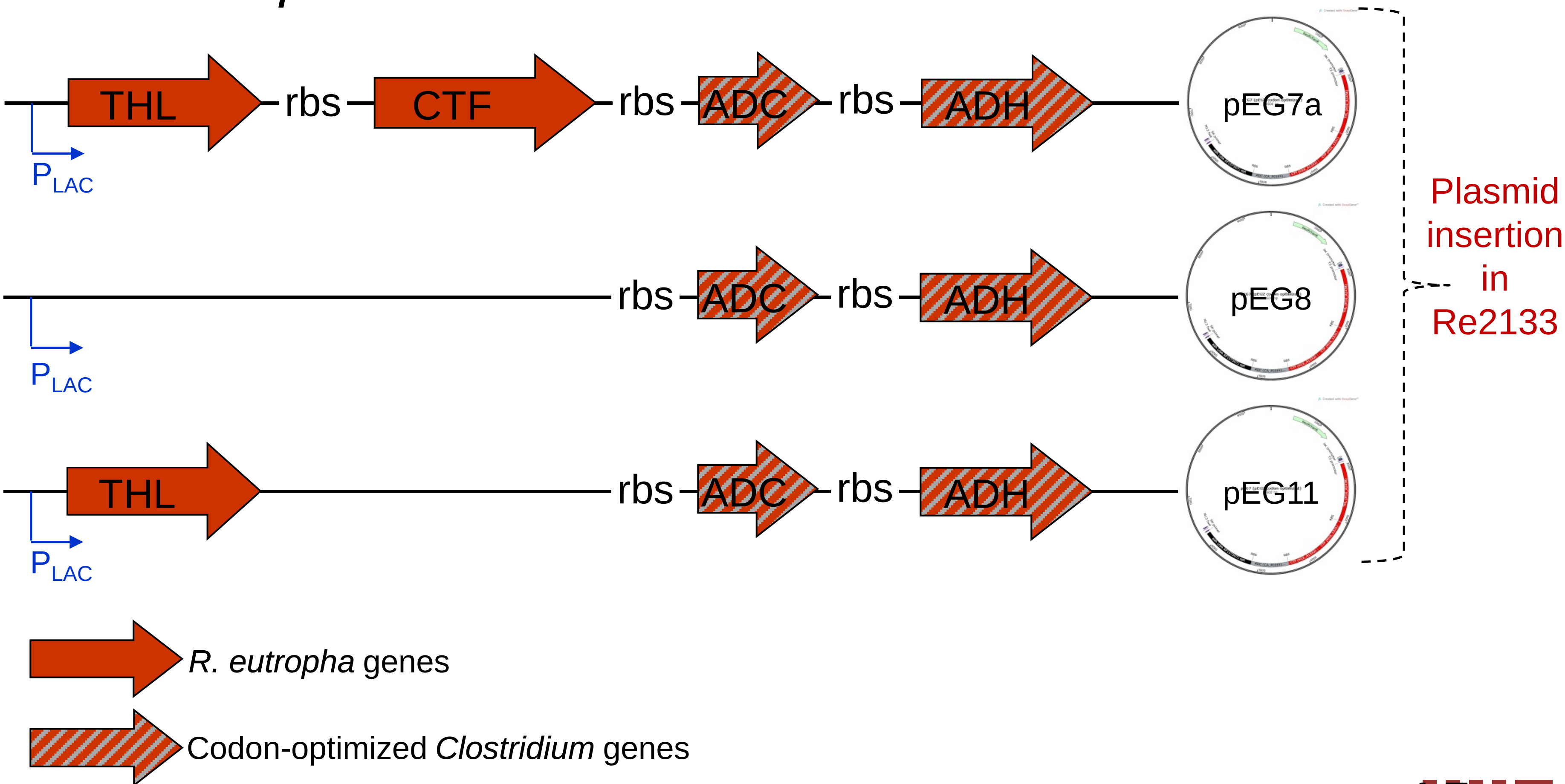


With the ADH from *R. eutropha* only about 80 mg/L of isopropanol are produced. Acetone is produced instead.

ADH from *R. eutropha:*
- low activity towards acetone
- but not suitable for isopropanol production

Strain constructions

- 3 - Is it necessary to overexpress the native genes from *R. eutropha* ?

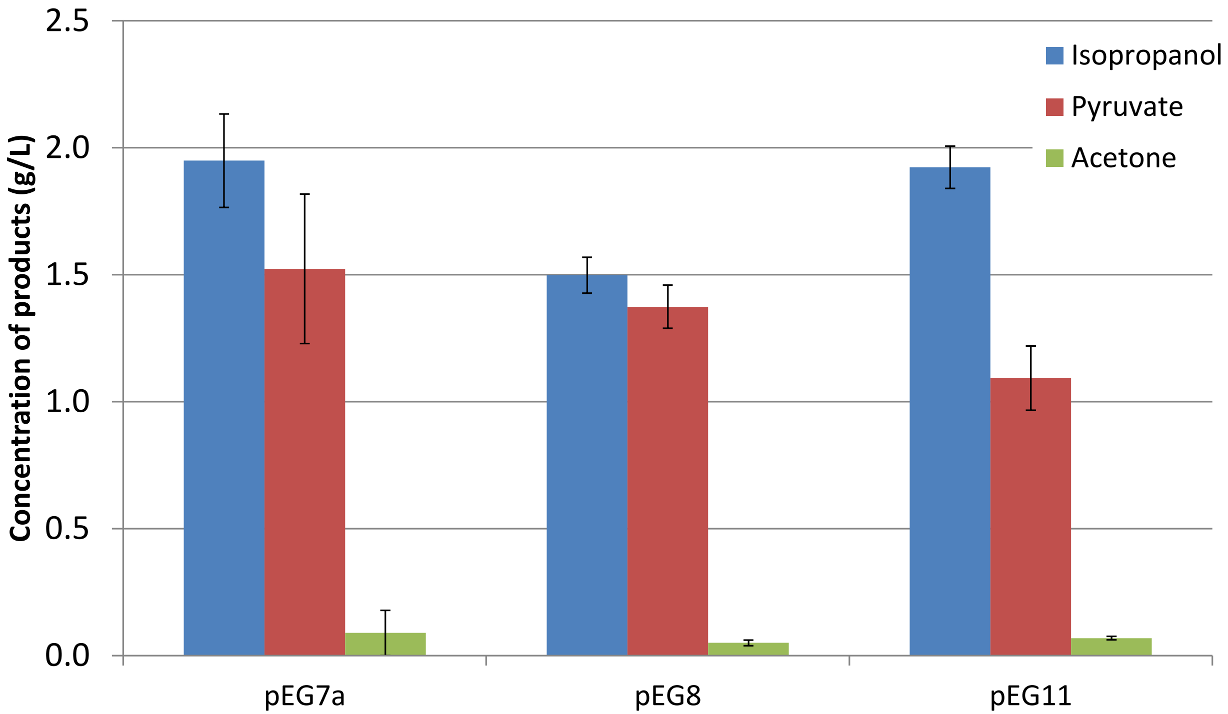


Strain Re2133: H16 ΔphaB1B2B3C1 Gen[r] *(Budde, C. F., A. E. Mahan, et al. (2010))*

Strain constructions

- 3 - Overexpression of *R. eutropha* native genes or not ?

Maximal isopropanol concentration and associated pyruvate and acetone concentration produced by strains Re2133/plasmid



THL/CTF/ADC/ADH /ADC/ADH **THL/** /ADC/ADH

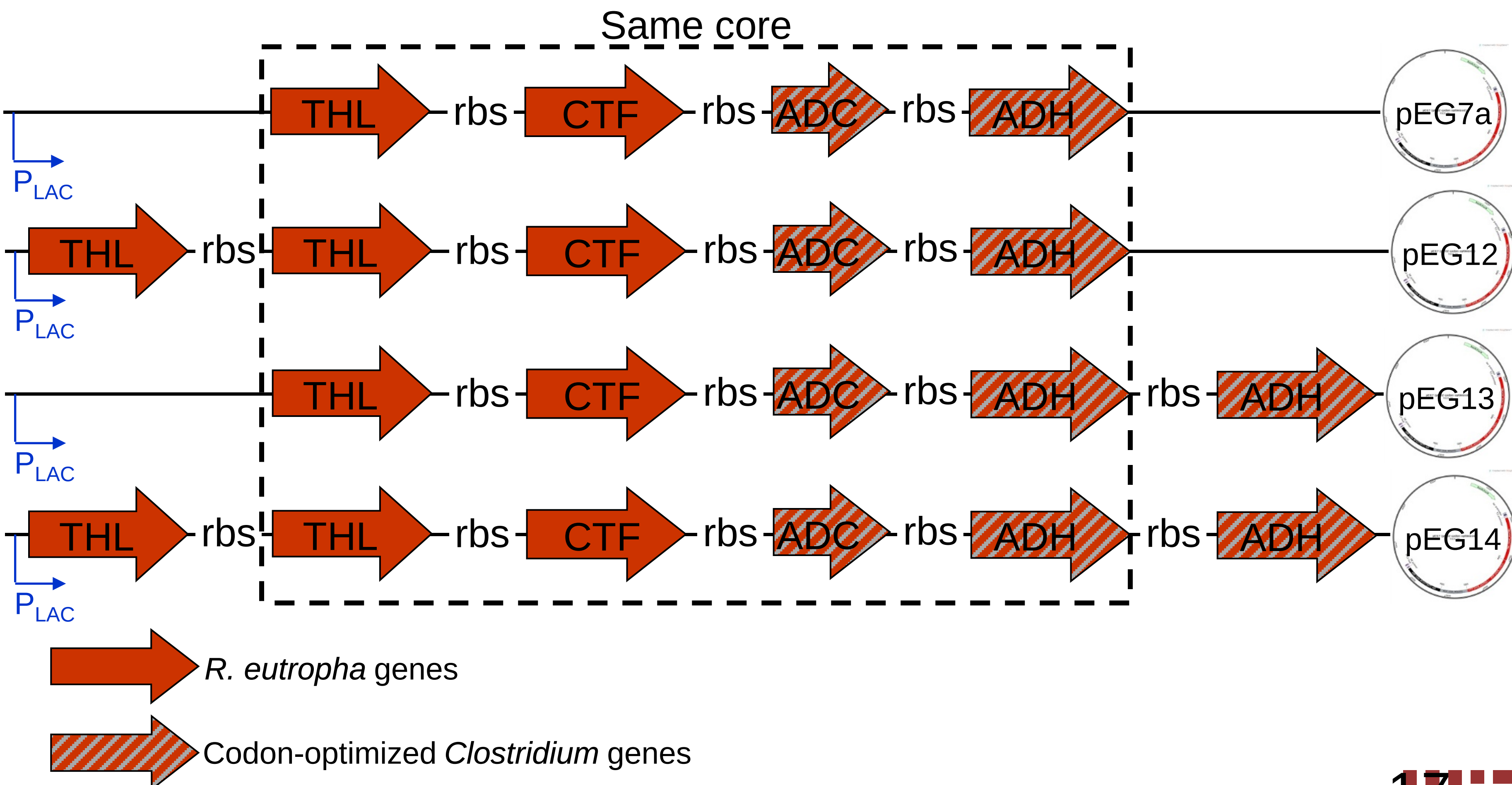
Without overexpression of THL and CTF: isopropanol production decreases by 1.3 ± 0.1 folds

The overexpression of the sole THL restore the level of isopropanol produced

The thiolase (THL) gene (*phaA*) has to be overexpressed on a plasmid but not the CoA-Transferase (CTF)

Strain constructions

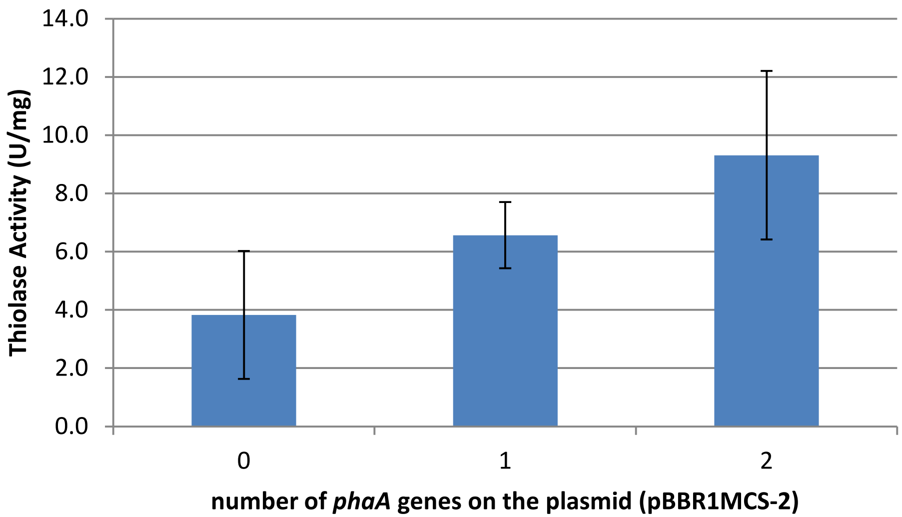
- 4 – Does gene duplication lead to higher isopropanol production ?



Strain Re2133: H16 ΔphaB1B2B3C1 Gen^r (*Budde, C. F., A. E. Mahan, et al. (2010)*)

Strain constructions

- 4 – Does gene duplication lead to higher isopropanol production ?
  - Thiolase (THL) gene (*phaA*) duplication on the plasmid
    - leads to higher specific thiolase activity



Each additional copy
=
increase of 2.7 U/mg

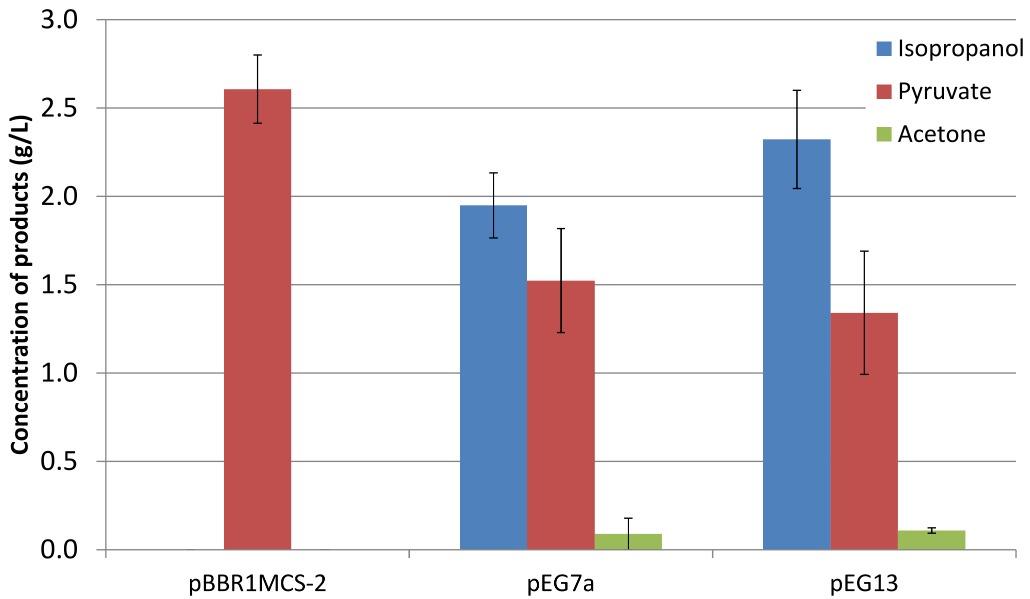
    - but does not increase isopropanol production

The condensation of two Acetyl-CoA into Acetoacetyl-CoA by the Thiolase is not anymore the limiting step

Strain constructions

- 4 – Does gene duplication lead to higher isopropanol production ?
  - Alcohol dehydrogenase (ADH) gene duplication on the plasmid
    - Increases the isopropanol production

Maximal isopropanol concentration and associated pyruvate and acetone concentration produced by strains Re2133/plasmid



