



WORLD BREWING CONGRESS

August 13–17, 2016 • Denver, Colorado, U.S.A.

#ElevateBeer



Asahi

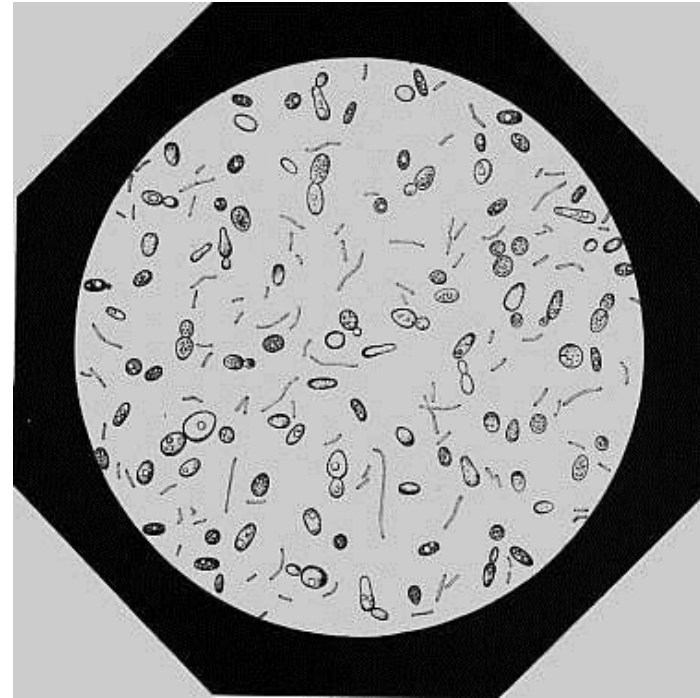
Recent Development in Detection and Identification Methods for Beer Spoilage Lactic Acid Bacteria -A Review

**Koji Suzuki, Masaki Shimokawa
and Hiromi Yamagishi
Quality Control Center
Asahi Breweries, Ltd.**

Back in 1870s



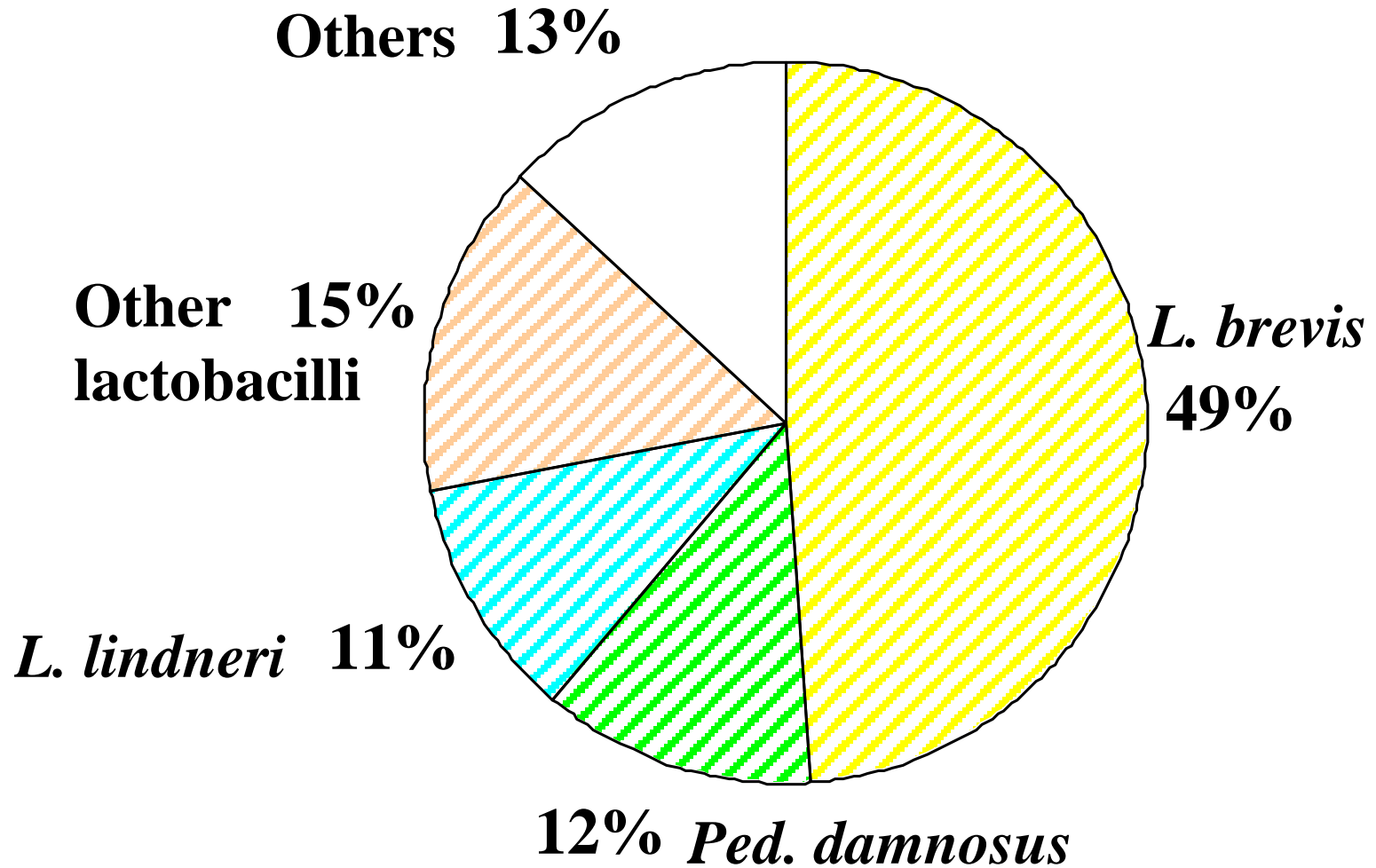
Louis Pasteur
Father of Microbiology



A drawing of spoiled beer
Etudes sur la bière (1876)

Just 140 years ago, beer spoilage *Lactobacillus*, later known as *L. pastorianus*, was first described by Pasteur.

Lactic acid bacteria (LAB) as major spoilers in beer



Brauwelt Back, W. (2003)

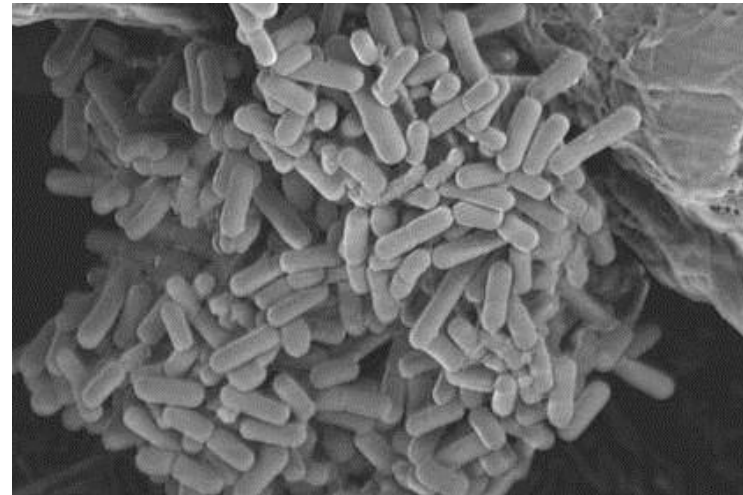
Beer spoilage incidents in Japan

1986 *Lactobacillus* sp.

