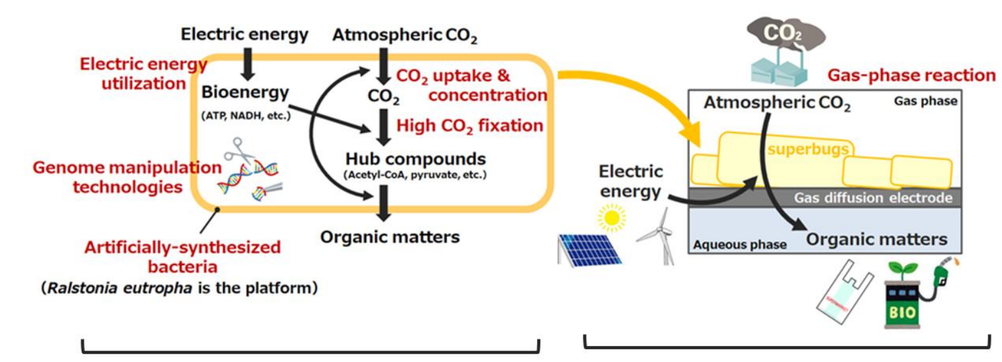
No. A-7-1E
PJ : Development of a bioprocess that uses electrical energy to fix atmospheric CO_2
Theme: Project summary

Organization: National Institute of Advanced Industrial Science and Technology (AIST)
Contact: Souichiro Kato (s.katou@aist.go.jp)

Summary of our project

■ Development of an innovative biotechnology for negative emission
■ Utilizing electric energy to convert atmospheric CO_2 into organic matters
■ More than 50 times more efficiently than plants (>50 kg-CO_2/m²/year)



"superbugs"
that use electric energy, uptake & concentrate atmospheric CO_2, and fix CO_2 with high efficiency.

"gas-phase reaction process"
that can effectively supply electricity, nutrients and CO_2 to superbugs.

R&D Items & Cooperation

■ Achievement goal (2022FY) : Demonstrate the feasibility of microbial CO_2 fixation by electricity using a gas-phase reactor

Project management, Synthetic microbiology (AIST)

①Genome manipulation technology
・Long DNA transfer technology
・Promoter library

②-4. Synthetic microbiology
・Create bacteria that can use electricity to efficiently produce organics

Genome manipulation technology ↓ / Gene info. ↑

Provide bacteria ↓ / Provide reactor technology ↑

Development of each property
②-1. Electricity utilization (Tokyo Tec. Inst.)
②-2. CO_2 uptake/concentration (Kobe Univ.)
②-3. CO_2 fixation (Tokyo Tec. Inst.)

Development of a gas-phase reactor
③-1. Reactor engineering (Nagoya Univ.)
③-2. Gas diffusion electrodes (Osaka Univ.)

1. Genome manipulation method

■ Target in this PJ : Development of genome manipulation method for *Ralstonia*

＊Genome manipulation method

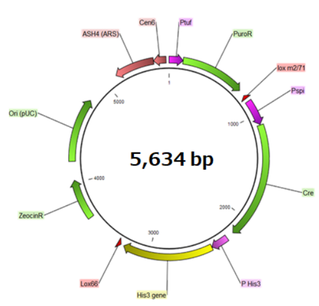
Objective: Develop a method that can introduce long DNA into the genome.

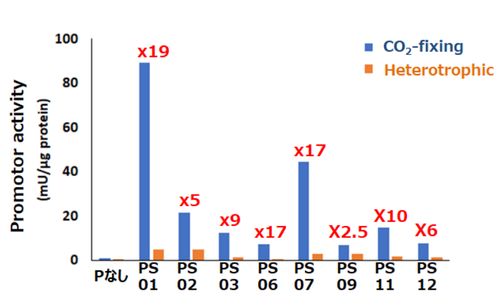
Achievements:
・Design a vector based on yeast artificial chromosome
・Gene introduction into the genome was achieved by CreLoxP method

＊Promotor library

Objective: Obtain promoters necessary to appropriately express the transgenes

Achievements:
・A simple promoter activity evaluation system was developed
・Identify 8 promoters that function specifically under CO_2-fixing conditions





2-1. Electricity-utilizing activity

■ Target in this PJ: Introducing a heterogeneous microbial electron transfer path in *Ralstonia* to confer electricity-utilizing activity

＊Introduction of electron transfer path

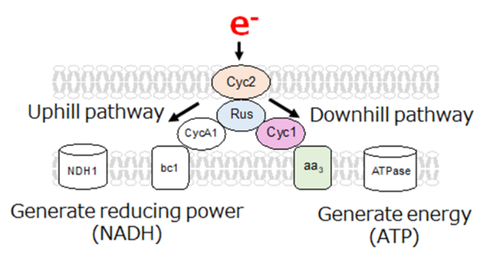
Objective: Introduce electron transfer path genes of *Acidithiobacillus*

Achievements:
・Uphill path & Up/Downhill paths were introduced into *Ralstonia*
・The expression was confirmed at RNA and protein level

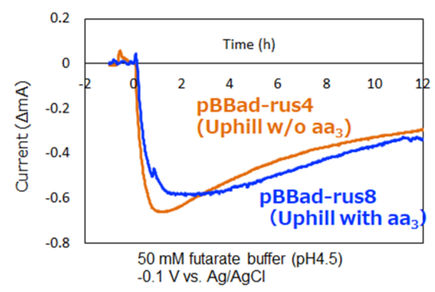
＊Electrochemical measurement

Objective: Demonstrate the electricity-utilizing activity of the *Ralstonia* strains

Achievements:
・Current consumption was observed in Uphill pass-introduced *Ralstonia* strains
・On-going for Up/Downhill paths mutants



Electron transfer path of *Acidithiobacillus*



2-2. CO_2 uptake/concentration

■ Target in this PJ: Introducing CO_2-fixing enzyme/-enrichment systems into *Ralstonia* to henhance their activities

＊Introduction of CO_2 enrichment system

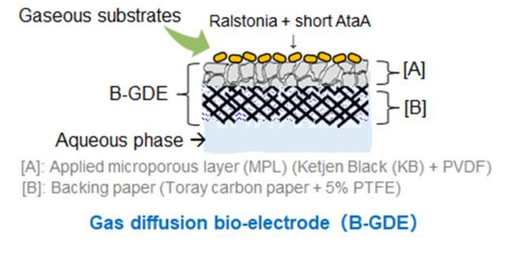
Objective: Introduce CO_2 enrichment systems of cyanobacteria into *Ralstonia*