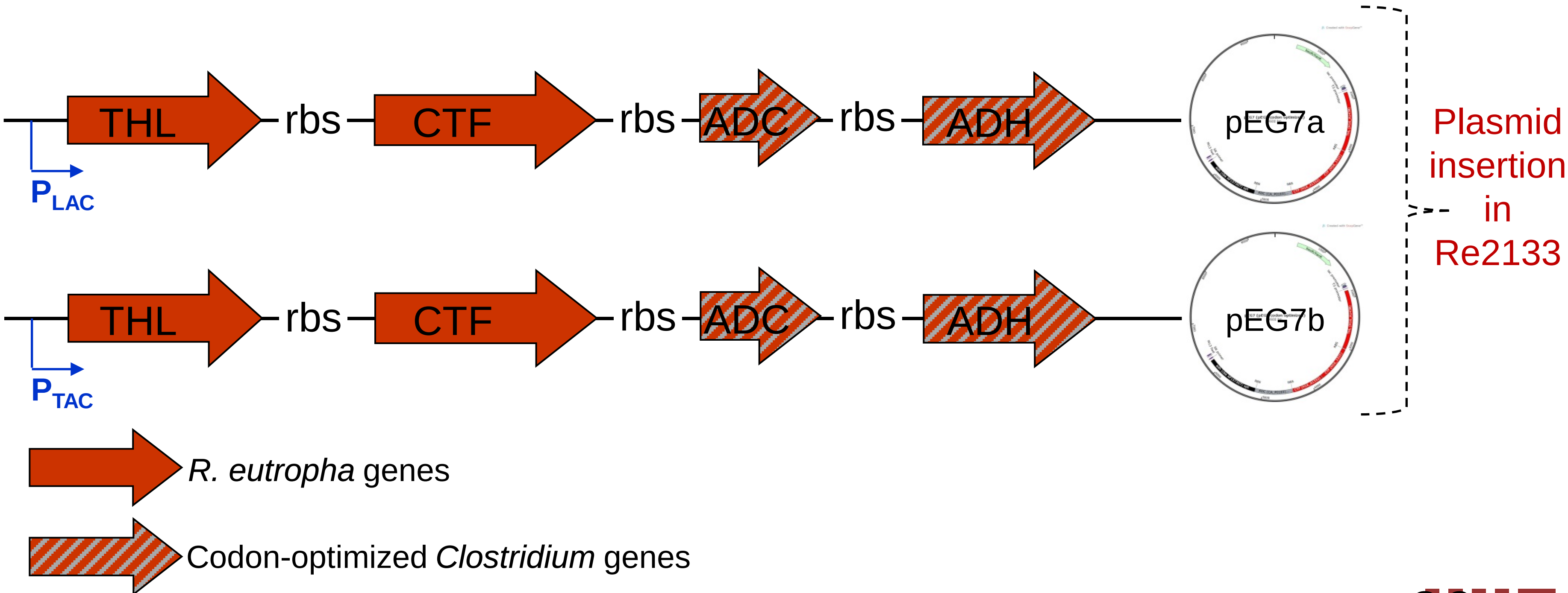
Empty plasmid | **1** copy of **ADH** gene | **2** copies of **ADH** gene

The Alcohol dehydrogenase is a limiting step for isopropanol production

Strain constructions

- 5 - Is the promoter strong enough to have an efficient ispopropanol production ?
  - P_{LAC} and P_{TAC} are constitutive promoter in *R. eutropha*
  - P_{TAC} shows 1.5 to 2 fold higher expression compared to P_{LAC}

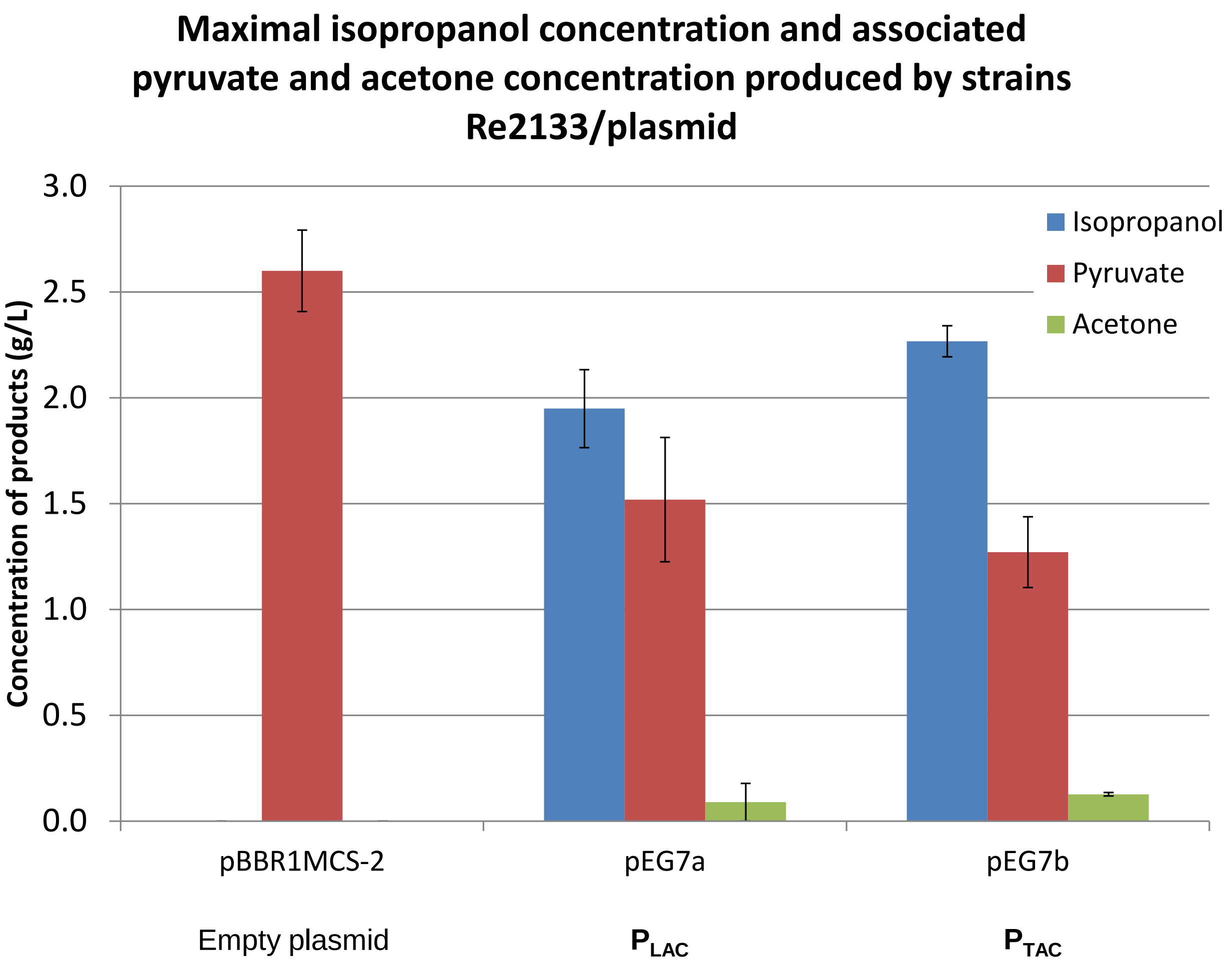
Fukui, T., Ohsawa, K., et al. (2010)



Strain Re2133: H16 ΔphaB1B2B3C1 Gen^{r} *(Budde, C. F., A. E. Mahan, et al. (2010))*

Strain constructions

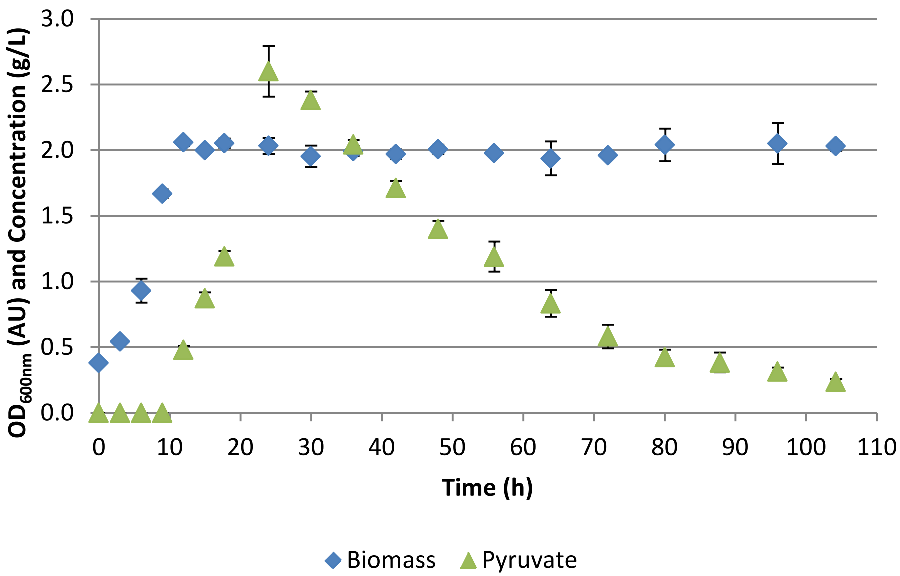
- 5 - P_{LAC} vs P_{TAC}



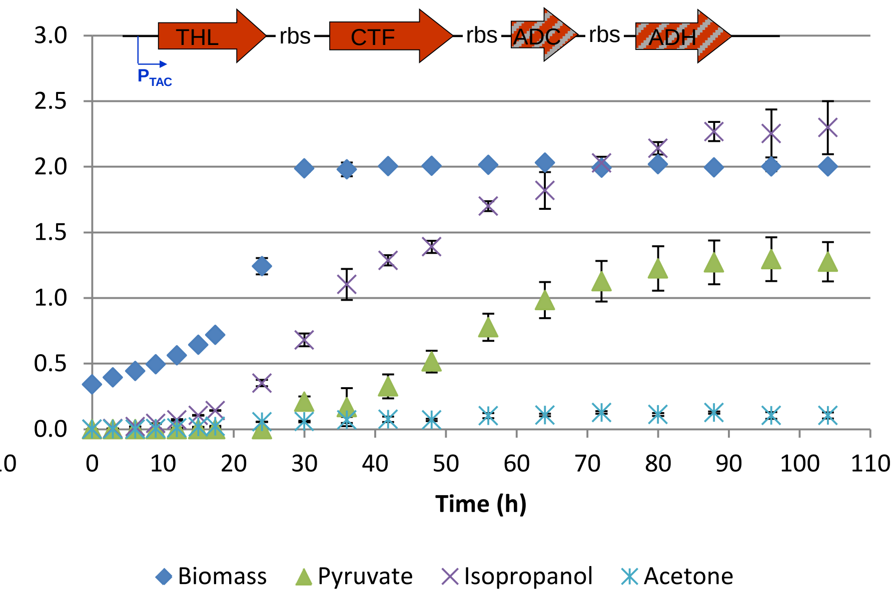
P_{TAC} used instead of P_{LAC} increases by 1.2 ± 0.1 folds the isopropanol production

Isopropanol production

Re2133/pBBR1MC-2 (Empty plasmid)



Re2133/pEG7b (P_{TAC} promoter)



$Y_{S,ISOPROP}$= 0.22 ± 0.01 Cmole.Cmole^{-1}

➔ 44% of the theoretical yield

Growth rate = 0.166 ± 0.002 h^{-1}

Growth rate = 0.052 ± 0.003 h^{-1}

Constitutive production of isopropanol decreases the growth rate by 3.2 folds
➔ An inducible system has to be used

Experiments done in flasks cultures (1 L) with 100 mL of Minimal Media (*Lu, J., C. Brigham, et al. (2012)*), nitrogen added to produce about 1 g/L of biomass, fructose as carbon source

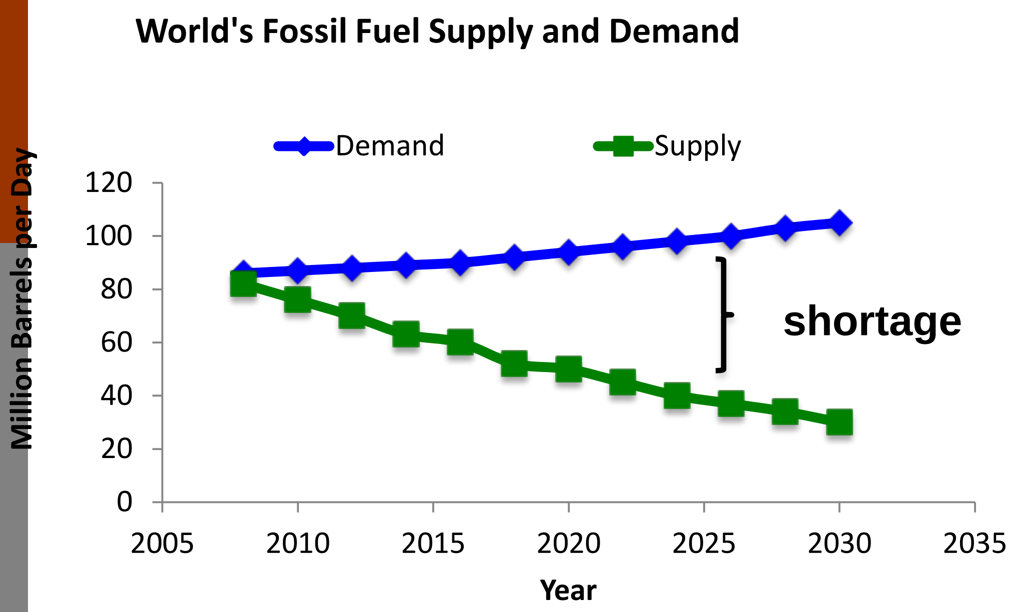
Conclusion and next challenge

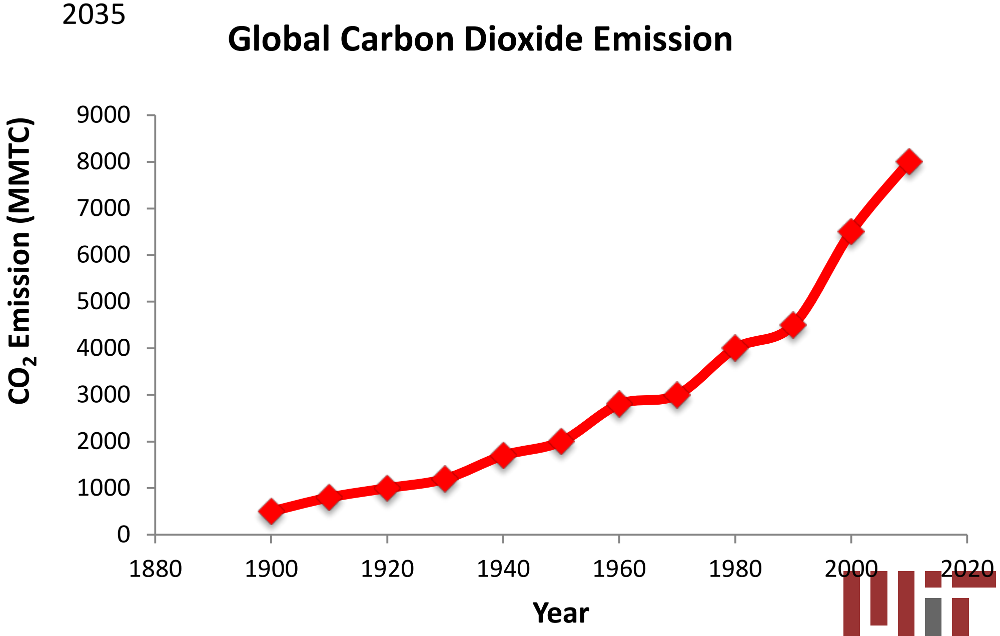
- First successful production of isopropanol by metabolically engineered *R. eutropha*
  - up to 2.4 g/L of isopropanol (with 1g/L of biomass)
  - the main by-product is pyruvate (due to a bottleneck in the isopropanol pathway)

Need to increase the carbon drive through the isopropanol pathway by fine-tuning of gene expression

- Coding Sequence optimization
- Ribosome binding site optimization
  - possibility to increase the translation initiation by 2 to 10000 times (according to the RBS calculator *Salis, Mirsky et al. 2009*)

Main Concerns





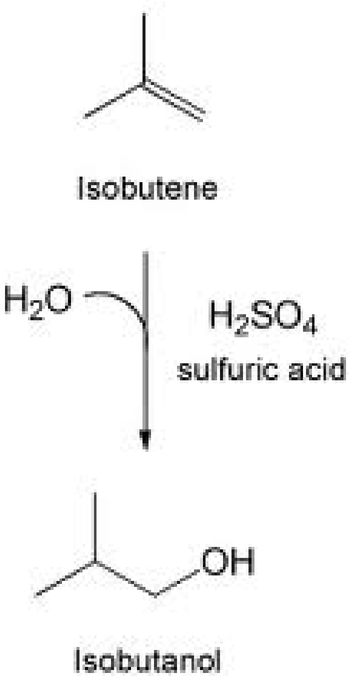
Alternative Biofuel

- Safe
- Sustainable
- From CO_2
- Does not threaten land reserved for food production
- Energy content similar to gasoline
- Compatible with the existing infrastructure