1989 *Lactobacillus paracollinoides*

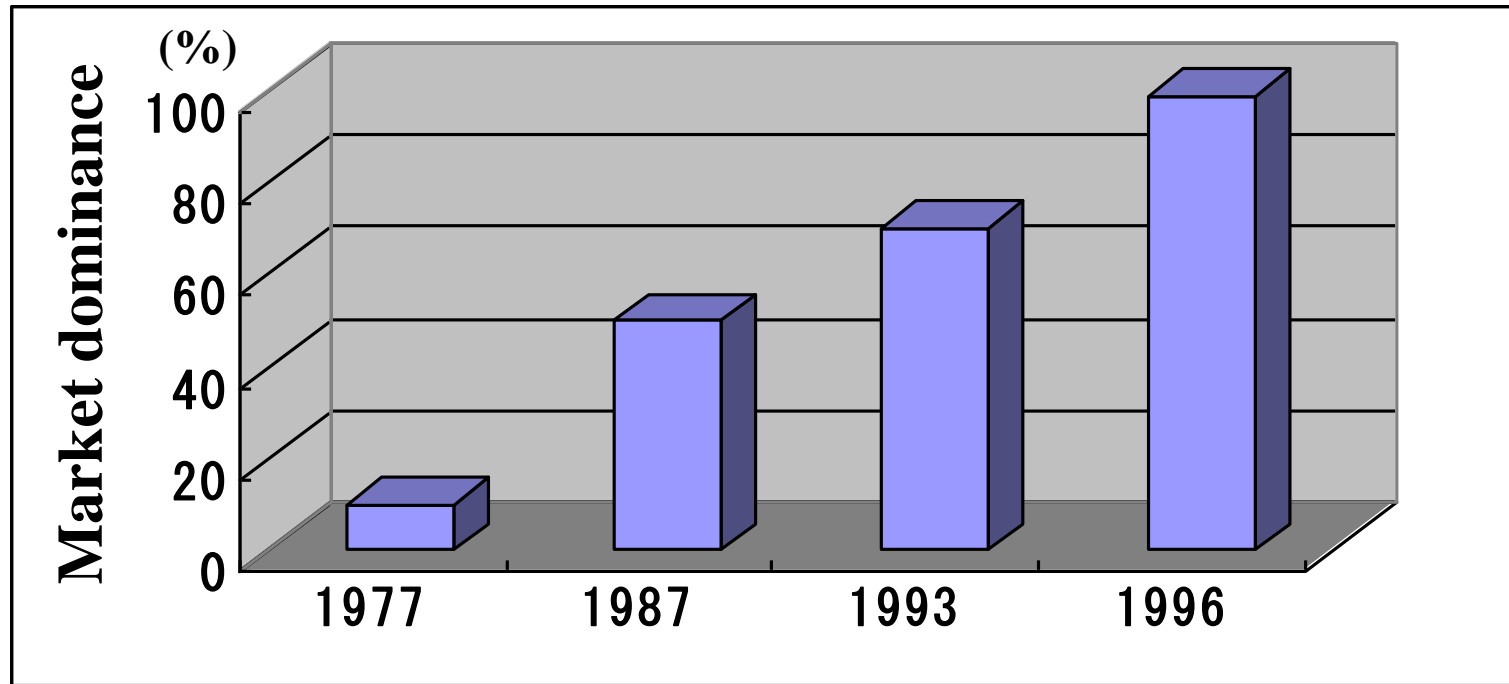
1991 *Lactobacillus lindneri*

etc.....



Major brewing companies in Japan experienced spoilage incidents during 1980 - 1995.

Rapid expansion of unpasteurized beer in Japan



No detection on quality control (QC) media

New species

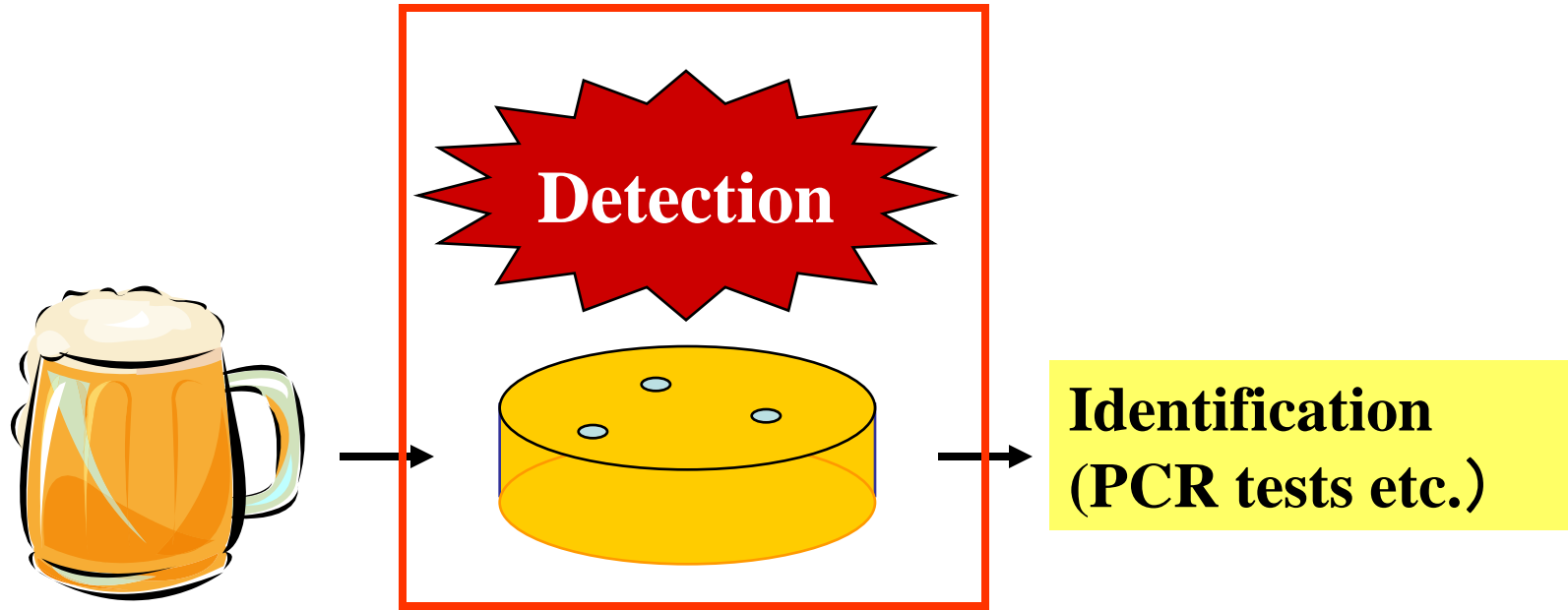
Issues addressed today

- 1. LAB strains hard to detect by QC media**
- 2. Emergence of new spoilage LAB species**

