Preservation and cultivation of Ammonia-Oxidizing Bacteria (AOB)

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Tips for Mainteinance of AOB

1. **Preservation of Ammonia-Oxidizing Bacteria (AOB)**

   L-drying and freezing method

2. **Purity check of culture of AOB**

   Introduction of one way ~ PCR-DGGE

3. **Cultivation of AOB**

   ~ bicarbonate contained in media of AOB ~

Background

Nitrification, the oxidation of ammonia to nitrate by chemolitotrophic nitrifying bacteria, is key process in the global cycling of nitrogen.

Nitrifying bacteria

Ammonia-Oxidizing Bacteria (AOB)

$$\text{Ammonia } (NH_3) \rightarrow \text{Nitrite } (NO_2^-)$$

Nitrite-Oxidizing Bacteria (NOB)

$$\text{Nitrite } (NO_2^-) \rightarrow \text{Nitrate } (NO_3^-)$$

Many strains have been isolated from ecosystem all over the world. However

Background

Only a few strains available in culture collections

Type strains

	ATCC	NCIMB	BCRC	ICMP	Univ. Hamburg
Nitrosococcus nitrosus	no culture isolated				
Nitrosococcus oceani	ATCC 19707	NCIMB 11848	BCRC 17464		
Nitrosospira briensis	no culture available				
Nitrosospira multiformis	ATCC 25196	NCIMB 11849			
Nitrosospira tenuis					Nv-1
Nitrosomonas aestuarii					Nm 36
Nitrosomonas communis					Nm 2
Nitrosomonas europaea	ATCC 25978	NCIMB 11850		ICMP 13139	
Nitrosomonas eutropha					Nm 57
Nitrosomonas halophila					Nm 1
Nitrosomonas marina					Nm 22
Nitrosomonas nitrosa					Nm 90
Nitrosomonas oligotropha					Nm 45
Nitrosomonas ureae					Nm 10

Non-type strains

ATCC: 5 strains NBRC: 1 strains

We were unable to find other strains

Allmost all the type strain are not in two different collections in two different countries

Background

Difficulty of handling and maintenance of pure culture of AOB

Growth monitoring of AOB

We have to measure the amount of nitrite production of AOB by colorimetric method.

Contamination detection of culture

Some strains can form colony, but the colony formations require several months.
Colony size of AOB is very small.

As for many researchers

Recognition that freezing and vacuum-drying are not suitable for AOB

Keeping isolates by serial transfer

Background

49 AOB strains possessed in NBRC

strain	source	strain	source
Nitrosovibrio sp. RY6A-2	rhizosphere soil	Nitrosomonas sp. IWT310	biological deodorizing equipment
Nitrosomonas sp. TNO615	marine	Nitrosolobus sp. HBN8222A	alkaline soil
Nitrosomonas sp. DYS323	biological deodorizing equipment	Nitrosolobus sp. HBN8226A	
Nitrosomonas sp. HTA6	rhizoplane	Nitrosolobus sp. CHIC5	
Nitrosomonas sp. IWT203	biological deodorizing equipment	Nitrosospira sp. FJI425	
Nitrosomonas sp. IWT305	biological deodorizing equipment		

Chemolithotrophic AOB are divided into two group.

- Betaproteobacterial AOB ・・・・・・・Monophyletic
  - Nitrosomonas
  - Nitrosospira
  - (Nitrosovibrio, Nitrosolobus)
- Gammaproteobacterial AOB
  - Nitrosococcus

Our purpose in this study is to make these strains available to the public in our NBRC culture collection.

1. Preservation of AOB

Known methods to maintain AOB

1. Serial transfer	
2. Storage under liquid paraffin	Nature (1957) 179: p789, Nature (1957) 179: p1200
3. Cold storage	
4. Cryopreservation	Bull Jpn Soc Microb Ecol (1994) 9: p119-123
5. freeze-drying	Soil Sci Plant Nutr (2004) 50: p777-781
6. L-drying	NBRC Method

Freezing, freeze-drying & L-drying are regular as long term storage.

L-drying

We tried some protective media to the strains unable to be preserved by our NBRC method.

Freezing

We confirmed whether the ordinary method (using glycerol and DMSO as cryoprotectant in deep freezer at -80 degree) was applicable to AOB.

1-1. L-drying preservation

NBRC method

12 kinds of protective media (SM1 – SM12) are prepared for various microorganisms.

SM1 : Standard

SM8 : For chemolithotroph

SM5 : For *Aquaspirillum* etc.

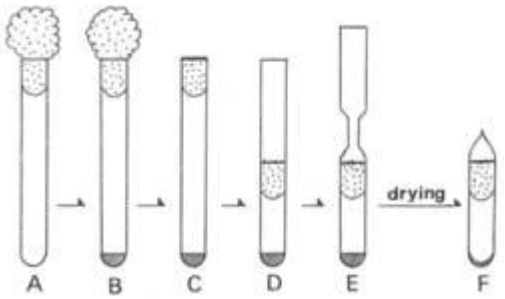
	SM1	SM8	SM5
Sodium glutamate	3 g	0.5 g	3 g
Adonitol	1.5 g	1.5 g	1.5 g
L-cystein monohydrate	0.05 g	0.01 g	—
ED·2HCl	—	—	0.4 g
Sorbitol	—	—	1 g
Potassium phospate (100 ml)	0.1 M	0.02 M	0.1 M

The media are based on combination of glutamete and phospate buffer. Adonitol and cystein are the mutation protective agent.

SM8 is protective medium for chemolithotroph.

The growth of AOB is inhibited at high concentration of cystein and phospate. So, in SM8, the amount of these compounds is limited.

Therefore, stability of preservation using SM8 is inferior to that of SM1.



1-1. L-drying preservation

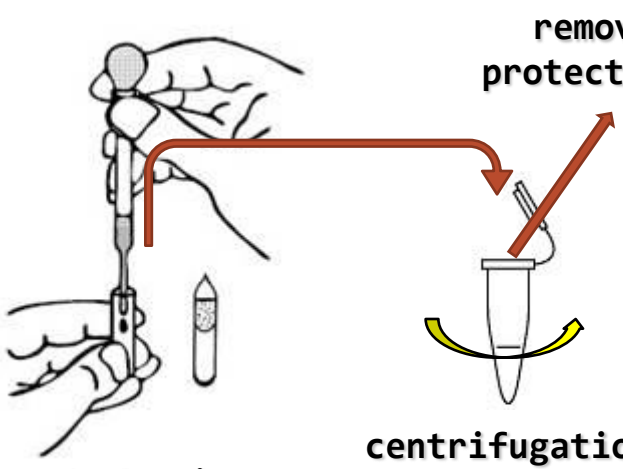
Some strains were unable
to be preserved using SM8

