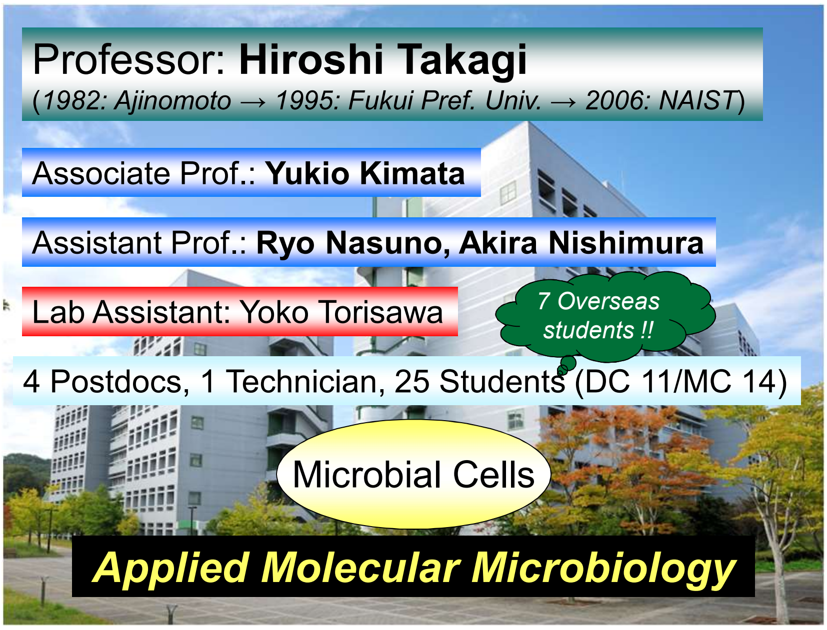
2020

Laboratory of Applied Stress Microbiology

Professor Hiroshi Takagi, Ph.D.

Originality !! NAIST *Serendipity !!*

The Takagi Laboratory



Professor: **Hiroshi Takagi**
(*1982: Ajinomoto → 1995: Fukui Pref. Univ. → 2006: NAIST*)

Associate Prof.: **Yukio Kimata**

Assistant Prof.: **Ryo Nasuno, Akira Nishimura**

Lab Assistant: Yoko Torisawa

7 Overseas students !!

4 Postdocs, 1 Technician, 25 Students (DC 11/MC 14)

Microbial Cells

Applied Molecular Microbiology

Microbial **Bioscience**

Screening (mutants, isolates)
Genomic information

Biochemistry & Physiology
Molecular Genetics & Cell Biology

Analysis and improvement of microbial functions

Gene engineering
Metabolic engineering

Protein engineering
Cell engineering

- Construction of useful microorganisms
- Production of valuable substances
- Development of promising technology

Food
Life

Environment
Energy

*Application to **Biotechnology***

***Yeast** is an important microorganism as …*

Model for "Higher Eukaryotes"

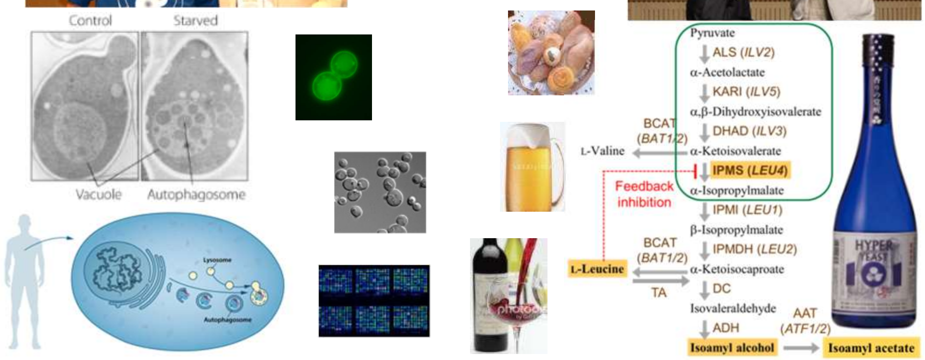
Autophagy

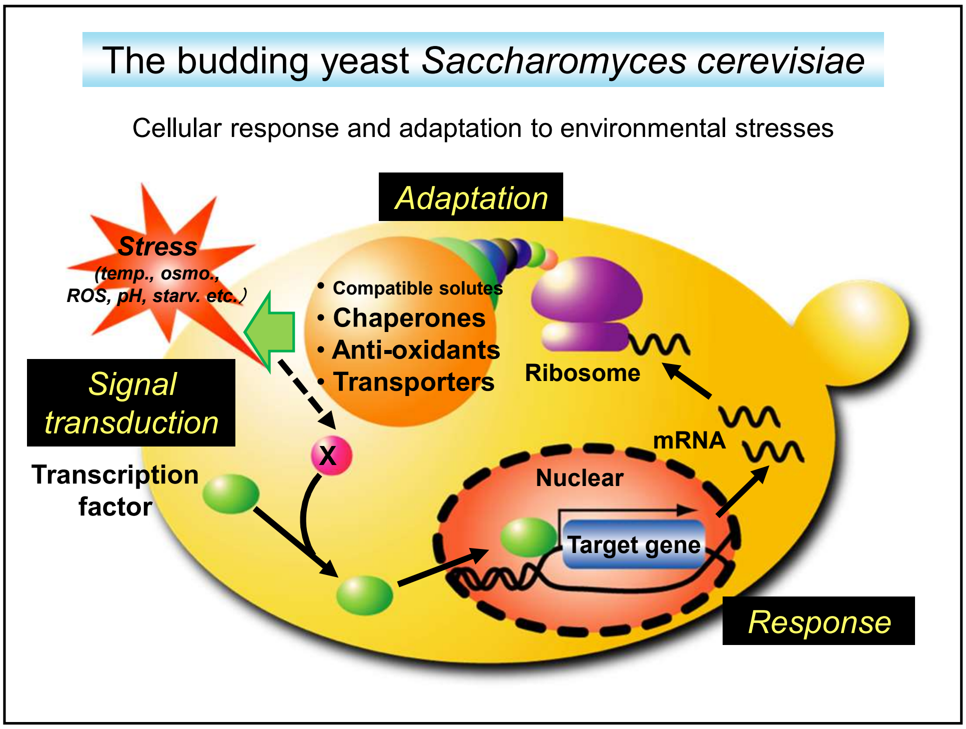
Novel Prize winner
Dr. Y. Ohsumi

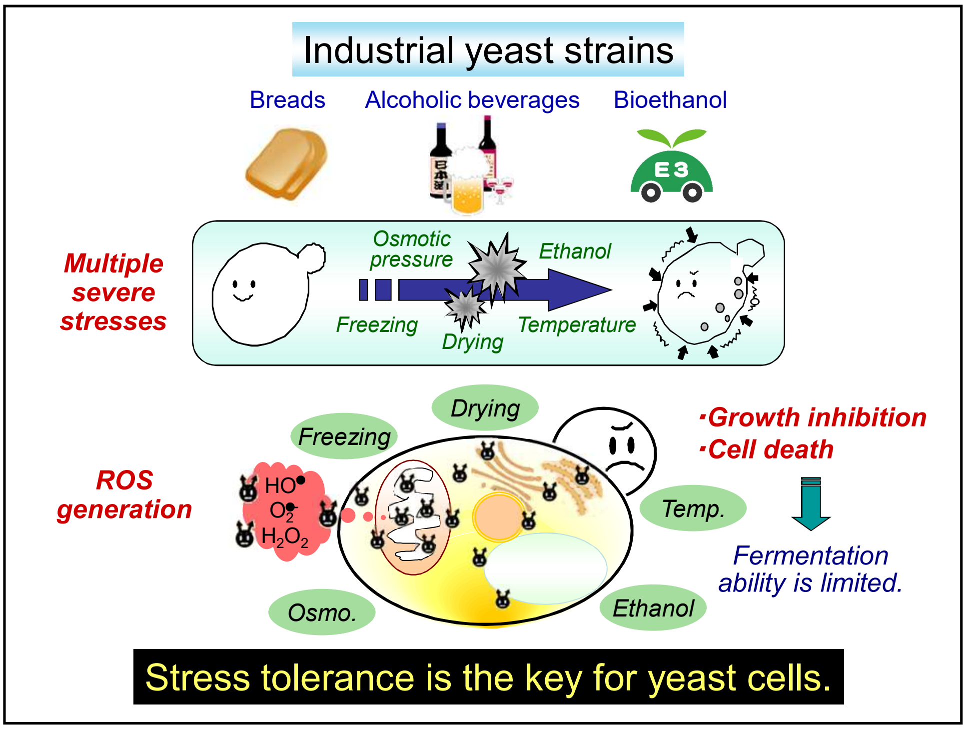
Awamori

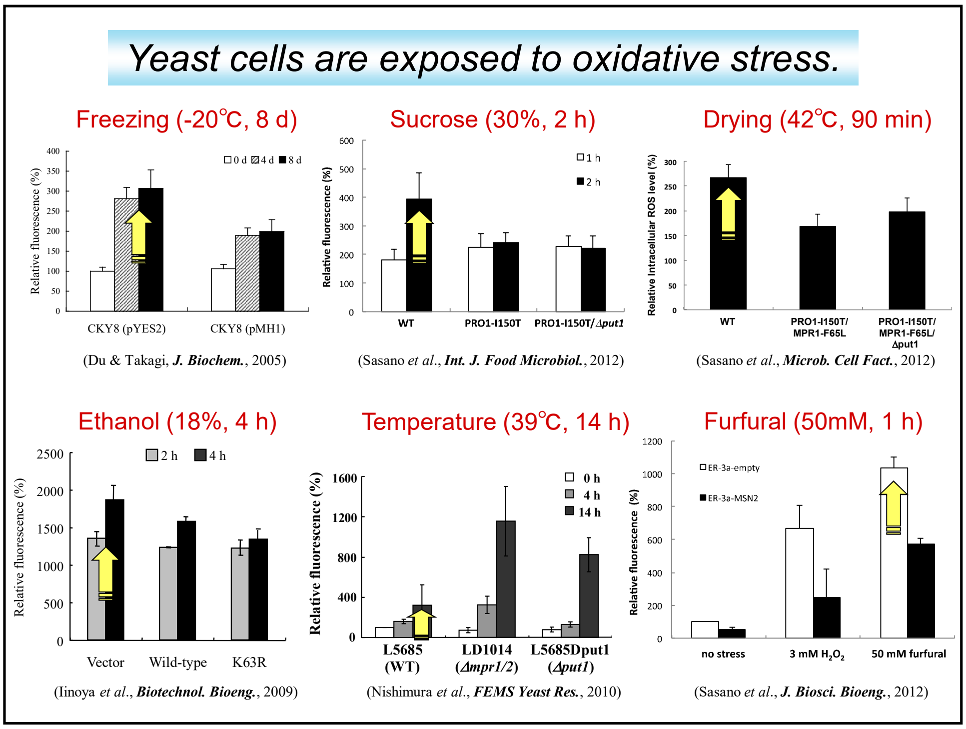
Alcohol beverage
brewed by my Yeast

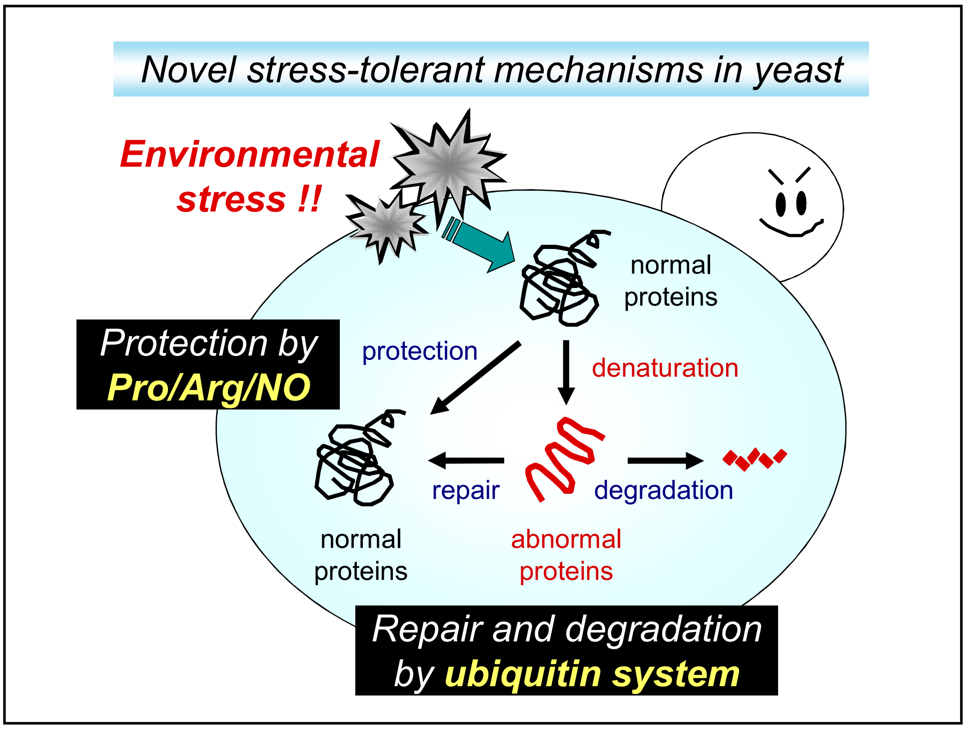
Good "Ethanol Producer"