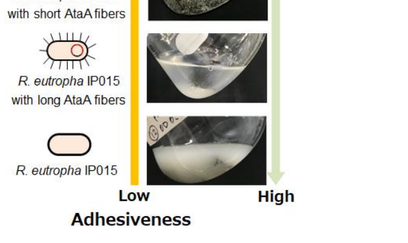
Achievements:
・A bicarbonate transport protein was adequately expressed in *Ralstonia*
・On-going for its activity measurements

＊High expression of CO_2-fixing enzyme

Objective: High expression of endogenous and exogenous CO_2-fixing enzyme (RuBisCO)

Achievements:
・High expression of endogenous RuBisCO resulted in higher activity/growth
・On-going for exogenous ones





2-3. Enhancement of CO_2 fixation

■ Target in this PJ: Enhancing CO_2 fixation by introducing a semi-artificial pathway

＊Construction of semi-artificial pathway

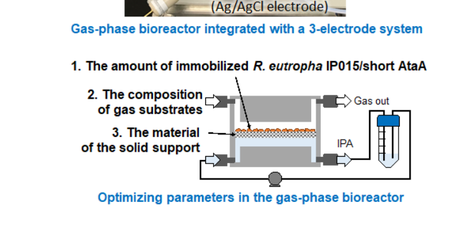
Objective: Introduce exogenous enzymes to make a semi-artificial CO_2 fixation pathway

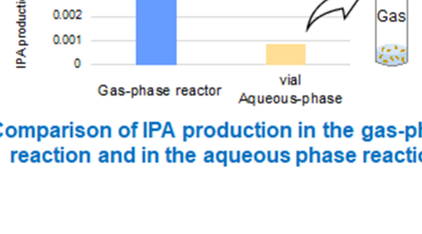
Achievements:
・The Em-rTCA pathway that functions by introducing 4 enzymes
・CO_2 fixation by the Em-rTCA pathway was confirmed by isotope experiments.

＊Improvement of CO_2-fixing enzymes

Oblective: Modify two CO_2-fixing enzymes to enjance Em-rTCA pathway

Achievements:
・Activity of CCR was enhanced by fusion with carbonic anhydrase
・Activity of PCC was enhanced by fusion with other bacterium's domain





CCR-CA fusion protein

CCR-CA has higher CO_2-fixing activity than original enzyme

3. Gas-phase reactor (1)

■ Target of this PJ : Establishing a gas-phase reactor to enhance CO_2 fixation

＊Gas diffusion electrode

Objective: Develop electrodes capable of supplying electricity, gas (CO_2), and liquid (nutrients) to *Ralstonia*

Achievements:
・Gas diffusion electrodes used in fuel cells were modified for bio-reactions
・Adjustment of resin/carbon mixing ratio in microporous layer, etc. enabled appropriate gas and liquid diffusivity

Gas diffusion bio-electrode (B-GDE)

＊Adhering ability of *Ralstonia*

Objective: Improve electrode adhesion in *Ralstonia* by introducing adhesive fibers

Achievements:
・Adhesiveness of *Ralstonia* was improved by introduction of *Acinetobacter*-derived adhesive fiber protein (Ata)

3. Gas-phase reactor (2)

■ Target of this PJ : Establishing a gas-phase reactor to enhance CO_2 fixation

＊Development of a gas-phase reactor

Objective: Develop electrodes capable of supplying electricity, gas, and liquid

Achievements:
・A lab-scale gas-phase reactor was developed to meet requirements

Gas-phase bioreactor integrated with a 3-electrode system

Optimizing parameters in the gas-phase bioreactor

＊Superiority of gas-phase reactors

Objective: Demonstrate a gas-phase reactor can enhance *Ralstonia* CO_2 fixation

Achievements:
・The production of isopropanol (IPA) from H_2/CO_2 was significantly increased by the gas-phase reaction

Comparison of IPA production in the gas-phase reaction and in the aqueous phase reaction

No. A-7-2E

PJ : Development of a bioprocess that uses electrical energy to fix atmospheric CO_2

Theme: Genome manipulation / Enhancement of CO_2 uptake

Organization: AIST, Kobe University

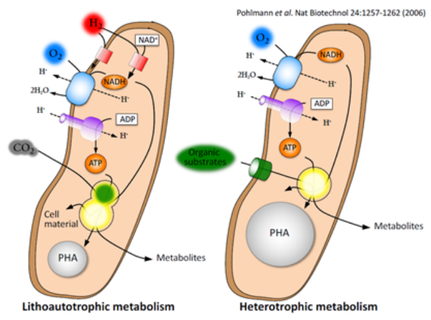
Contact: Souichiro Kato (s.katou@aist.go.jp), Hiroki Ashida (hiroki_ashida@people.kobe-u.ac.jp)

1. Genome manipulation method

■**Backgrounds:**
Ralstonia is a well-known biopolymer-producing bacterium, but the large-scale genome manipulation technology required for this PJ has not been established

■**Target in this PJ**
Establish basic technology for genome manipulation for *Ralstonia*, including long DNA transfer technology

■**R&D items**
＊Improvement of long DNA manipulation and introduction of Cre-LoxP system to enable to introduce long DNA strands equivalent to several hundred kb
＊Development of promoter libraries necessary to adequately express each gene



***Ralstonia eutropha* H16**

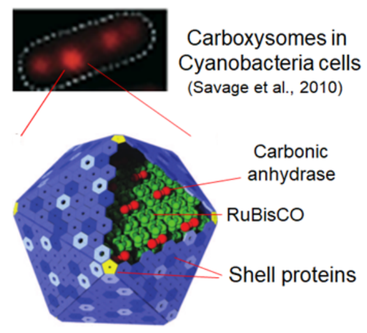
■**Achievements:**
①Long DNA manipulation methods for *Ralstonia*
②Comprehensive gene expression analysis under CO_2-fixing conditions
③Identify promoters working under CO_2-fixing conditions

2. CO_2 uptake/concentration

■**Backgrounds:**
Ralstonia has the ability to fix CO_2 by the Calvin-Benson cycle using RuBisCO, but its activity is low

■ **Target in this PJ**
Introducing an inorganic carbon enrichment system into *Ralstonia* to provide CO2 uptake and enrichment capacity

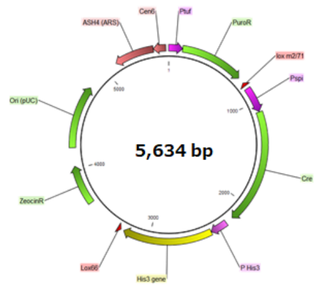
■**R&D Items**
＊Introduce CO_2 transport to provide inorganic carbon uptake and intracellular concentrating ability.
＊Enhance CO_2 fixing capacity through high expression of endogenous and exogenous RuBisCO