B2	++
EGT404	++
DYS317	++
CNS332	–
DYS323	–
IWT203	++
IWT310	++
NIWC6	–
NIWC9	+
HTA10	+
HTA25	+

CNS332
DYS323
NIWC6

We tried some protective media

NG

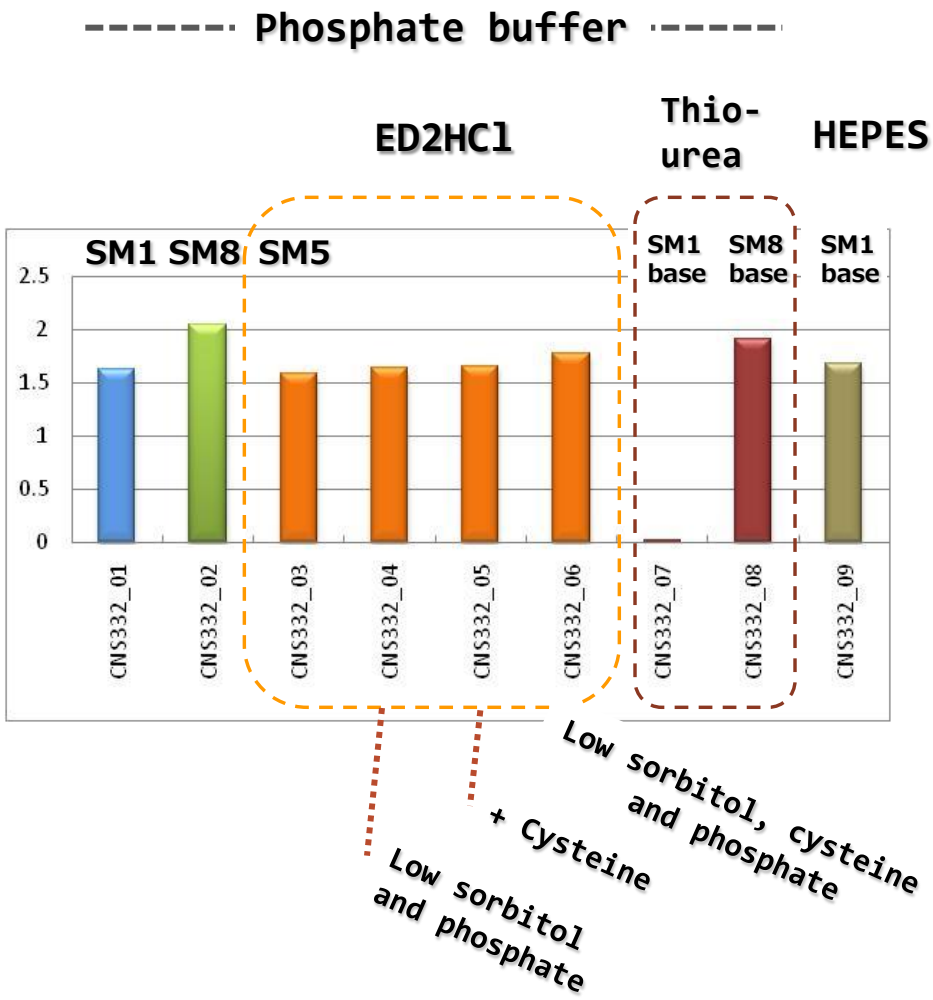


cultivation

OK

1-1. L-drying preservation

Strain CNS332



Growth yield after rehydration and cultivation for 2 weeks

01 SM1 : Standard

02 SM8 : For chemolithotroph

03 SM5 : For *Aquaspirillum*
ED2HCl in place of cysteine

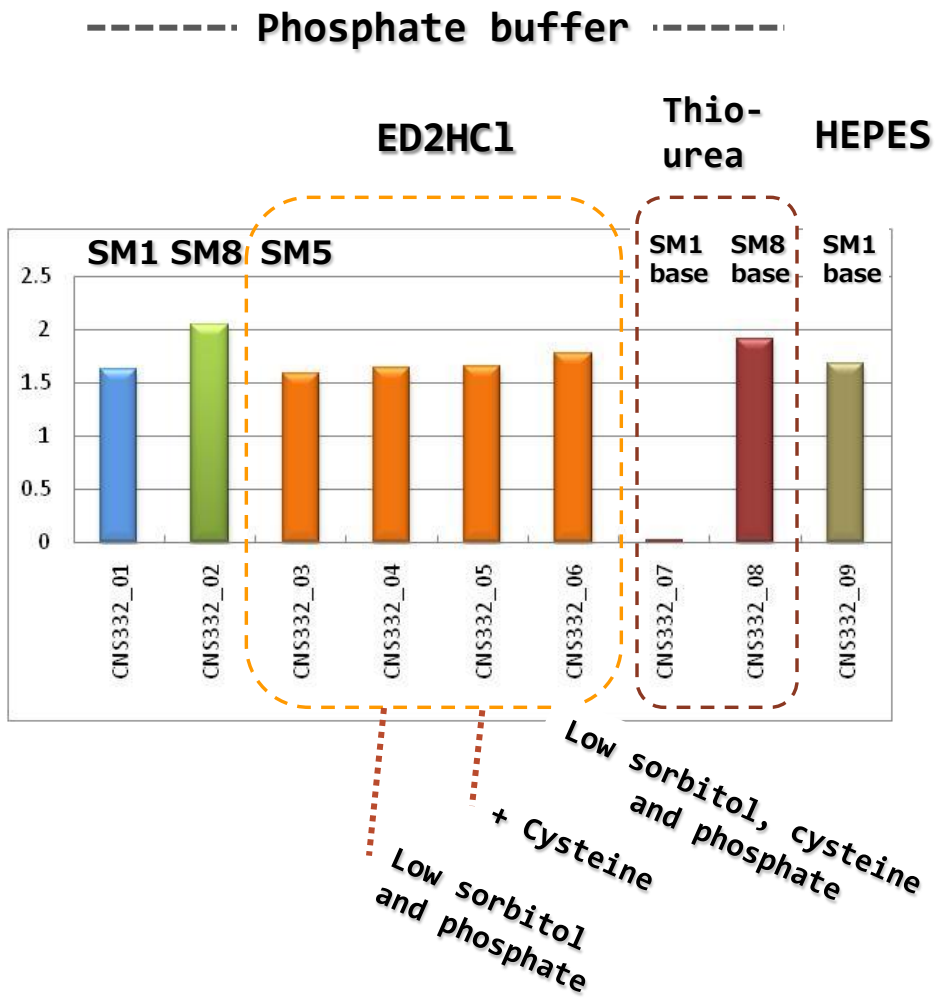
04
05
06
} SM5 modified
Ingredients conc. are different