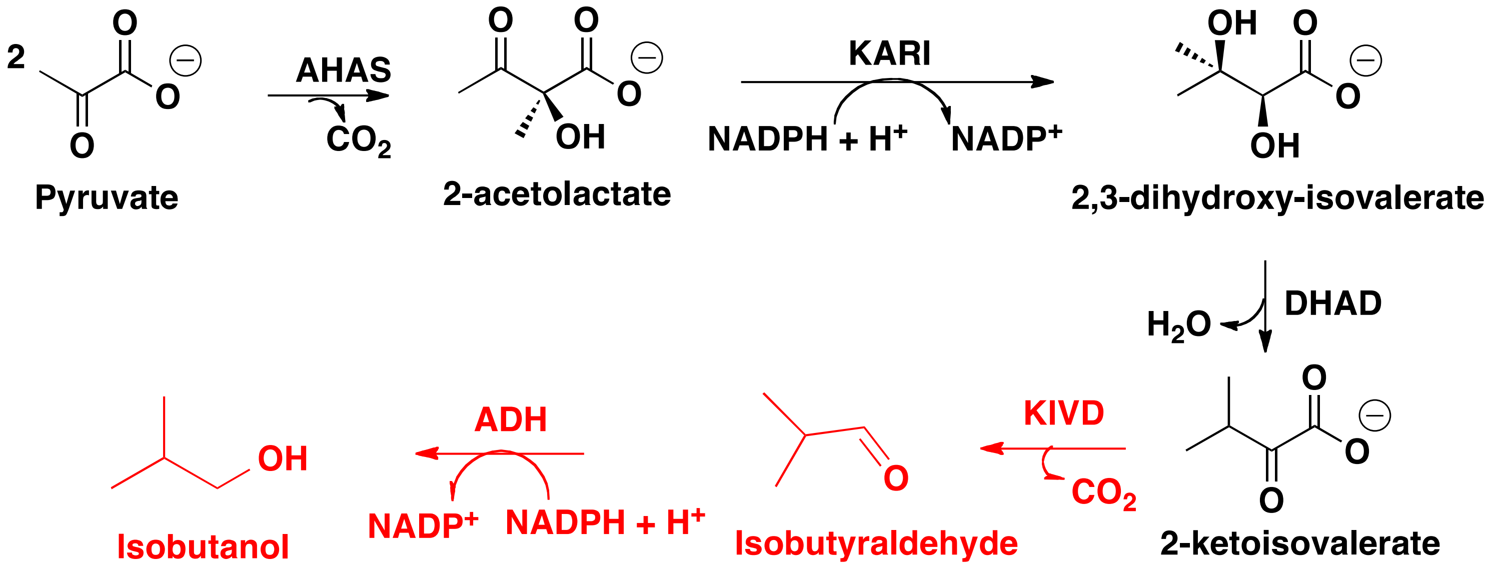
Production of isobutanol from CO_2, O_2, and H_2 in *Ralstonia eutropha*

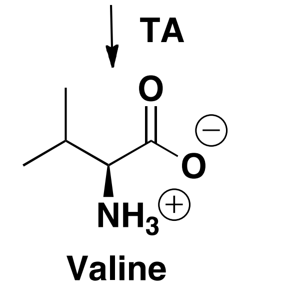
How’s Isobutanol Synthesized?

Chemical



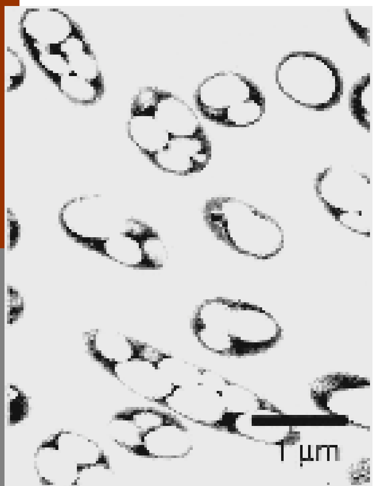
Biological



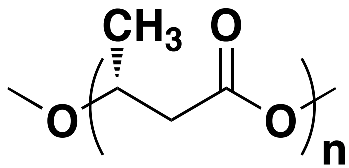


AHAS: acetohydroxyacid synthase
KARI: ketoacid reductoisomerase
DHAD: dihydroxyacid dehydratase
TA: transaminase
KIVD: ketoisovalerate decarboxylase
ADH: alcohol dehydrogenase

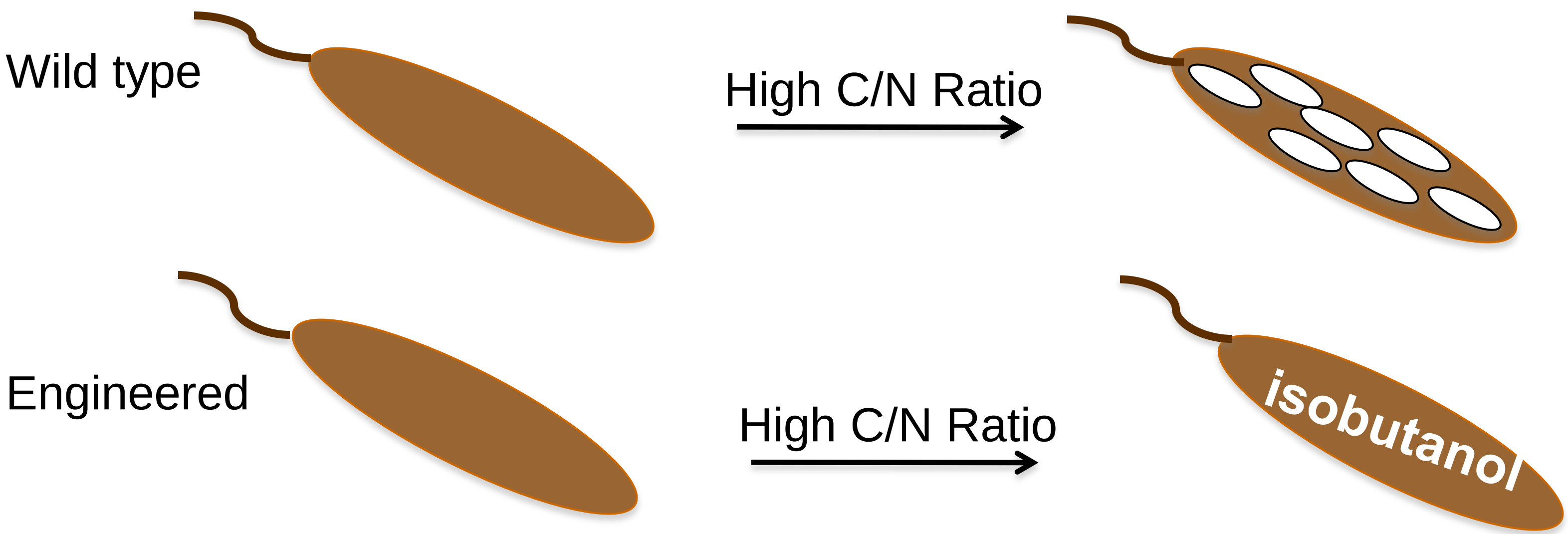
Why *Ralstonia eutropha*?



TEM image by Dr. Gregory York

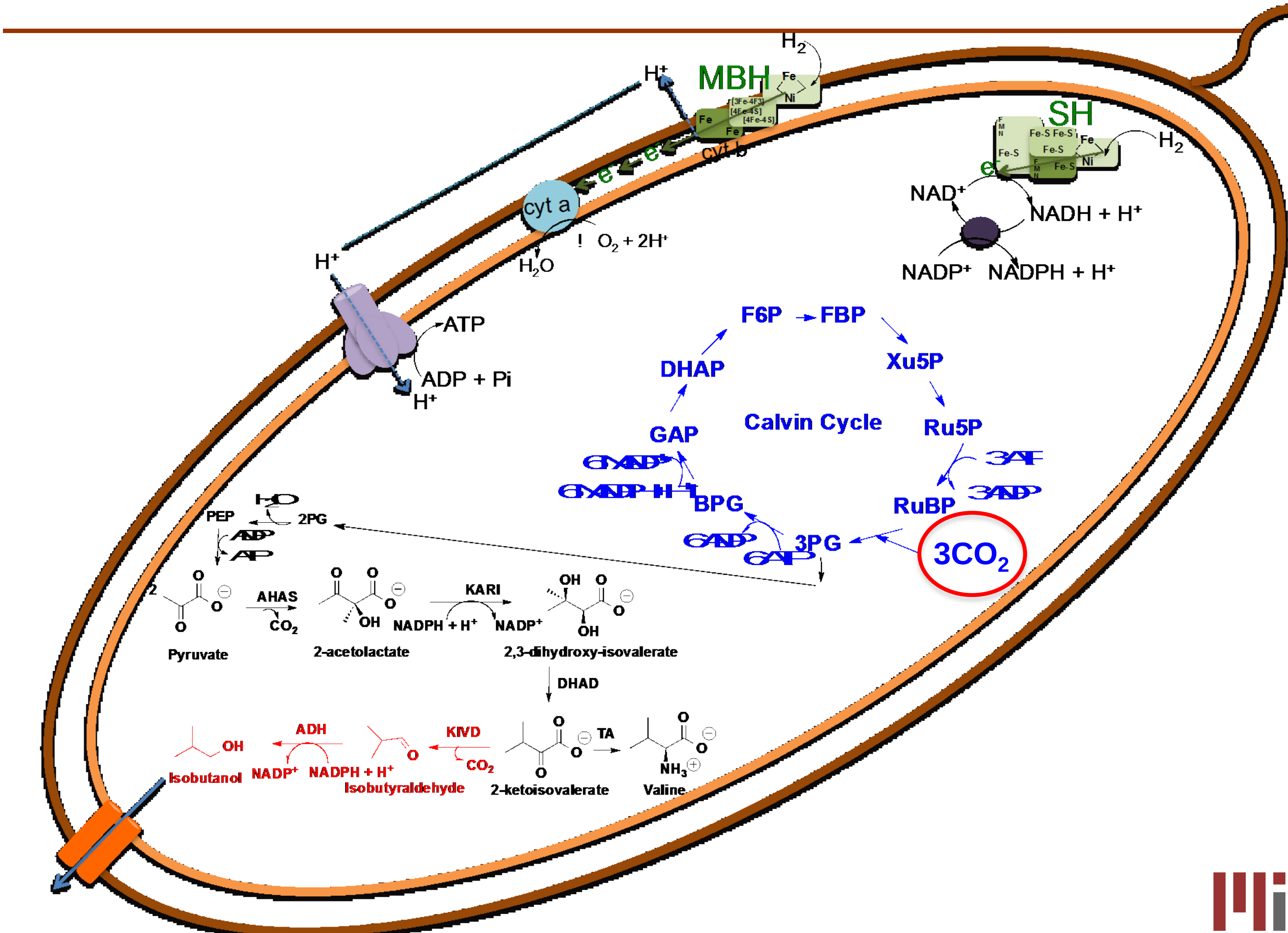


Polyhydroxybutyrate (PHBs)

