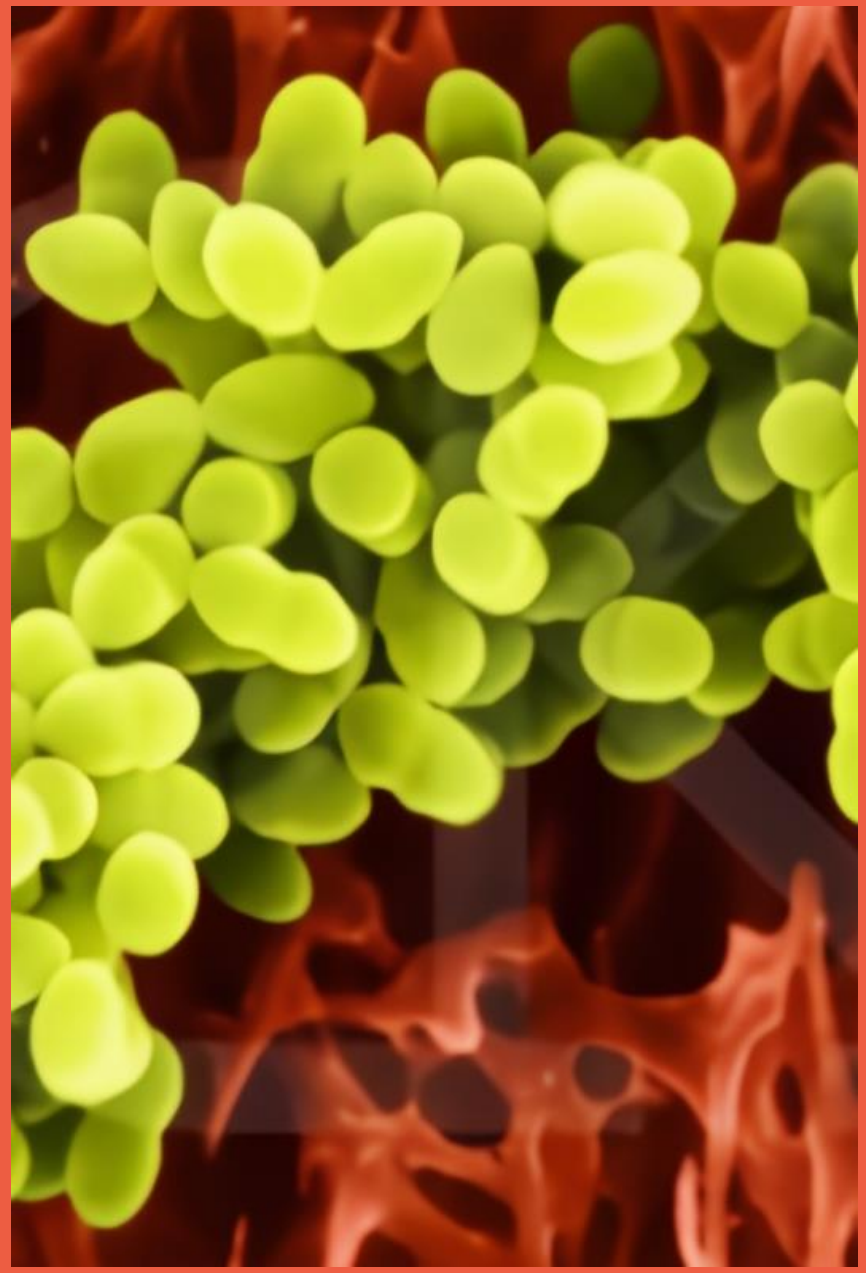
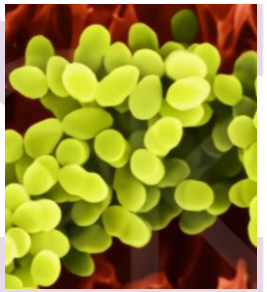
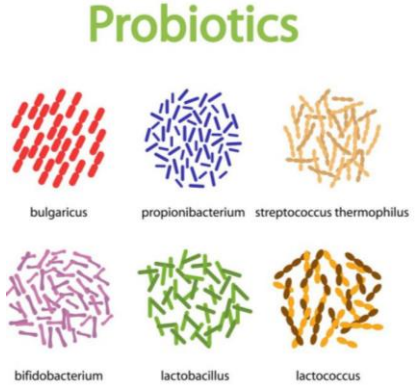
Lactococcus lactis, a promising and efficient factory for bioproduction of functional proteins

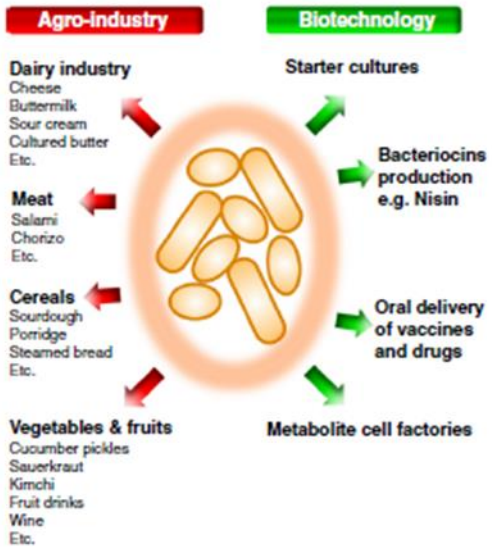
Annie FRELET-BARRAND



The Gram^{+} bacteria *Lactococcus lactis*







- Naturally present in cheese and fermented food
- Anaerobic facultative aerobic
- Culture in fermentation +/- respiration; Optimal growth at 30°C