07 SM1
08 SM8
} Thiourea in place of Adonitol

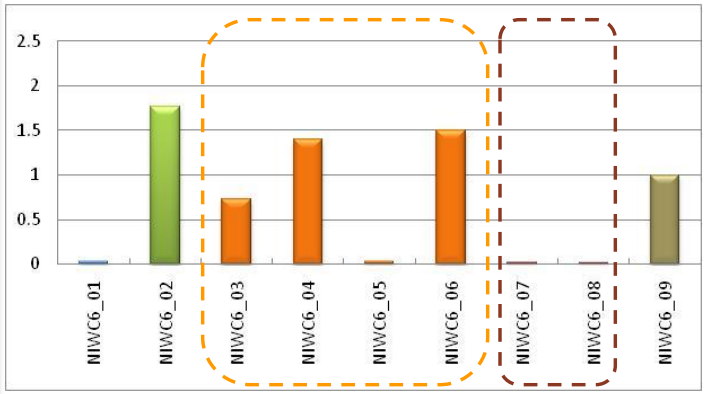
09 SM1 HEPES in place of phosphate

1-1. L-drying preservation

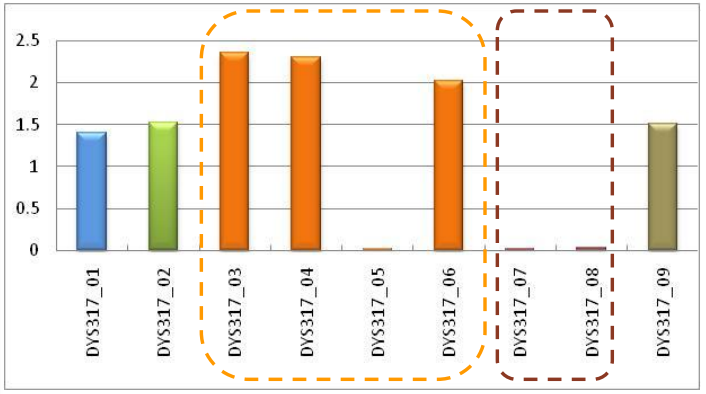
Strain CNS332



Strain NIWC6



Strain DYS317



1-1. L-drying preservation

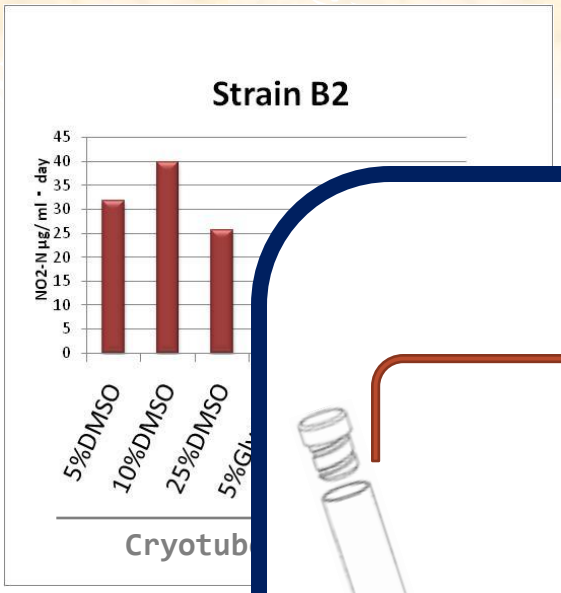
As for L-drying preservaion of AOB,

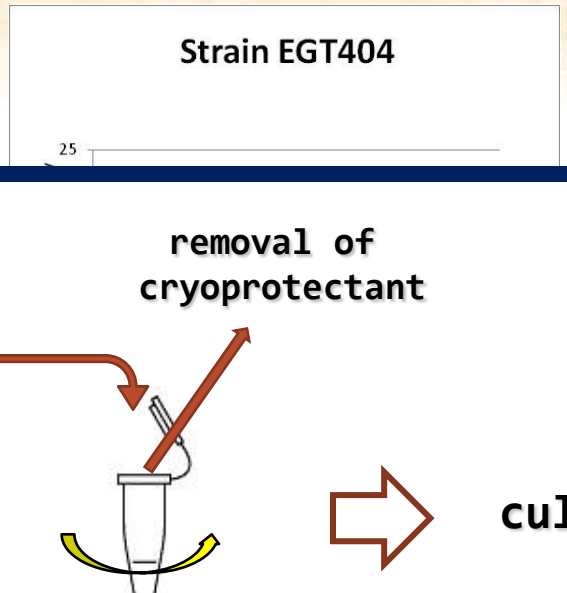
It is effective for recovery to remove the protective media.

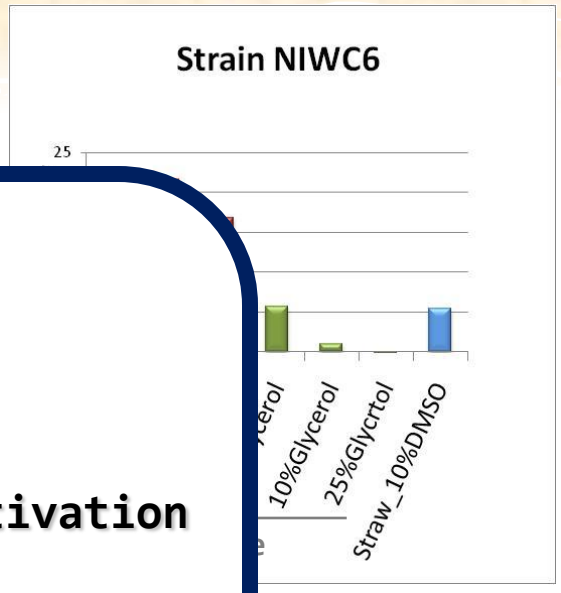
AOB can be stably preserved using SM8.

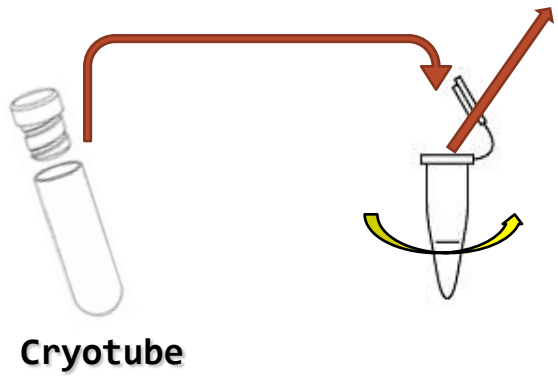
Ethylenediamine dihydrochloride and HEPES are effective.

1-2. Cryopreservation



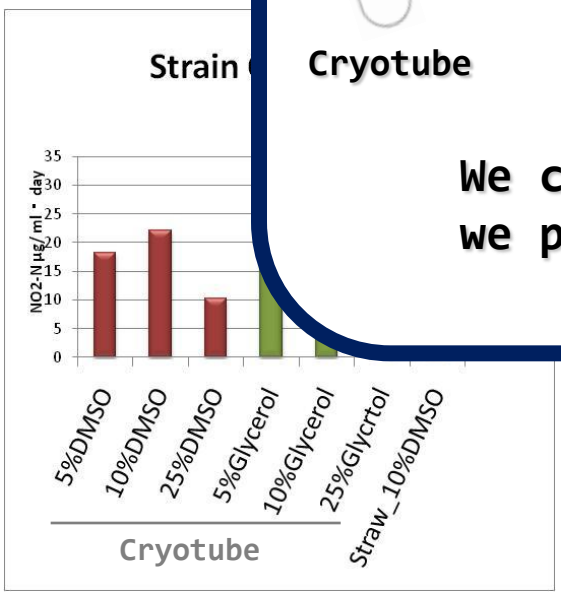


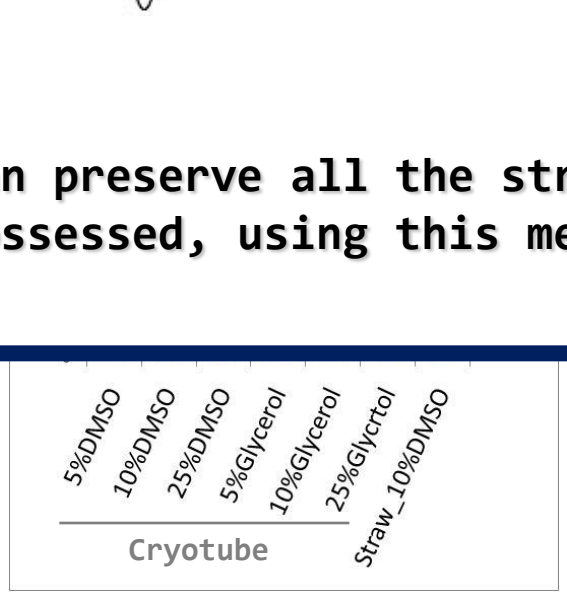




cultivation

We can preserve all the strains
we possessed, using this method.