■**Achievements:**
①Generation of Ralstonia strains highly expressing endogenous/exogenous RuBisCO
②Enhanced CO_2 fixation activity by high expression of RuBisCO
③Expression of exogenous bicarbonate transport proteins

1-①. Long DNA manipulation methods

＊**Long DNA handling methods**

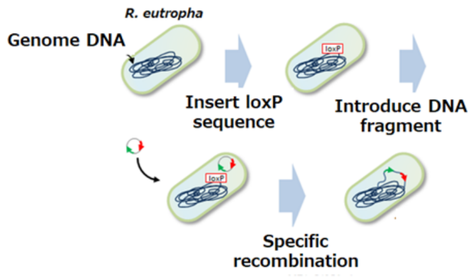


・Design a vector based on yeast artificial chromosome (can handle hundreds kpp DNA)

・Examine vector introduction method by electroporation

・Disruption of restriction enzyme genes (Improve recombination efficiency by 50~ fold)

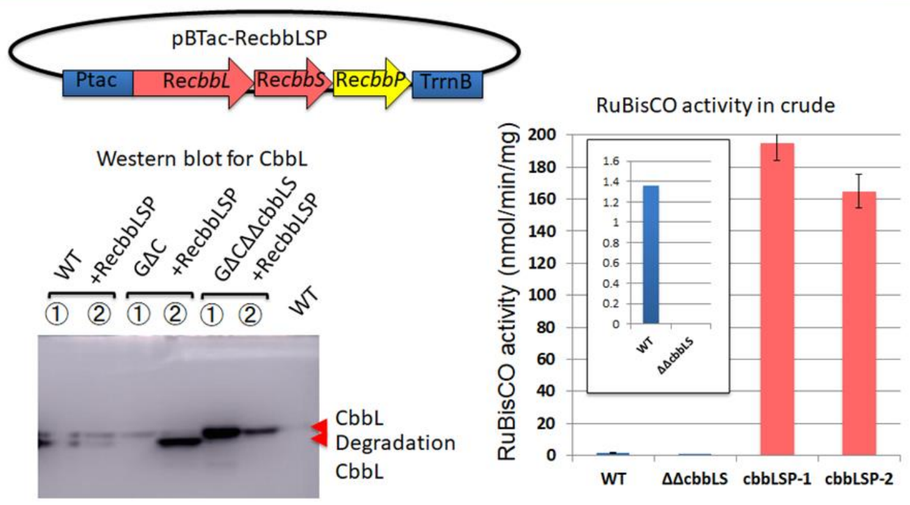
＊**Methods for genome insertion**



・The efficient genome insertion method (CreLoxP) method was applied for the first time to *Ralstonia*

・Successful insertion of long DNA strands exceeding tens of kbp into the genome

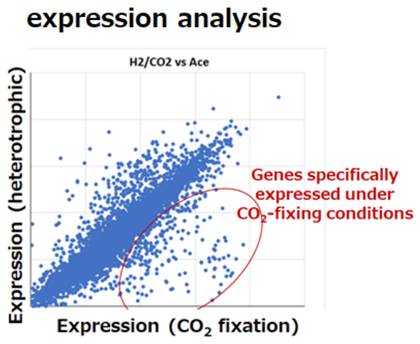
2-①. RuBisCO high expressing strains



＊High expression of endogenous RuBisCO increased CO_2 fixation activity
＊On-going for exogenous RuBisCO with high activity

1-②. Gene expression analysis

＊**Comprehensive gene expression analysis**



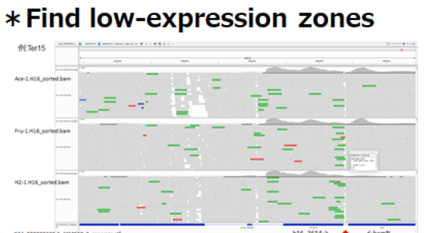
・Identified a group of genes whose expression is significantly upregulated under CO_2-fixing conditions

＊**Find promoter candidates**

	Expression level			Fold change		
	H2/CO2	Ace	Fru	H2/Ace	H2/Fru	
cbb_C2	7581	21	168	368	45	Chr_2のcbb
hox_pla	2138	11	23	189	95	NAD-reducing hydrogenase
selB_C2	647	5	18	125	35	
ttt_C2	362	2	4	159	88	tripartite tricarboxylate transporter substrate binding protein

Candidates for promoters specifically working under CO_2-fixing conditions

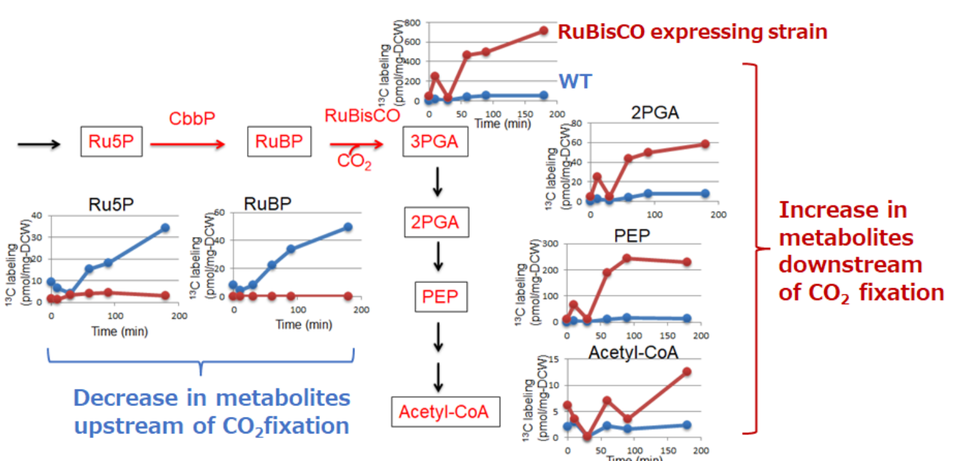
＊**Find low-expression zones**



Candidates for terminators, and sites for DNA insertion

2-②. CO2 fixation by increased RuBisCO

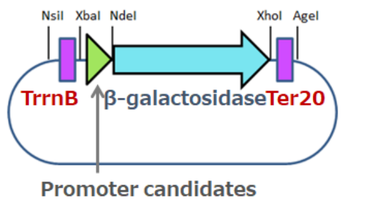
■Calvin-Benson cycle metabolites measured by dynamic metabolomics

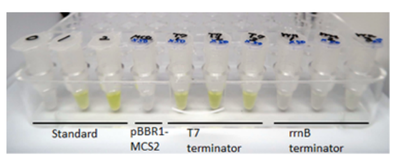


＊The rate-limiting CO_2 fixation reaction was enhanced by high expression of endogenous RuBisCO