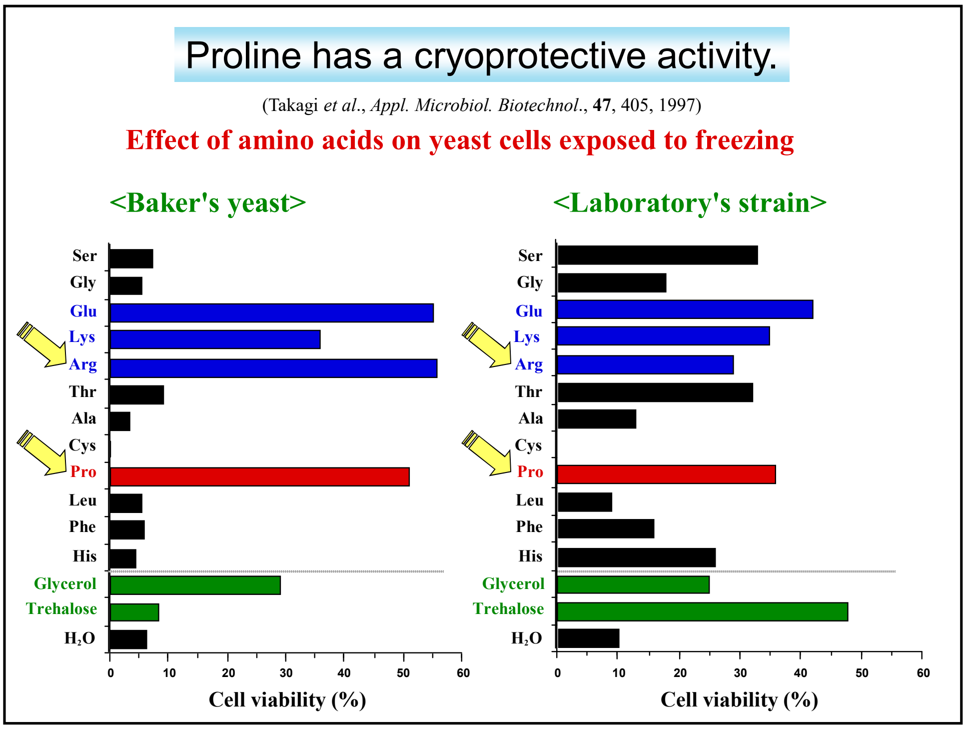


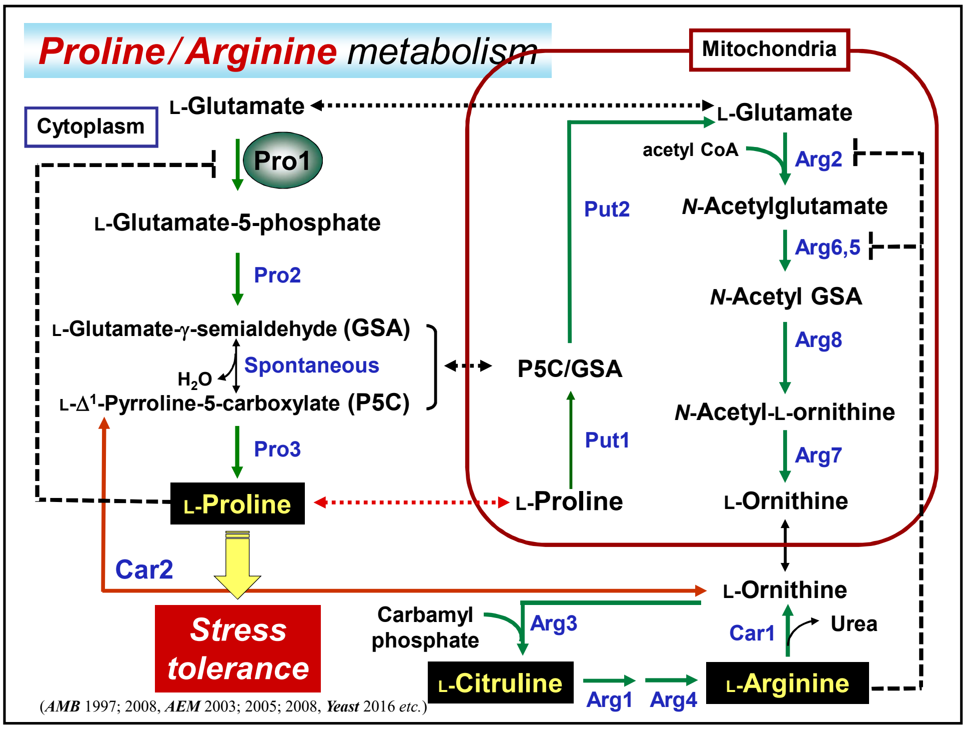

The budding yeast *Saccharomyces cerevisiae*
Cellular response and adaptation to environmental stresses
Adaptation
Stress
(temp., osmo., ROS, pH, starv. etc.)
• Compatible solutes
• Chaperones
• Anti-oxidants
• Transporters
Ribosome
Signal transduction
mRNA
Transcription factor
X
Nuclear
Target gene
Response


Industrial yeast strains
Breads
Alcoholic beverages
Bioethanol
E3
Multiple severe stresses
Osmotic pressure
Ethanol
Freezing
Drying
Temperature
ROS generation
HO•
O2•
H2O2
Freezing
Drying
Temp.
Osmo.
Ethanol
·Growth inhibition
·Cell death
Fermentation ability is limited.
Stress tolerance is the key for yeast cells.


Yeast cells are exposed to oxidative stress.
Freezing (-20℃, 8 d)
Sucrose (30%, 2 h)
Drying (42℃, 90 min)
(Du & Takagi, J. Biochem., 2005)
(Sasano et al., Int. J. Food Microbiol., 2012)
(Sasano et al., Microb. Cell Fact., 2012)
Ethanol (18%, 4 h)
Temperature (39℃, 14 h)
Furfural (50mM, 1 h)
(Iinoya et al., Biotechnol. Bioeng., 2009)
(Nishimura et al., FEMS Yeast Res., 2010)
(Sasano et al., J. Biosci. Bioeng., 2012)


Novel stress-tolerant mechanisms in yeast
Environmental stress !!
normal proteins
Protection by Pro/Arg/NO
protection
denaturation
repair
degradation
normal proteins
abnormal proteins
Repair and degradation by ubiquitin system

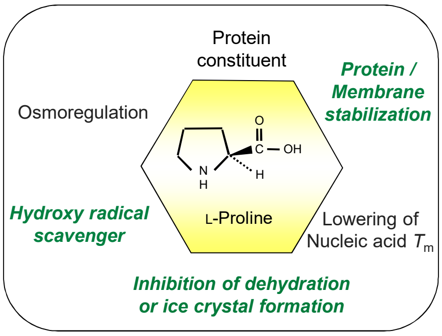

Proline has a cryoprotective activity.
(Takagi et al., Appl. Microbiol. Biotechnol., 47, 405, 1997)
Effect of amino acids on yeast cells exposed to freezing
<Baker's yeast>
<Laboratory's strain>
Ser
Gly
Glu
Lys
Arg
Thr
Ala
Cys
Pro
Leu
Phe
His
Glycerol
Trehalose
H_2O
0 10 20 30 40 50 60
Cell viability (%)


Proline/Arginine metabolism
Mitochondria
Cytoplasm
L-Glutamate
Pro1
L-Glutamate-5-phosphate
Pro2
L-Glutamate-γ-semialdehyde (GSA)
H_2O
Spontaneous
L-Δ1-Pyrroline-5-carboxylate (P5C)
Pro3
L-Proline
Car2
Stress tolerance
L-Glutamate
acetyl CoA
Arg2
Put2
N-Acetylglutamate
Arg6,5
N-Acetyl GSA
Arg8
P5C/GSA
Put1
N-Acetyl-L-ornithine
Arg7
L-Proline
L-Ornithine
L-Ornithine
Carbamyl phosphate
Arg3
Car1
Urea
L-Citruline
Arg1
Arg4
L-Arginine
(AMB 1997; 2008, AEM 2003; 2005; 2008, Yeast 2016 etc.)

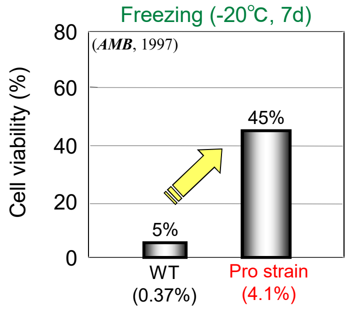
Proline *confers stress tolerance on yeast cells.*

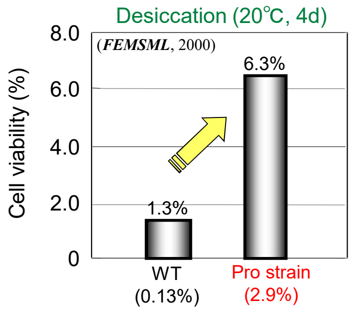
- In response to osmotic stresses, many bacterial and plant cells accumulate proline.
- Yeast cells induce glycerol or trehalose synthesis, but do NOT increase proline level.

Physiological functions



Proline-accumulating mutants were isolated among proline analogue-resistant mutants.





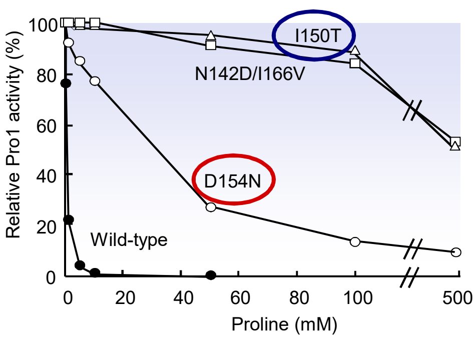
Pro1 *variants with enhanced* ***proline****-synthetic activity*

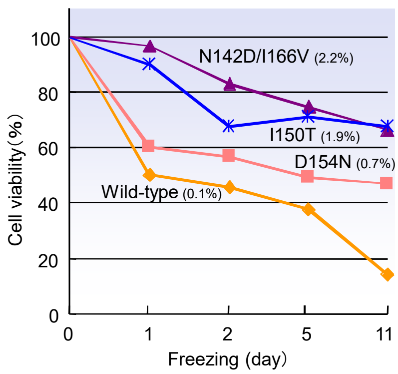
(Sekine *et al.*, ***Appl. Environ. Microbiol.***, **73**, 4011, 2007)

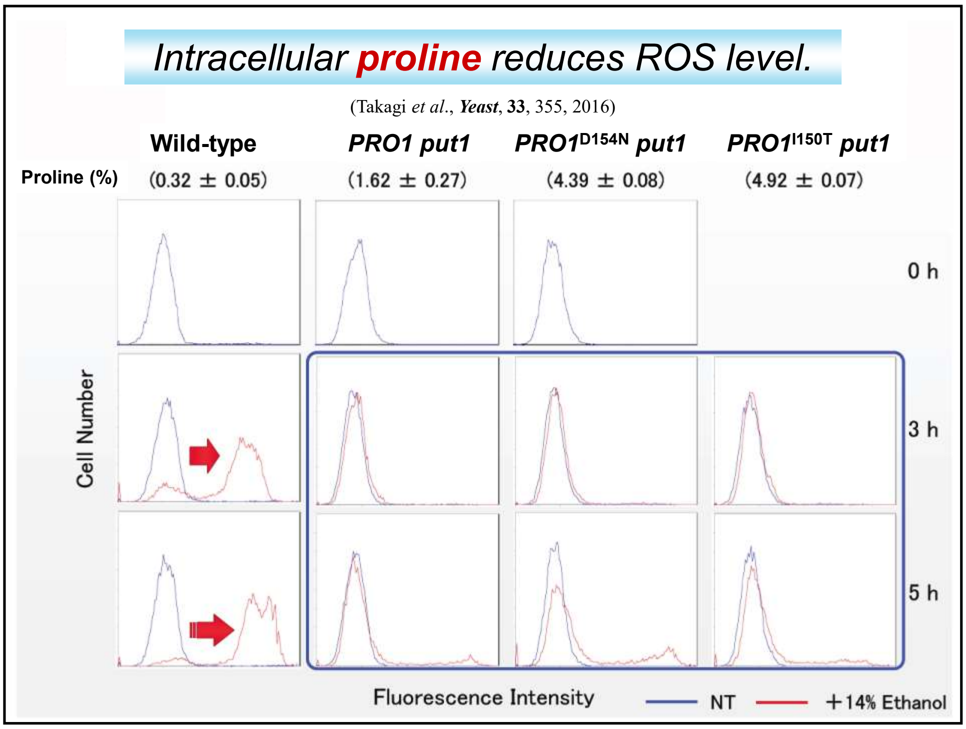
γ-Glutamyl kinase (Pro1)

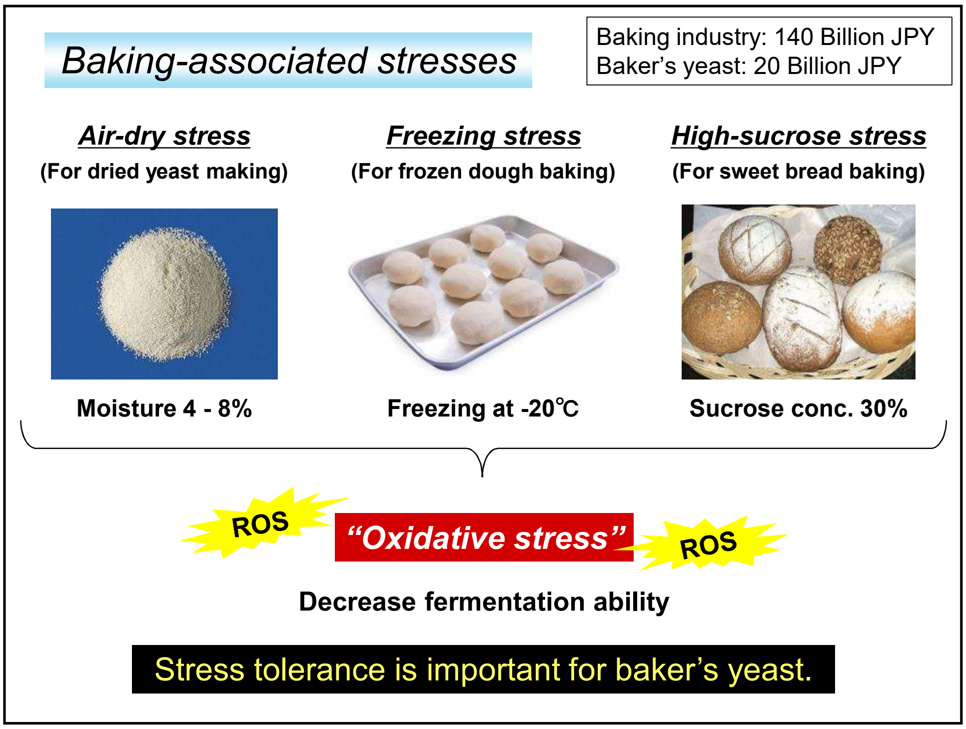
the rate-limiting enzyme in proline synthesis

PCR random mutagenesis in ***PRO1*** → Isolation of **AZC-resistant** clones






Intracellular proline reduces ROS level.
(Takagi et al., Yeast, 33, 355, 2016)
Wild-type
PRO1 put1
PRO1D154N put1
PRO1I150T put1
Proline (%)
(0.32 ± 0.05)
(1.62 ± 0.27)
(4.39 ± 0.08)
(4.92 ± 0.07)
0 h
3 h
5 h
Cell Number
Fluorescence Intensity
NT
+14% Ethanol


Baking-associated stresses
Baking industry: 140 Billion JPY
Baker's yeast: 20 Billion JPY
Air-dry stress
(For dried yeast making)
Freezing stress
(For frozen dough baking)
High-sucrose stress
(For sweet bread baking)
Moisture 4 - 8%
Freezing at -20℃
Sucrose conc. 30%
ROS
"Oxidative stress"
ROS
Decrease fermentation ability
Stress tolerance is important for baker's yeast.