stored in deep
)C)

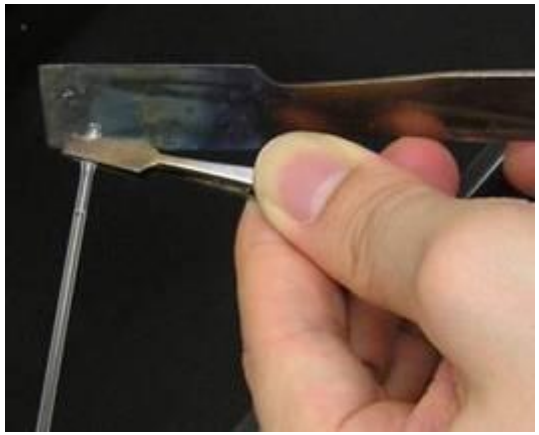
O (tube)
O (tube)
O (tube)
lycerol (tube)
Glycerol (tube)
25% Glycerol (tube)

Stored in LN vapor phase
in the tank

10% DMSO (straw)

1-2. Cryopreservation

Rapid freezing using straw

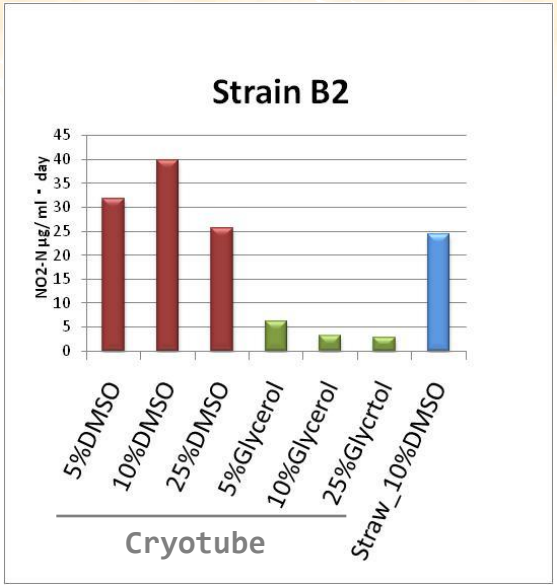


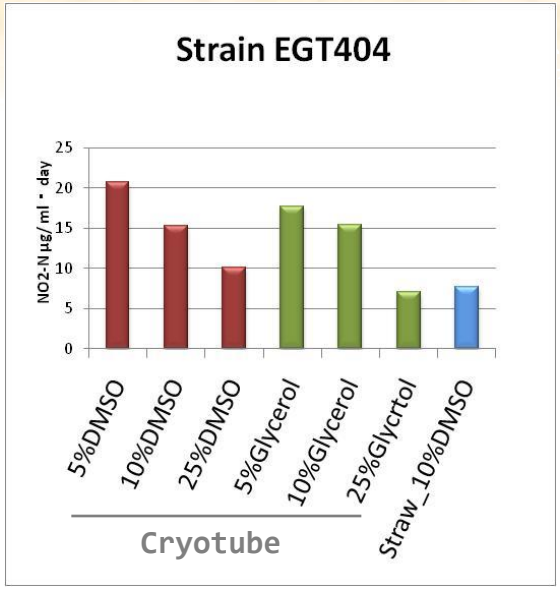
0.25 ml Straw 133 mm * 1.6 mm (ID)
Ionomer resin (cryo bio system)

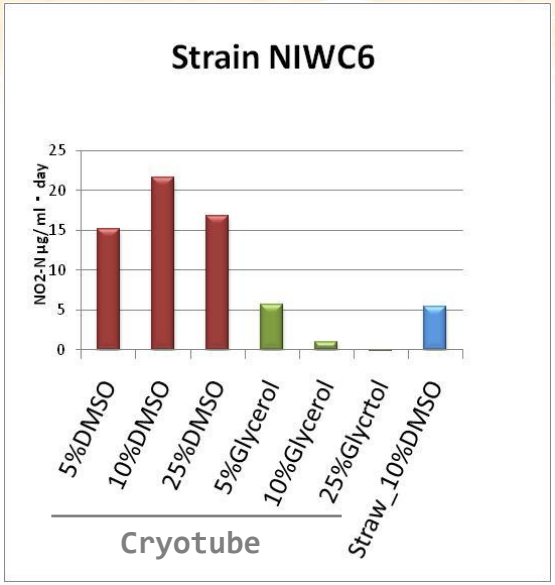
Rapidly-frozen by plunging into liquid nitrogen → **Stored in LN gas phase in the tank**

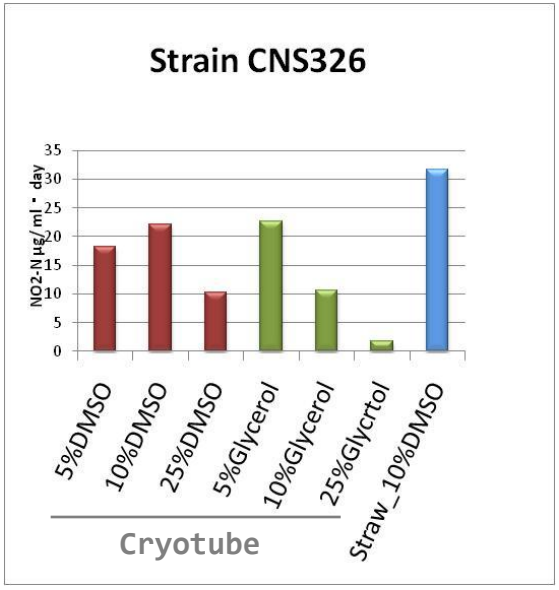
The result indicates the importance of dehydration taking place during slow freezing.