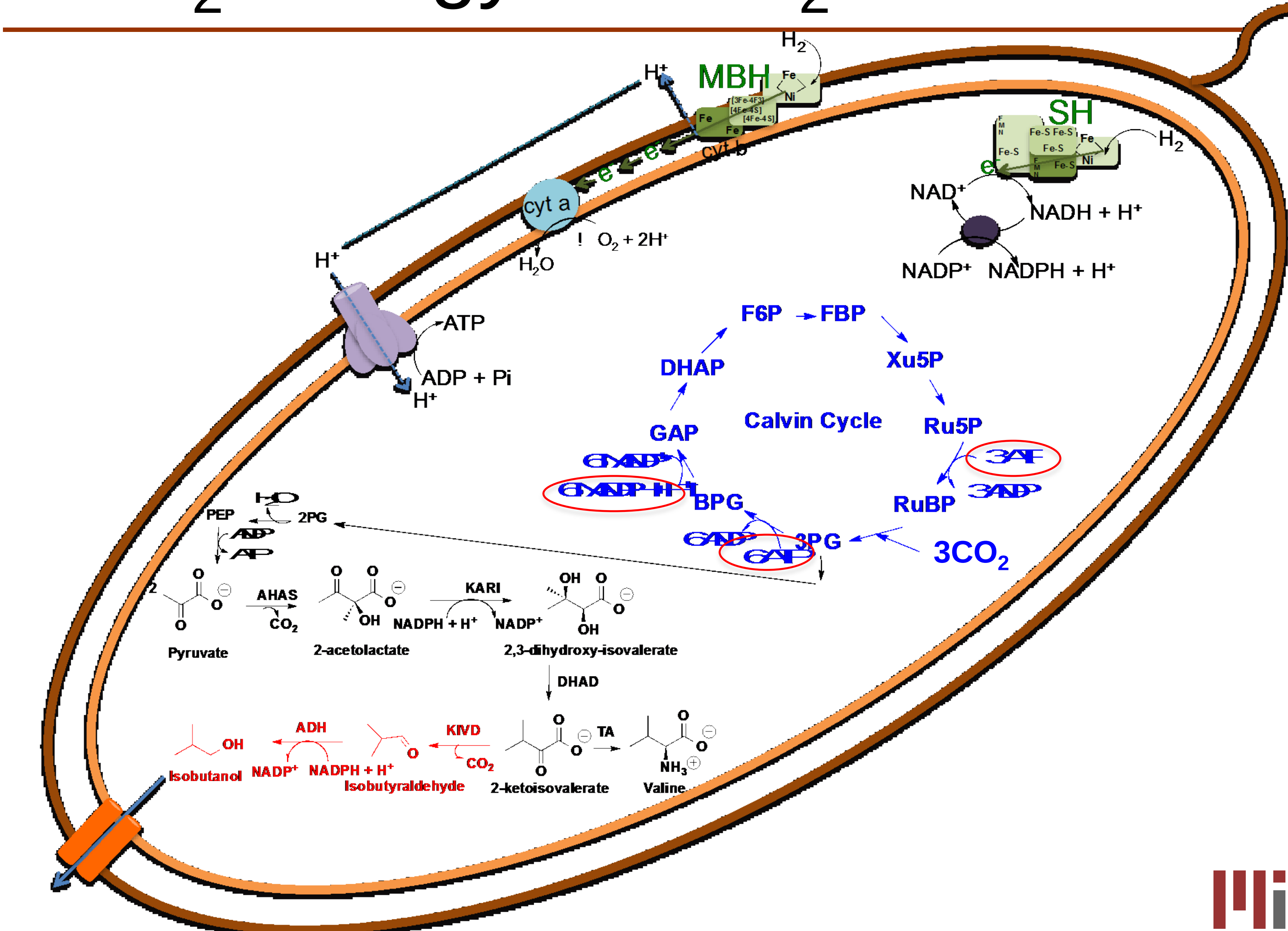


Slide modified from CB

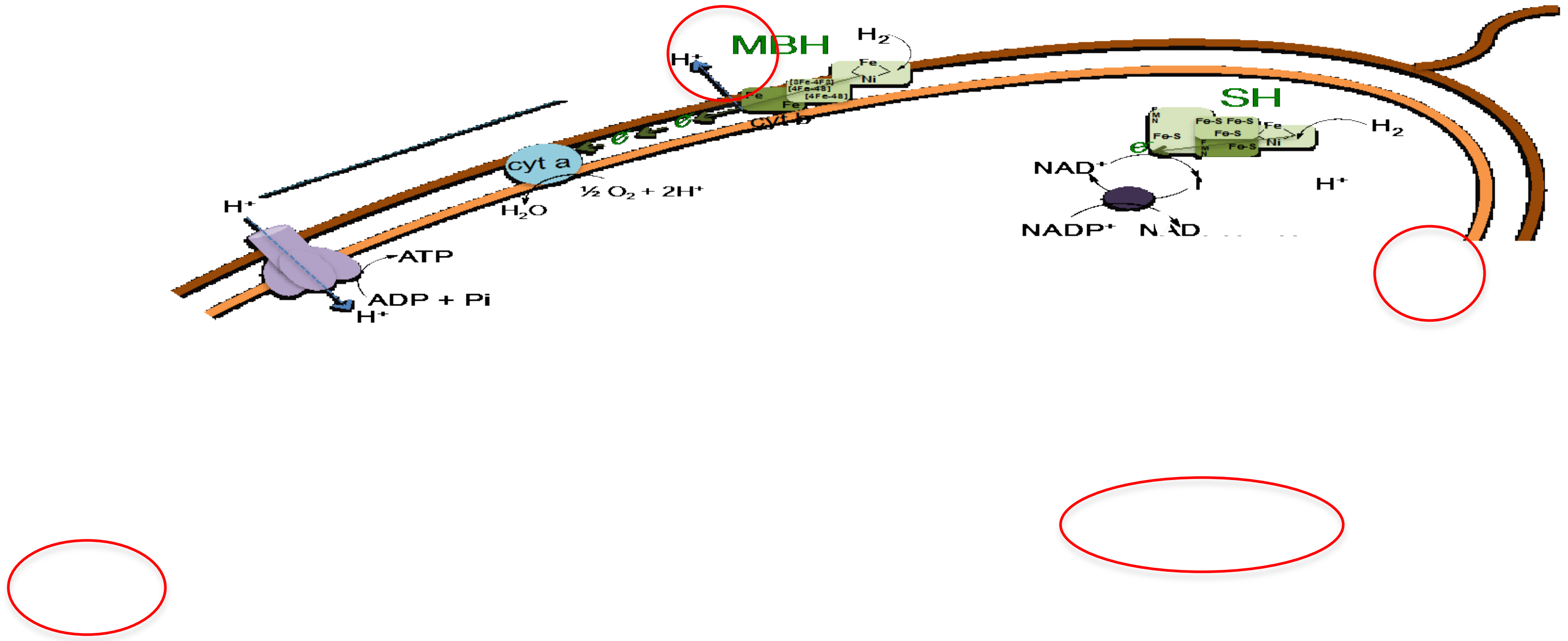
Capable of Fixing Carbon Dioxide



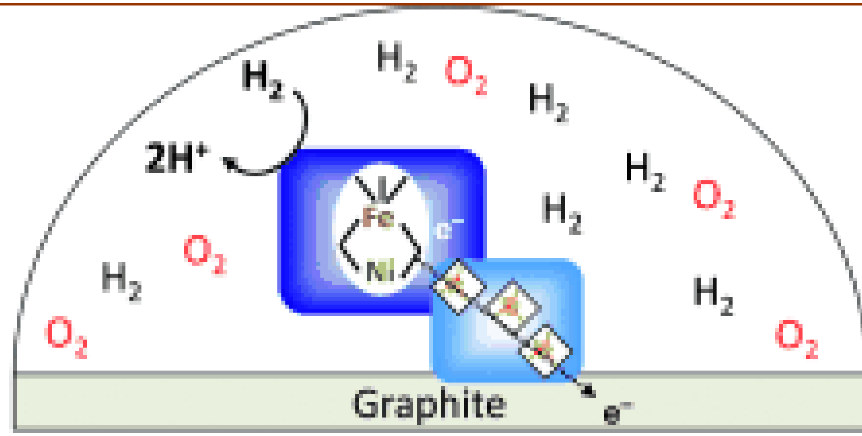
H_2 Energy for CO_2 Fixation

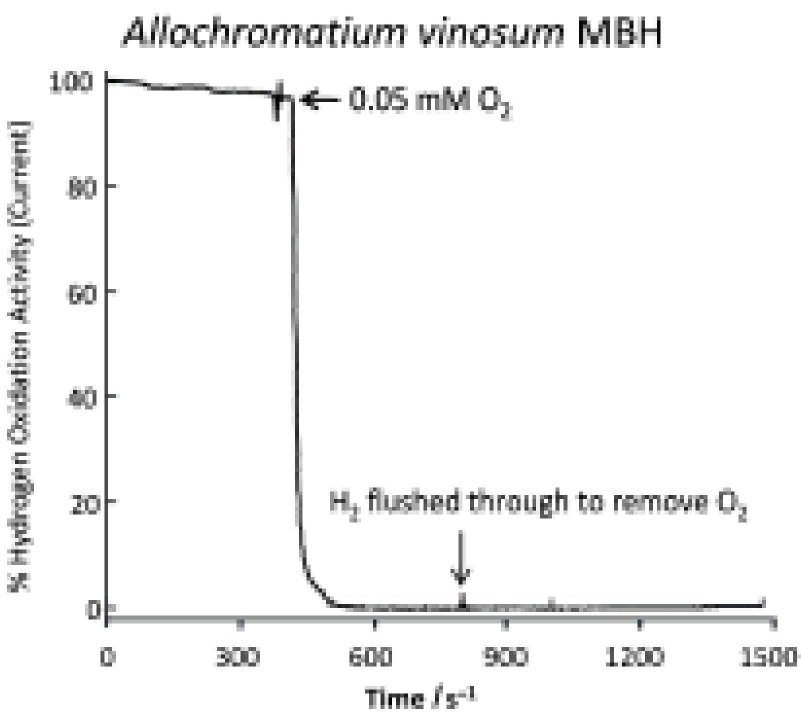


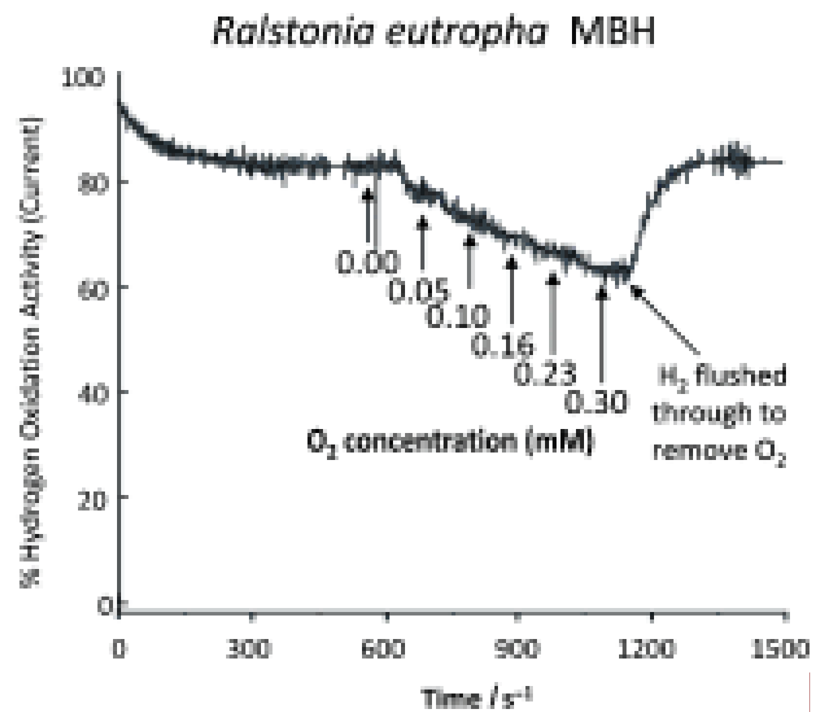
Extremely Unique Hydrogenases



Hydrogenases Tolerate O_2

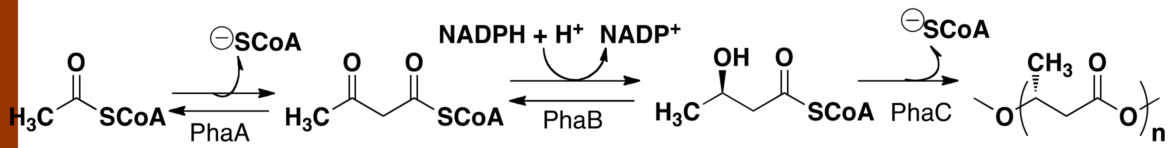




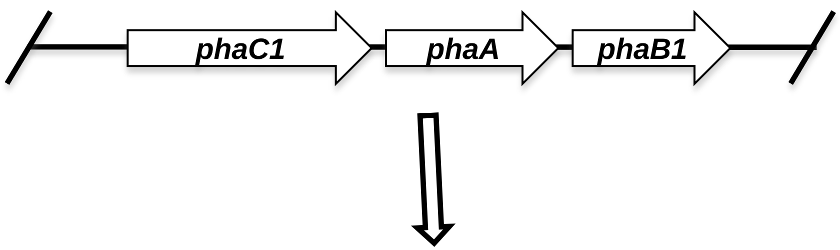


Lenz, et al. Chempyschem 2010

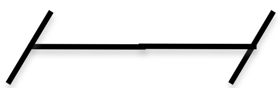
Δ*phaCAB* Strain Secreted Pyruvate



R. eutropha **H16** (chromosome 1)



R. eutropha ΔphaCAB (chromosome 1)



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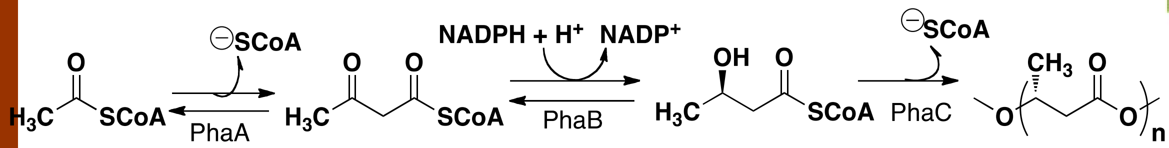
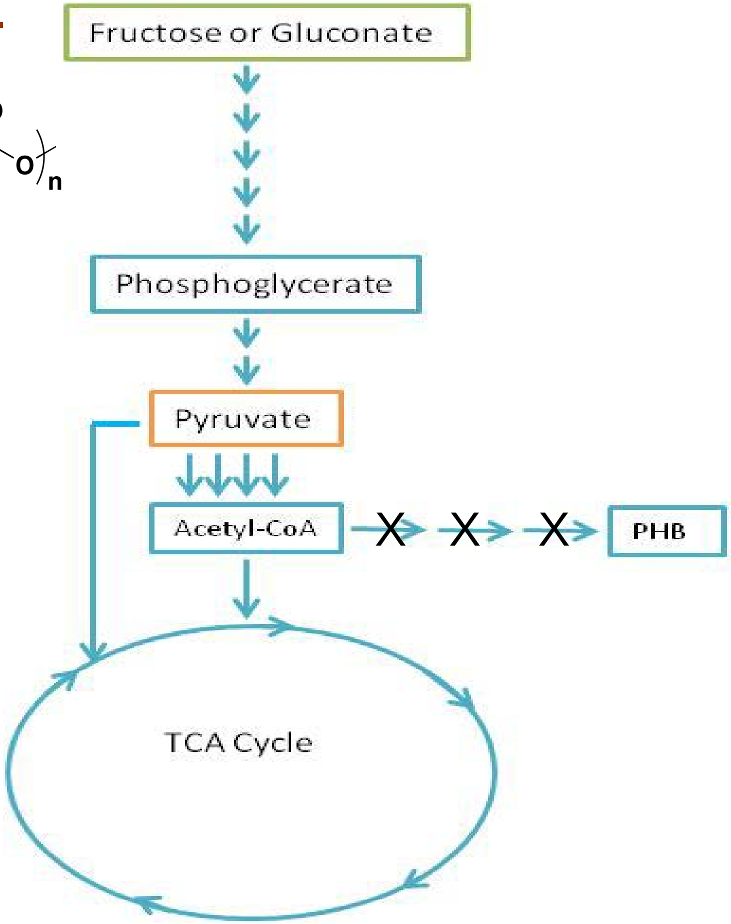
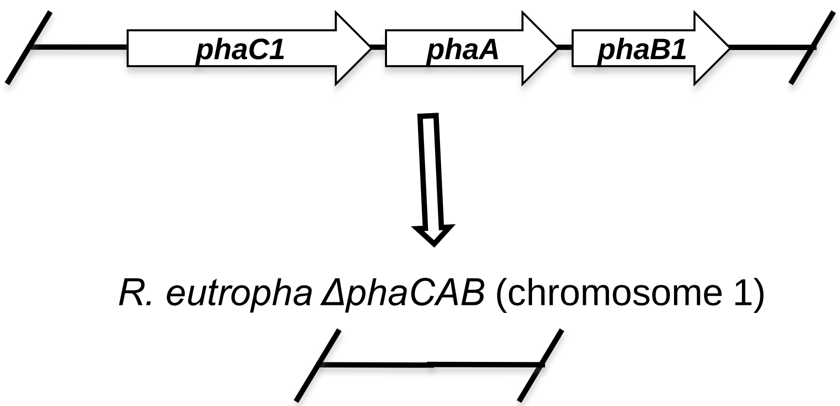
Δ*phaCAB* Strain Secreted Pyruvate

2





R. eutropha **H16** (chromosome 1)



R. eutropha ΔphaCAB (chromosome 1)

Strains	Residual CDW	[Gluc] (% *w/v*)	[Pyr] (% *w/v*)	[PHB] (% CDW)	[NADH] (pmol/CFU)	[NADPH] (pmol/CFU)
H16	1.1±0.1	1.2±0.03	0	83±2.9	0.01±0	7.5E−5±1E−5
Re2061	1.3±0.2	0.7±0.04	0.6±0.01	0	0.04±0.002	8.2E−5±0.7E−5
p value	NA	6.5E−5	NA	NA	1.3E−5	0.36

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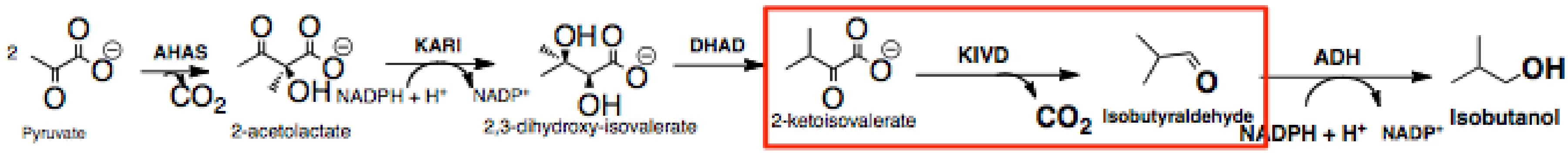
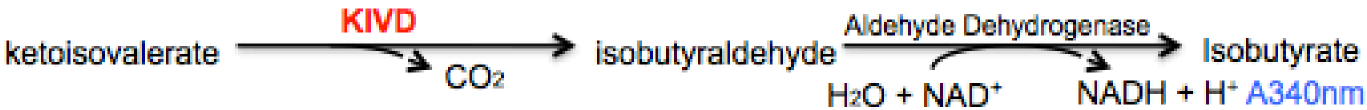
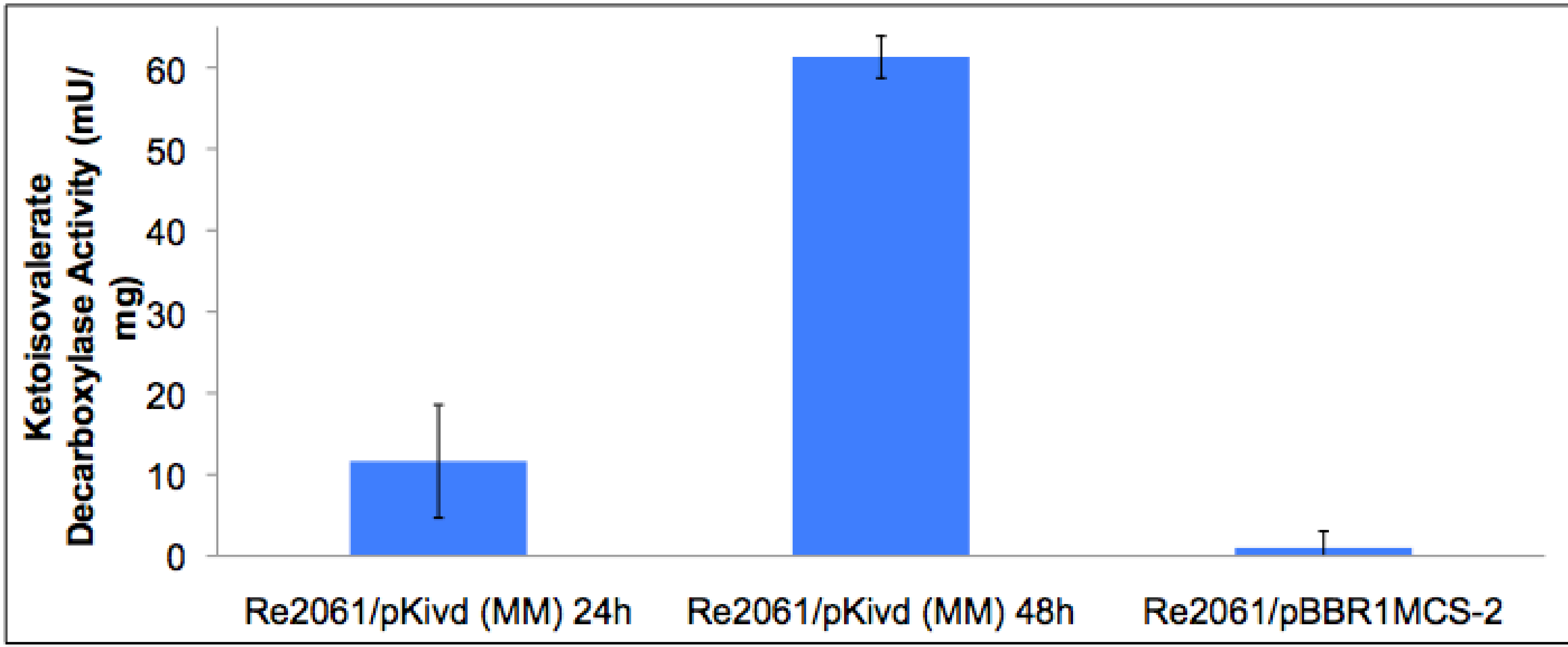
Active Ketoisovalerate Decarboxylase



- Ketoisovalerate decarboxylase (KIVD) from *Lactococcus lactis*
- *kivd* gene was incorporated in a broad-host-range-cloning vector pBBR1MCS-2