Proline *increased fermentation ability in frozen dough.*

(Kaino *et al.*, ***Appl. Environ. Microbiol.***, **74**, 5845, 2008)

with Dr. Shima (NFRI)

bread dough

bread-making flour	100 g
yeast (66% moisture basis)	4 g
sucrose	5 g
NaCl	2 g
water	68 ml

↓

CO_2 gas production

↓

prefermentation (30℃, 120 min)

↓

freezing (-20℃, 9 days)

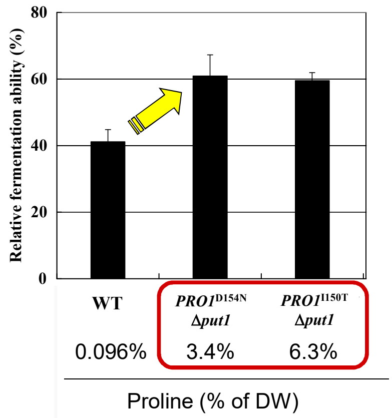
↓

thawing (30℃, 30 min)

↓

CO_2 gas production

Freezing stress tolerance test



also in sweet dough and after air-drying.

(Sasano *et al.*, ***Int. J. Food Microbiol.***, **138**, 181, 2010; **152**, 40, 2012)

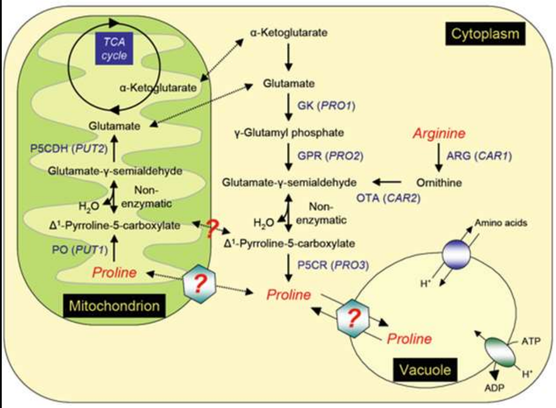
① Proline

Appl. Microbiol. Biotechnol., **47**, 405, 1997; **79**, 273, 2008, **81**, 211, 2008; *FEMS Microbiol. Lett.*, **184**, 103, 2000; *Appl. Environ. Microbiol.*, **69**, 212, 2003; **69**, 6527, 2003; **71**, 8656, 2005; **73**, 4011, 2007; **74**, 5845, 2008; *J. Biosci. Bioeng.*, **94**, 2002; **100**, 538, 2005; **103**, 277, 2007; **116**, 576, 2013; *Biosci. Biotech. Biochem.*, **73**, 2131, 2009; **76**, 454, 2012; *Int. J. Food Microbiol.*, **152**, 40, 2012; **238**, 233, 2016; *J. Gen. Appl. Microbiol.*, **62**, 132, 2016; *Yeast*, **33**, 353, 2016; *FEBS Lett.*, **590**, 2906, 2016; *Microbial Cell*, **3**, 522, 2016; **6**, 482, 2019.

＜ So far ＞

★ Proline protects yeast cells from various stresses as a ROS scavenger *!!*

★ Desensitization of feedback inhibition of γ-glutamyl kinase (Pro1) enhances proline accumulation and stress tolerance *!!*



＜ Current projects ＞

- Novel physiological functions (ribosomal autophagy, life span)
- Transport to mitochondria/vacuole
- Functional analysis of GK and PO

⬇

Breeding of novel stress-tolerant yeast strains with Pro engineering

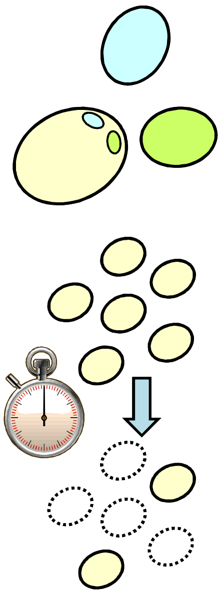
Lifespan of yeast cells

Replicative lifespan

- Is defined as the number of daughter cells produced from a mother cell before dying (Ave. 25).
- Is similar to the aging of mitotically active cells in multicellular organisms.
  - fibroblast, epithelium etc.

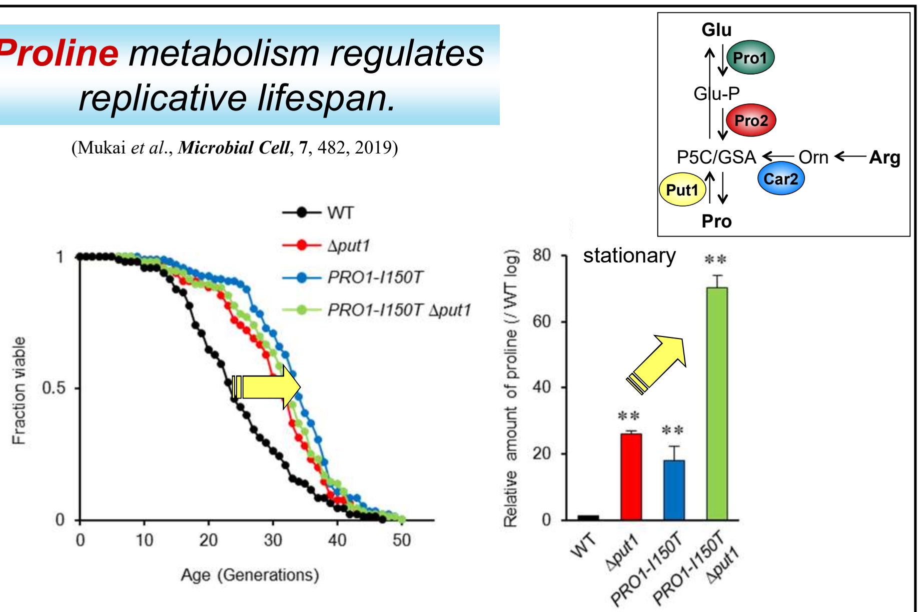
Chronological lifespan

- Is defined as the time non-dividing cells in a stationary phase culture remain viable.
- Is proposed as a model for the aging of post-mitotic tissues in mammals.
  - nerve cell, myocardial cell *etc*.



Proline metabolism regulates replicative lifespan.

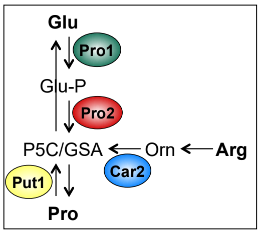
(Mukai *et al*., ***Microbial Cell***, 7, 482, 2019)

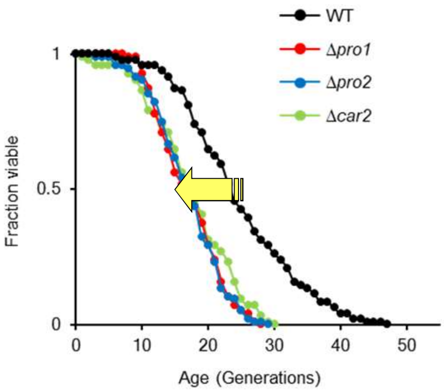


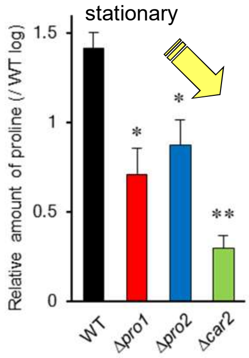
Extended replicative lifespan observed in yeast cells with increased proline content

***Proline** metabolism regulates replicative lifespan.*

(Mukai *et al*., ***Microbial Cell***, 7, 482, 2019)







Shortened replicative lifespan observed in yeast cells with reduced proline content

Application to industrial yeasts

Replicative lifespan

Beer yeast

(For harvesting and reusing)

https://www.vectorstock.com/royalty-free-vector/beer-brewing-process-infographic-in-flat-style-vector-5771537

Chronological lifespan

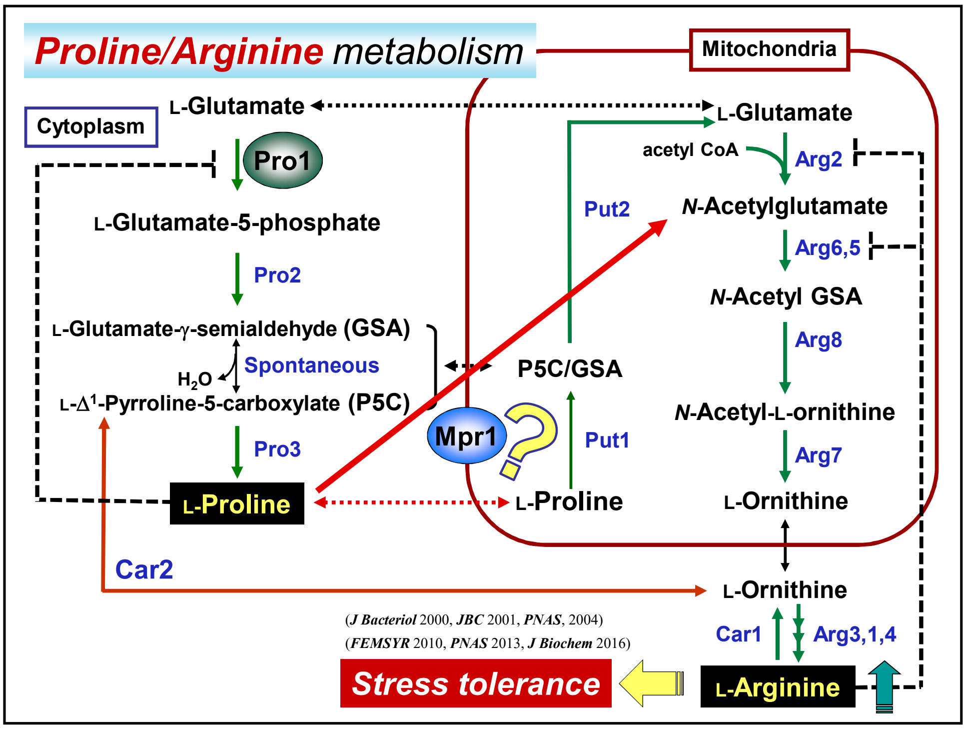
Air-dry stress

(For dried yeast making)

Baker's yeast

Freezing stress

(For frozen dough baking)



*Construction of **Mpr1 variants***

Wild-type Mpr1

PCR random mutagenesis

Mpr1 variants with enhanced activity and stability (K63R / F65L)

(Iinoya *et al.*, ***Biotechnol. Bioeng.***, **103**, 341, 2009)

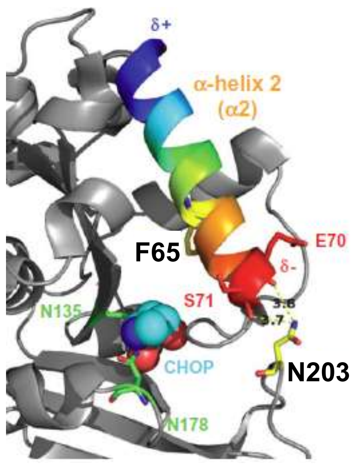
Crystal structure of Mpr1

(Nasuno *et al.*, ***Proc,. Natl. Acad. Sci. USA***, **110**, 12821, 2013)

Structure-based molecular design

Mpr1 variants with enhanced stability (N203K / N203R)

(Nasuno *et al.*, ***J. Biochem.***, **159**, 271, 2016)



Stabilization of α-helix 2 containing Phe65 by Asn203

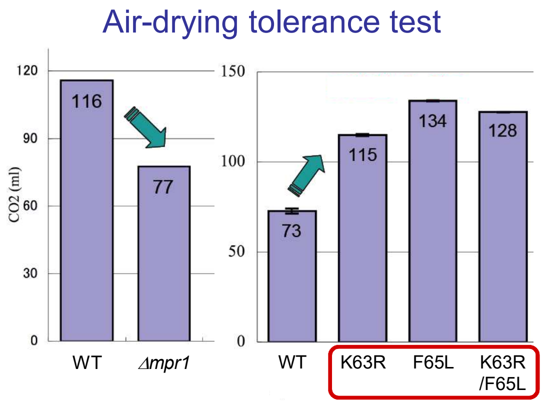
Self-cloning baker's yeasts that express ***Mpr1 variant***

(Sasano *et al.*, ***Int. J. Food Microbiol.***, **138**, 181, 2010)

cultivation in cane molasses medium (30℃, 24 hr)
↓
air-drying (37℃, 4 h)
↓
bread dough

bread-making flour	100 g
yeast (66% moisture basis)	4 g
sucrose	5 g
NaCl	2 g
water	65 ml

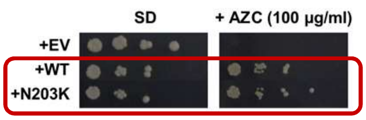
↓
CO_2 gas production



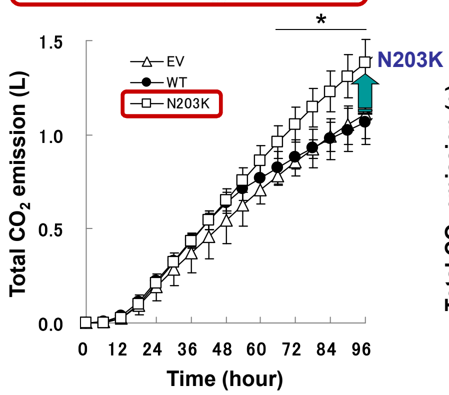
Increased fermentation ability after air-drying stress