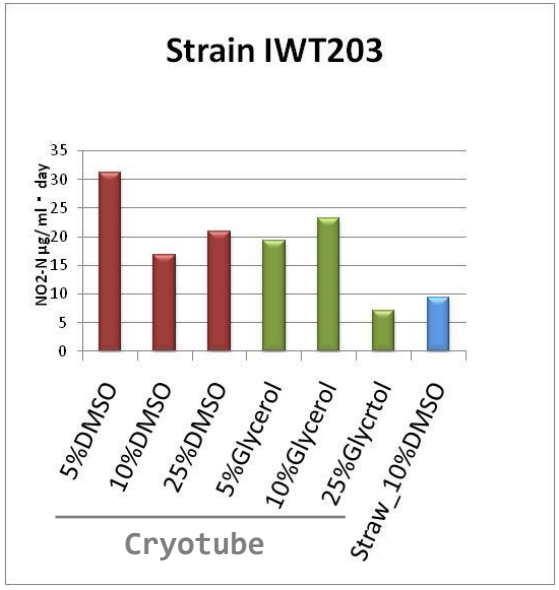
1-2. Cryopreservation











Freezed and stored in deep freezer (-80C)

- 5% DMSO (tube)
- 10% DMSO (tube)
- 25% DMSO (tube)
- 5% Glycerol (tube)
- 10% Glycerol (tube)
- 25% Glycerol (tube)

Stored in LN vapor phase in the tank

- 10% DMSO (straw)

1-2. Cryopreservation

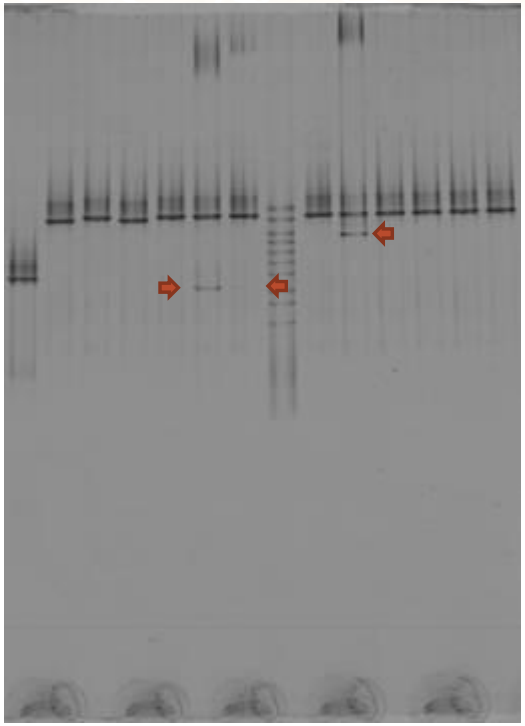
As for cryopreservaion of AOB

The method using 5-10% DMSO as cryoprotectant and freezing in -80C is effective.

The recovery of AOB was improved by removal of DMSO before cultivation.

10% DMSO (straw)

2. Purity check of culture of AOB



Combination of

1 microscopy

2 plate cultivation with some different media

3 sequencing

4 DGGE

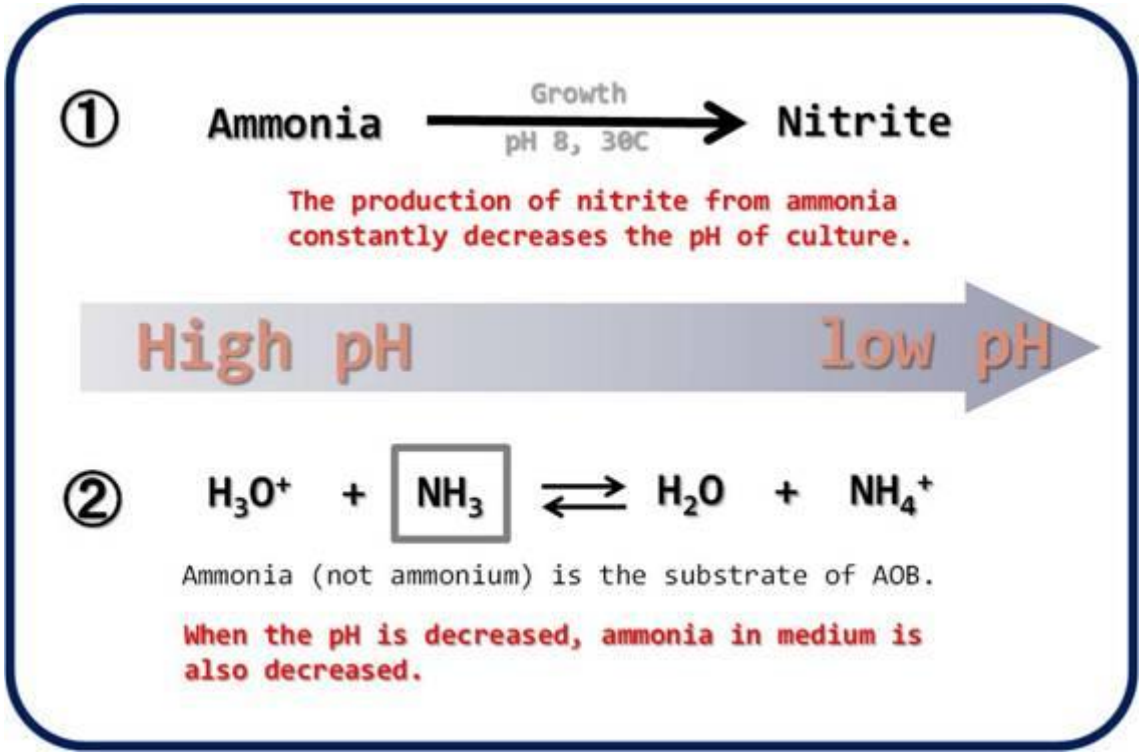
Actually, by DGGE we could detect contamination that we can't detect by other methods.

DGGE condition:

Primer	Sequence	Annealing positions	Target	Annealing temp	Amplicon length (bp)	DGGE condition
518R	ATTACCGCGGCTGCTGG	518-534	V3	65→55℃, -0.5℃/cycle	194	10%, 30-70%, 1120 V･h
- 357F-GC	CCT ACG GGA GGC AGC AG	341-357				