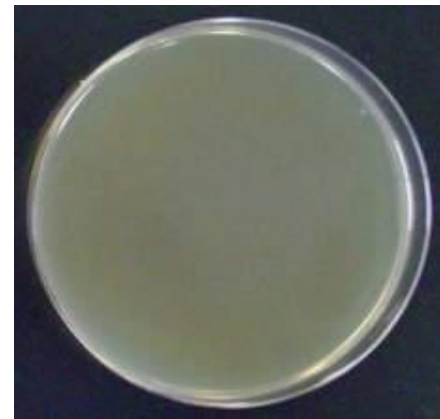
Part 1

**LAB strains hard to detect
by QC media**

Microbiological QC tests - Features and problems -

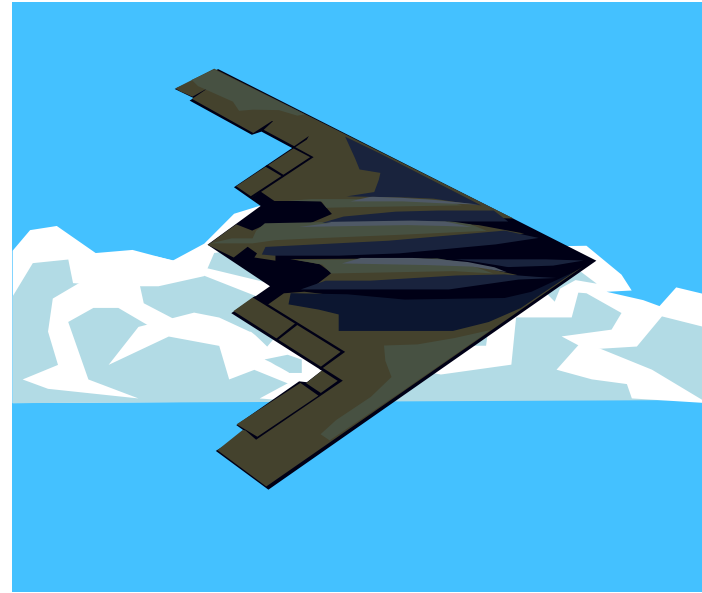


**Easy-to-culture
spoilage LAB**



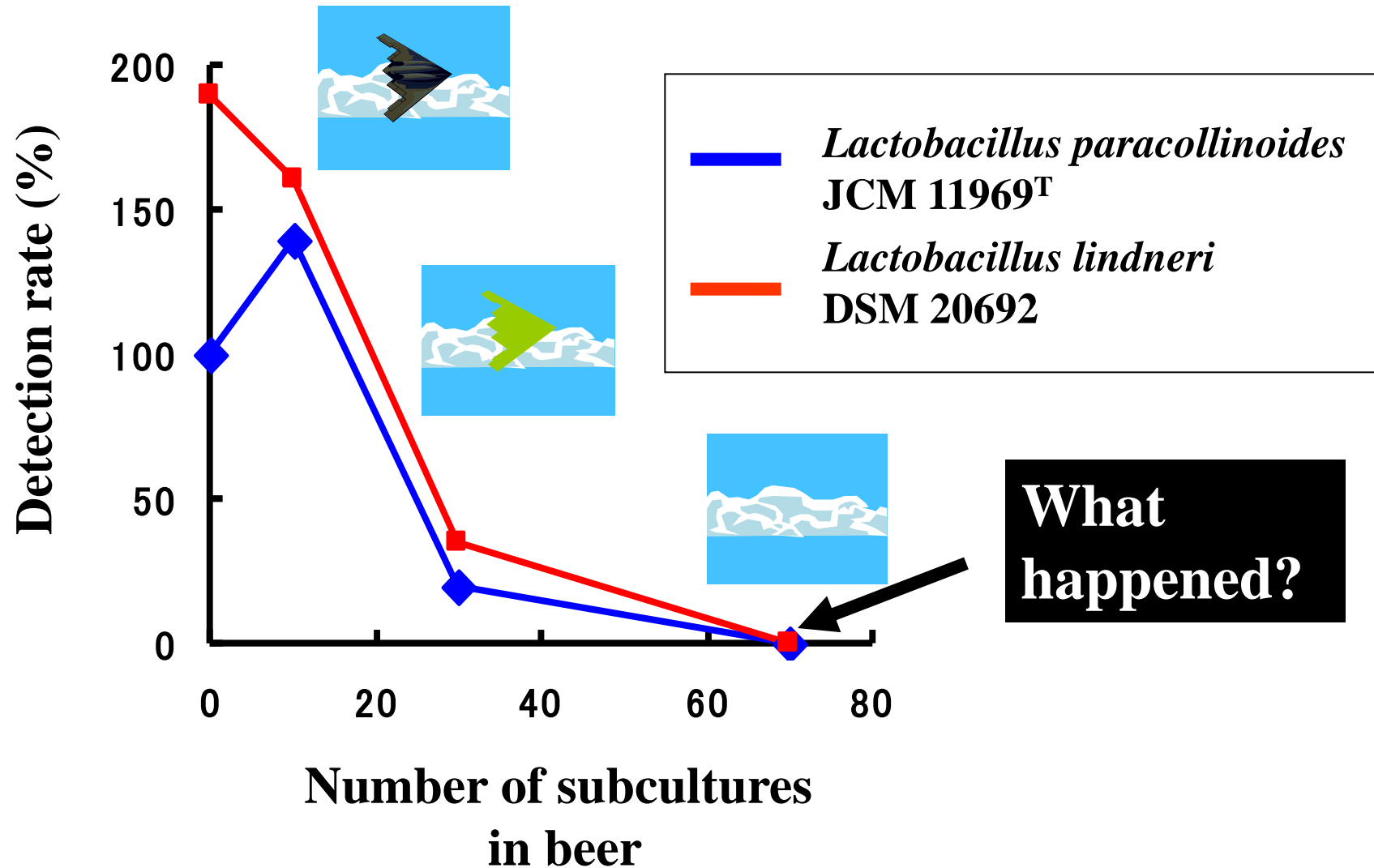
**Hard-to-culture
spoilage LAB**

Stealth bomber in the brewing industry?



Enigma: Visible and invisible strains even among the identical species.

Culturability lost through beer adaptation



“Die-hard Beer Lover” Hypothesis

**Beer is my beloved wife (husband)!!!
and I am too attached to her (him)!!!**



**Beer-specific
growth factors**

pH

**Sometimes too much nutrient
is not good for them.**

Development of an advanced beer-spoiler detection (ABD) medium

<Medium compositions>

MRS broth (powder)	2.61g
---------------------------	--------------

Sodium acetate	0.5g
-----------------------	-------------

Cycloheximide	10mg
----------------------	-------------

Agar	15g
-------------	------------

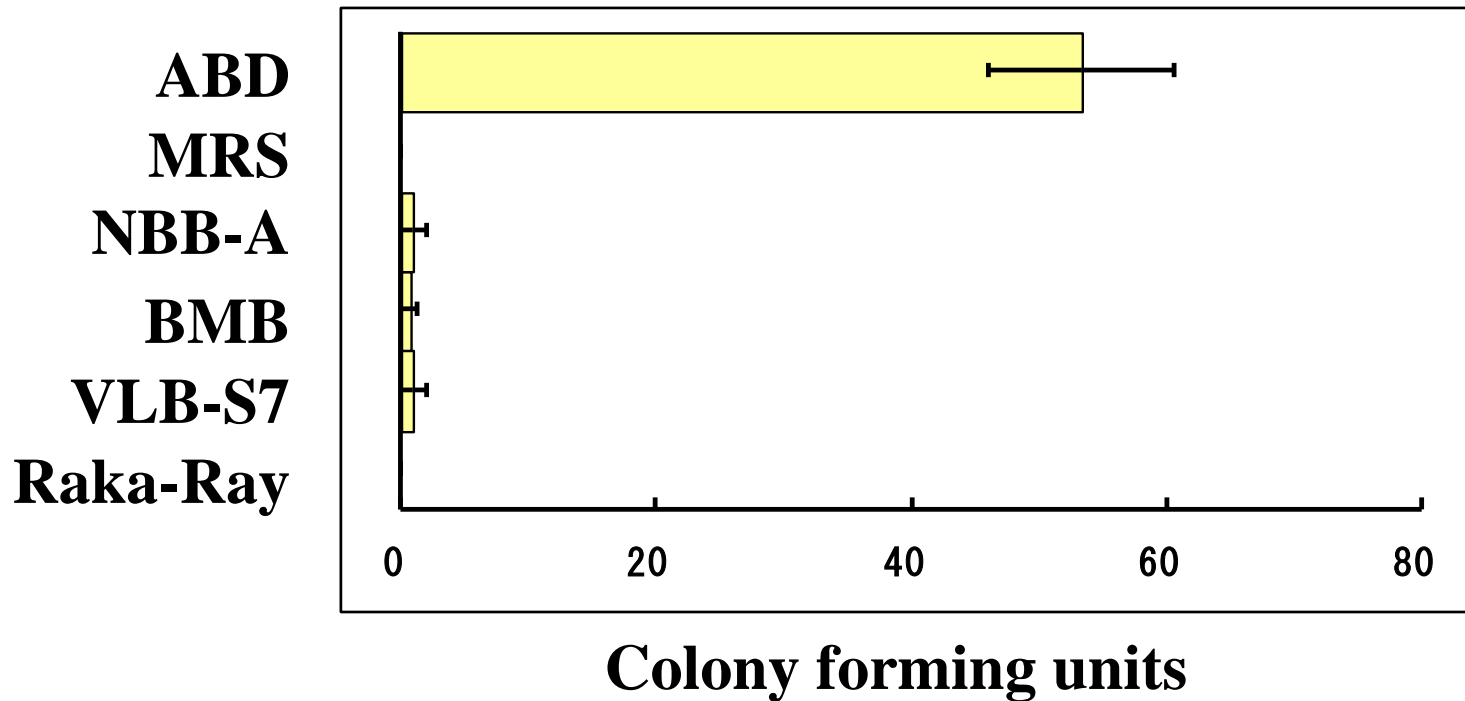
Beer (pilsner-type) 1000ml	
-----------------------------------	--

pH 5.0

The medium contains only 0.26% MRS (de Man, Rogosa and Sharpe) broth in beer and its pH is adjusted to 5.0 to detect hard-to-culture LAB strains, as well as easy-to-culture ones.