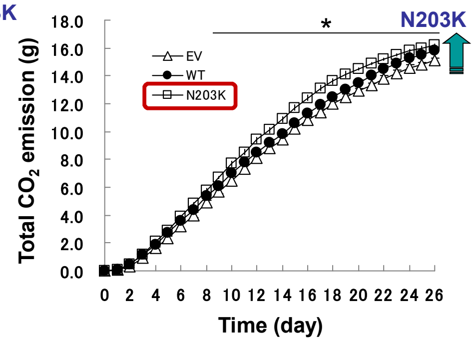
Sake yeasts that express ***Mpr1 variant***

(Ohashi *et al.*, ***J. Ind. Microbiol. Biotechnol.***, **46**, 1039, 2019)

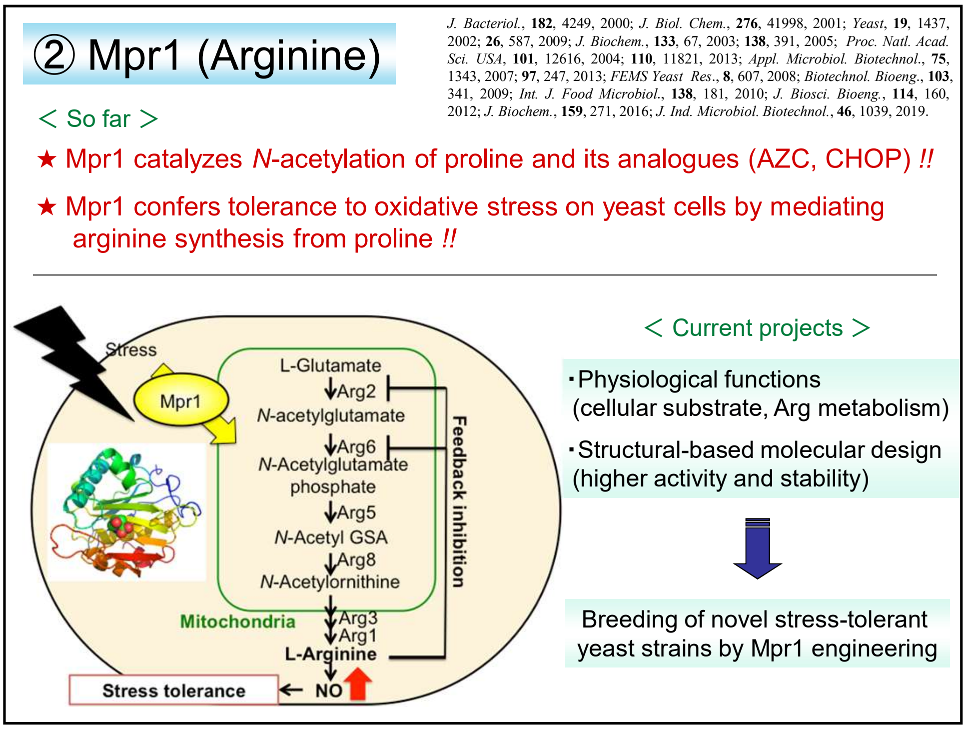


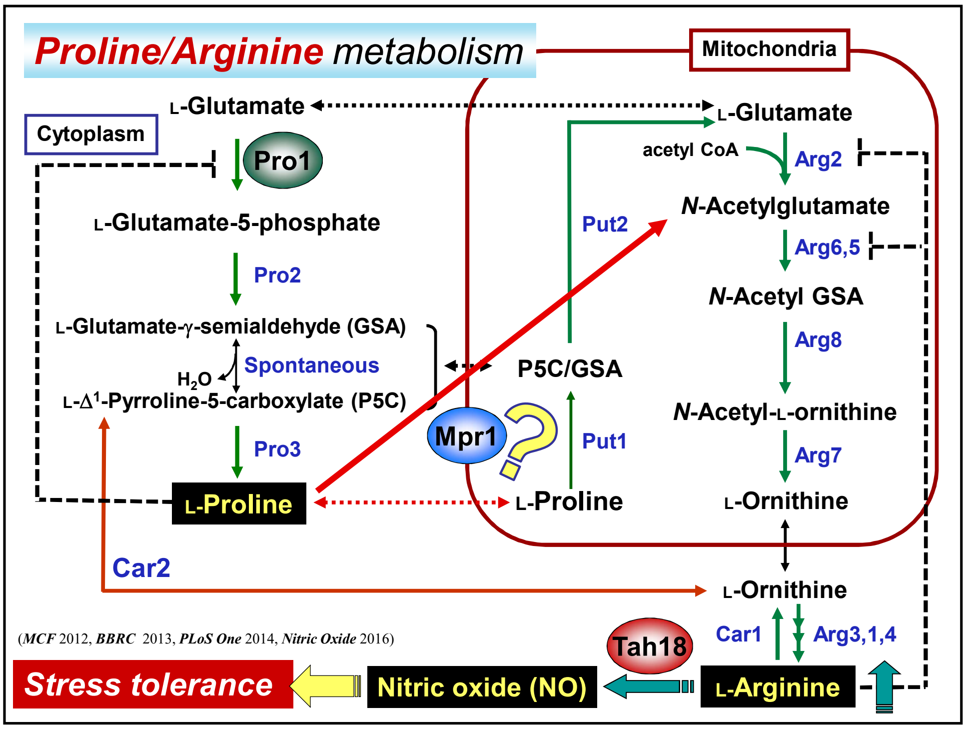
- Pregelatinized rice: 36.4 g
- Dried koji: 9.6 g
- Water containing yeast cells: 87 ml
- Temp.: 15℃, 26 days (n=3)





produced a higher ethanol (17.1%) than other strains (16.1%)


② Mpr1 (Arginine)
J. Bacteriol., 182, 4249, 2000; J. Biol. Chem., 276, 41998, 2001; Yeast, 19, 1437, 2002; 26, 587, 2009; J. Biochem., 133, 67, 2003; 138, 391, 2005; Proc. Natl. Acad. Sci. USA, 101, 12616, 2004; 110, 11821, 2013; Appl. Microbiol. Biotechnol., 75, 1343, 2007; 97, 247, 2013; FEMS Yeast Res., 8, 607, 2008; Biotechnol. Bioeng., 103, 341, 2009; Int. J. Food Microbiol., 138, 181, 2010; J. Biosci. Bioeng., 114, 160, 2012; J. Biochem., 159, 271, 2016; J. Ind. Microbiol. Biotechnol., 46, 1039, 2019.
＜ So far ＞
★ Mpr1 catalyzes N-acetylation of proline and its analogues (AZC, CHOP) !!
★ Mpr1 confers tolerance to oxidative stress on yeast cells by mediating arginine synthesis from proline !!
Stress
Mpr1
L-Glutamate
Arg2
N-acetylglutamate
Arg6
N-Acetylglutamate phosphate
Arg5
N-Acetyl GSA
Arg8
N-Acetylornithine
Mitochondria
Arg3
Arg1
L-Arginine
Feedback inhibition
Stress tolerance
NO
＜ Current projects ＞
・Physiological functions (cellular substrate, Arg metabolism)
・Structural-based molecular design (higher activity and stability)
Breeding of novel stress-tolerant yeast strains by Mpr1 engineering


Proline/Arginine metabolism
Mitochondria
Cytoplasm
L-Glutamate
Pro1
L-Glutamate-5-phosphate
Pro2
L-Glutamate-γ-semialdehyde (GSA)
Spontaneous
H_2O
L-Δ1-Pyrroline-5-carboxylate (P5C)
Pro3
L-Proline
Car2
L-Glutamate
acetyl CoA
Arg2
Put2
N-Acetylglutamate
Arg6,5
N-Acetyl GSA
Arg8
P5C/GSA
Mpr1
Put1
N-Acetyl-L-ornithine
Arg7
L-Proline
L-Ornithine
L-Ornithine
Car1
Arg3,1,4
Tah18
(MCF 2012, BBRC 2013, PLoS One 2014, Nitric Oxide 2016)
Stress tolerance
Nitric oxide (NO)
L-Arginine

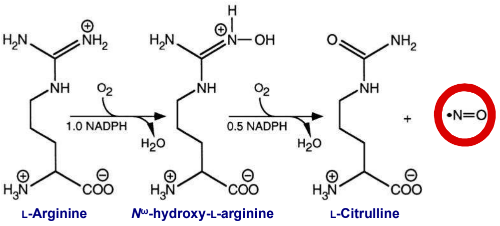
Role and synthesis of **NO** in organisms

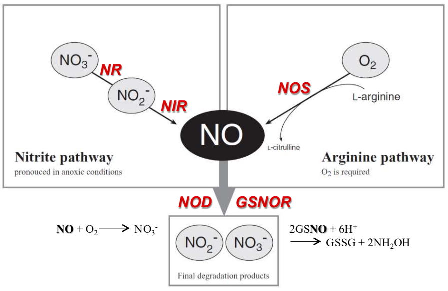
1992: Science
"Molecule of the Year"
1998: Nobel Prize
Drs. Murad, Ignarro, Furchgott

Toxic air pollutant (exhaust gas, photochemical smog, greenhouse gas, acid rain)

Mammals	blood pressure regulation, neural transmission, infection, inflammation *etc.*	**NOS**
Plants, Algae	germination, stomata regulation, pathogen response, drought tolerance *etc.*	**NR**
Bacteria	biofilm formation, antibiotic resistance, radiation resistance, antioxidation *etc.*	**NR/NOS**
Fungi	nitrate respiration, conidiation, infection *etc.*	**NIR**
Yeasts	high hydrostatic pressure resistance, copper resistance *etc.*	**?**

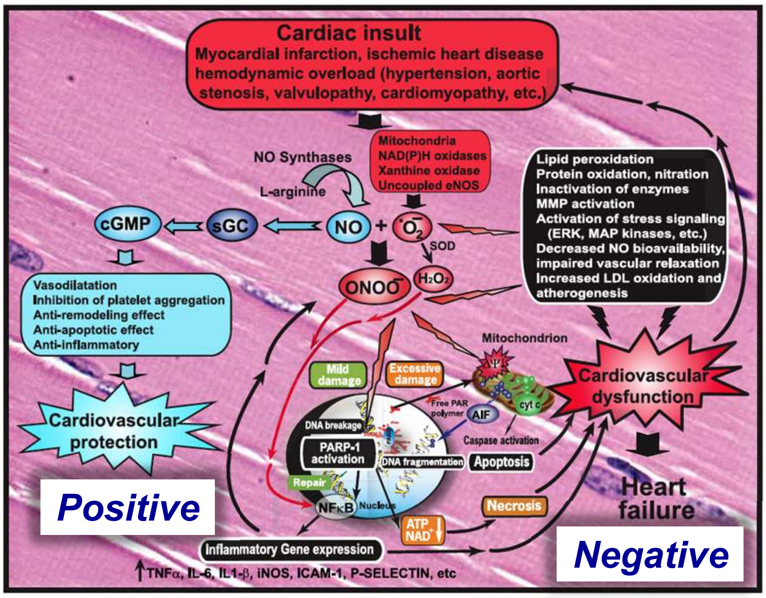
NO Synthase (NOS)





(Yamasaki, *Plant Cell Environ.*, 2005)

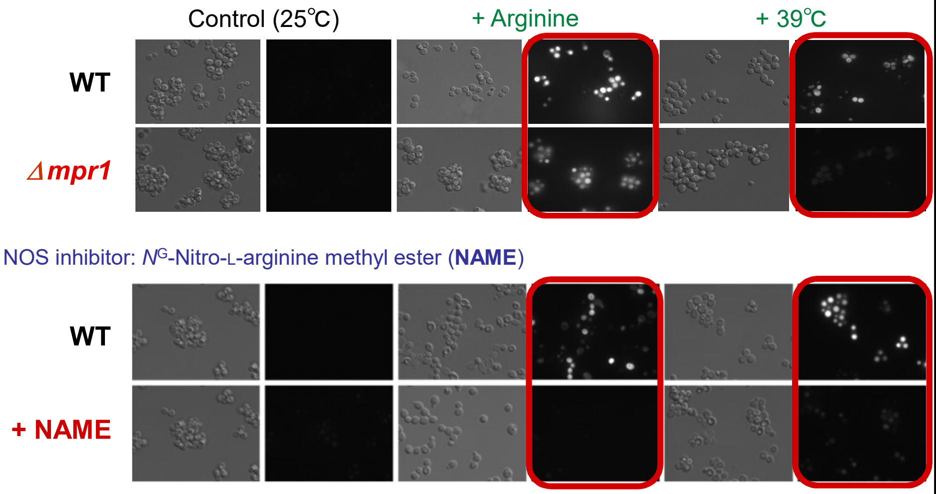
Dual effects of **NO** in organisms



(Pacher *et al.*, *Physiol. Rev.*, 2007)