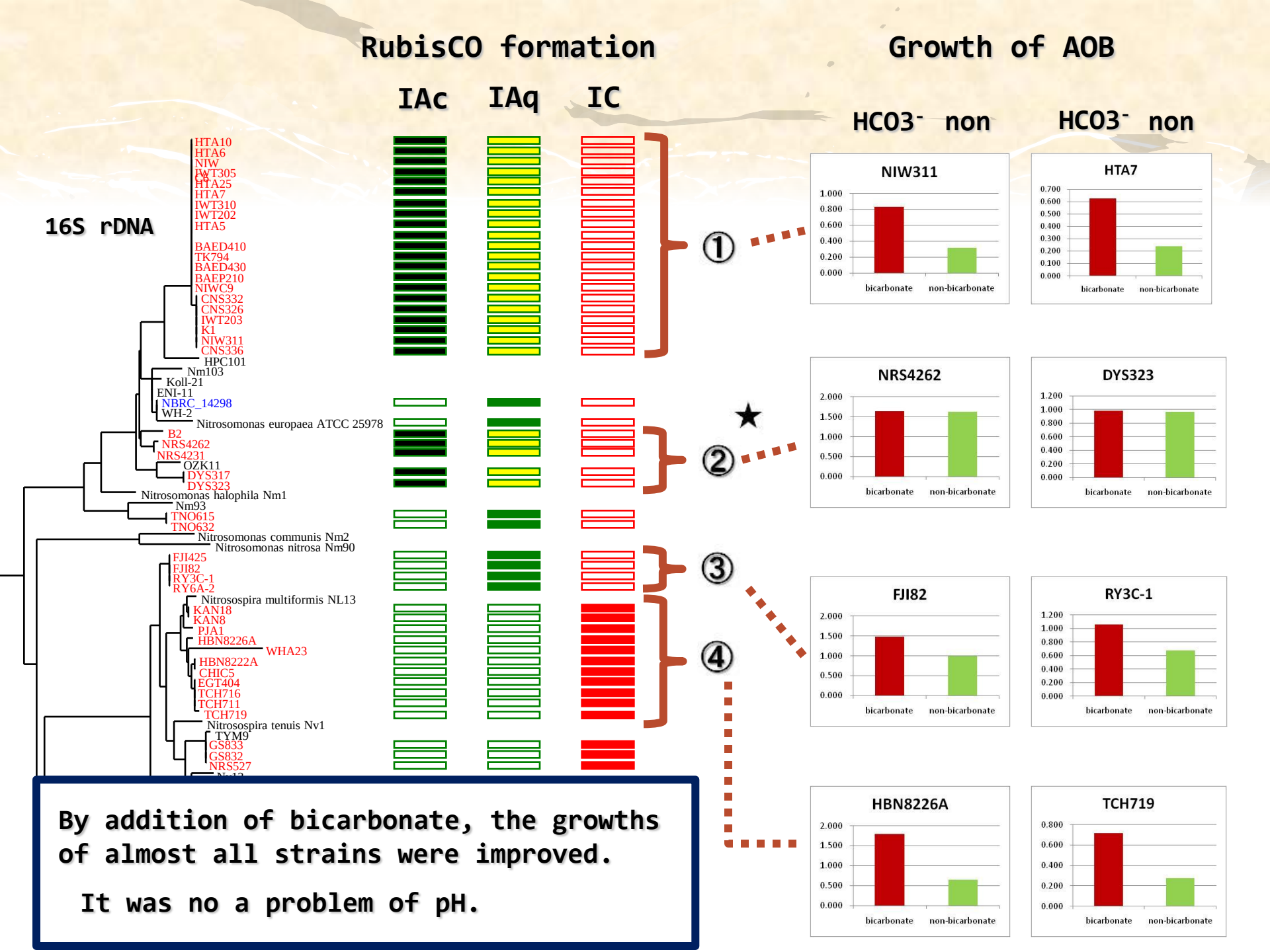
3. Cultivation of AOB

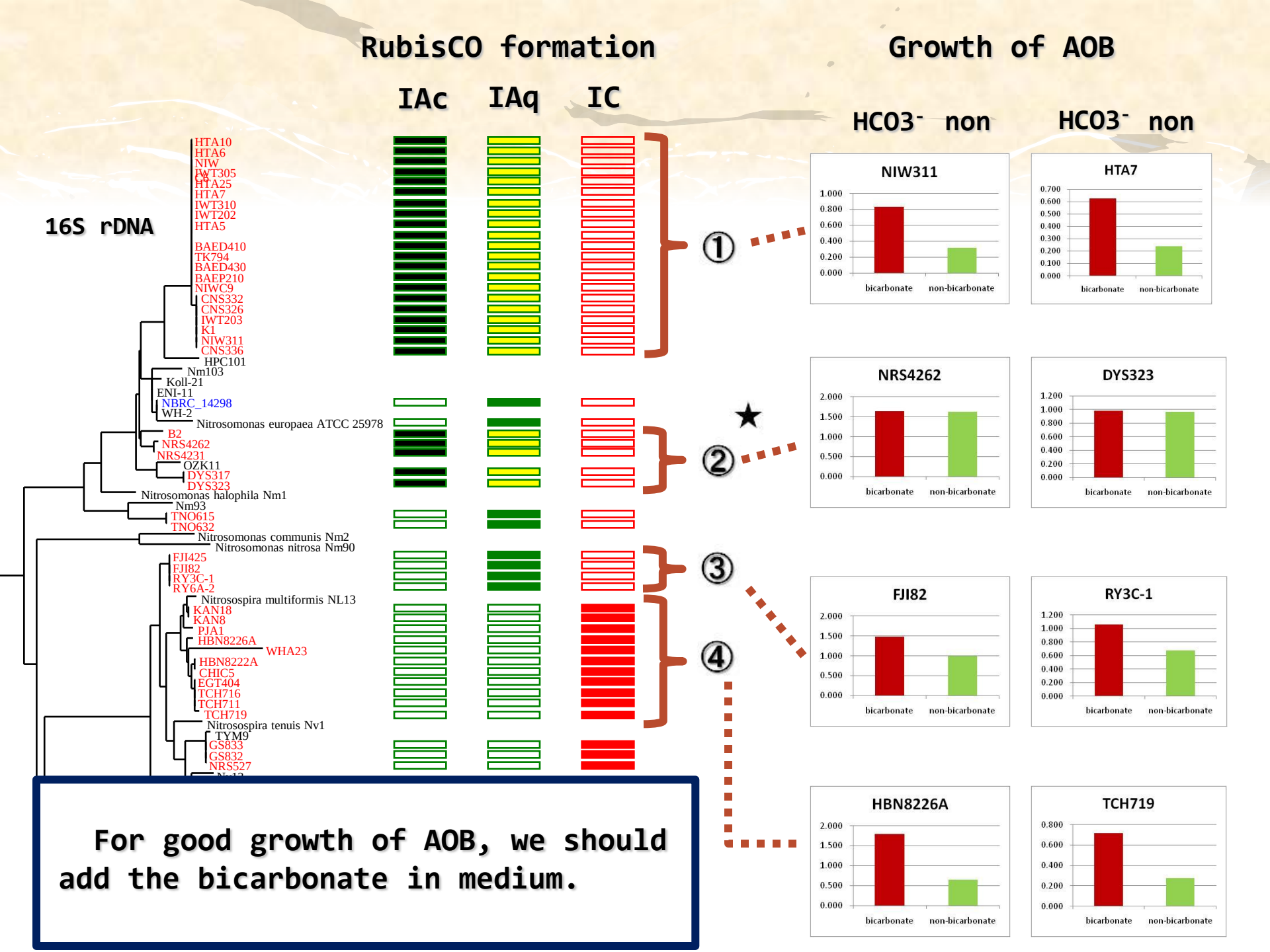
For growth of AOB, it is important to keep the pH constant.



Buffering agent
- Bicarbonate
- Carbonate
- HEPES

We know by experience that the growth of AOB is improved by addition of bicarbonate.