Comparison between ABD and other QC media

**Hard-to-culture
L. lindneri strain**

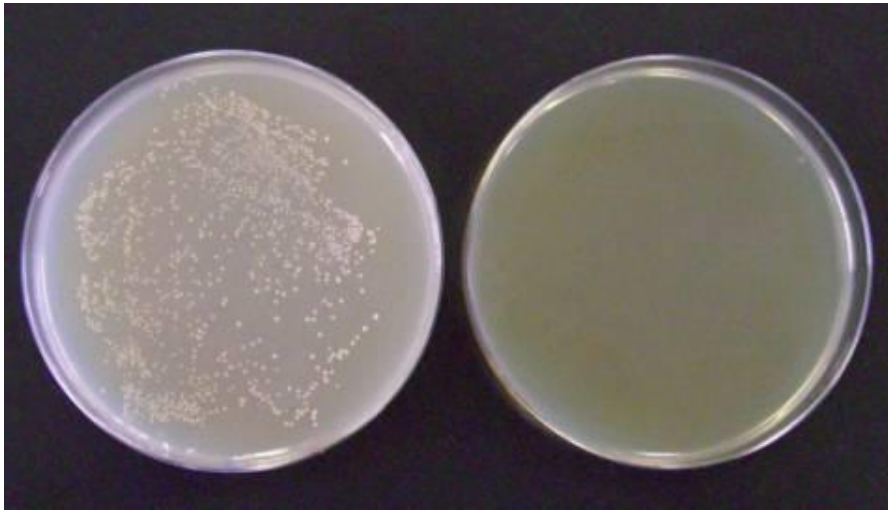


Anaerobic incubation at 25°C for 14 days

Survey of brewing environments

ABD

MRS



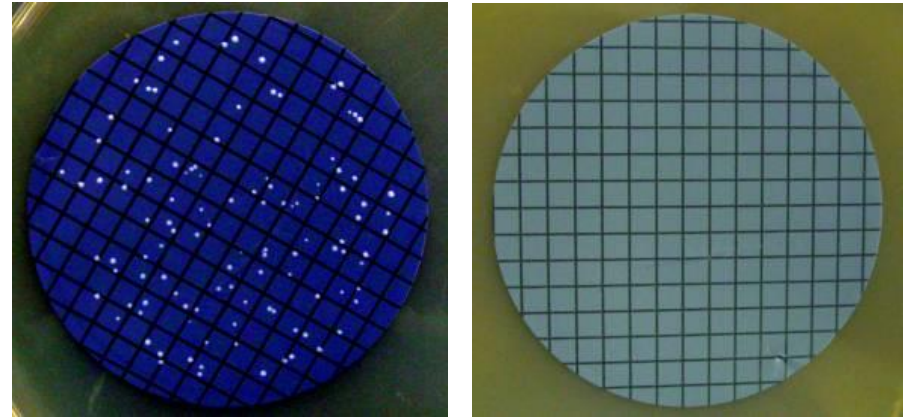
2408CFU

0CFU

Recycled container
L. paracollinoides

ABD

MRS



148CFU

0CFU

Imported beer
L. lindneri

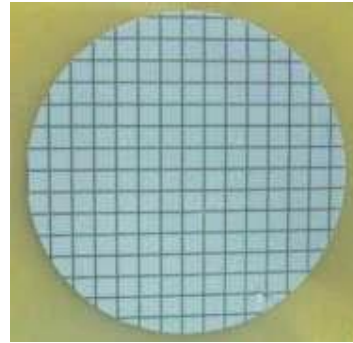
56% cases surveyed in stealth mode

More rapid approach needed

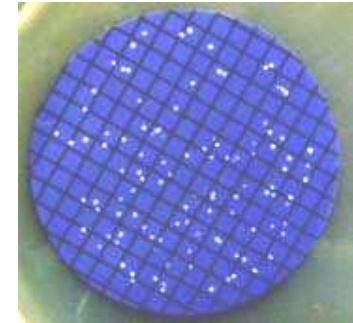
Beer filtration



**Culture
on ABD**

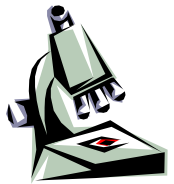
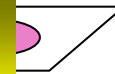


Identification

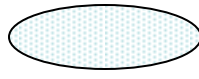
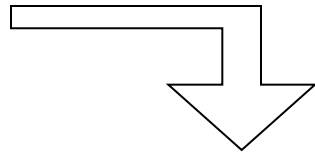


- Microscopic observations
- Gram staining
- Catalase activities
- PCR tests etc....

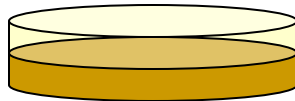
**About 100,000,000 cells are needed
for human eyes to detect them...
(3 - 14 days)**



Microcolony method



PC membrane



μ Finder Inspection System

Beer filtration



Incubation



3 days

CFDA staining



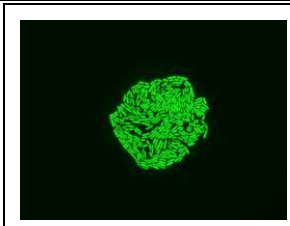
30-60 min

Detection

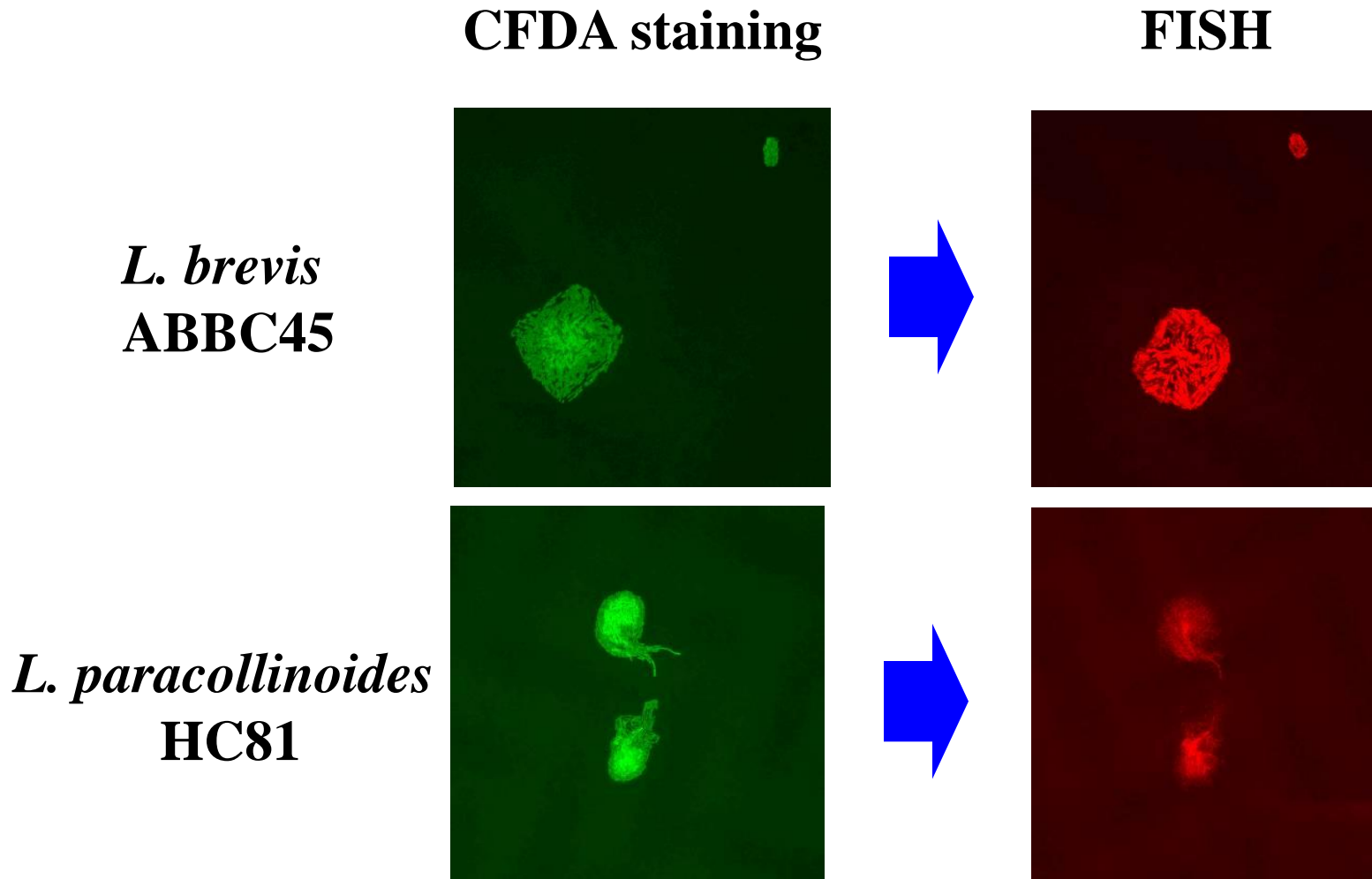
15 min

ABD medium

10 - 100 cells
are enough!

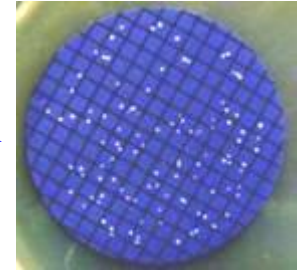


Species identification by fluorescence *in situ* hybridization (FISH) method

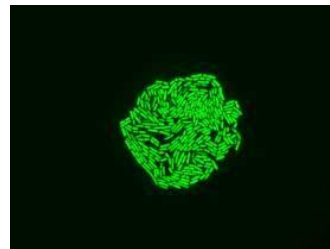
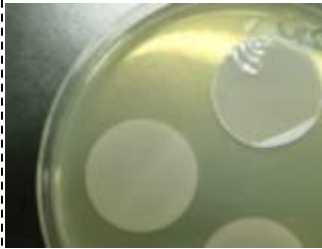


Asano, S., Iijima, K., Suzuki, K., Motoyama, Y. *et al.*, *J. Biosci. Bioeng.* (2009)
Yasuhara, T., Motoyama, Y. *et al.*, *J. Am. Soc. Brew. Chem.* (2001)

Advantages of microcolony approach



Culture



Microcolony

Short time incubation (~ 3days + ca 3hours)

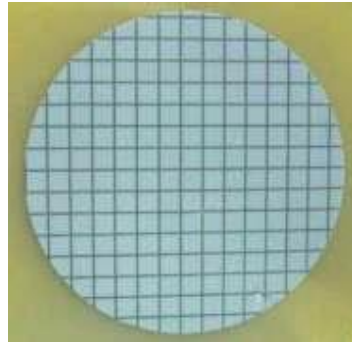
In some urgent cases, more rapid methods may be needed !