1-③. Promotor library

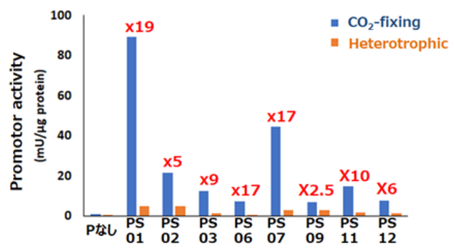
＊**Evaluation of promoters**





・Develop an evaluation system that can easily quantify promoter activity calorimetrically

＊**Promoter library**

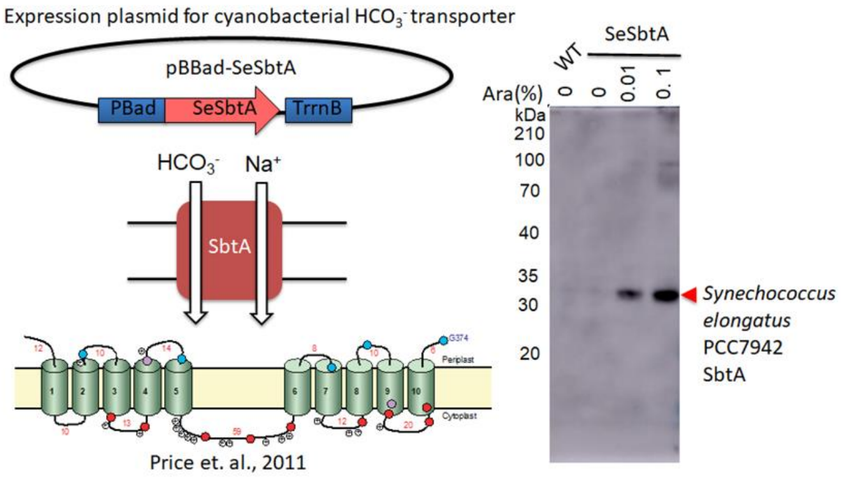


・Identify 8 promoters that function specifically under CO_2-fixing conditions

Using these results, we are now constructing an artificial synthetic strain with high electrical availability, high CO_2 uptake and fixation capacity, and electrode attachment ability.

2-③. Bicarbonate transporter

Expression plasmid for cyanobacterial HCO_3^- transporter



＊Successful expression of a bicarbonate transporter from cyanobacteria
＊On-going for its activity measurements

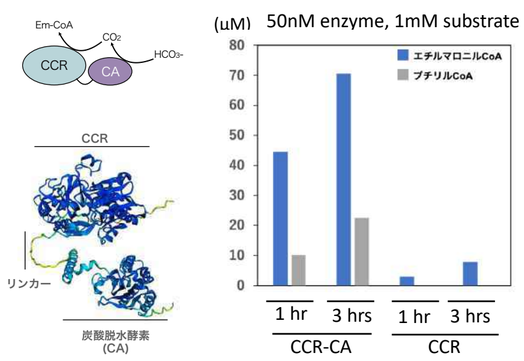
No. A-7-3E
PJ :電気エネルギーを利用し大気CO2を固定するバイオプロセスの研究開発
Theme: Enhancement of CO2 fixing enzyme ability and providing electrotrophy

Organization: Tokyo Institute of Technology
Contact: Kosuke Fujishima (fuji@elsi.jp)

■Aim of the project :
Ralstonia eutropha H16 (Ralstonia) has been previously used as a model organism for bioplastic and other material/compound producing microbe. Here, we will search and create a novel CO2-fixing enzyme which play a key role in the Ethylmalonyl-CoA pathway. In addition, a series of genes corresponding to the electron-transfer system in the cell membrane from the iron-oxidizing bacterium *Acidithiobacillus ferrooxidans* will be introduced to validate the electrotrophic activity of Ralstonia.

< Enhancement of CO2 fixing enzyme ability >

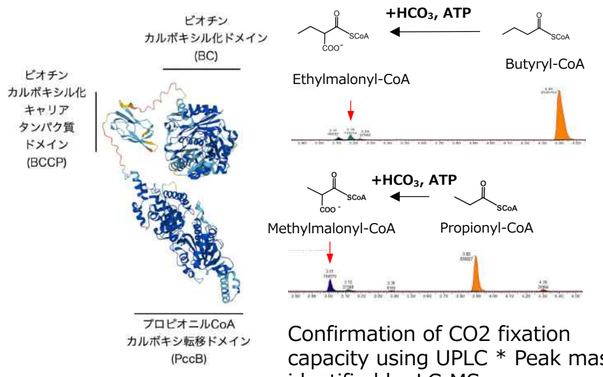
Crotonyl-CoA carboxylase/reductase (ccr) from Methylorubrum is fused with diatom derived monomeric carbonic anhydrase (CA)



CCR-CA fusion protein (CCR_CA)

Artificially generated CCR-CA fusion protein showed higher activity than natural CCR under atmospheric CO2

Engineering *Rhodopseudomonas* derived long chain carboxylase (LCC) tp create novel Propionyl/Ethylmalonyl-CoA carboxylase



Domain-fused propionyl-CoA carboxylase (LCC_PCCB)

Confirmation of CO2 fixation capacity using UPLC * Peak mass identified by LC-MS

LCC_PCCB contributes to two CO2 fixation reactions in the semi-artificial synthetic pathway

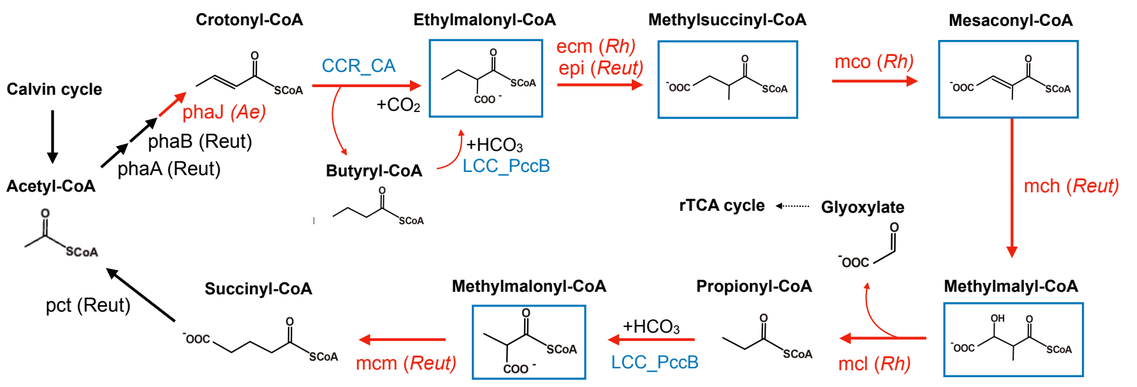
- The CCR-CA fusion protein was found to be more active than natural CCR under low CO2 concentrations.
- Artificial domain-fused carboxylase can synthesize methylmalonyl- and ethylmalonyl-CoA via HCO3 fixation