RubisCO formation
Growth of AOB
IAc
IAq
IC
HCO3- non
HCO3- non
16S rDNA
HTA10
HTA6
NIW
IWT305
HTA25
HTA7
IWT310
IWT202
HTA5
BAED410
TK794
BAED430
BAEP210
NIWC9
CNS332
CNS326
IWT203
K1
NIW311
CNS336
HPC101
Nm103
Koll-21
ENI-11
NBRC_14298
WH-2
Nitrosomonas europaea ATCC 25978
B2
NRS4262
NRS4231
OZK11
DYS317
DYS323
Nitrosomonas halophila Nm1
Nm93
TNO615
TNO632
Nitrosomonas communis Nm2
Nitrosomonas nitrosa Nm90
FJI425
FJI82
RY3C-1
RY6A-2
Nitrosospira multiformis NL13
KAN18
KAN8
PJA1
HBN8226A
WHA23
HBN8222A
CHIC5
EGT404
TCH716
TCH711
TCH719
Nitrosospira tenuis Nv1
TYM9
GS833
GS832
NRS527
①
②
③
④
★
NIW311
HTA7
NRS4262
DYS323
FJI82
RY3C-1
HBN8226A
TCH719
bicarbonate
non-bicarbonate
By addition of bicarbonate, the growths of almost all strains were improved.
It was no a problem of pH.


RubisCO formation
Growth of AOB
IAc
IAq
IC
HCO3- non
HCO3- non
16S rDNA
HTA10
HTA6
NIW
IWT305
HTA25
HTA7
IWT310
IWT202
HTA5
BAED410
TK794
BAED430
BAEP210
NIWC9
CNS332
CNS326
IWT203
K1
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FJI425
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KAN18
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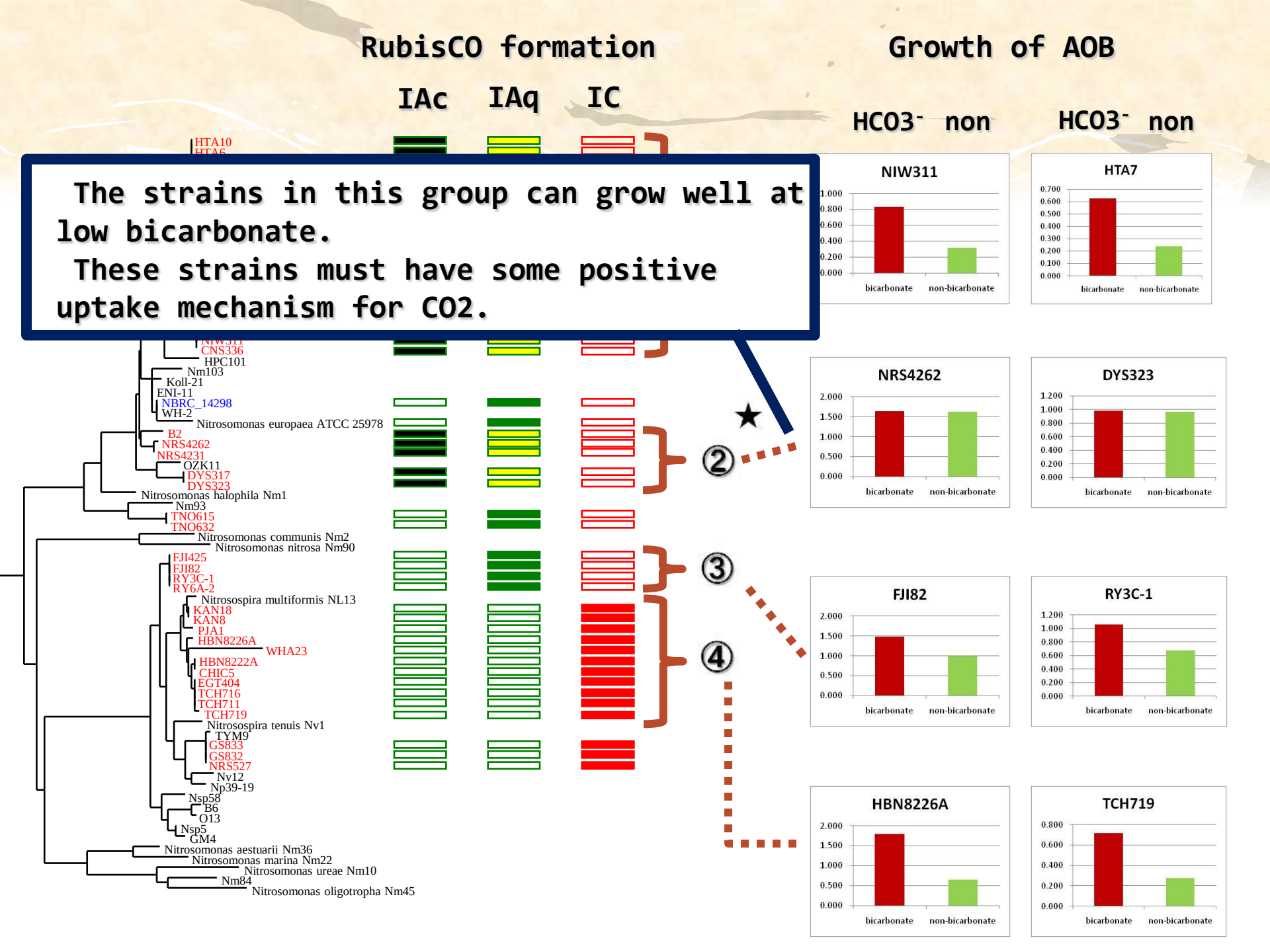
WHA23
HBN8222A
CHIC5
EGT404
TCH716
TCH711
TCH719
Nitrosospira tenuis Nv1
TYM9
GS833
GS832
NRS527
①
②
③
④
★
NIW311
HTA7
NRS4262
DYS323
FJI82
RY3C-1
HBN8226A
TCH719
bicarbonate
non-bicarbonate
For good growth of AOB, we should add the bicarbonate in medium.


RubisCO formation
IAc
IAq
IC
Growth of AOB
HCO3- non
HCO3- non
The strains in this group can grow well at low bicarbonate.
These strains must have some positive uptake mechanism for CO2.
HTA10
HTA6
NIW311
CNS336
HPC101
Nm103
Koll-21
ENI-11
NBRC_14298
WH-2
Nitrosomonas europaea ATCC 25978
B2
NRS4262
NRS4231
OZK11
DYS317
DYS323
Nitrosomonas halophila Nm1
Nm93
TNO615
TNO632
Nitrosomonas communis Nm2
Nitrosomonas nitrosa Nm90
FJI425
FJI82
RY3C-1
RY6A-2
Nitrosospira multiformis NL13
KAN18
KAN8
PJA1
HBN8226A
WHA23
HBN8222A
CHIC5
EGT404
TCH716
TCH711
TCH719
Nitrosospira tenuis Nv1
TYM9
GS833
GS832
NRS527
Nv12
Np39-19
Nsp58
B6
O13
Nsp5
GM4
Nitrosomonas aestuarii Nm36
Nitrosomonas marina Nm22
Nitrosomonas ureae Nm10
Nm84
Nitrosomonas oligotropha Nm45
②
③
④
NIW311
HTA7
NRS4262
DYS323
FJI82
RY3C-1
HBN8226A
TCH719
bicarbonate
non-bicarbonate

As for the
the format

RubisCO is a k

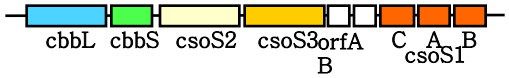
The enzyme exi
combination in a

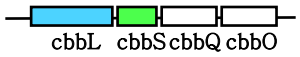
Carboxysomes are bacterial microcompartments that contain RubisCO enzyme.