Why not eliminate culturing process?

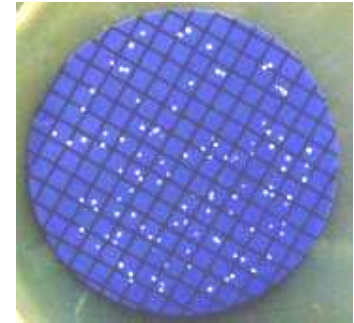
Beer filtration



Culture

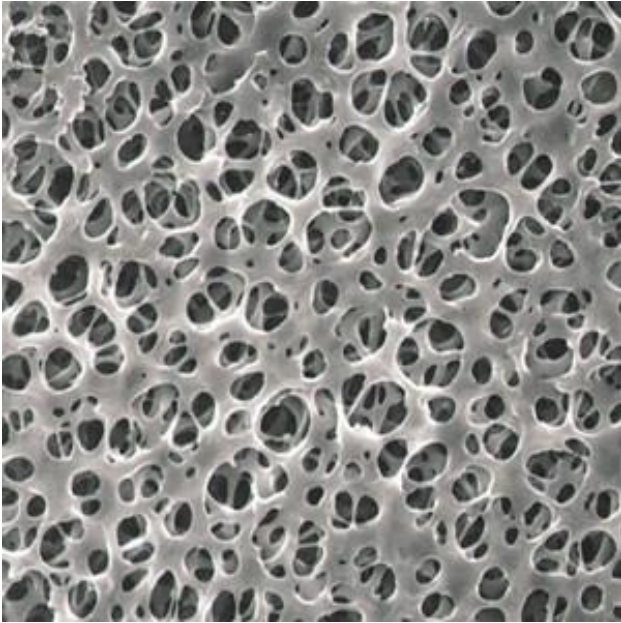


Identification

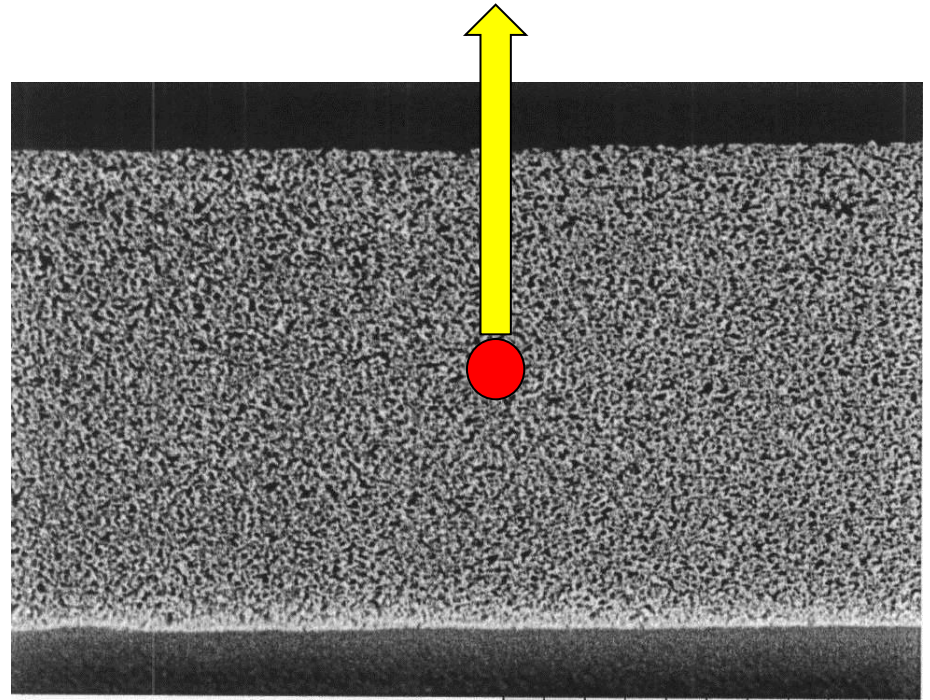


**A single cell must be recovered
from membrane matrix**

Membrane filter magnified by electron microscope



Surface structure



Cross section

Membrane filters are *ca* 150 μ m thick with a mesh-like structure. It is never an easy task to recover one tiny bacterial cell (2 μ m) trapped deeply within the membrane matrix.

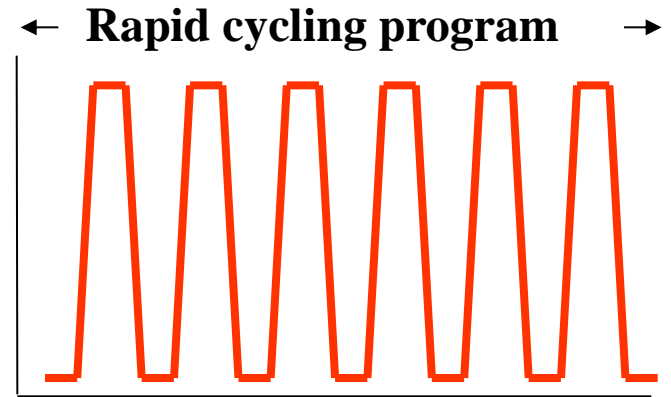
Pressure cycling technology (PCT)



Barocycler™

300 MPa

0.1 MPa

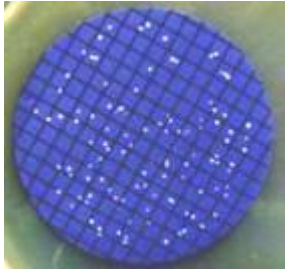


0.1 MPa → 300 MPa : less than 3sec
300 MPa → 0.1 MPa : less than 1sec

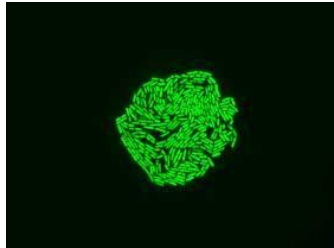
The cells and DNA of beer spoilage LAB are efficiently released from the membrane matrix.

As a result, 12 species of beer spoilage LAB can be detected at a single cell/300ml level without culturing.

Culture-independent direct method

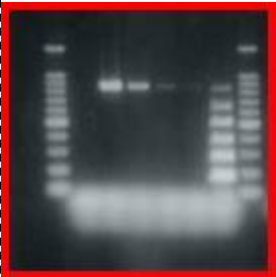


Culture



Microcolony

Short time incubation (~ 3days)



**Direct detection
(8hrs)**

Details presented by Mr. Shimokawa at BCOJ Symposium (Aug. 14).