U = μmol product formed per minute

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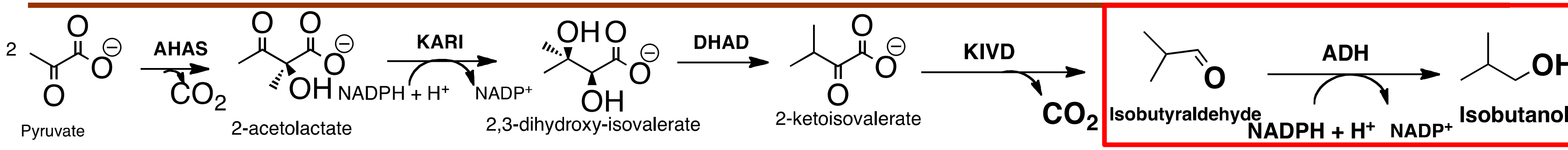
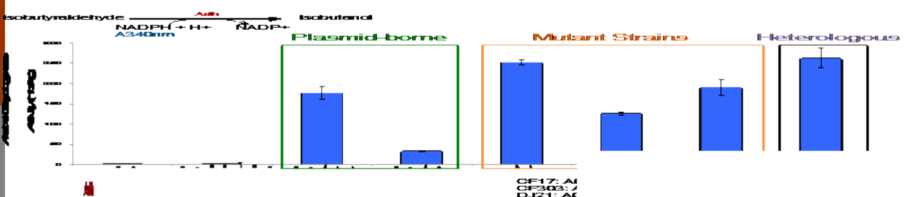
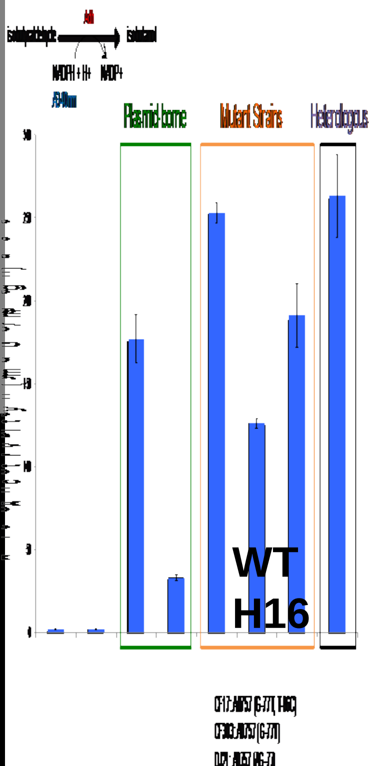
Mutant Strains with Active ADH







= µmol product formed per minute

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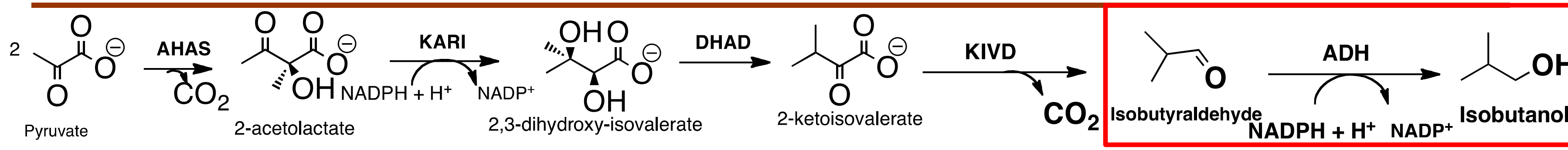
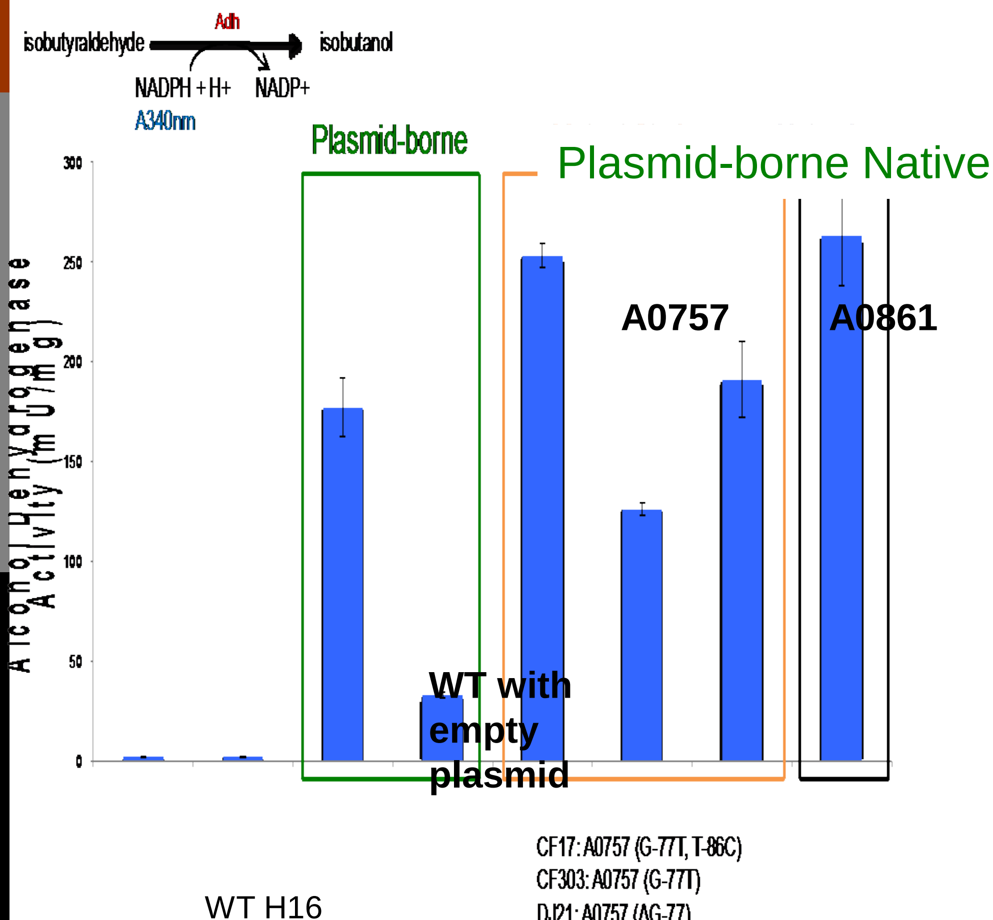
Mutant Strains with Active ADH





= μmol product formed per minute

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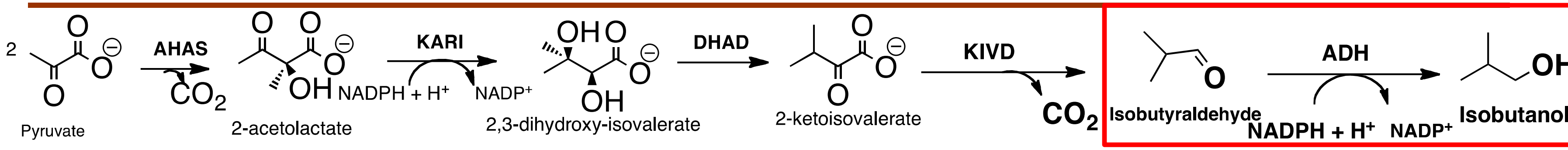
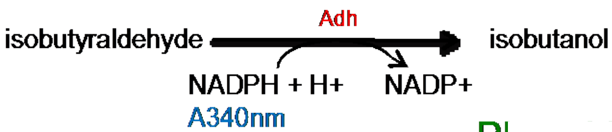
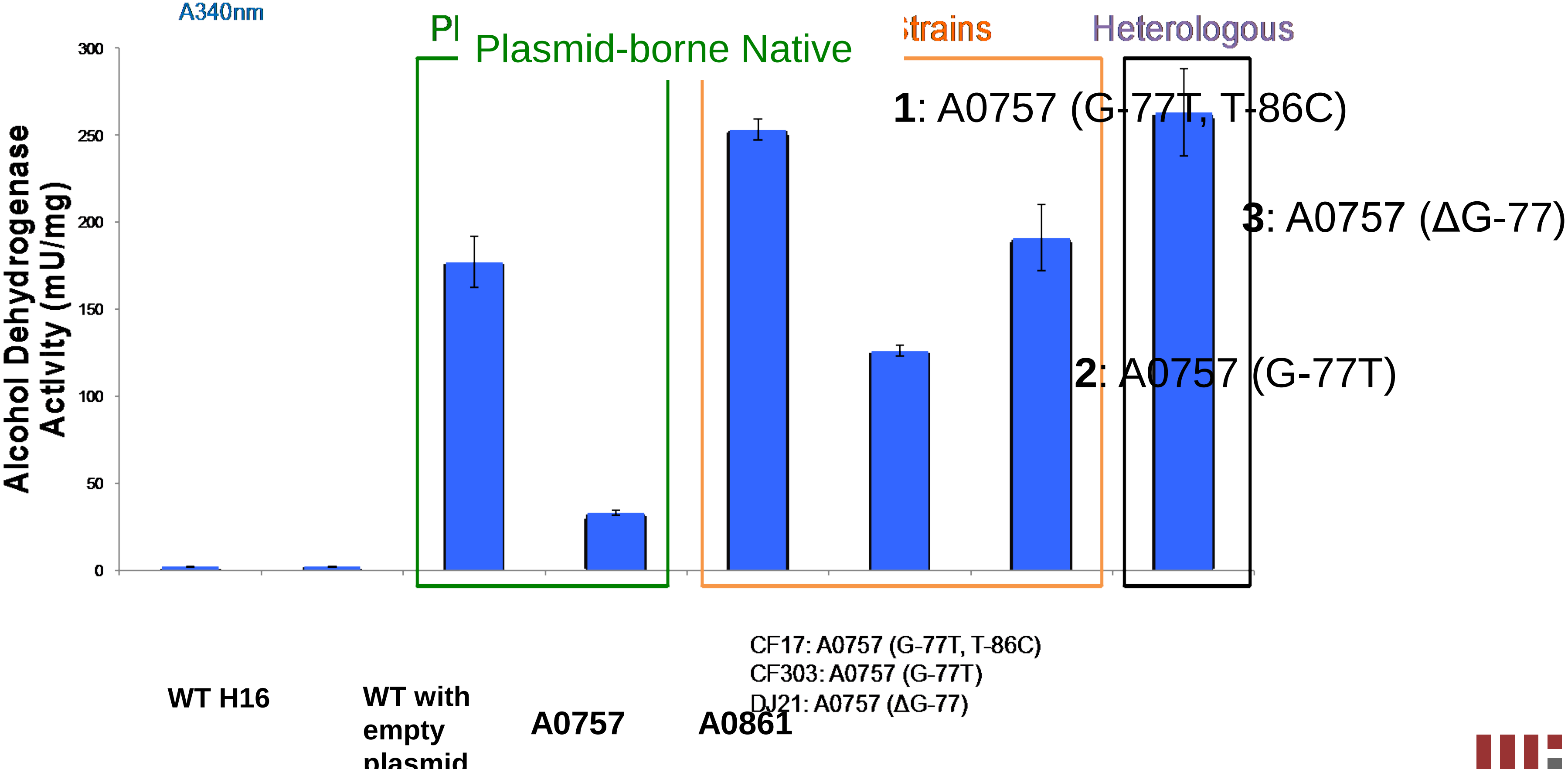
Mutant Strains with Active ADH







] = µmol product formed per minute

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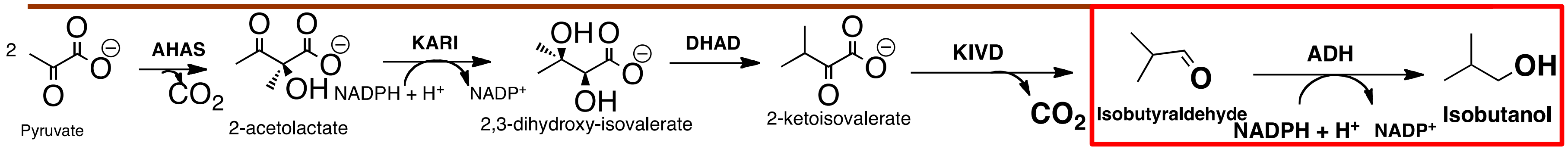
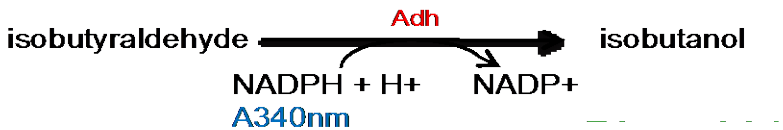
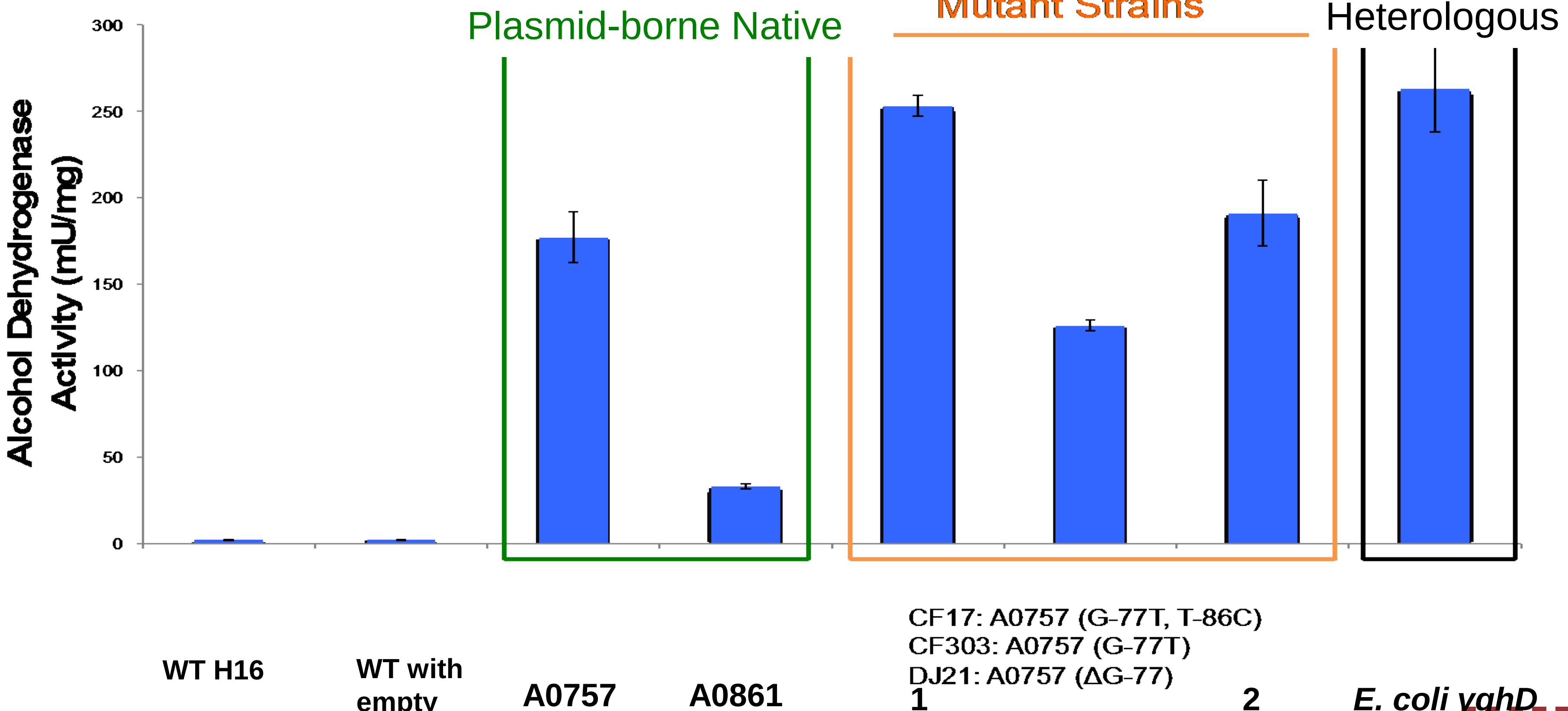
Mutant Strains with Active ADH







A0757: 1.(G-77T, T-86C); 2.(G-77T); 3.(ΔG-77)