- Model bacteria for fundamental research
- « Generally regarded as safe » by FDA (QPS by EFSA)

→ agro industry + biotechnology (fermented food)

- Intensive studies (physiology, genetics, genomics) → genome sequenced

- Production of heterologous proteins

→ Antigen/therapeutic protein delivery - **MEDECINE / HEALTH**

→ BIOTECHNOLOGY = Soluble and membrane proteins (MPs)

- Promoter-based expression (Pontes *et al.*, 2011)

Strategies for gene cloning & expression

- Classical : between *Nco*I (*N*-ter) and another site of the MCS (*C*-ter) (Kunji *et al.*, 2003)
- Other strategies :
  - LIC (ligation independent cloning) and VBEx (vector backbone exchange) (Geerstma and Poolman, 2007)
  - Compatible with Gateway (Frelet-Barrand *et al.*, 2010)
  - Lactococcus vectors with Gateway cassettes for expression of soluble proteins (Douillard *et al.*, 2010)
  - Vectors for co-expression (Berlec et al., 2018, 2021)

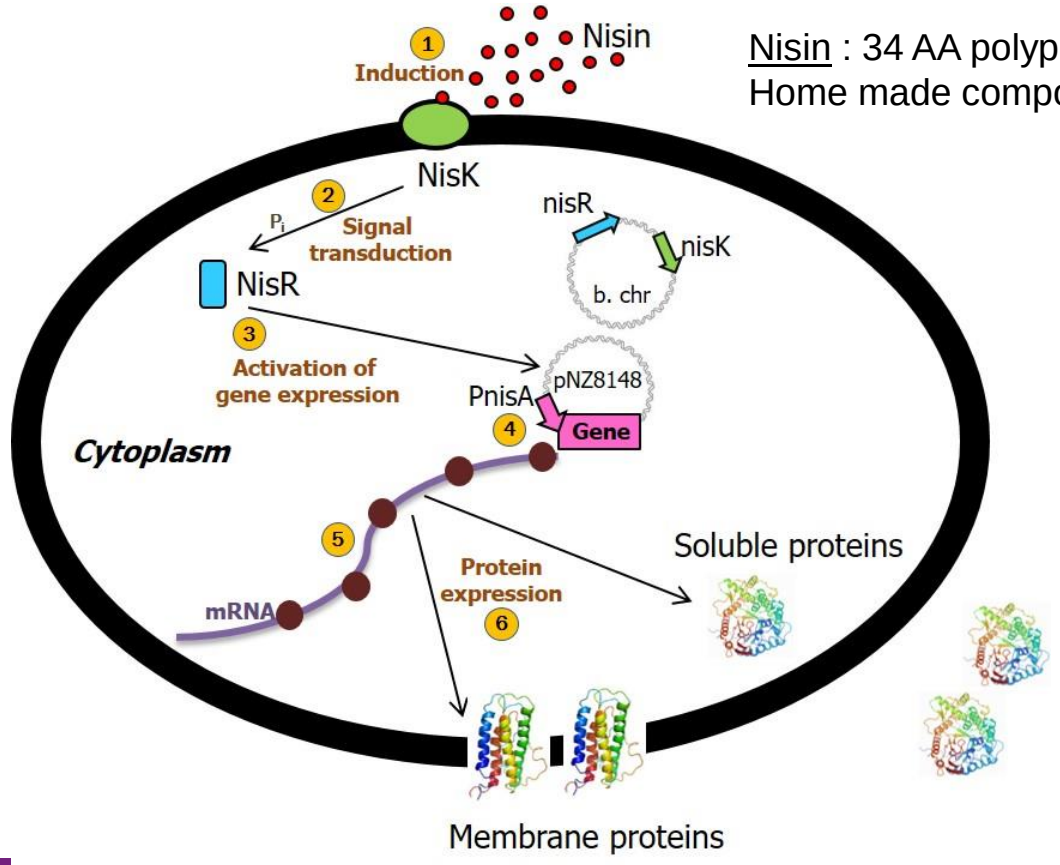
- Evolved *L. lactis* strains: fusion of GFP and antibiotic resistance (Linares et al., J Mol Biol, 2010)
- New strain: *L. lactis M4* (Noreen *et al.*, *Microb Cell Fact*, 2011)