Part 2

Emergence of new beer spoilage species

Beer spoilage LAB species

Genus *Lactobacillus*

L. brevis

L. lindneri

L. paracollinoides

L. backi

L. paucivorans

L. rossiae

L. casei

Other lactobacilli

Genus *Pediococcus*

Ped. damnosus

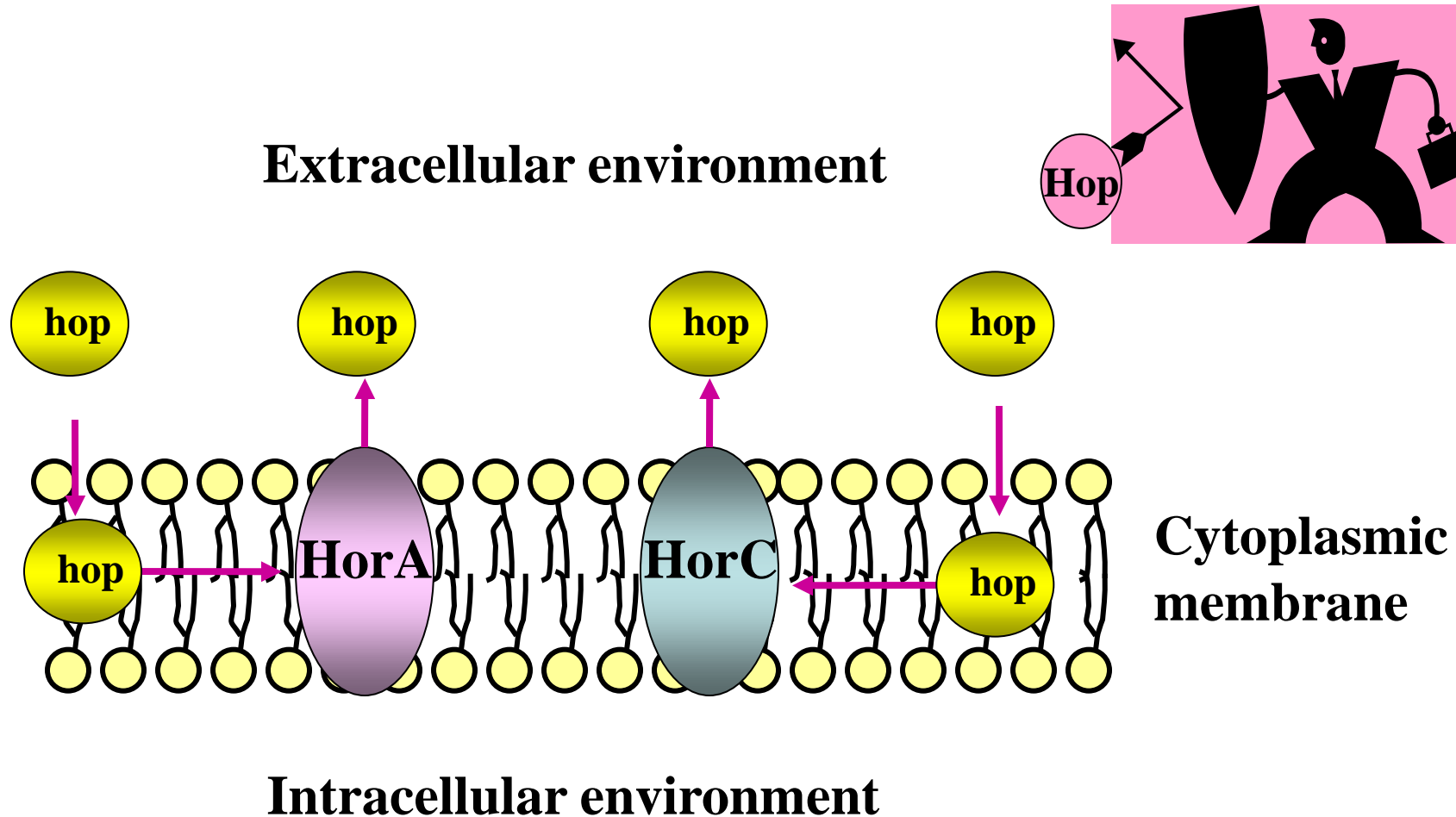
Ped. claussenii

Ped. inopinatus

Other pediococci

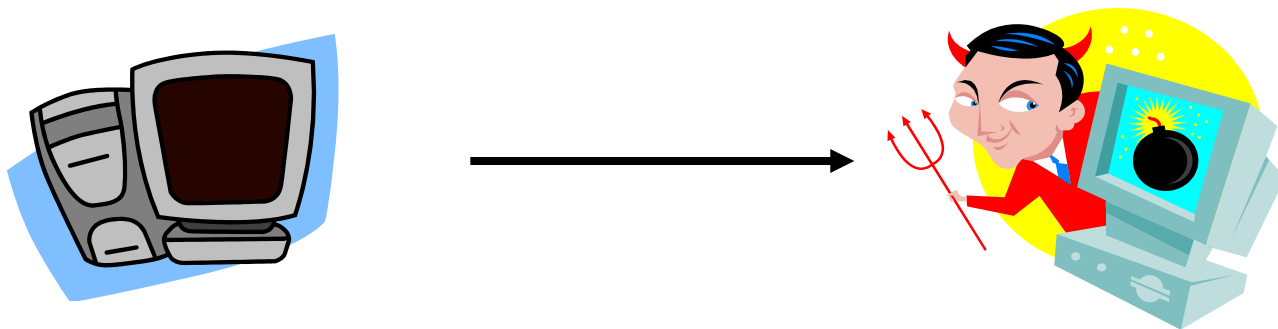
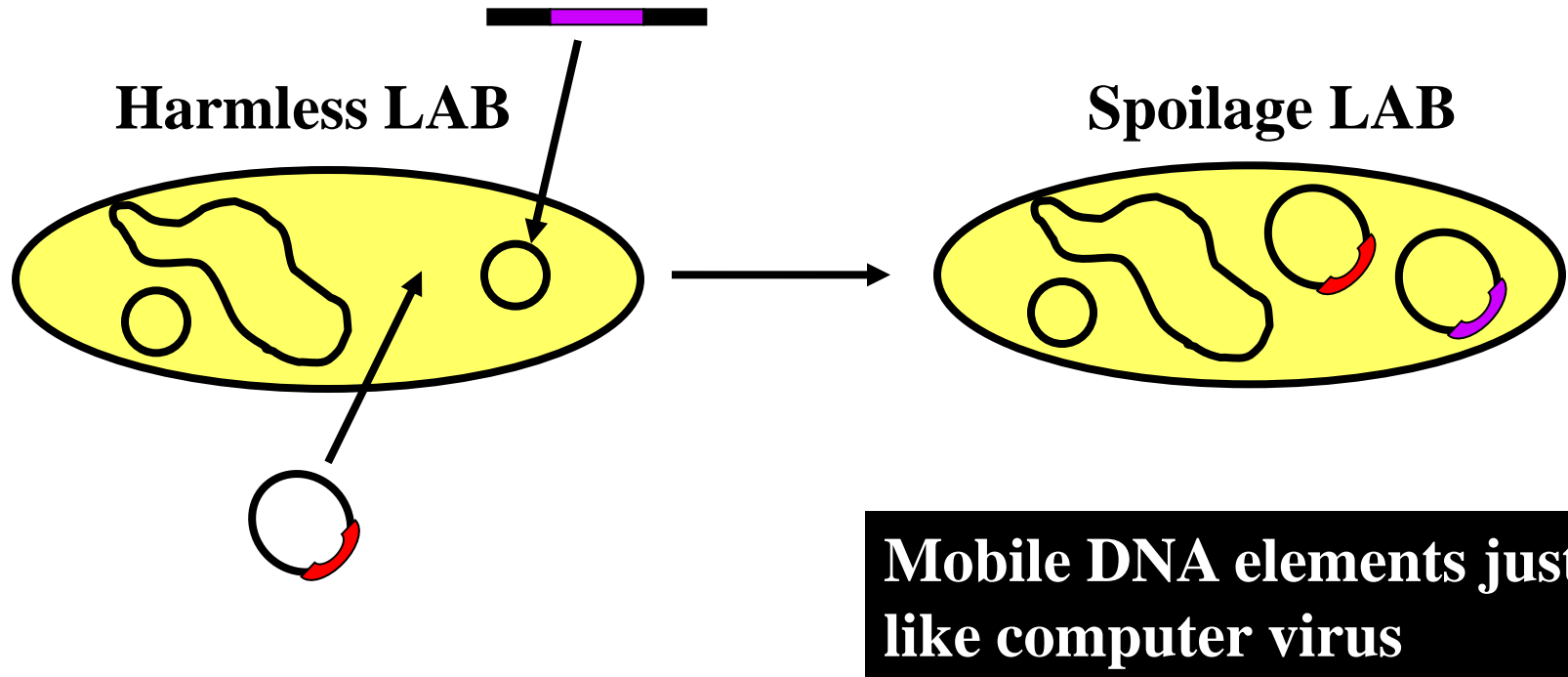
Currently more than 10 LAB species have been reported to spoil beer and new species are emerging.