These compartments are thought to concentrate CO2 to overcome the inefficiency of RubisCo.

carboxysomal RubisCO

Green-like RubisCO

IAc 

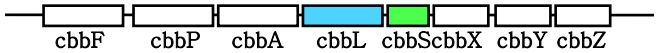
IAq 

Detectable By PCR

non-primers

Detectable By PCR

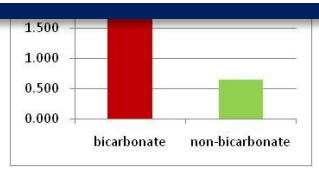
Red-like RubisCO

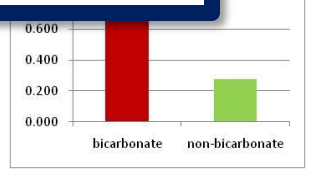
IC 

Detectable By PCR

16S

Nitrosomonas aestuarii Nm36
Nitrosomonas marina Nm22
Nitrosomonas ureae Nm10
Nm84
Nitrosomonas oligotropha Nm45

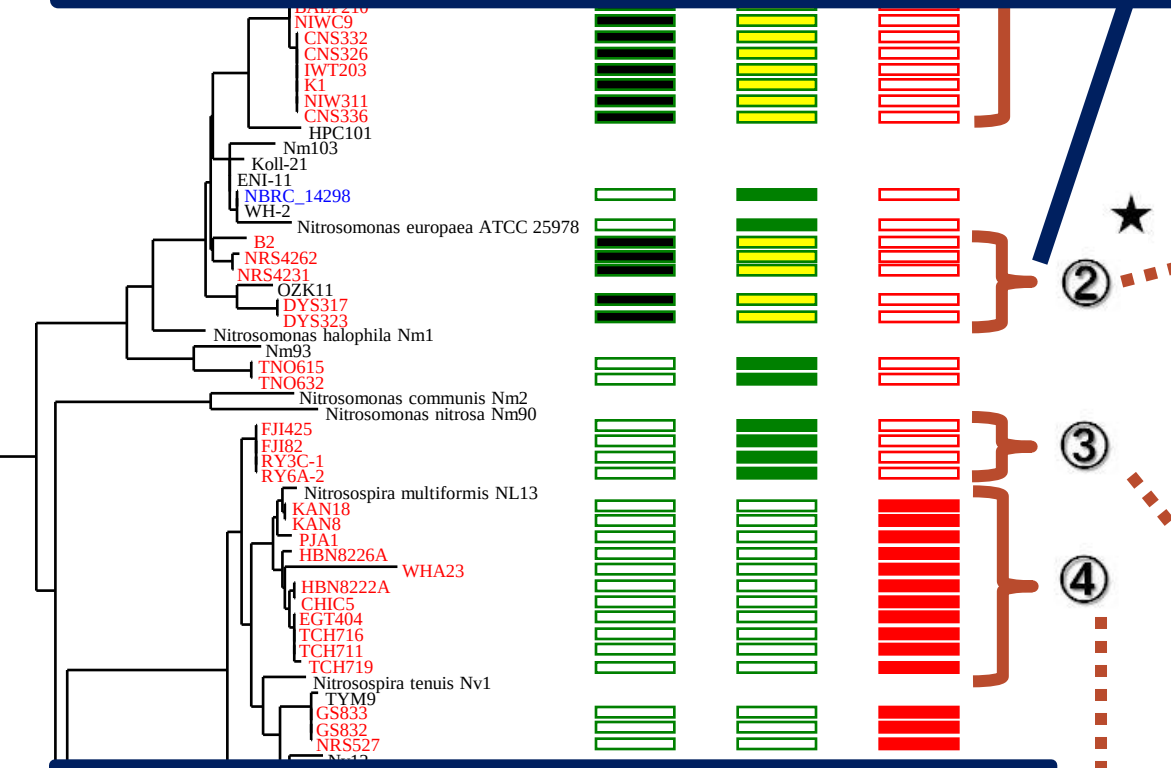


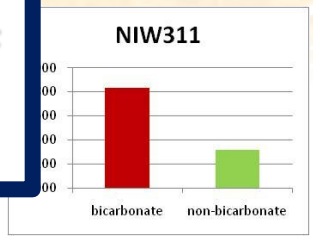


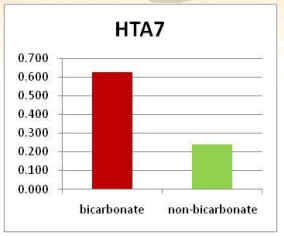
RubisCO formation

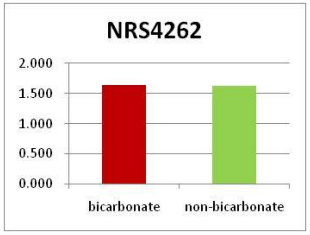
Growth of AOB

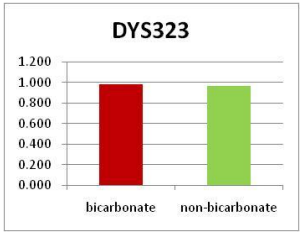
These strains in this group can grow well at low bicarbonate condition by function of CO2 concentrating mechanism in carboxysome.

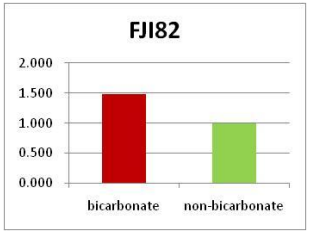


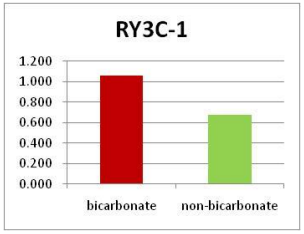


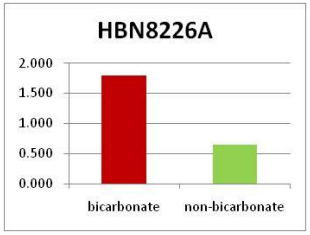


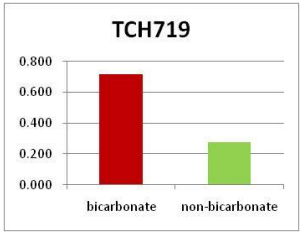












Each group is characterized by their formation of RubisCO.

Conclution

Preservation:

We could preserve all the AOB strains we possessed by freezing using DMSO and L-drying using SM8 medium.

In both methods, the recovery of AOB was improved by removal of protective media before cultivation.

Purity check:

We should check the purity of culture by combination of some methods.

DGGE is effective as one of purity check methods.

Cultivation:

By addition of bicarbonate, the growths of almost all strains were improved, however the effect of bicarbonate is different among the phylogenetic groups of AOB.

We suspect that the difference among group may be related to their RubisCO formations.