*High-temp. stress induces NOS activity-dependent **NO** synthesis.*

NO-sensitive probe: Diaminofluorescein (DAF)-FM diacetate

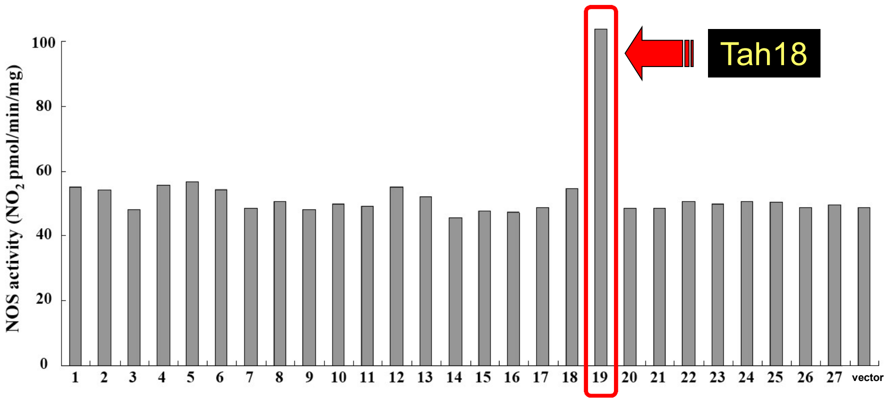


*What is the **NOS**-like protein in yeast ??*

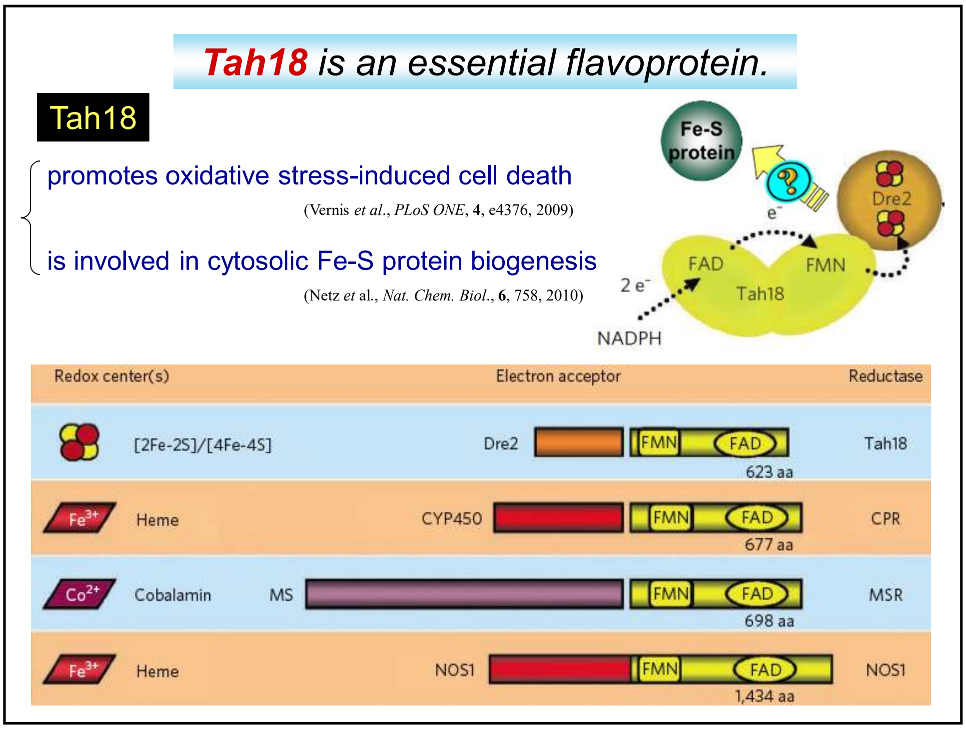
No mammalian NOS orthologues in the genome

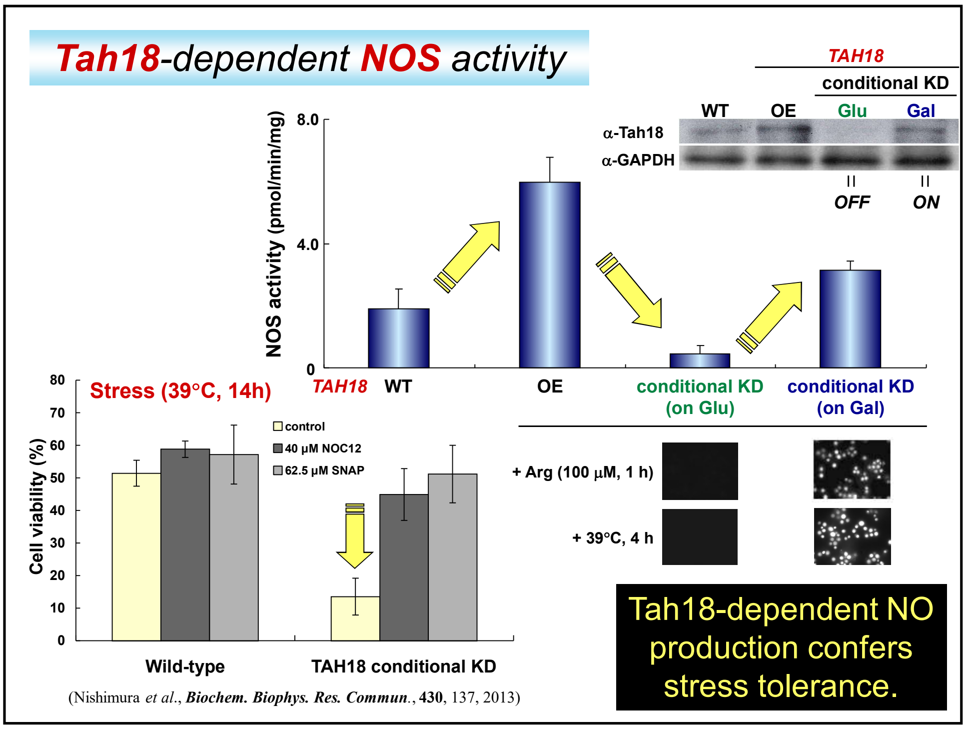
Oxidoreductase genes: 275 → **Unknown** genes 27

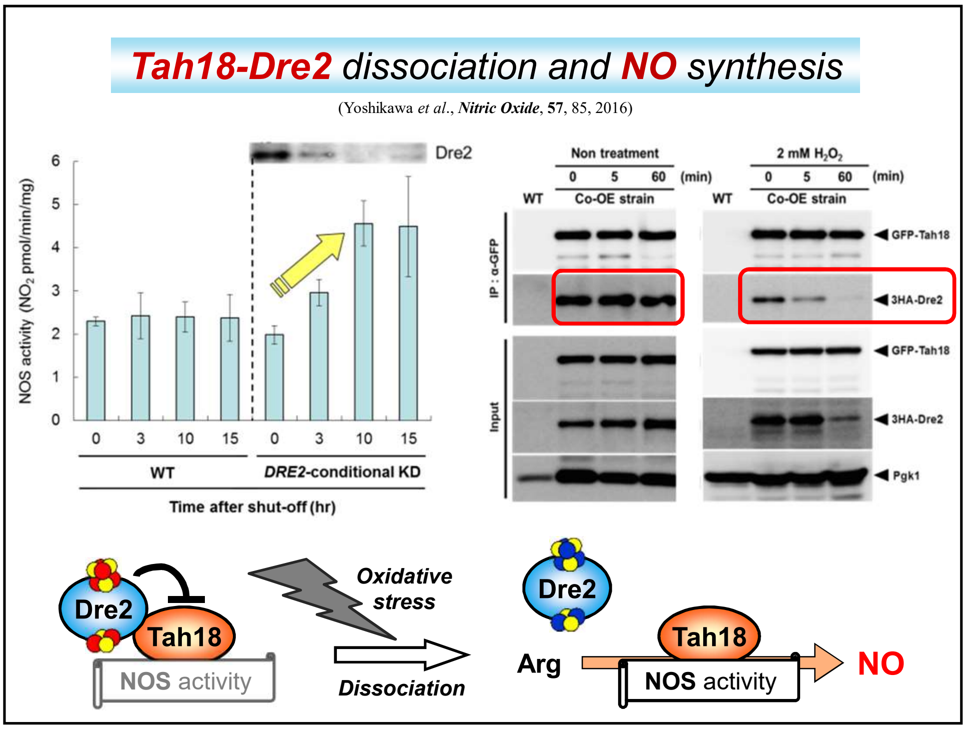
Gene expression in *E. coli* → NO_2^- production in crude extract

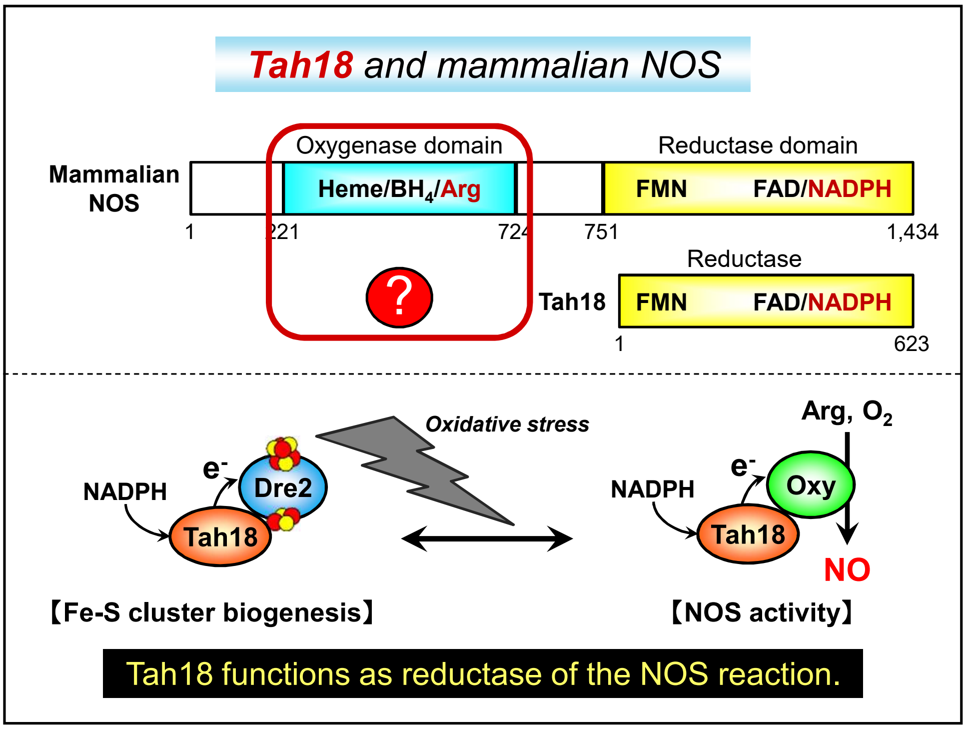


(Nishimura *et al.*, *Biochem. Biophys. Res. Commun.*, **430**, 137, 2013)

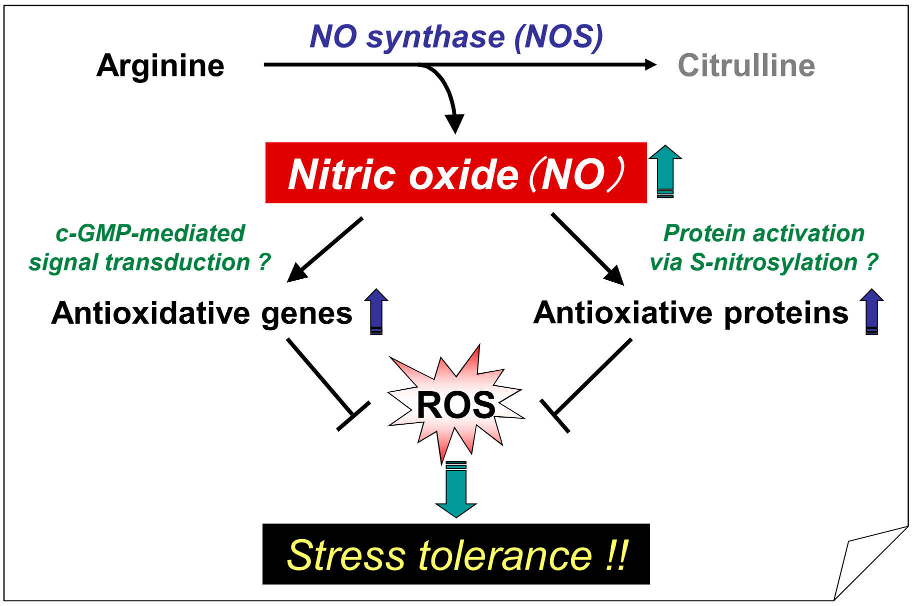

Tah18 is an essential flavoprotein.
Tah18
promotes oxidative stress-induced cell death
(Vernis et al., PLoS ONE, 4, e4376, 2009)
is involved in cytosolic Fe-S protein biogenesis
(Netz et al., Nat. Chem. Biol., 6, 758, 2010)
Fe-S protein
e⁻
Dre2
FAD
FMN
Tah18
2 e⁻
NADPH
Redox center(s)
Electron acceptor
Reductase
[2Fe-2S]/[4Fe-4S]
Dre2
623 aa
Tah18
Heme
CYP450
677 aa
CPR
Cobalamin
MS
698 aa
MSR
Heme
NOS1
1,434 aa
NOS1


Tah18-dependent NOS activity
TAH18
conditional KD
WT
OE
Glu
Gal
α-Tah18
α-GAPDH
OFF
ON
NOS activity (pmol/min/mg)
8.0
4.0
0
TAH18
WT
OE
conditional KD (on Glu)
conditional KD (on Gal)
+ Arg (100 μM, 1 h)
+ 39°C, 4 h
Stress (39°C, 14h)
Cell viability (%)
80
70
60
50
40
30
20
10
0
control
40 μM NOC12
62.5 μM SNAP
Wild-type
TAH18 conditional KD
(Nishimura et al., Biochem. Biophys. Res. Commun., 430, 137, 2013)
Tah18-dependent NO production confers stress tolerance.


Tah18-Dre2 dissociation and NO synthesis
(Yoshikawa et al., Nitric Oxide, 57, 85, 2016)
Dre2
NOS activity (NO_2 pmol/min/mg)
0
3
10
15
WT
DRE2-conditional KD
Time after shut-off (hr)
Non treatment
2 mM H_2O_2
(min)
WT
Co-OE strain
IP : α-GFP
Input
GFP-Tah18
3HA-Dre2
Pgk1
Dre2
Tah18
NOS activity
Oxidative stress
Dissociation
Arg
NO


Tah18 and mammalian NOS
Mammalian NOS
Oxygenase domain
Heme/BH_4/Arg
Reductase domain
FMN
FAD/NADPH
1
221
724
751
1,434
Reductase
Tah18
623
?
Oxidative stress
NADPH
e^-
Dre2
Tah18
【Fe-S cluster biogenesis】
Arg, O_2
Oxy
NO
【NOS activity】
Tah18 functions as reductase of the NOS reaction.

Antioxidative mechanism by ***NO***

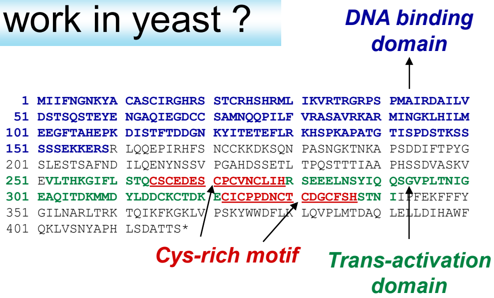


How does **NO** work in yeast ?