Hop resistance mechanisms by efflux pumps

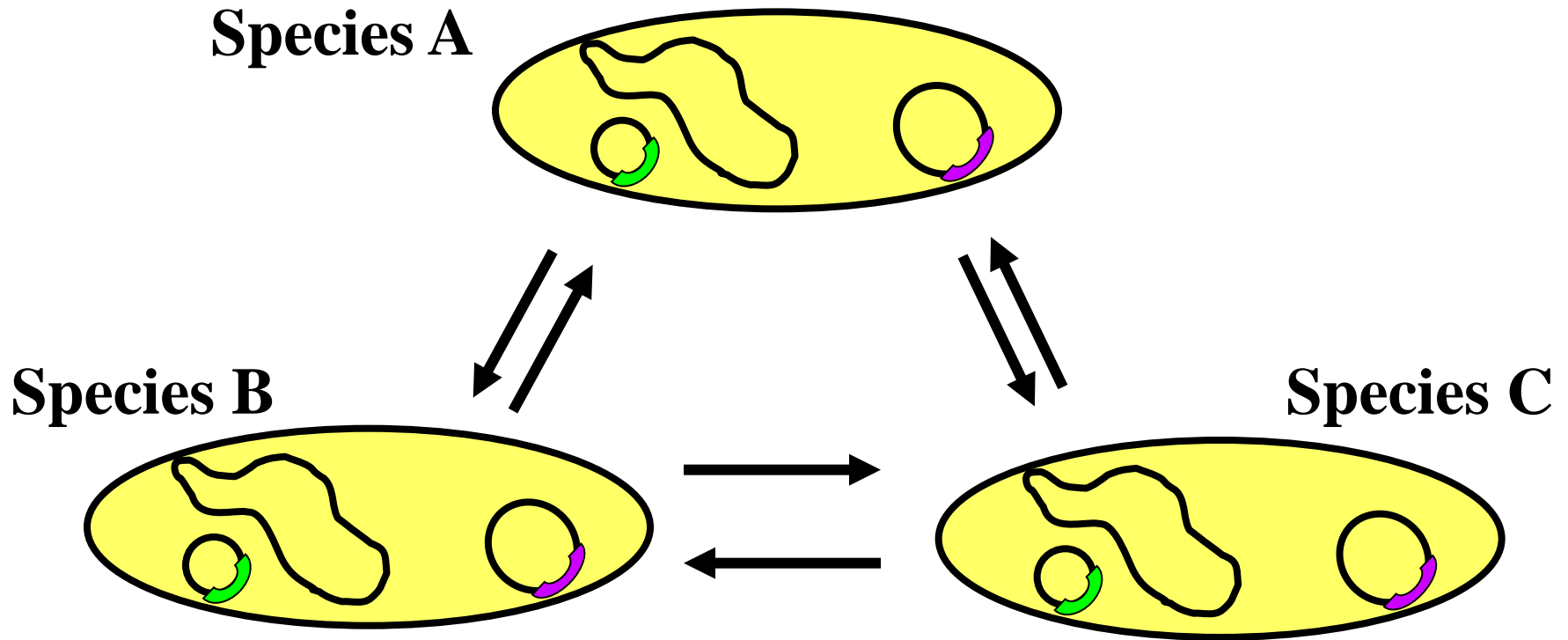


Hop-resistance genes, *horA* and *horC*, seem to be acquired through horizontal gene transfer.

What is horizontal gene transfer?



Interspecies exchanges of hop-resistance genes



 Hop resistance genes, such as
 *horA* and *horC*.

Comprehensive detection of beer spoilage LAB

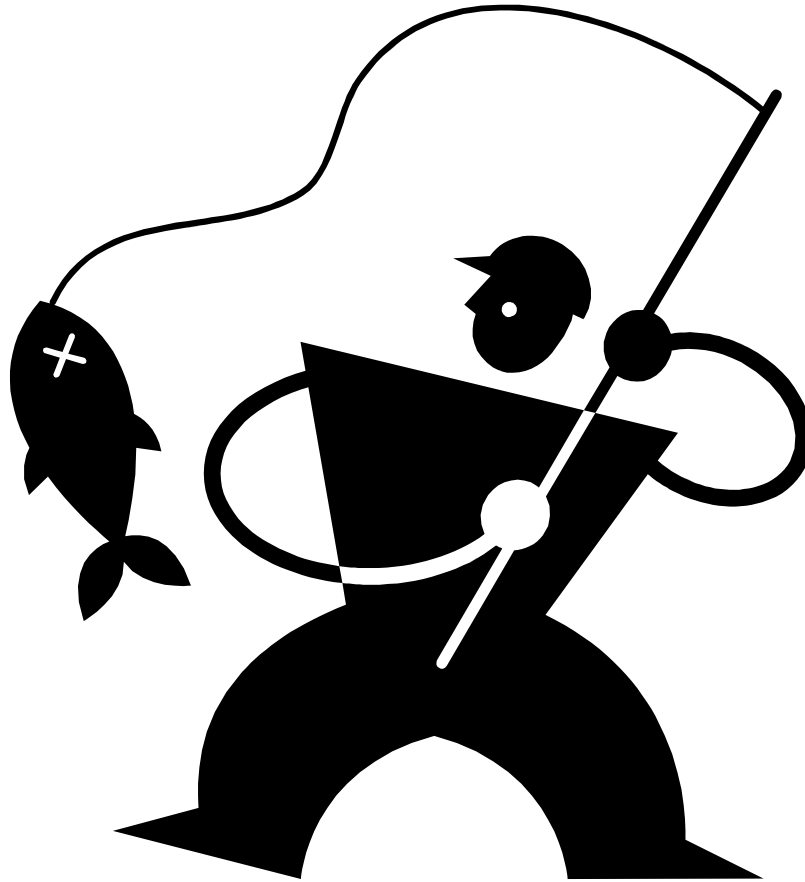
***horA* positive: 93.2% (Total 82 strains)**
***L. brevis* 35, *L. paracollinoides* 28,
L. lindneri 9, *L. backi* 2, *Ped. damnosus* 8**

***horC* positive: 97.7% (Total 86 strains)**
***L. brevis* 35, *L. paracollinoides* 30,
L. lindneri 10, *L. backi* 3, *Ped. damnosus* 8**

***horA* or *horC*: 100% (Total 88 strains)**
***L. brevis* 36, *L. paracollinoides* 30,
L. lindneri 11, *L. backi* 3, *Ped. damnosus* 8**

 Positive strains
 Negative strains

We can target these genetic markers for determining spoilage ability of LAB!



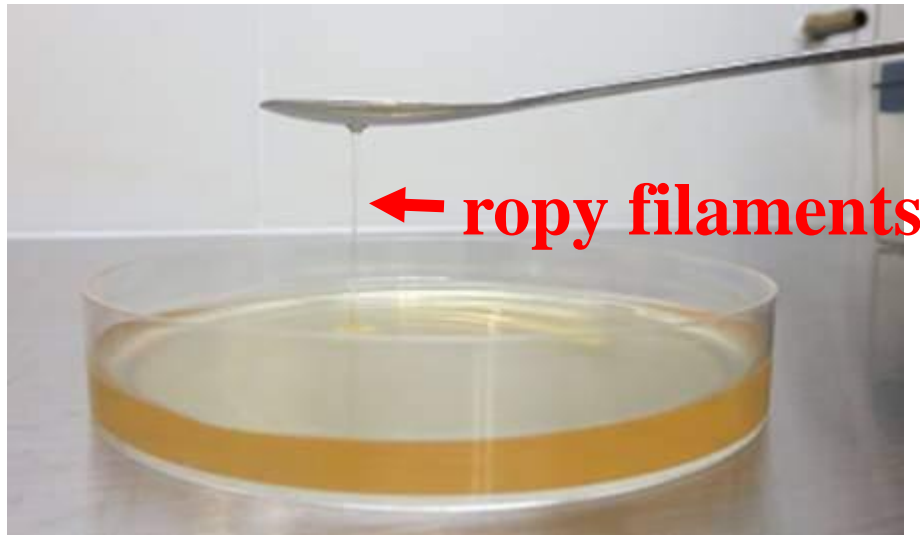
Seven new LAB species discovered since 2000 have been shown to carry *horA* or *horC*.

Another new threat?

Spread of a new type of beer spoilage LAB

LAB with ropy phenotype

**Beer becomes viscous and slimy like a jelly.
This is called ropiness in the brewing industry.**

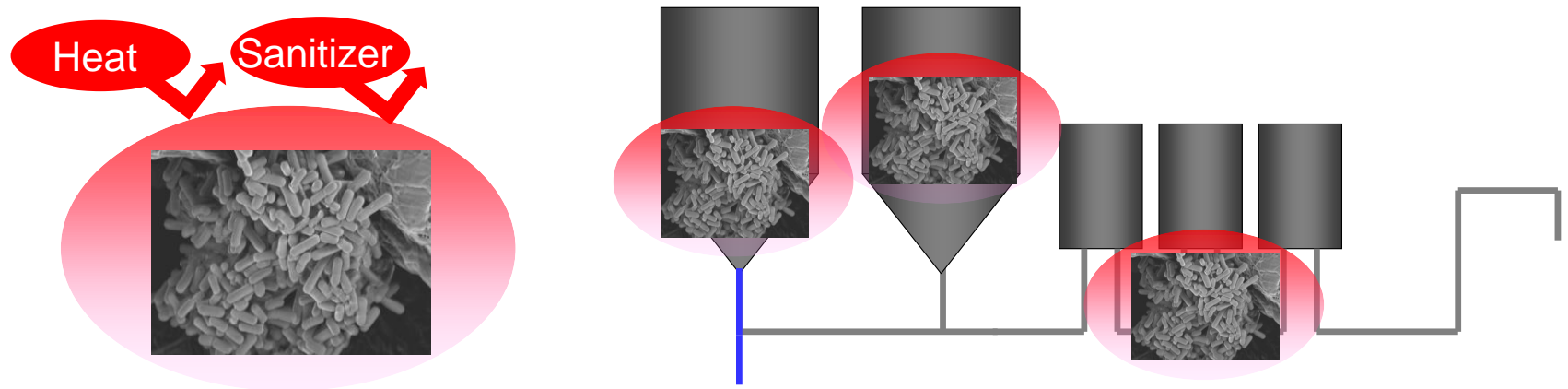


The ropiness used to be rare but this type of spoilage seems to be on the rise in Europe.

Emergence of a new type of LAB

Ropy LAB with extracellular polysaccharides (EPS)

- EPS acts as a protective barrier against various stress factors.
- High resistance against heat (up to 25PU) and sanitizers.