< *In vitro* validation of enzymatic reactions contributing to a semi-artificial CO2 fixation circuits>

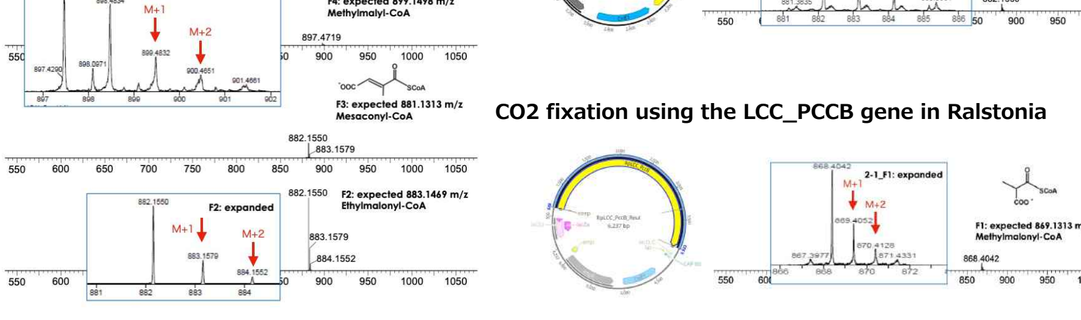
Intermediate acylated CoA
LC-MS同定 + 分取済

: Reaction confirmed in Ralstonia
: *in vitro* validated reaction

青字：novel enzyme made in this PJ
赤字：Enzyme purified this PJ



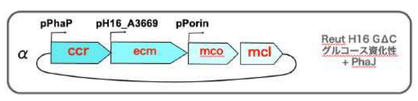
A group of proteins isolated and purified for activity confirmation and synthesis of intermediate acyl CoAs.



- A total of seven reactions of the ethylmalonyl-CoA pathway in the semi-artificial synthetic circuit, from crotonyl-CoA to succinyl-CoA, were successfully verified to proceed *in vitro*, including enzymes from Ralstonia.
- We succeeded in synthesizing and preparative isolation of five non-promotional intermediate acyl-CoA compounds, which are industrially difficult to synthesize.

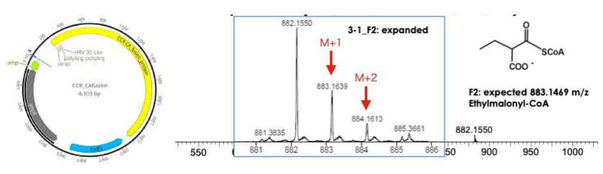
< Metabolic analysis of genes related to ethylmalonyl CoA circuit >

Metabolic analysis of Reut introduced with four genes related to the Em-CoA circuit.



CO2 uptake was confirmed by LC-MS of the exact mass of acyl CoA compounds in cell extracts using Ralstonia with carbon isotope C13 labeled carbonate added to the culture medium.

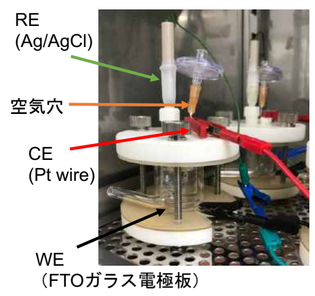
CO2 fixation using the CCR-CA gene in Ralstonia

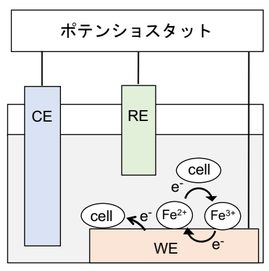


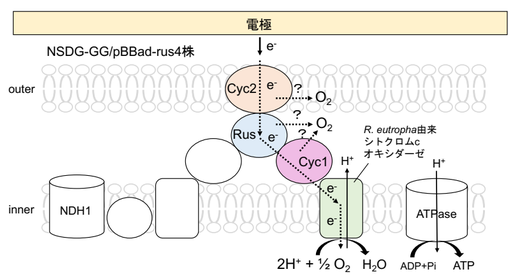
CO2 fixation using the LCC_PCCB gene in Ralstonia

- We prepared Reut strains transfected with four genes (ccr, ecm, mco, mcl) and artificial enzymes (CCR-CA, LCC_PCCB) created in this PJ, and added carbon isotope (C13)-labeled CO2 during culture, and identified acyl CoA compounds (M+1, M+2) associated with isotope-labeled Em-CoA circuit in the cell extracts by LC-MS.

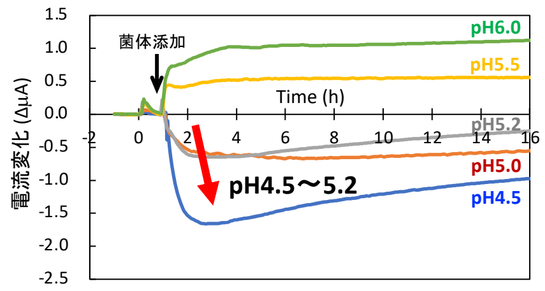
< Electrochemical measurements of the *Ralstonia* strains after introduction of electron transfer pathway>



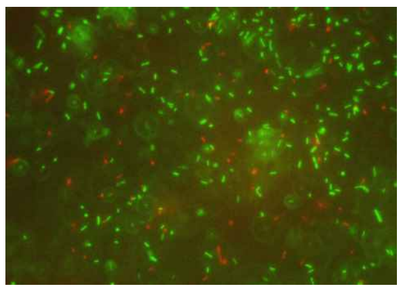




Electron transfer introduced株 (pBBad-rus4)



50 mM Phthalate, -0.1～-0.19 v (vs. Ag/AgCl)