= μmol product formed per minute

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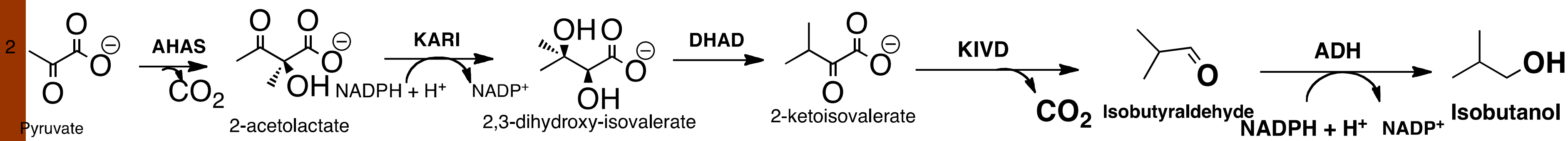
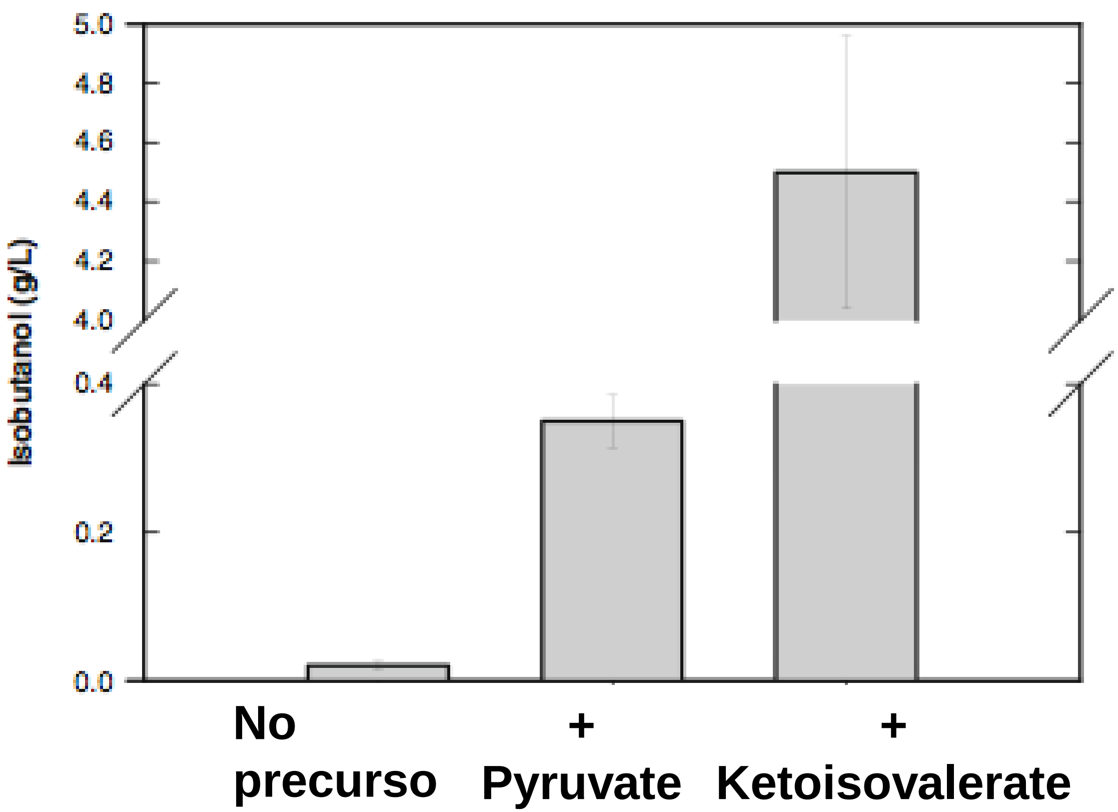
De novo Isobutanol Production



ΔphaCAB/p*kivd* & *adh*



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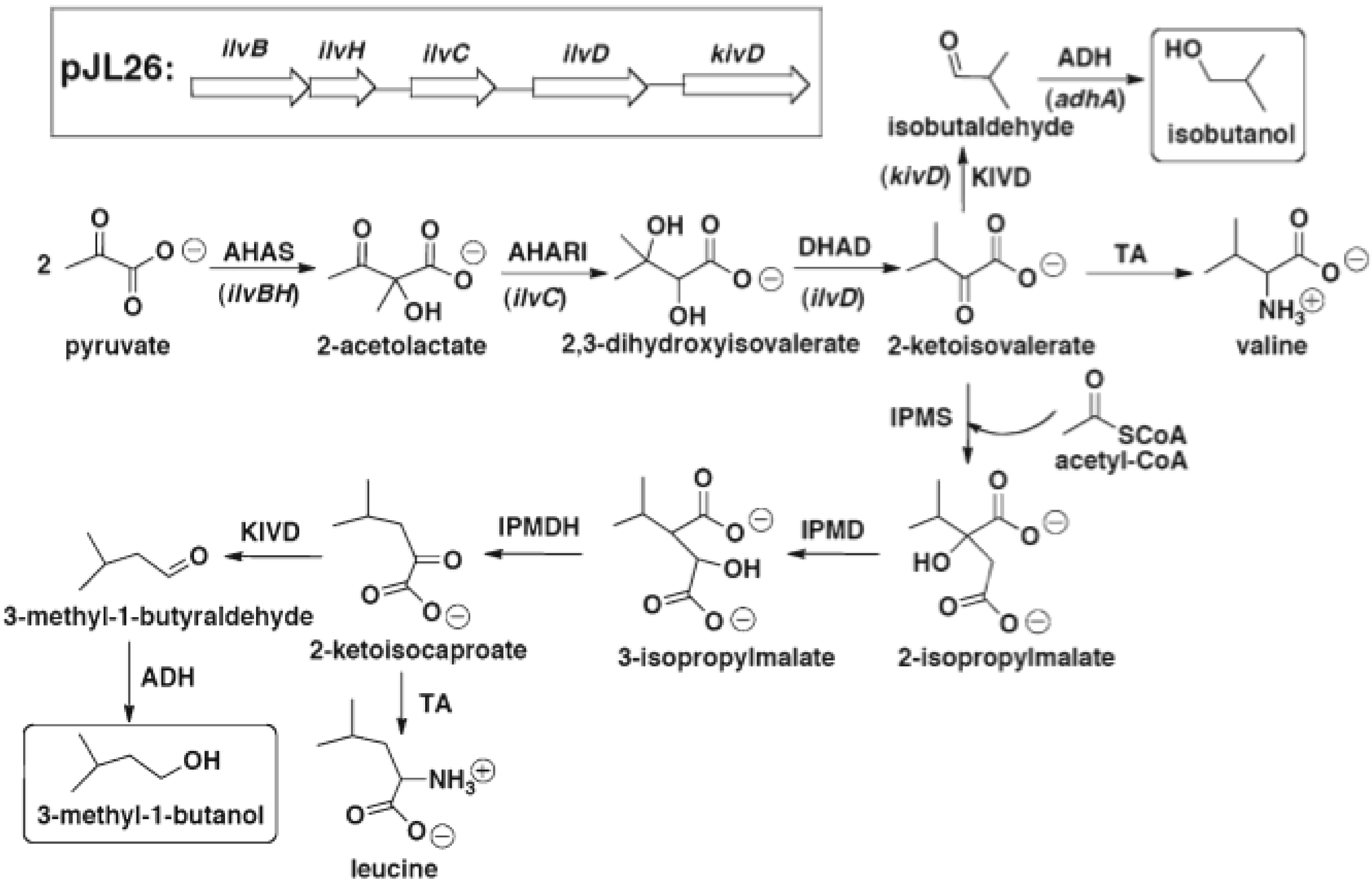
Constructed Isobutanol Production Operon



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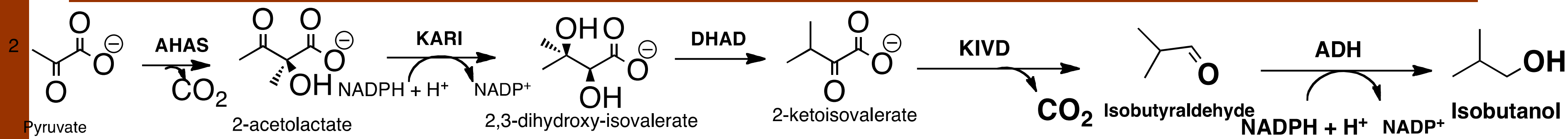
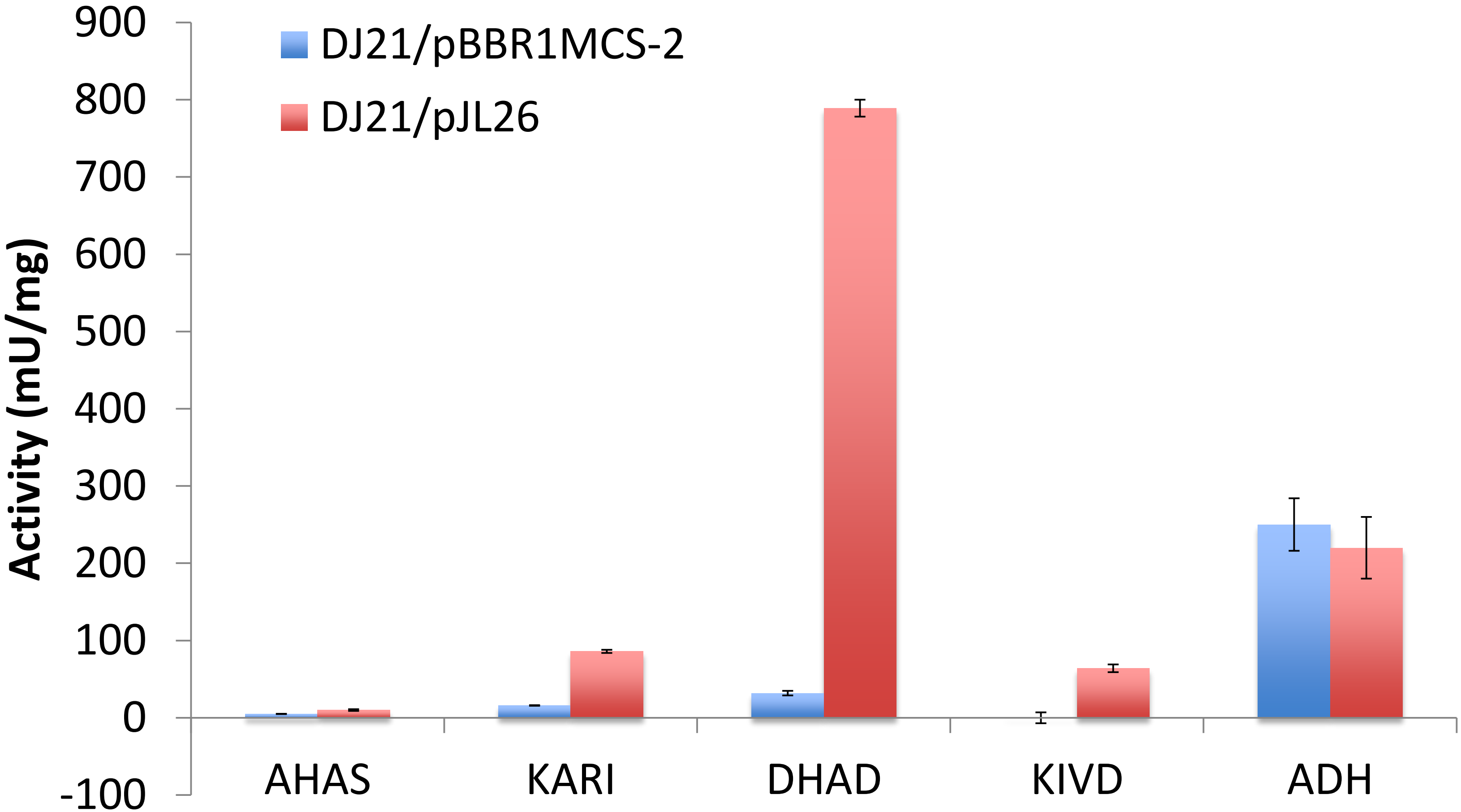
Pathway Enzyme Activities





U = μmol product formed per minute

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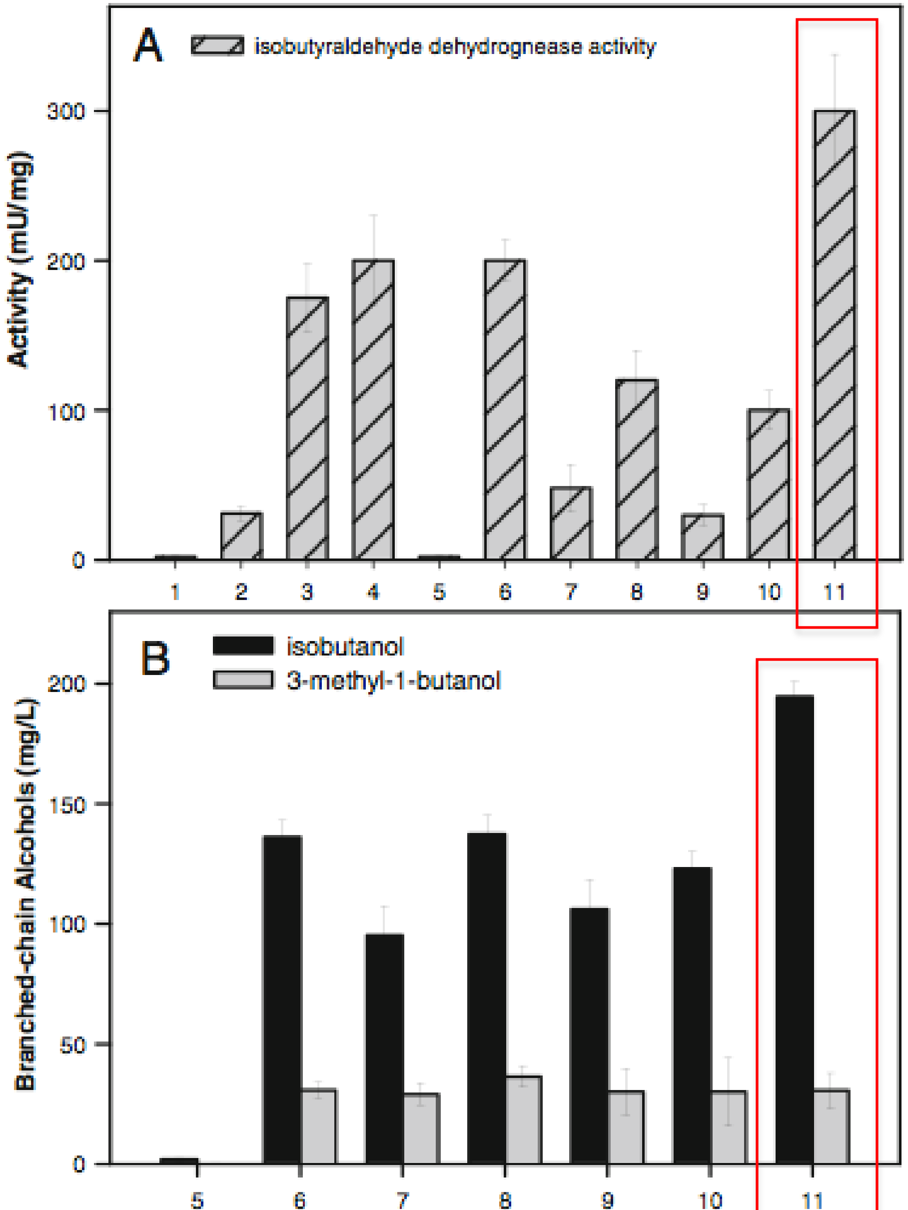
Increased Production



11
DJ21/pJL26

- DJ21 has constitutively expressed *adh*
- Highest ADH activity
- Highest production of isobutanol