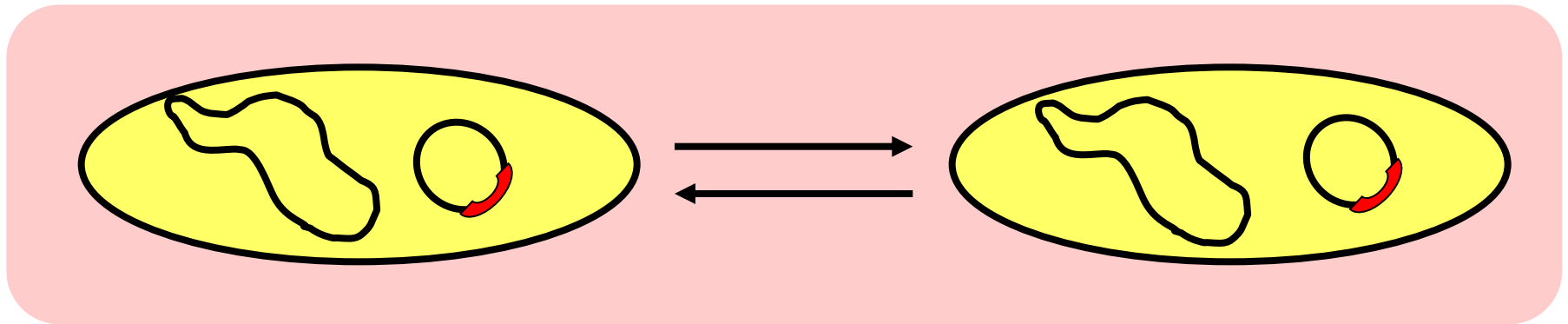
One of the most difficult beer spoilers to eradicate !!!

Discrimination of EPS-producing LAB is important.

Interspecies exchanges of slime-producing genes

Beer spoilage
L. brevis

Beer spoilage
Ped. damnosus
Ped. claussenii

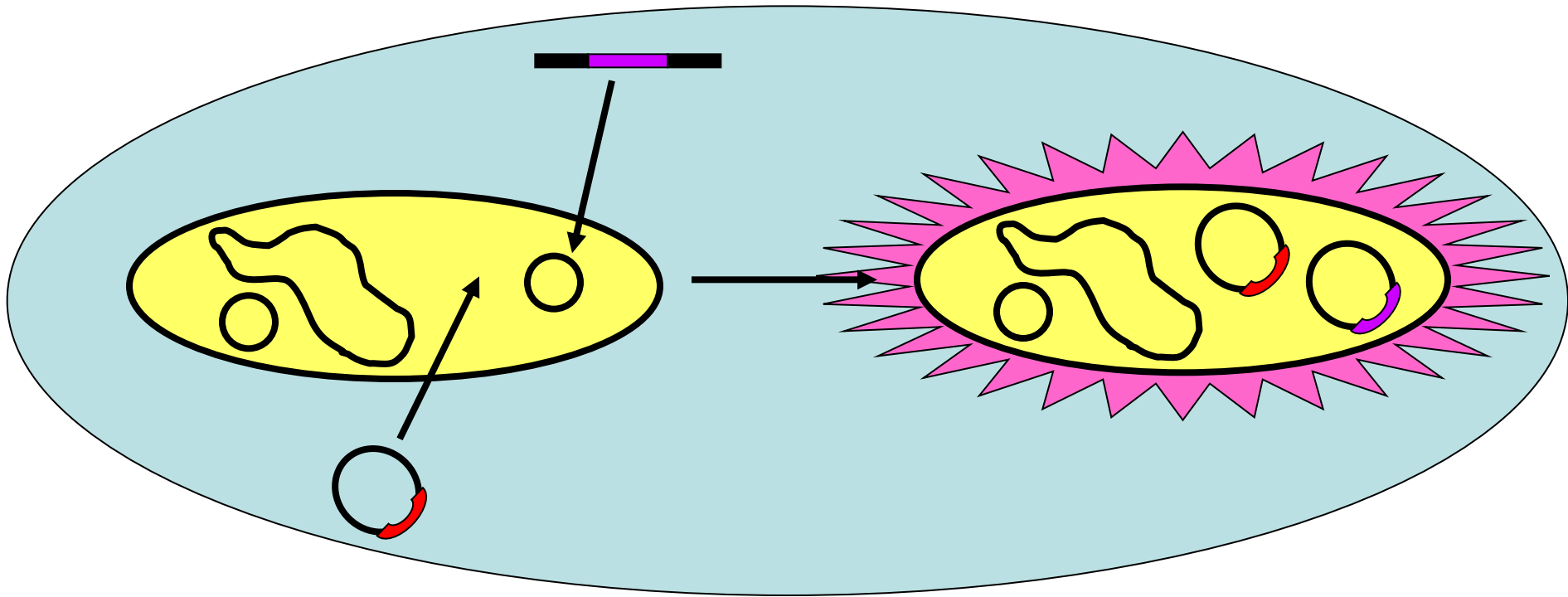


 *gtf* (glycosyltransferase) gene

Different beer spoilage LAB species possess the *gtf* genes with 98 - 99% nucleotide identities.

Beer spoilage LAB in evolution

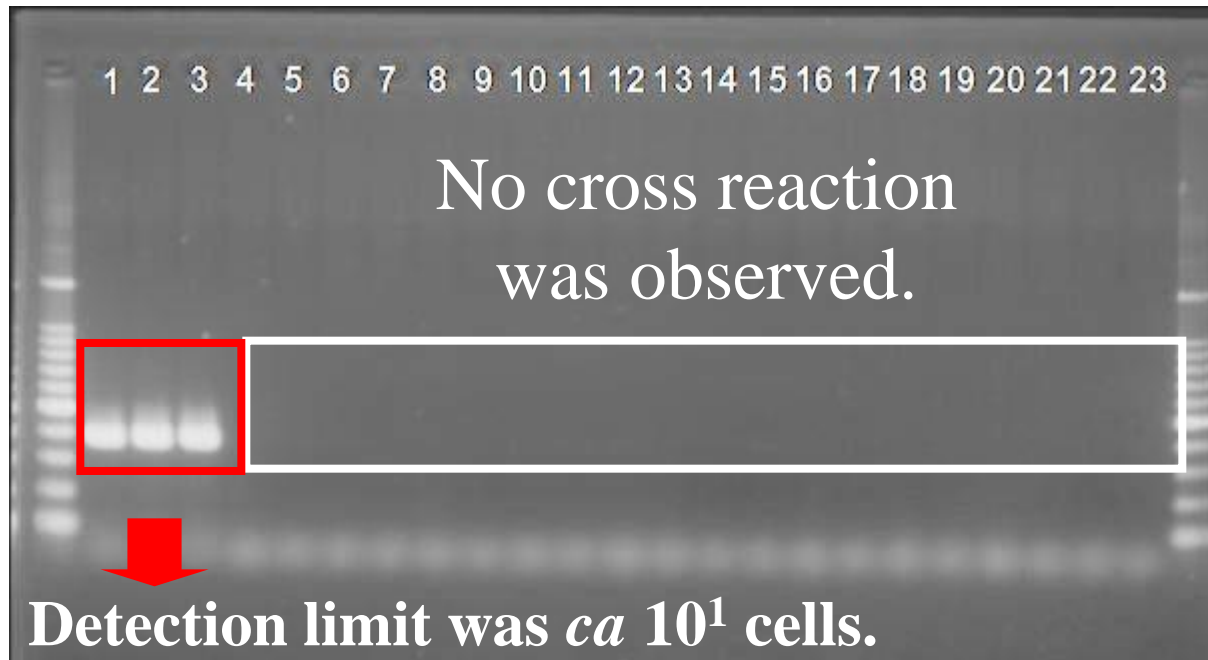
- Hypothesis -



Acquisitions of advantageous genes

**Adaptive processes to counteract human efforts,
such as pasteurization and CIP?**

Evaluation of specificity



1. Ropy *L. brevis*
2. Ropy *Ped. damnosus*
3. Ropy *Ped. claussenii*
- 4-22. Non-ropy strains of beer spoilage LAB

The *gtf*-specific PCR was shown to detect ropy LAB strains specifically, independent of species.

The case for optimism ?



**Beer jelly,
anyone ???**

Immunostimulatory action

Anti-tumor effect

Cholesterol reduction

EPS produced by LAB have several health benefits !

Summary



- 1. Hard-to-culture strains can be detected by using beer-based media or the pressure cycling technology.**
- 2. New beer spoilage LAB species can be identified by targeting hop resistant genes, such as *horA* and *horC*.**
- 3. New threats are constantly emerging and we need to deal with them.**

This year we are celebrating 21st anniversary of no microbiological incidents by Japanese major brewers !

*Thank you for
your kind attention !*