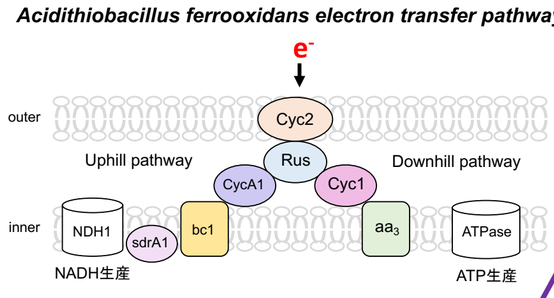


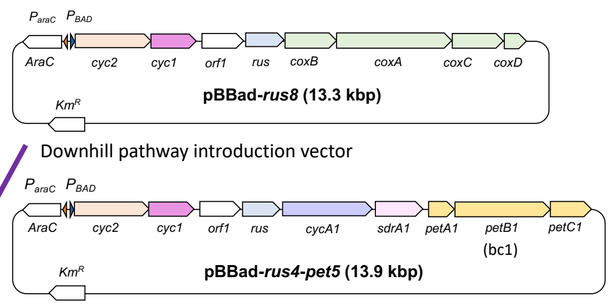
50mM. Phthalate(pH4.5), -0.1V vs Ag/AgCl for 4h
LIVE/DEAD BacLight used

- Detection of cathode current by the engineered Ralstonia strain

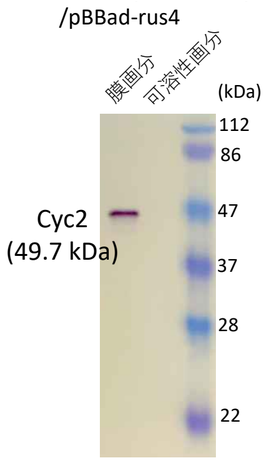
<Protein expression and localization of electron transfer related genes as well as introduction of both Downhill and Uphill pathways>

***Acidithiobacillus ferrooxidans* electron transfer pathway**



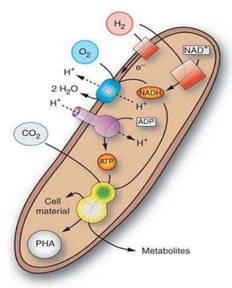


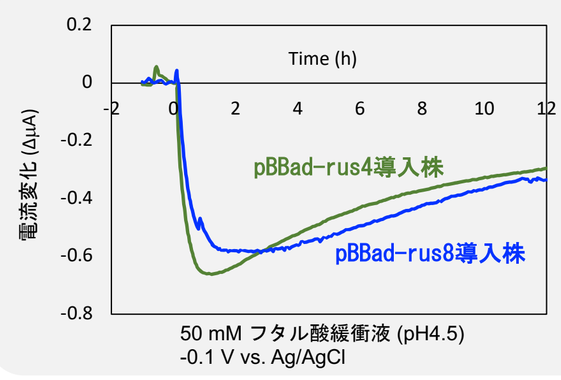
R. eutropha H16GΔ*phaC* /pBBad-rus4



Western blot analysis using Anti-Cyc2 peptide antibody

Ralstonia



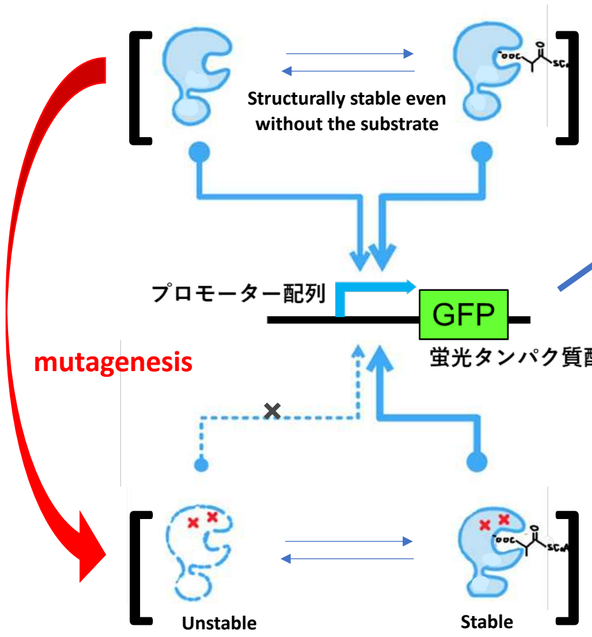


※No significant differences were observed in *rus8-transgenic strains compared to rus4-transgenic strains.
Rus4-pet5 transgenic strains will be measured in the near future

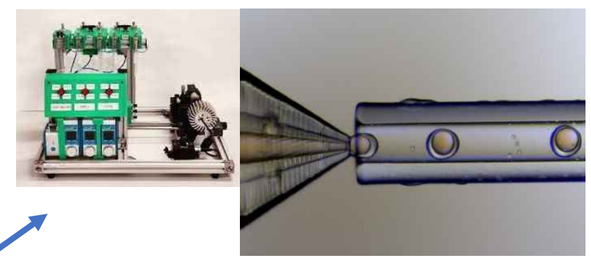
- Preparation and transduction of inducible expression vectors, confirmation of Cyc2 expression by Western blotting.

<Development of protein biosensor to screen for high activity LCC_PCCB>

Screening strategy of a protein sensor (mmd_LuxR) for sensing methylmalonyl-CoA



Double emulsion generator



- A transcription factor-fused methylmalonyl-CoA decarboxylase that is conformationally stable only in the presence of ethylmalonyl-CoA, a substrate of LCC_PCCB, was designed

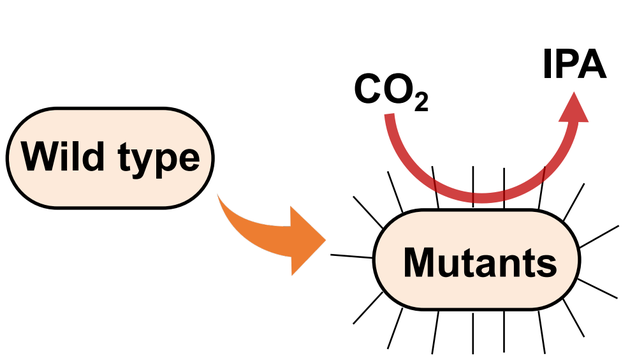
No. A-7-4E
PJ : R&D of bioprocesses using electrical energy for atmospheric CO_2 fixation
Theme: Construction of microbial gas-phase reactors for CO_2 bioconversion

Organization: Nagoya University and Osaka University
Contact: Prof K. Hori (khori@chembio.nagoya-u.ac.jp) and Prof S. Nakanishi (nakanishi@chem.es.osaka-u.ac.jp)

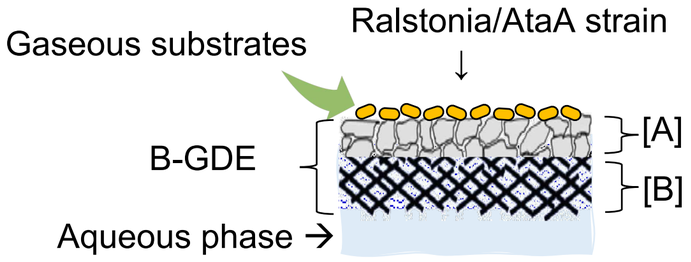
The goal of this project

To enhance the rate of CO_2 fixation using *Ralstonia* in a gas-phase bioreactor integrated with a gas-diffusion bioelectrode.