S-Nitroso-*N*-acetylpenicillamine (**SNAP**)

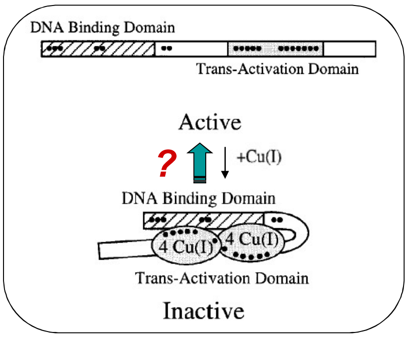
NO upregulates the expression of genes involved in **copper uptake**.

	10 min	30 min	60 min
CTR1	2.1	3.6	5.8
CTR3	0.96	1.3	1.7
FRE1	2.2	4.6	6.9
FRE7	2.7	7.8	19
IRC7	1.1	1.4	1.7
REE1	1.2	1.4	2.4

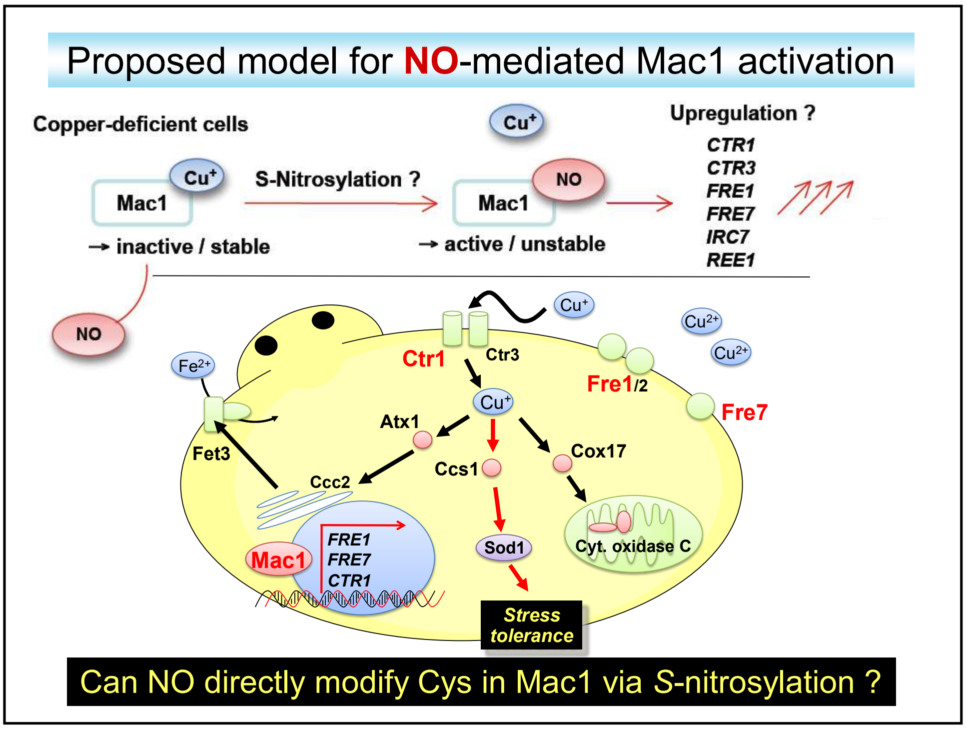


The transcription factor Mac1

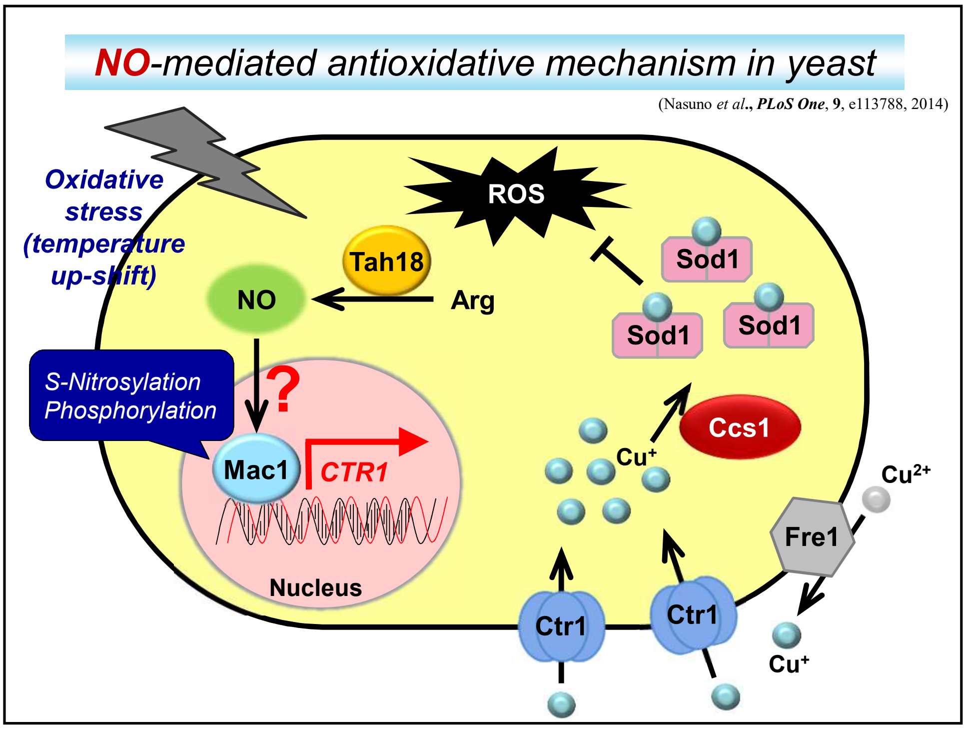
- activates genes involved in Cu^{+} uptake.
- is involved in stress resistance.
- is inactivated by Cu^{+} binding.
- is activated under Cu^{+} starvation condition.



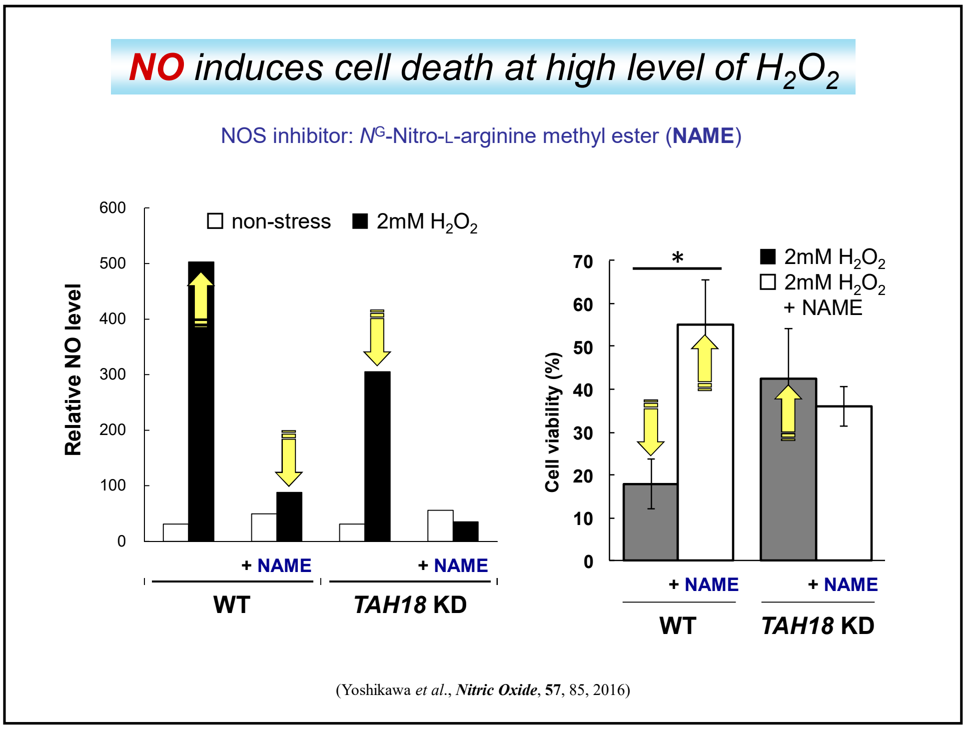
(*EMBO J*, **17**, 5400, 1998)



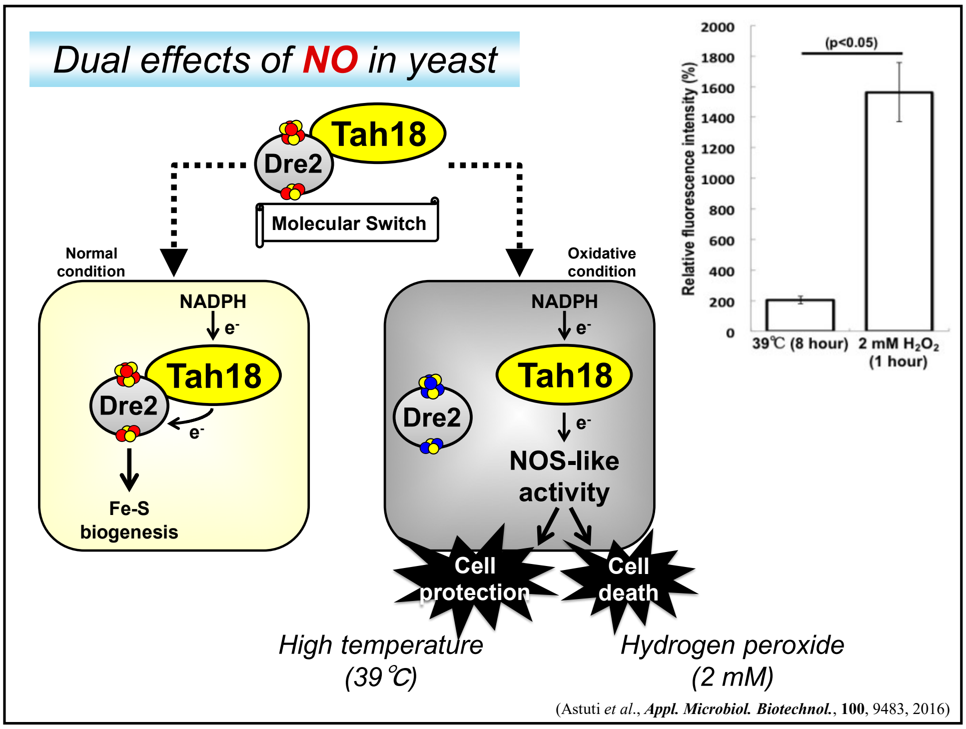
Proposed model for NO-mediated Mac1 activation
Copper-deficient cells
Cu+
Mac1
S-Nitrosylation ?
NO
→ inactive / stable
→ active / unstable
Upregulation ?
CTR1
CTR3
FRE1
FRE7
IRC7
REE1
Cu2+
Fe2+
Ctr1
Ctr3
Fre1/2
Fre7
Atx1
Fet3
Ccc2
Ccs1
Cox17
Sod1
Cyt. oxidase C
Stress tolerance
Can NO directly modify Cys in Mac1 via S-nitrosylation ?



NO-mediated antioxidative mechanism in yeast
(Nasuno et al., PLoS One, 9, e113788, 2014)
Oxidative stress (temperature up-shift)
ROS
Tah18
NO
Arg
Sod1
S-Nitrosylation
Phosphorylation
?
Mac1
CTR1
Nucleus
Ccs1
Cu+
Cu2+
Fre1
Ctr1



NO induces cell death at high level of H2O2
NOS inhibitor: NG-Nitro-L-arginine methyl ester (NAME)
non-stress
2mM H2O2
Relative NO level
600
500
400
300
200
100
0
+ NAME
+ NAME
WT
TAH18 KD
Cell viability (%)
70
60
50
40
30
20
10
0
*
2mM H2O2
2mM H2O2 + NAME
+ NAME
+ NAME
WT
TAH18 KD
(Yoshikawa et al., Nitric Oxide, 57, 85, 2016)



Dual effects of NO in yeast
Tah18
Dre2
Molecular Switch
Normal condition
Oxidative condition
NADPH
e-
Tah18
Dre2
e-
Fe-S biogenesis
NADPH
e-
Tah18
Dre2
e-
NOS-like activity
Cell protection
Cell death
High temperature (39℃)
Hydrogen peroxide (2 mM)
Relative fluorescence intensity (%)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
(p<0.05)
39℃ (8 hour)
2 mM H2O2 (1 hour)
(Astuti et al., Appl. Microbiol. Biotechnol., 100, 9483, 2016)

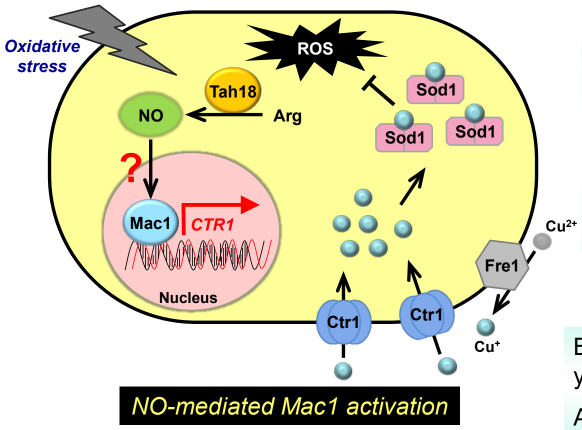
③ Nitric oxide

FEMS Yeast Res., **10**, 687, 2010; *Microb. Cell Fact.*, **11:**40 doi:10.1186/1475-2859-11-40, 2012: *Proc. Natl. Acad. Sci. USA*, **110**, 11821, 2013; *Biochem. Biophys. Res. Commun.*, **430**, 137, 2013 ; *PLoS One*, **9**, e113788, 2014; *Nitric Oxide-Biol. Chem.*, **52**, 29, 2016; **57**, 85, 2016; *Appl. Microbiol. Biotechnol.*, **100**, 9483, 2016; *Med. Mycol.* **59**, E63-E70, 2018; *Sci. Rep.*, **10**, 6015. doi: 10.1038/s41598-020-62890-3; *Anal. Biochem.*, doi: 10.1016/j.ab.2020.113707.