~180 mg/L isobutanol
~30 mg/L 3-methyl-1-butanol

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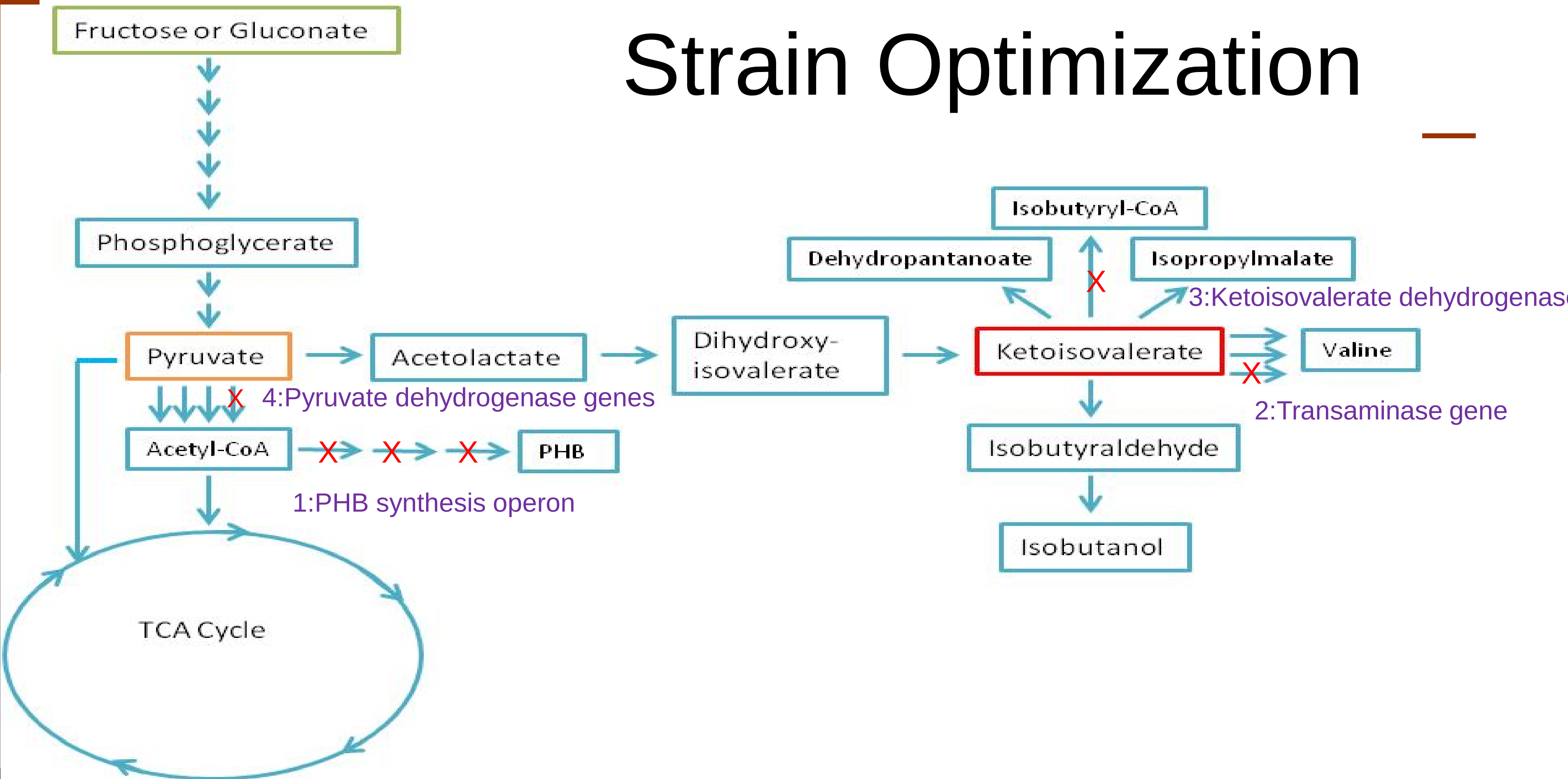
Strain Optimization



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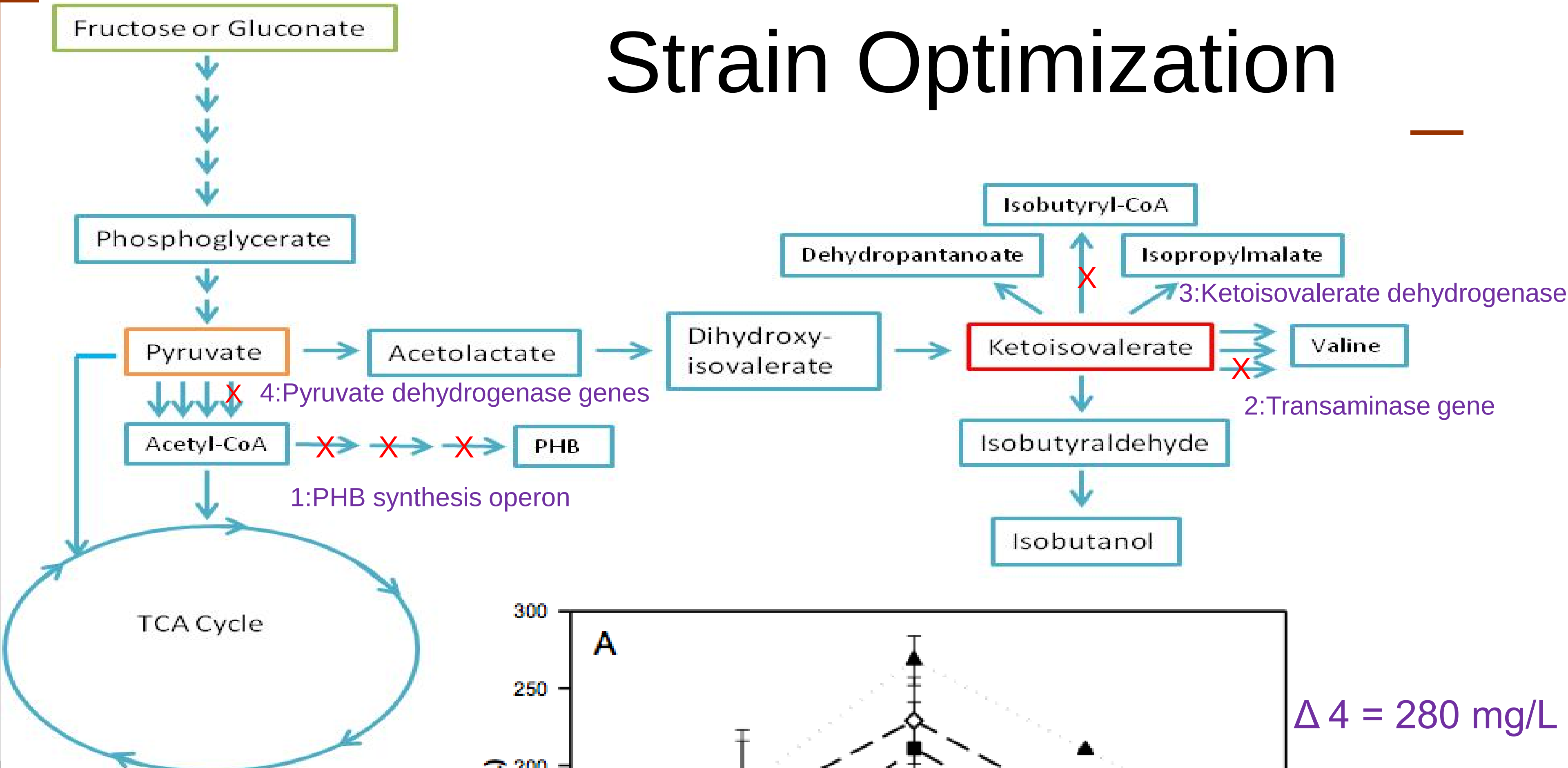
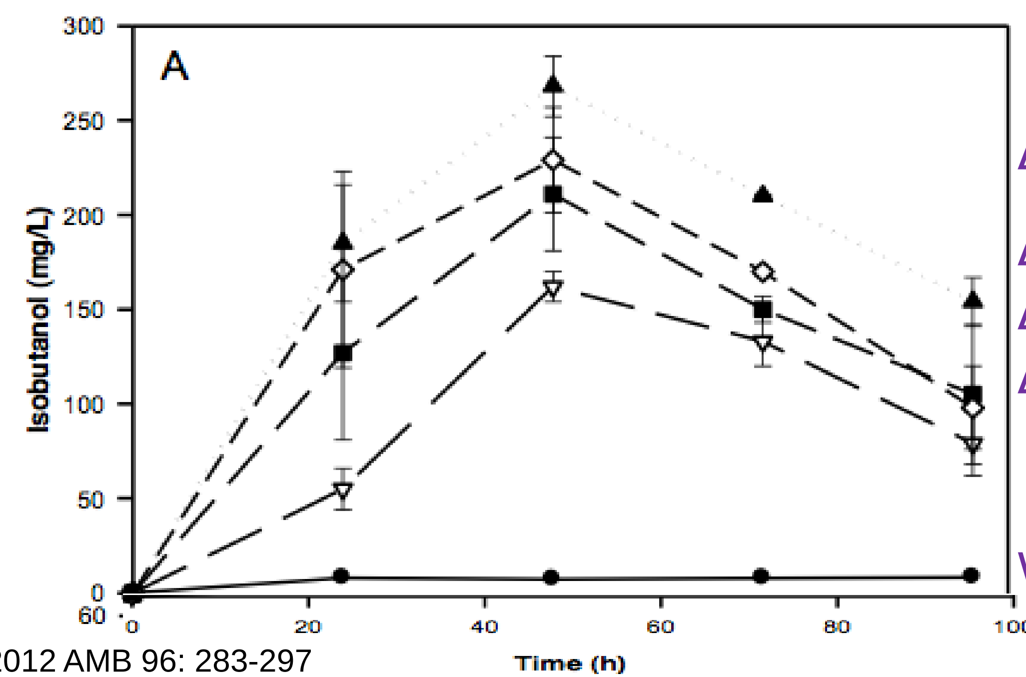
Strain Optimization





Δ 4 = 280 mg/L

Δ 3 = 220 mg/L

Δ 2 = 200 mg/L

Δ 1 = 150 mg/L

WT = 0 mg/L

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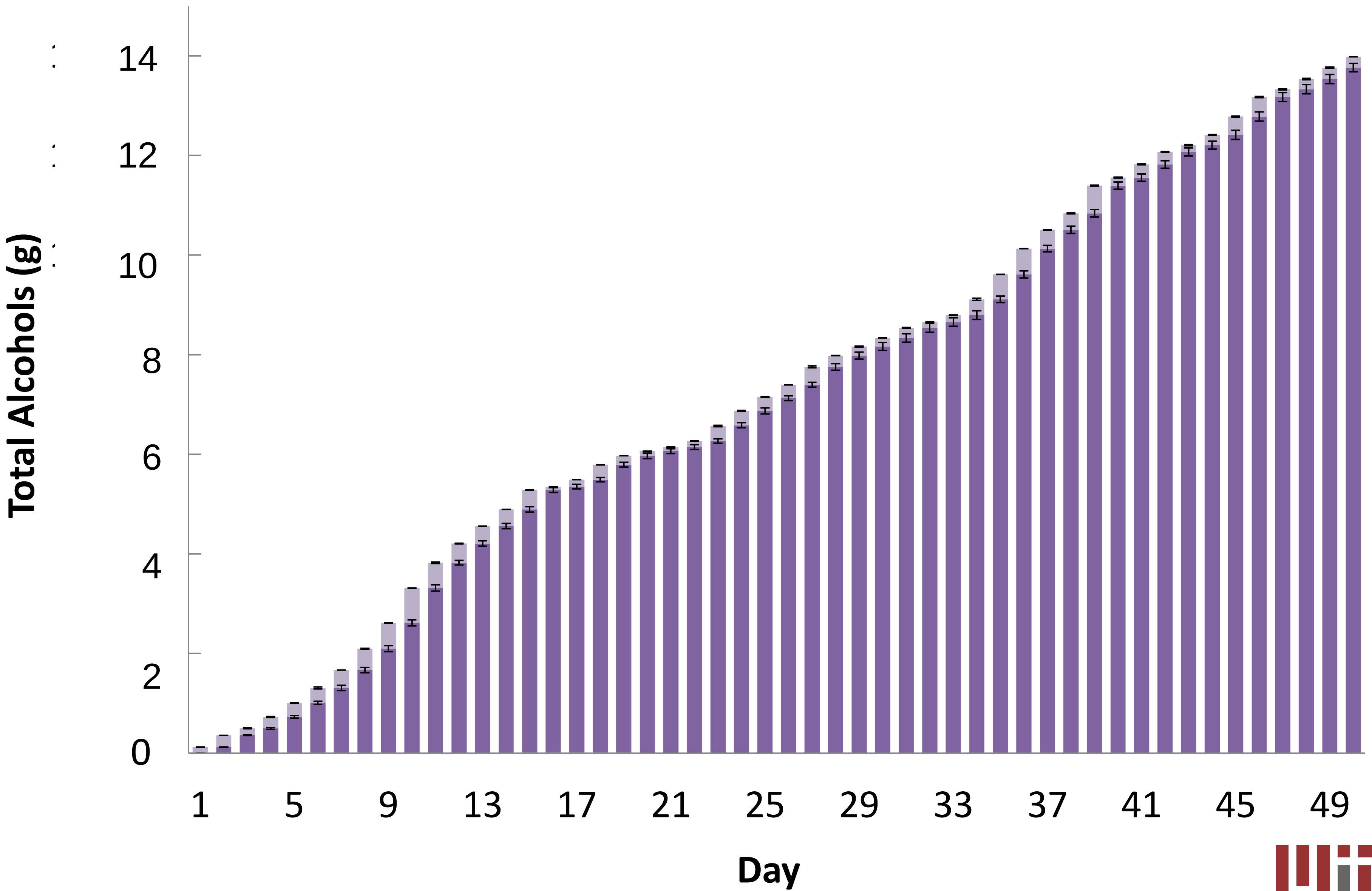
Semi-Continuous Culture



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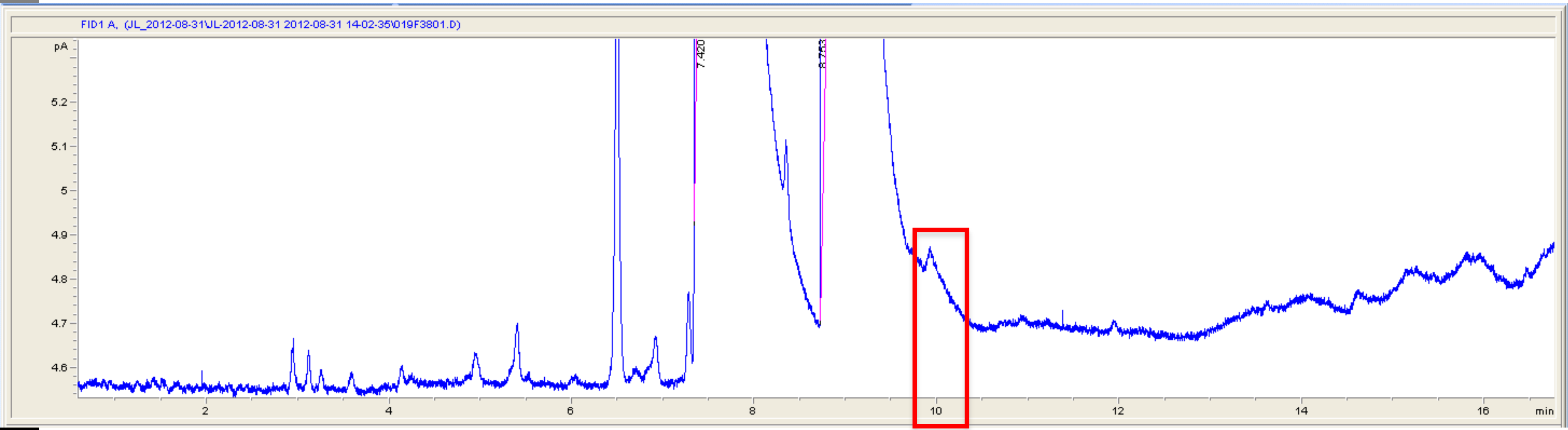
Autotrophic Production

- Re2425/pJL26
- Minimal Media with CO_2, O_2, H_2
- Isobutanol ~3 mg/L
- No 3-methyl-1-butanol



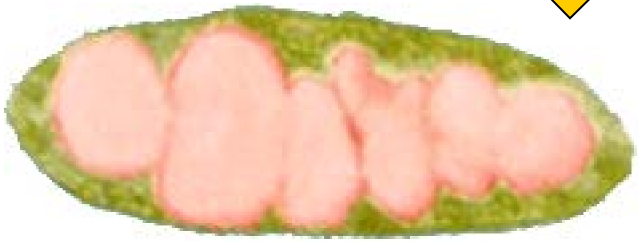
With CG

Construction of *R. eutropha* strains that produce PHA containing high %HHx monomers

Purpose

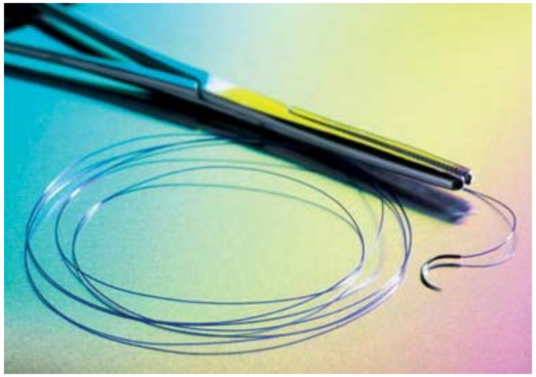
Natural feedstocks (palm oil, CPKO, RBD palm oil, etc.)