① *Ralstonia*/AtaA strain construction

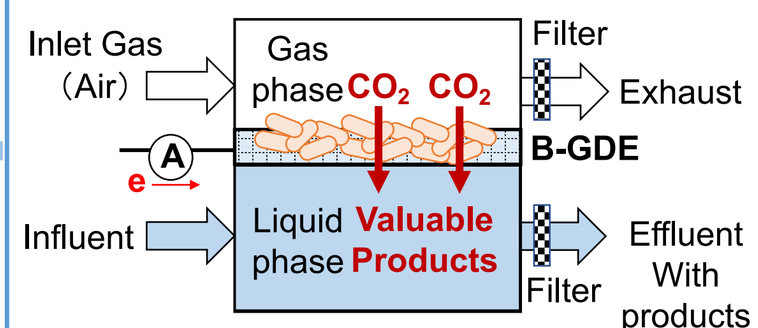


② Electrode development

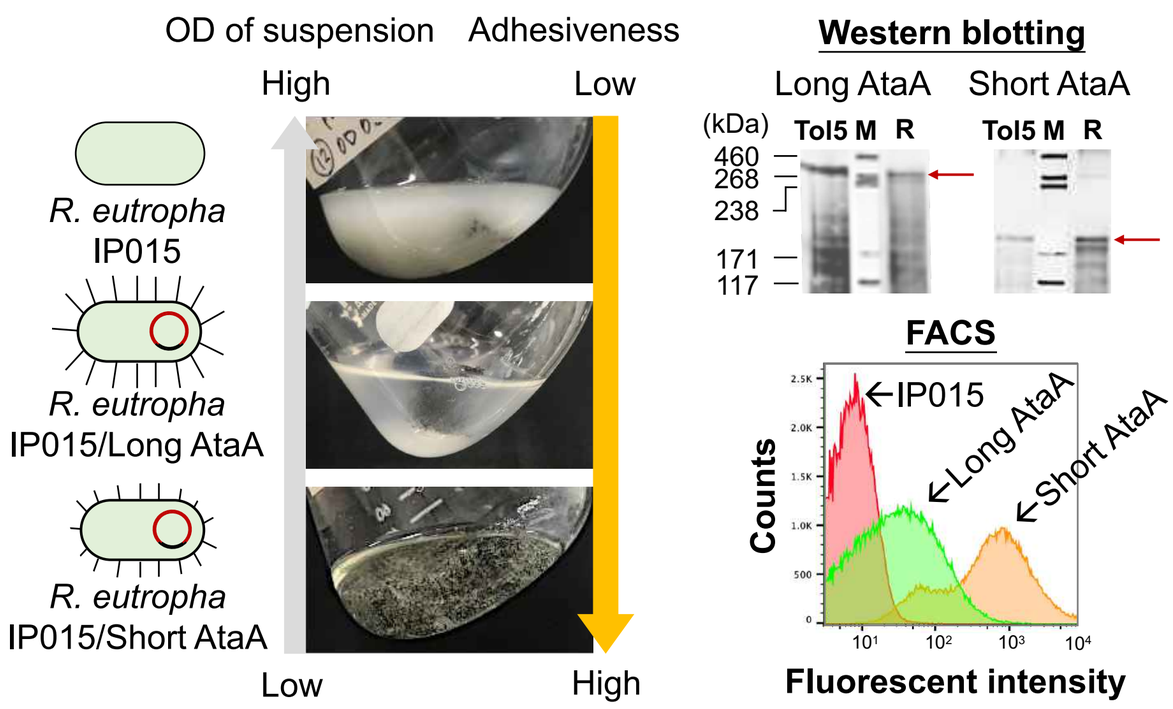


[A]: Applied microporous layer (MPL)
(Ketjen Black (KB) + PVDF)
[B]: Backing paper (Toray carbon paper
+ 5% PTFE)

③ Reactor development



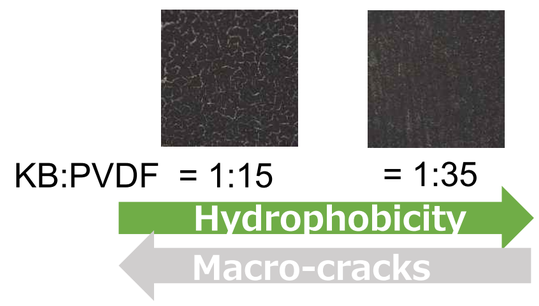
Construction of an engineering *Ralstonia* for high adhesiveness



AtaA, a trimeric autotransporter adhesin from *Acinetobacter* sp. Tol 5

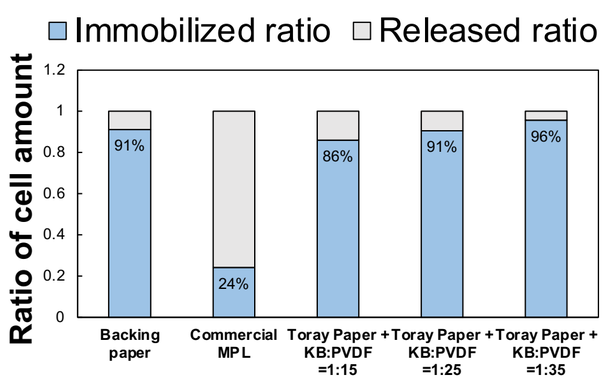
		Fiber length (nm)	Fiber size (kDa)	Vector
Long AtaA	Full-length (FL)	261	359	pBBad
Short AtaA	40% of FL	100	144	pBBad

Electrode development for cell immobilization

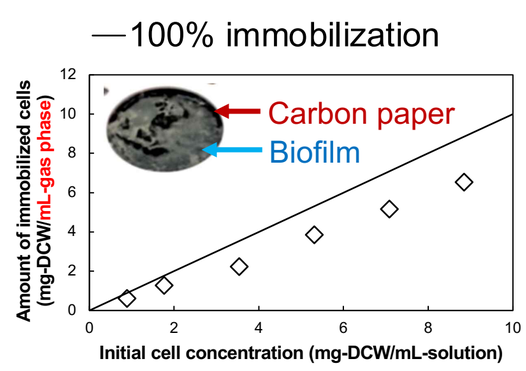


MPLs with various compositions

Availability of water penetration (KB+PVDF=1:35)