＜ So far ＞

★ NO is produced from Arg through the Tah18-dependent activity *!!*

★ NO confers oxidative stress tolerance on yeast cells by enhancing Sod1 activity through the activation of Mac1 *!!*



NO-mediated Mac1 activation

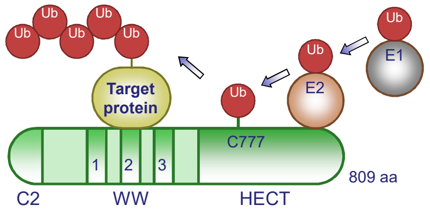
＜ Current projects ＞

- Regulation of synthesis (Tah18-Dre2 interaction)
- Physiological roles (cell protection vs. cell death)
- Protein modifications (*S*-nitrosylation, nitration)

Breeding of novel stress-tolerant yeast strains with NO engineering

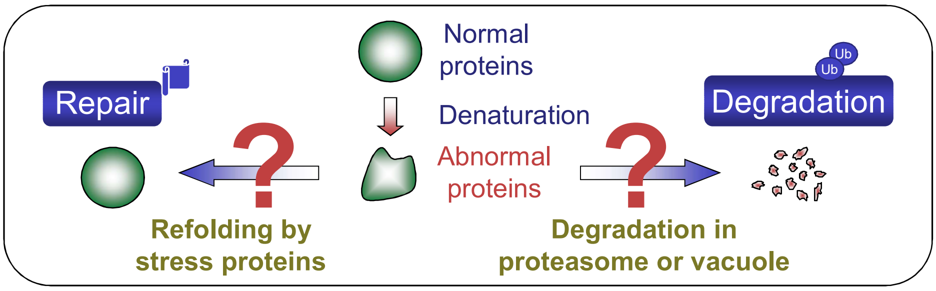
Application to pathogenic yeasts

Rsp5 is an essential E3 ubiquitin ligase.



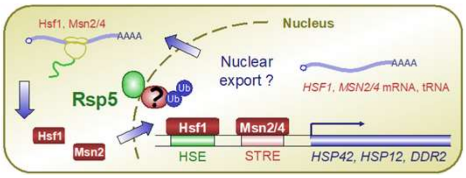
Rsp5 participates in many events through ubiquitination of target proteins;

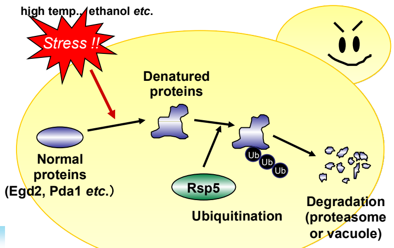
endocytosis of plasma membrane permeases, mitochondrial inheritance, degradation of the large subunit of RNA pol. II, biosynthesis of unsaturated fatty acids, actin cytoskelton organization, sporulation, ER-associated degradation etc.



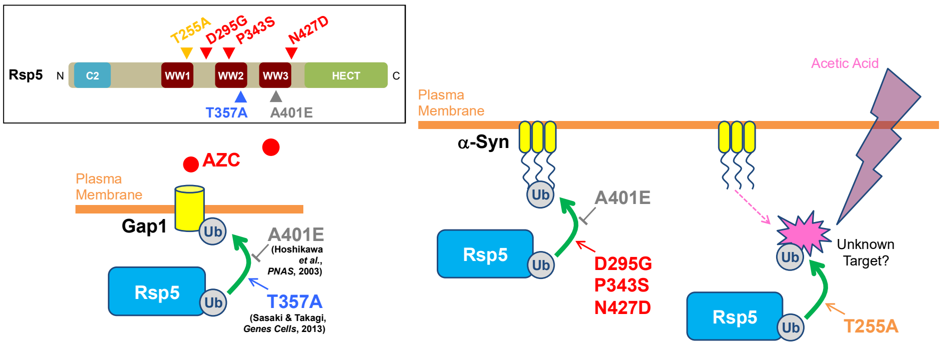
Rsp5 may be involved in repair / degradation of abnormal proteins.

Rsp5 Involved in repair / degradation of abnormal proteins





Required for degradation of Gap1



④ Ubiquitin system

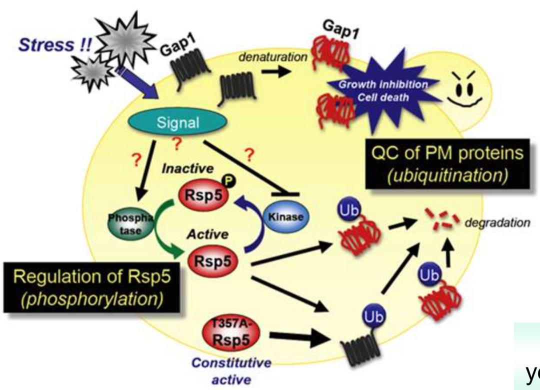
Proc. Natl. Acad. Sci. USA, **100**, 11505, 2003; *FEBS Lett.*, **580**, 3433, 2006; *Biosci. Biotech. Biochem.*, **70**, 2762, 2006; **73**, 2268, 2009; *FEMS Microbiol. Lett.*, **277**, 70, 2007; *Genes Cells*, **13**, 105, 2008; *FEMS Yeast Res.*, **9**, 73, 2009; **14**, 567, 2014; **17**, fox083, 2017; *FEBS J.*, **276**, 5287, 2009 ; *J. Brew. Distill.*, **3**, 1, 2012, *Genes Cells*, **18**, 459, 2013; *Eukaryot. Cell*, **13**, 1191, 2014; *J. Biochem.*, **157**, 251, 2015; *Appl. Environ. Microbiol.*, **84**, e00406-18, 2018.

＜ So far ＞

★ Rsp5 is involved in quality control of plasma membrane proteins *!!*

★ Rsp5 activity is regulated by phosphorylation of a conserved Thr357 *!!*

＜ Current projects ＞



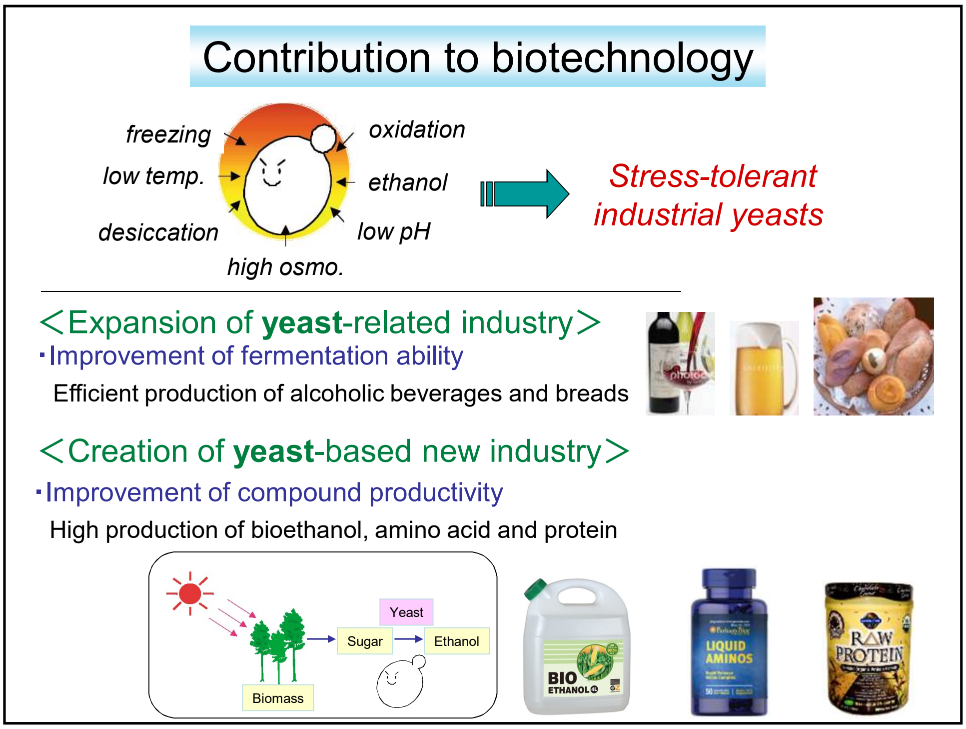
・Recognition and degradation of abnormal proteins by Rsp5

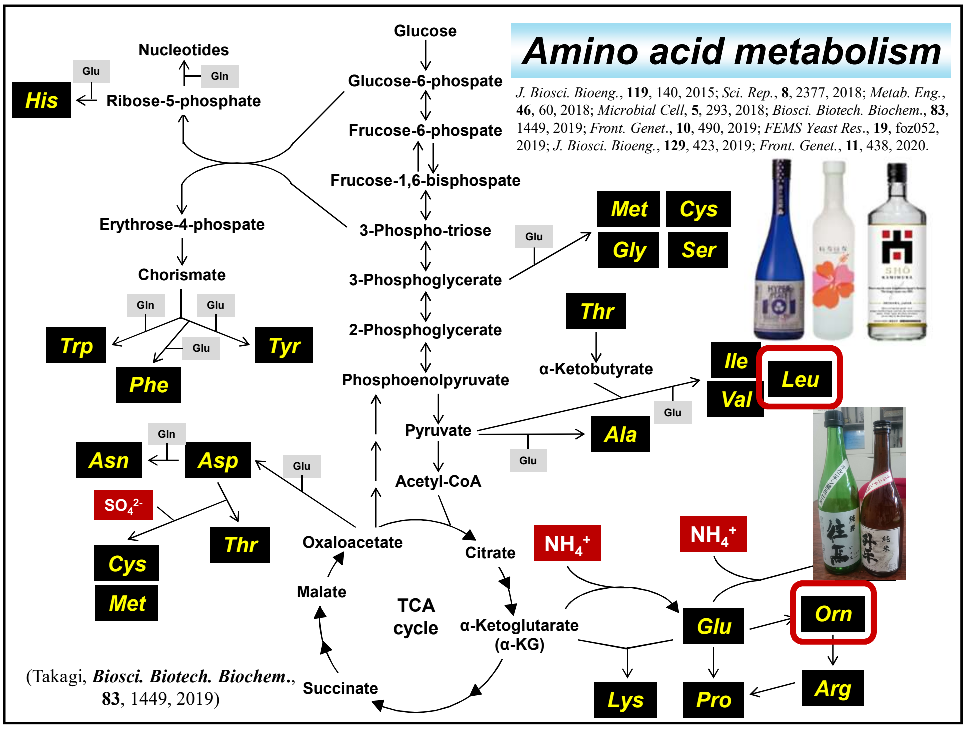
・Functional improvement of Ub-system (Rsp5)

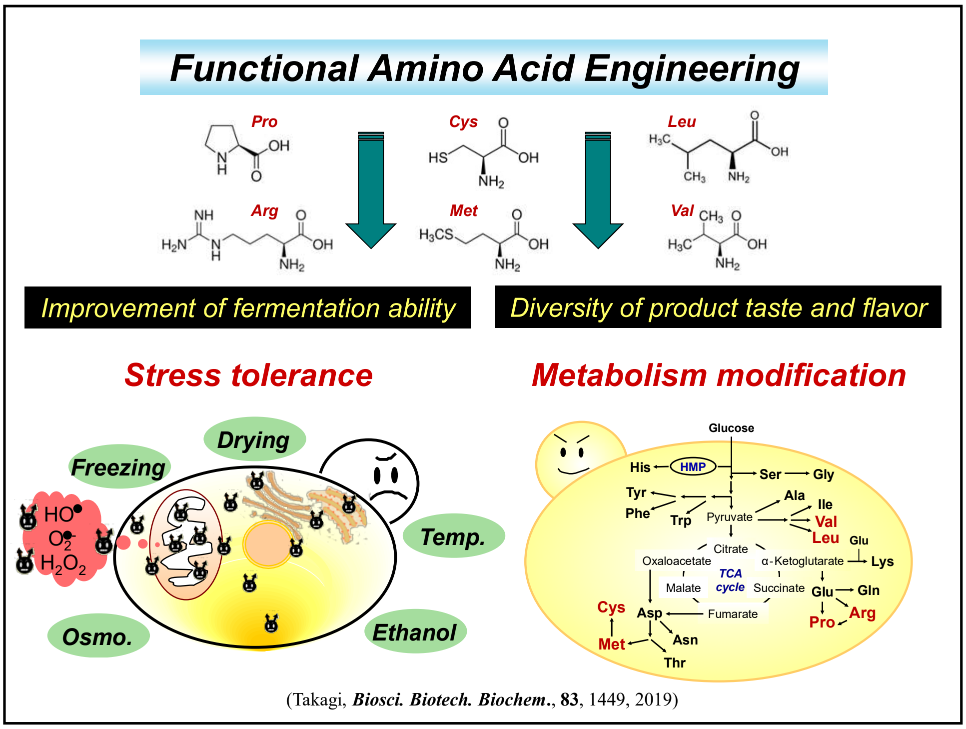
・Regulation of the Rsp5 activity via phosphorylation