Waste streams (mixed organic acids)



Ralstonia eutropha

Household and industrial goods



Medical goods

Images: www.amrit-cwt.com/services.html; www.chemistrystore.com; G. York; Metabolix

Production Targets

- Production of P(HB-*co*-HHx) from palm oil and palm oil products
  - Production goal: > 50 g/L PHA
  - Total PHA CDW: > 50%
  - Polymer composition goal: > 10 mol% HHx
- Production of P(HB-*co*-HV) from mixed acids
  - Mixed acids derived from treated POME
- Target applications
  - Packaging and casings
  - Plastic bags
  - Oil-absorbing facial tissue
  - Biomedical

What we know

- *R. eutropha* grows using plant oils as the sole carbon source
- *R. eutropha* produces large quantities of intracellular polyhydroxyalkanoate (PHA)
- *R. eutropha* can be genetically manipulated
- Therefore, *R. eutropha* can be used as an industrial PHA production strain

Heterologous PHA synthases in *R. eutropha*

TABLE 2. Compositions of PHAs from strains grown on different carbon sources[b]

Carbon source	Strain	PHA (% of CDW)	PHA composition (mol%)[a]			
			HB	HV	HHx	HHp
Fructose	H16	75 ± 3	100			
	Re2000	79 ± 2	100			
	Re2001	39 ± 1	100			
Hexanoate	H16	49 ± 2	99.61 ± 0.01		0.39 ± 0.01	
	Re2000	51 ± 1	88.5 ± 0.2		11.5 ± 0.2	
	Re2001	48 ± 2	81.1 ± 0.4		18.9 ± 0.4	
Heptanoate	H16	52 ± 3	62.6 ± 0.5	37.4 ± 0.5		0
	Re2000	62 ± 1	40.4 ± 0.3	59.6 ± 0.3		tr
	Re2001	48 ± 6	25.2 ± 1.1	72.9 ± 1.6		1.9 ± 0.5
Octanoate	H16	66 ± 3	100		tr	
	Re2000	66 ± 2	93.44 ± 0.08		6.56 ± 0.08	
	Re2001	42 ± 4	89.6 ± 0.3		10.4 ± 0.3	

[a] Abbreviations: HB, 3-hydroxybutyrate; HV, 3-hydroxyvalerate; HHx, 3-hydroxyhexanoate; HHp, 3-hydroxyheptanoate; tr, trace amounts.
[b] PHA produced by H16 and recombinant *R. eutropha* strains expressing *phaC1*$_{Ra}$ and *phaC2*$_{Ra}$ was analyzed after the strains were grown for 60 h on 2% fructose or 0.4% fatty acids. All media contained 0.05% NH_4Cl. The values reported are averages from triplicate cultures ± SDs.

Data: Budde, *et al.* 2011 *Appl Environ. Microbiol.*

Re2000 = *R. eutropha* Re1034 containing *phaC1* from *R. aethirivorans*
Re2001 = *R. eutropha* Re1034 containing *phaC2* from *R. aethirivorans*

Conclusion: Recombinant strains of *R. eutropha* grown using fatty acids as the sole carbon source produce PHA containing HHx.

Rhodococcus aetherivorans

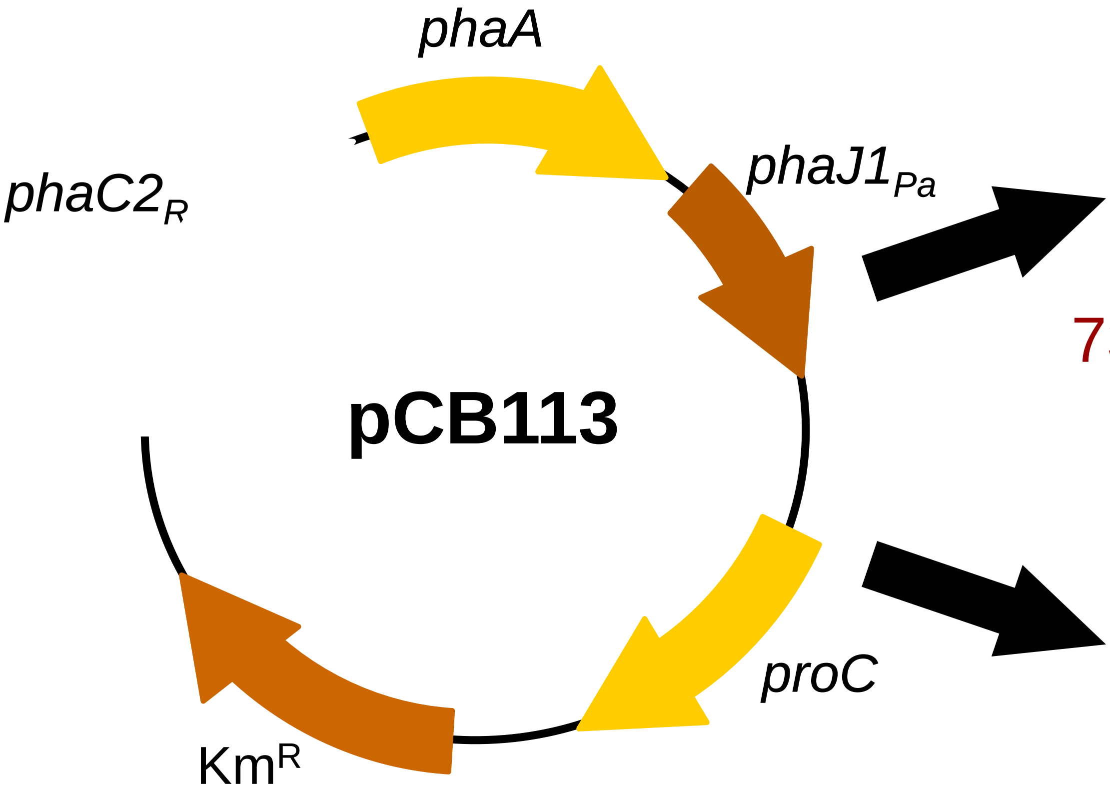
- Originally isolated from toluene-contaminated water (Chartrain, *et al*. 1998)
- Known for:
  - Growth on phenolic compounds
  - Conversion of indene to indanol (Buckland, et al, 1999)
- Genome contains 3 PHA synthase genes
- Use of *phaC1*$_{Ra}$ for P(HB-*co*-HHx) biosynthesis is novel
  - Typically, *A. caviae* synthase used for copolymer

Strains for Synthesis of P(HB-*co*-HHx)

PHA production in palm oil shake flask cultures

PHA Biosynthetic Operon in Genome	PHA (% of CDW)	HHx (mol%)
phaC1 *phaA* *phaB1*	79%	0%
phaA *phaB1*	50%	2%
phaC1 *phaA* *ΔphaB*	22%	2%
phaA *ΔphaB*	26%	31%
phaA *phaJ1*	40%	22%

Strains for Synthesis of P(HB-*co*-HHx)



Strain Re2058
Δ*phaC1*
Δ*proC*

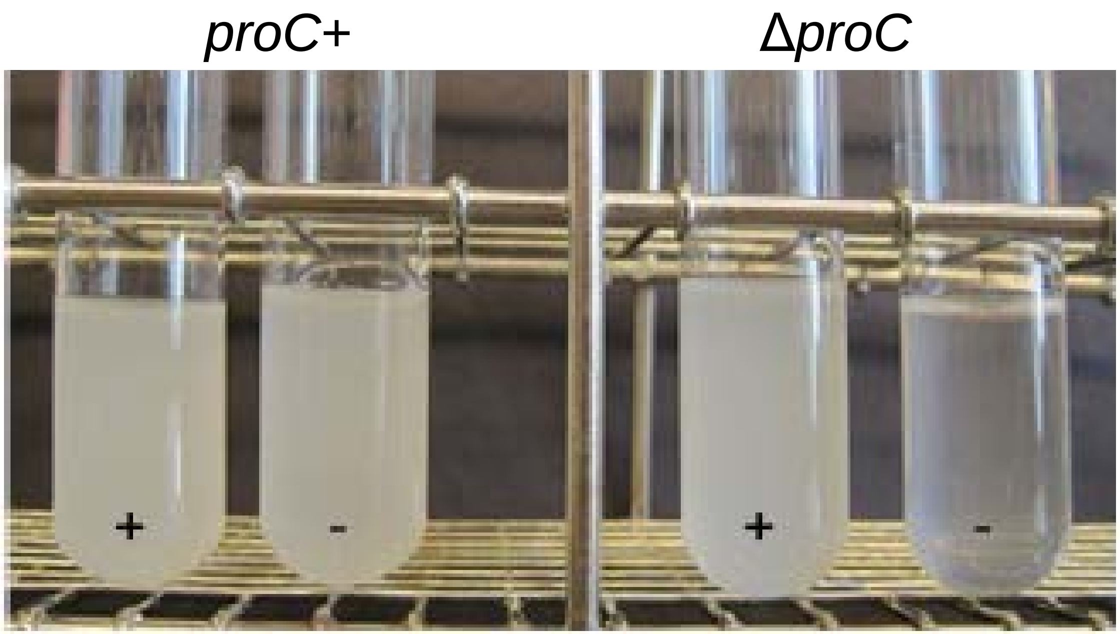
73% PHA, 13 mol% HHx (flask cultures)

Strain Re2160
Δ*phaC1*
Δ*phaB1,2,3*
Δ*proC*

64% PHA, 24 mol% HHx (flask cultures)

Goals for %HHx content exceeded

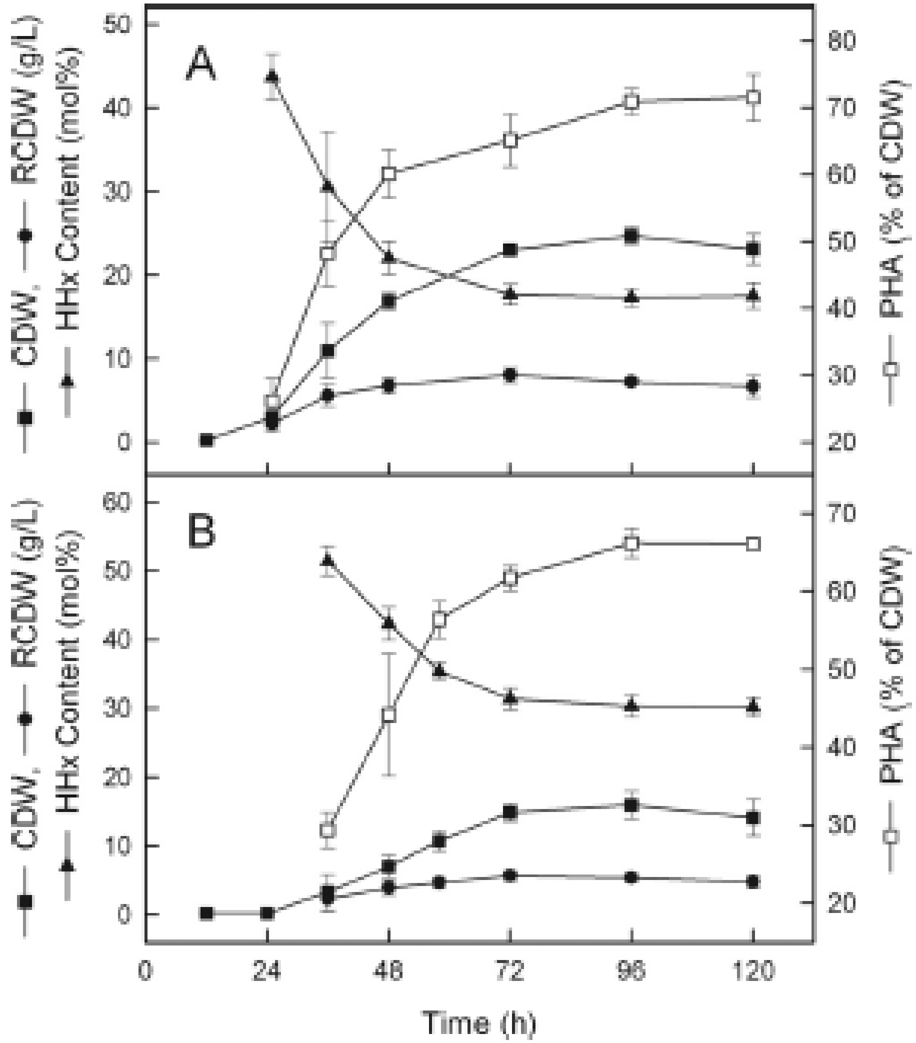
Plasmid stability – use of *proC* "addiction system"



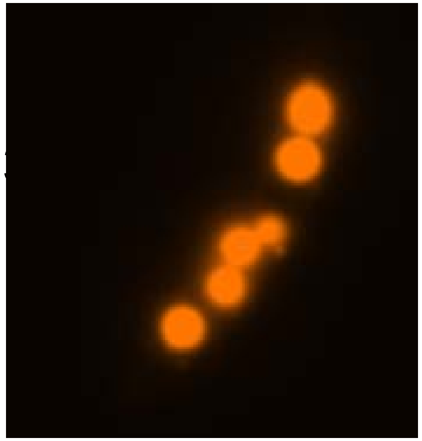
+ 0.2% proline added to media
- no proline added

A Δ*proC* strain cannot grow in minimal medium without exogenously added proline. The addition of a plasmid (in this case pCB113) with a *proC* gene present will alleviate this auxotrophy. This phenomenon creates an "addiction system" where it is in the strain's best interest to keep the plasmid even in the absence of antibiotic selection.

Palm oil fermentation results



A = Re2058/pCB11
ΔphaC1ΔproC



M. Prophete

B = Re2160/pCB113
ΔphaC1ΔphaB123ΔproC

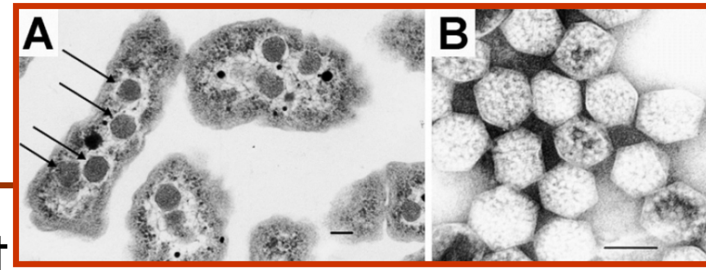
Data: Budde, *et al.* 2011 *Appl Environ. Microbiol.*

Batch fermentations carried out using palm oil as the sole carbon source

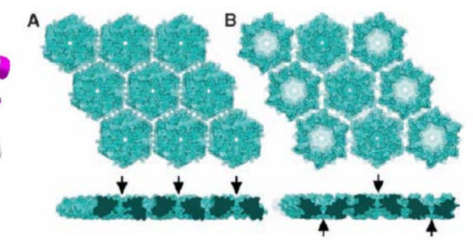
Conclusions

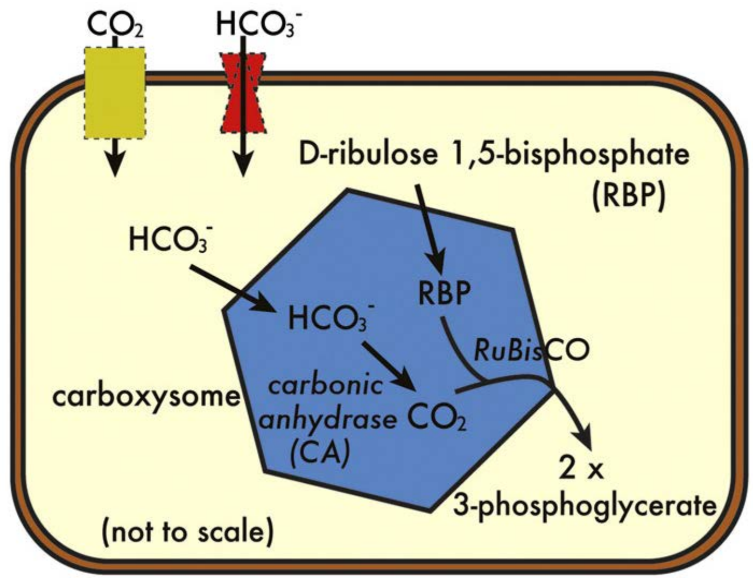
- Use of a heterologous *phaC* gene in *R. eutropha* results in HHx incorporation
  - Gene dosage increases the %HHx content
- Plasmid stabilization system established
  - Metabolic addiction system
  - Using *proC* gene
- Up to 73% (w/w) PHA containing 13-24 mol% HHx produced with new strains
  - Exceeds goals
  - Re2058/pCB113 and Re2160/pCB113
  - Strains transferred to MMBPP colleagues

Carboxysome



- Bacterial microcompartment
- Polyhedral in shape
- Contains CO_2 fixation enzymes: carbonic anhydrase and RuBisCO
- Shell subunits are small, homologous proteins which form hexamers or pentamers
- Role of carboxysome
  - Optimize carbon fixation by localizing and concentrating CO_2 and RuBisCO
    - Because RuBisCO lacks specificity for CO_2 and O_2
    - Additionally CO_2 has poor solubility





Other Types of Carbon fixation

- The Calvin (CBB) cycle
  - Wasteful O_2 reaction
- Reductive TCA cycle (‘Reverse TCA’)
  - O_2-sensitive
- Reductive acetyl-CoA (Wood-Ljungdahl)
  - O_2-sensitive
- 3-Hydroxypropionate/4-hydroxybutyrate
  - O_2-sensitive, found in Archaea
- 3-hydroxypropionate
  - No steps affected by O_2

Conclusions

- Very versatile platform
- Need to manage reducing power
- Increasing opportunities for metabolic engineering
  - Four step process:
    - 1. Make model from fundamental principles
    - 2. Manipulate model
    - 3. Measure data from model
    - 4. Mine data for process development