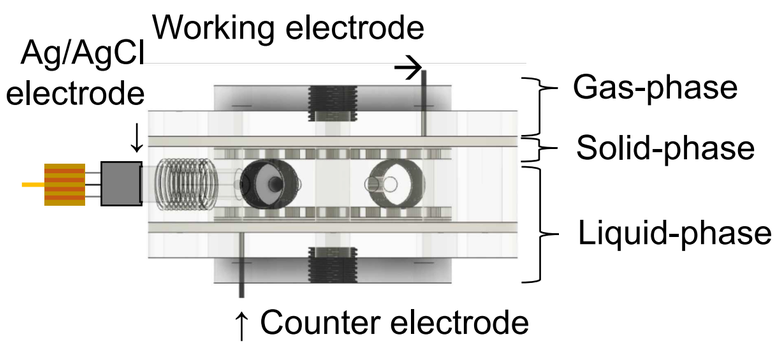


Immobilization efficiency on various layers of carbon paper

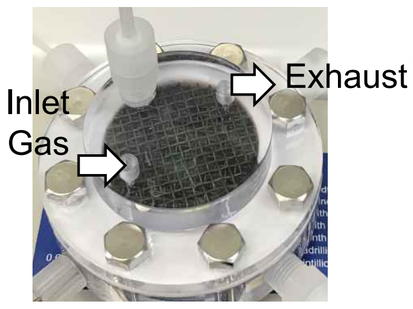


Effects of initial cell concentrations on immobilization efficiency

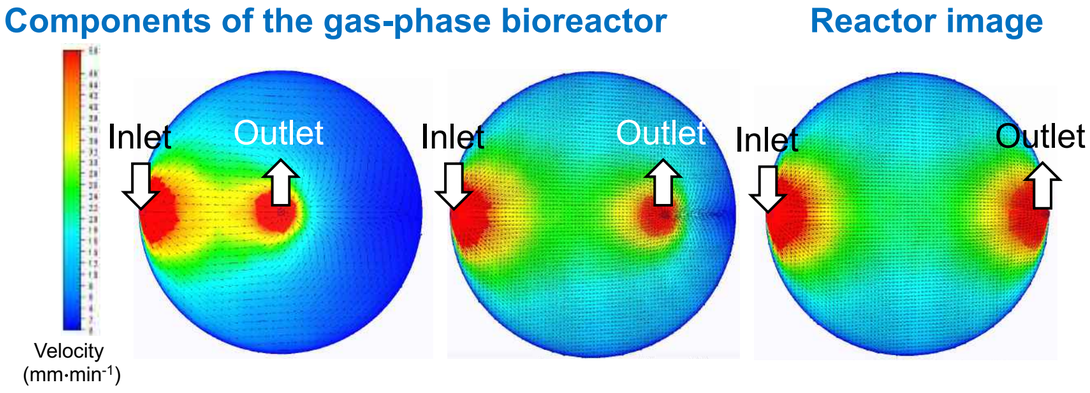
Reactor development for CO_2 bioconversion



Components of the gas-phase bioreactor

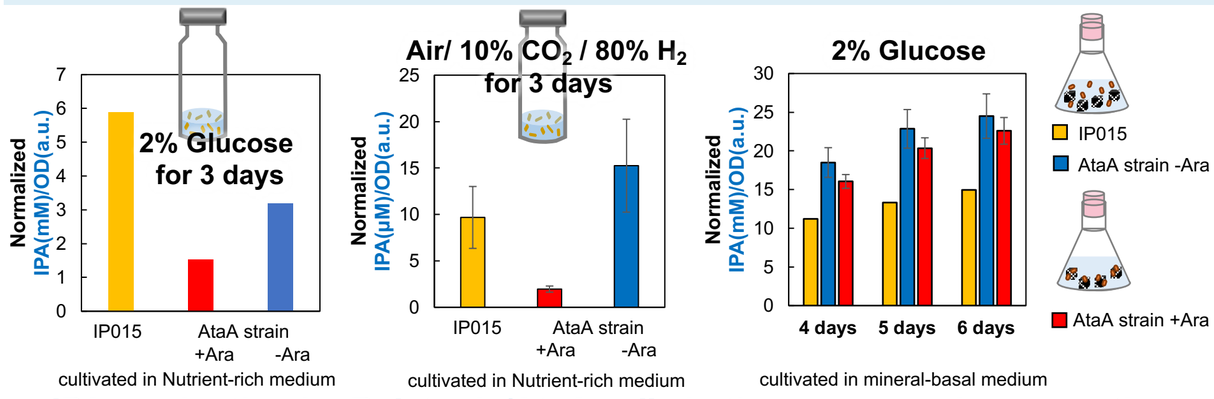


Reactor image

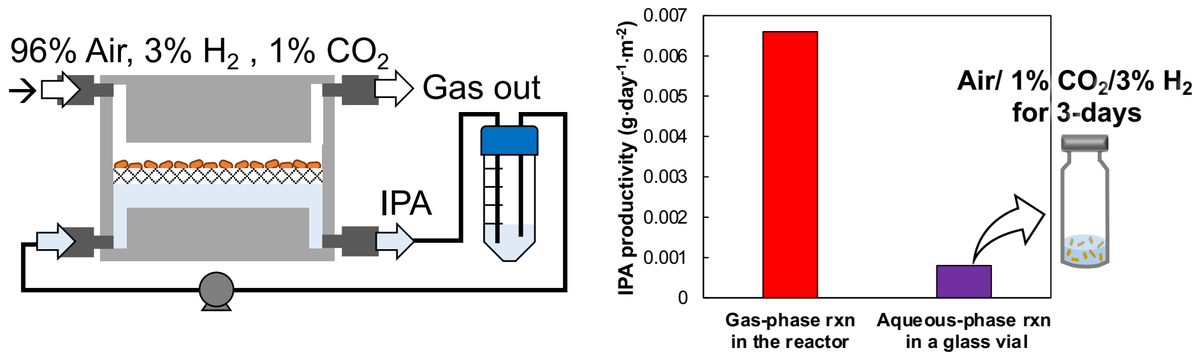


Optimization of entrance/exit positions for uniform distribution of the gas flow pattern in the gas chamber using Computational Fluid Dynamics

IPA production by Ralstonia/AtaA strain



IPA production by Ralstonia/AtaA cells in the aqueous phase reactions



Comparison of IPA production in the gas-phase reaction and in the aqueous-phase reaction

Conclusion

1. Short AtaA fiber with high adhesivity was successfully expressed on the surface of *Ralstonia eutropha* IP015 cells.
2. An optimized B-GDE with a modified layer of MPL shows the functions to control water penetration and enhance the cell immobilized amount.
3. IPA production is higher in the gas-phase reaction when using 1% CO_2 and 3% H_2 in the air, compared to that in the aqueous-phase reaction.