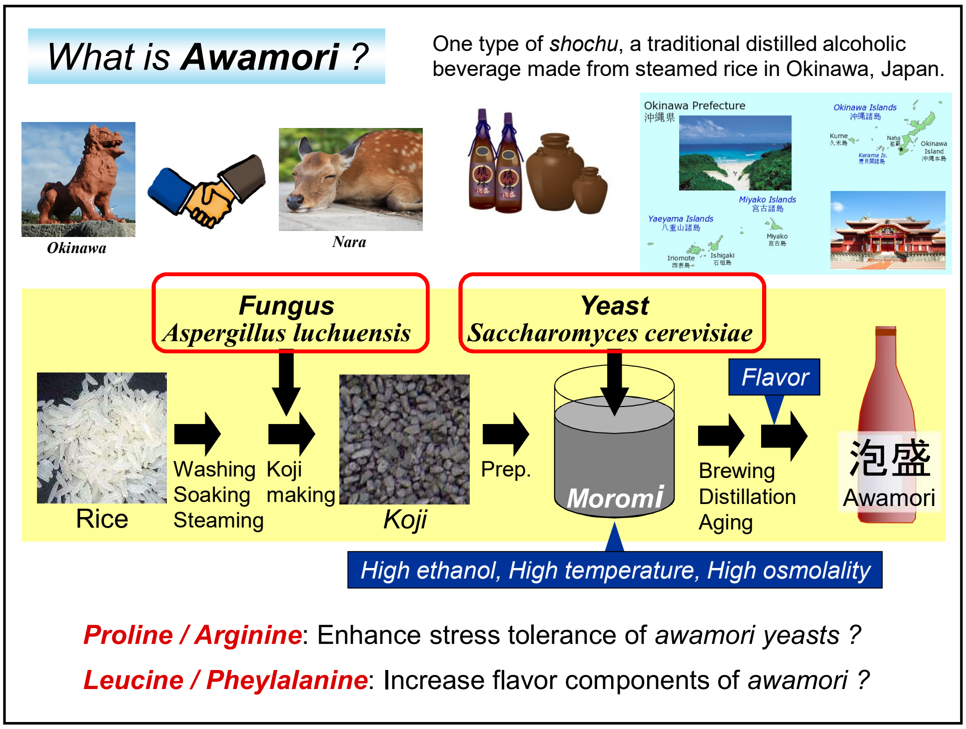
⬇

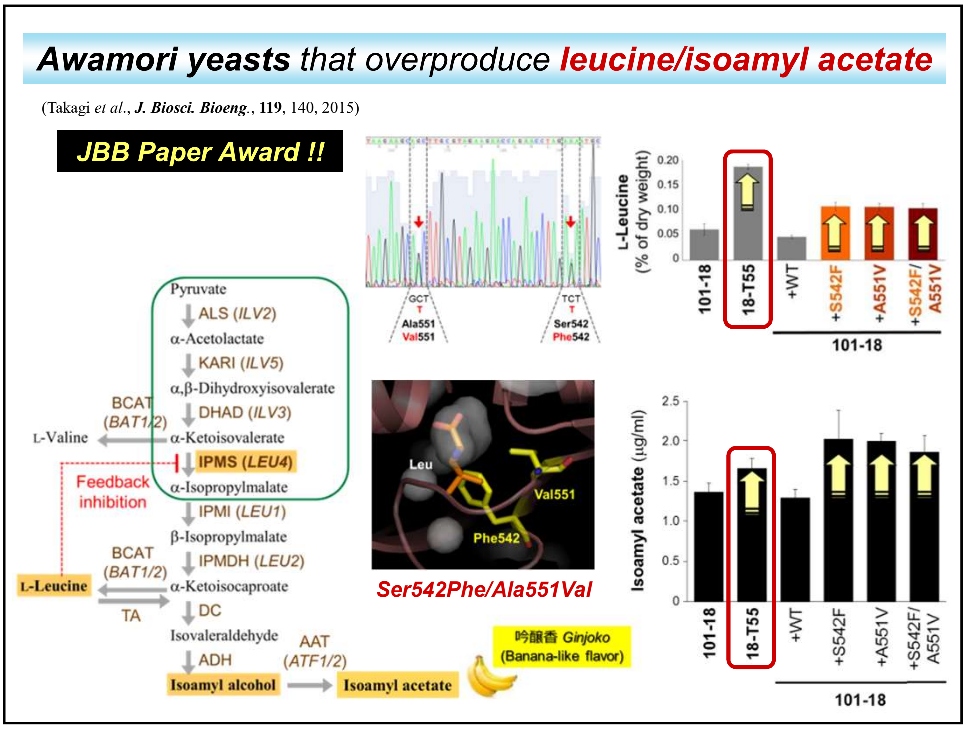
Breeding of novel stress-tolerant yeast strains with improved Ub-system


Contribution to biotechnology
freezing
low temp.
desiccation
high osmo.
oxidation
ethanol
low pH
Stress-tolerant industrial yeasts
＜Expansion of yeast-related industry＞
・Improvement of fermentation ability
Efficient production of alcoholic beverages and breads
＜Creation of yeast-based new industry＞
・Improvement of compound productivity
High production of bioethanol, amino acid and protein
Yeast
Sugar
Ethanol
Biomass
BIO ETHANOL
LIQUID AMINOS
RAW PROTEIN


Amino acid metabolism
J. Biosci. Bioeng., 119, 140, 2015; Sci. Rep., 8, 2377, 2018; Metab. Eng., 46, 60, 2018; Microbial Cell, 5, 293, 2018; Biosci. Biotech. Biochem., 83, 1449, 2019; Front. Genet., 10, 490, 2019; FEMS Yeast Res., 19, foz052, 2019; J. Biosci. Bioeng., 129, 423, 2019; Front. Genet., 11, 438, 2020.
Glucose
Glucose-6-phospate
Frucose-6-phospate
Frucose-1,6-bisphospate
3-Phospho-triose
3-Phosphoglycerate
2-Phosphoglycerate
Phosphoenolpyruvate
Pyruvate
Acetyl-CoA
Nucleotides
Ribose-5-phosphate
Erythrose-4-phospate
Chorismate
His
Trp
Tyr
Phe
Asn
Asp
Thr
Cys
Met
Gly
Ser
α-Ketobutyrate
Ile
Leu
Val
Ala
Glu
Gln
SO$_4^{2-}$
Oxaloacetate
Citrate
Malate
TCA cycle
α-Ketoglutarate (α-KG)
Succinate
NH$_4^+$
Orn
Arg
Lys
Pro
(Takagi, Biosci. Biotech. Biochem., 83, 1449, 2019)

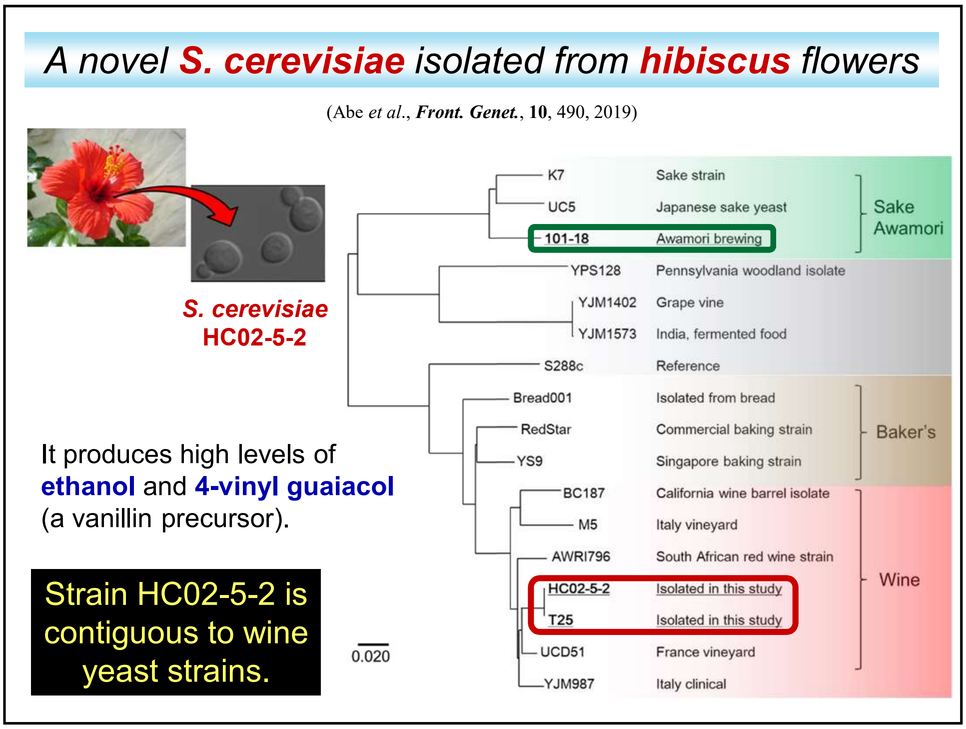

Functional Amino Acid Engineering
Pro
Cys
Leu
Arg
Met
Val
Improvement of fermentation ability
Diversity of product taste and flavor
Stress tolerance
Metabolism modification
Drying
Freezing
Temp.
Osmo.
Ethanol
HO•
O_2^-
H_2O_2
Glucose
His
HMP
Ser
Gly
Tyr
Phe
Trp
Pyruvate
Ala
Ile
Val
Leu
Citrate
Oxaloacetate
α-Ketoglutarate
Glu
Lys
TCA cycle
Malate
Succinate
Gln
Cys
Asp
Fumarate
Pro
Arg
Met
Asn
Thr
(Takagi, Biosci. Biotech. Biochem., 83, 1449, 2019)

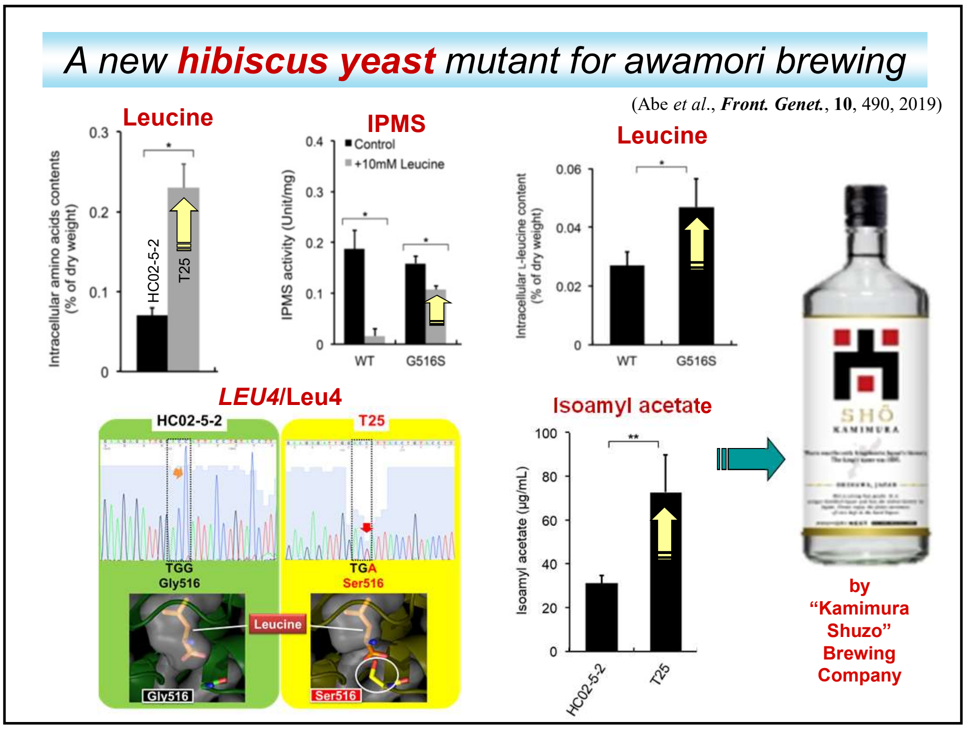

What is Awamori ?
One type of shochu, a traditional distilled alcoholic beverage made from steamed rice in Okinawa, Japan.
Okinawa
Nara
Okinawa Prefecture
沖縄県
Okinawa Islands
Miyako Islands
Yaeyama Islands
Fungus
Aspergillus luchuensis
Yeast
Saccharomyces cerevisiae
Flavor
Rice
Washing
Soaking
Steaming
Koji
making
Koji
Prep.
Moromi
Brewing
Distillation
Aging
泡盛
Awamori
High ethanol, High temperature, High osmolality
Proline / Arginine: Enhance stress tolerance of awamori yeasts ?
Leucine / Pheylalanine: Increase flavor components of awamori ?



Awamori yeasts that overproduce leucine/isoamyl acetate
(Takagi et al., J. Biosci. Bioeng., 119, 140, 2015)
JBB Paper Award !!
Pyruvate
ALS (ILV2)
α-Acetolactate
KARI (ILV5)
α,β-Dihydroxyisovalerate
DHAD (ILV3)
α-Ketoisovalerate
IPMS (LEU4)
α-Isopropylmalate
IPMI (LEU1)
β-Isopropylmalate
IPMDH (LEU2)
α-Ketoisocaproate
DC
Isovaleraldehyde
ADH
Isoamyl alcohol
AAT (ATF1/2)
Isoamyl acetate
BCAT (BAT1/2)
L-Valine
Feedback inhibition
L-Leucine
TA
GCT T Ala551 Val551
TCT T Ser542 Phe542
Leu
Val551
Phe542
Ser542Phe/Ala551Val
吟醸香 Ginjoko (Banana-like flavor)
L-Leucine (% of dry weight)
0.20
0.15
0.10
0.05
0
101-18
18-T55
+WT
+S542F
+A551V
+S542F/A551V
101-18
Isoamyl acetate (μg/ml)
2.5
2.0
1.5
1.0
0.5
0

On-sale from May 20, 2016
革新的な泡盛酵母「101H酵母」使用
HYPER YEAST 101 !!
"Shinzato Shuzo" Brewing Company
共同開発
合名会社新里酒造
奈良先端科学技術大学院大学
(株)バイオジェット
琉球大学農学部
伝統技術と先端技術による
香りの覚醒
Awakening Fragrance !!
HYPER YEAST 101
琉球泡盛 HYPER YEAST 101
atmospheric/vacuum distillation 50%/50% alcohol 35% 720ml ¥2,000


A novel S. cerevisiae isolated from hibiscus flowers
(Abe et al., Front. Genet., 10, 490, 2019)
S. cerevisiae
HC02-5-2
It produces high levels of ethanol and 4-vinyl guaiacol (a vanillin precursor).
Strain HC02-5-2 is contiguous to wine yeast strains.
0.020
K7 Sake strain
UC5 Japanese sake yeast
101-18 Awamori brewing
Sake
Awamori
YPS128 Pennsylvania woodland isolate
YJM1402 Grape vine
YJM1573 India, fermented food
S288c Reference
Bread001 Isolated from bread
RedStar Commercial baking strain
YS9 Singapore baking strain
Baker's
BC187 California wine barrel isolate
M5 Italy vineyard
AWRI796 South African red wine strain
HC02-5-2 Isolated in this study
T25 Isolated in this study
UCD51 France vineyard
YJM987 Italy clinical
Wine


A new hibiscus yeast mutant for awamori brewing
(Abe et al., Front. Genet., 10, 490, 2019)
Leucine
Intracellular amino acids contents (% of dry weight)
0.3
0.2
0.1
0
HC02-5-2
T25
IPMS
Control
+10mM Leucine
IPMS activity (Unit/mg)
0.4
0.3
0.2
0.1
0
WT
G516S
Leucine
Intracellular L-leucine content (% of dry weight)
0.06
0.04
0.02
0
WT
G516S
LEU4/Leu4
HC02-5-2
T25
TGG
Gly516
TGA
Ser516
Leucine
Gly516
Ser516
Isoamyl acetate
Isoamyl acetate (μg/mL)
100
80
60
40
20
0
HC02-5-2
T25
SHŌ
KAMIMURA
by "Kamimura Shuzo" Brewing Company