Strains and plasmid

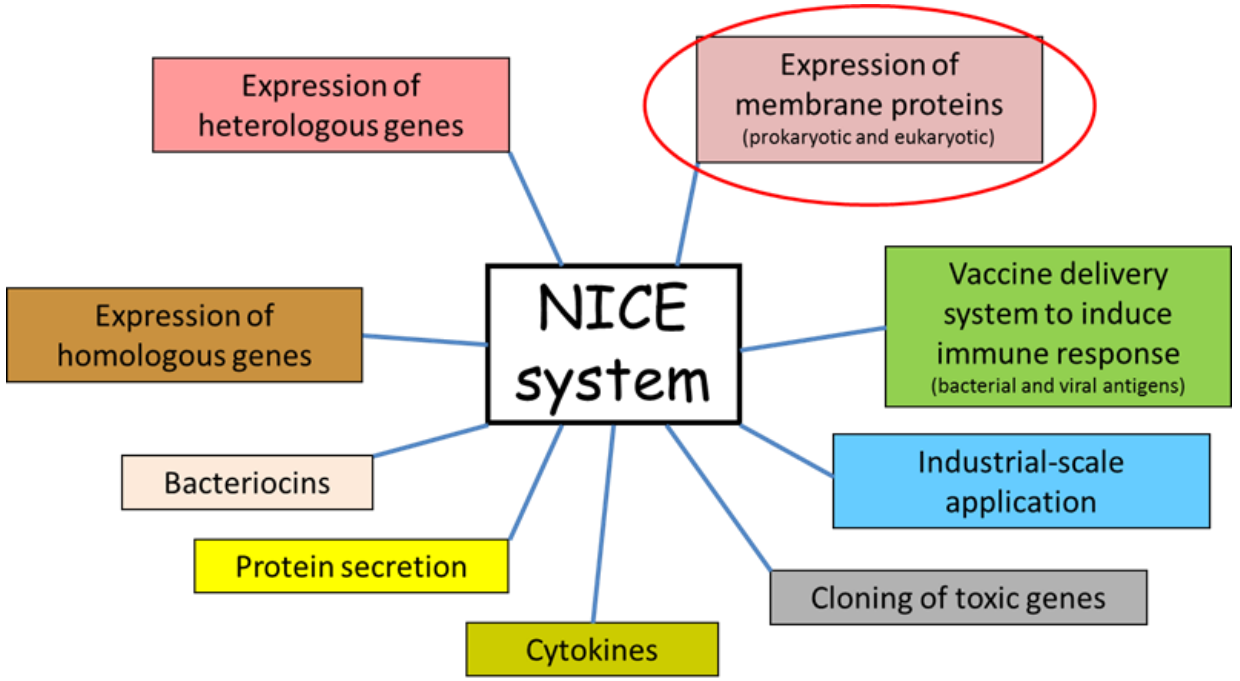
- NIZO strains
  - *Lactococcus lactis* NZ9700 : nisin producing strain
  - *Lactococcus lactis* NZ9000 : model strain MG1363 with *nis* R and *nis* K genes integrated in the chromosome. Host strain for nisin inducible vectors
- Plasmid
  - pNZ8148: vector with *cat*-gene, *nisA*-promoter and small multicloning site with *Nco*I site for translational fusions
    - Low copy compared to *E. coli* vectors

Versatile and tightly controlled NICE system to express proteins

NICE : Nisin Induced Controlled gene Expression



Nisin : 34 AA polypeptide antibiotic // Induction with subinhibitory concentrations
Home made compound



Frelet-Barrand, 2022, MDPI Biomolecules

Lactococcus lactis

for soluble protein expression

Table 1 List of industrial enzymes and compounds produced from various *Lactococcus lactis* strains

Industrial type & products	Applications/functions	*Lactococcus lactis* strain
Compounds		
Lactic acid	Preservative, flavouring, polylactic acid, plastic, emulsifier, moisturizer	All strains
Acetoin/diacetyl	Flavouring	CRL264
L-alanine	Sweetener	$AlaDH^{+}LDH^{-}$
Linalool	Flavouring	NZ9000
Germacrene D	Antimicrobial, insecticidal, pheromones	NZ9000
β-Sesquiphellandrene	Antimicrobial, antioxidant, anticancer	NZ9000
Hyaluronic acid	Cosmetics, medical	NZ9020
Vitamins		
Folate (B11)	Health supplements	NZ9000
Riboflavin (B12)	Health supplements	NZ9000
Biofuels		
Ethanol	Energy source	CS4435
Peptides		
Bacteriocin	Anti-microbial, preservative	NZ9000
Brazzein	Sweetener	*N/S*
Mabinlin II	Sweetener	*N/S*
Nisin Z	Food preservative	F44
Enzymes		
β-Cyclodextrin glucanotransferase	Starch degradation	NZ9000
Coumarate CoA ligase (4CL)	Metabolic engineering	FI9974
Alcohol acyltransferase (SAAT)	Metabolic engineering	NZ9000
Linalool/nerolidol synthase (FaNES)	Metabolic engineering	NZ9000
Sesquiterpene synthase	Metabolic engineering	NZ9000
3-Hydroxy-3-methylglutaryl CoA reductase (HMGR)	Metabolic engineering	NZ9000
Bile salt hydrolase (BSH)	Intestinal metabolism, probiotics	NZ3900
Acid urease	Urea hydrolysis	*N/S*

N/S not specified

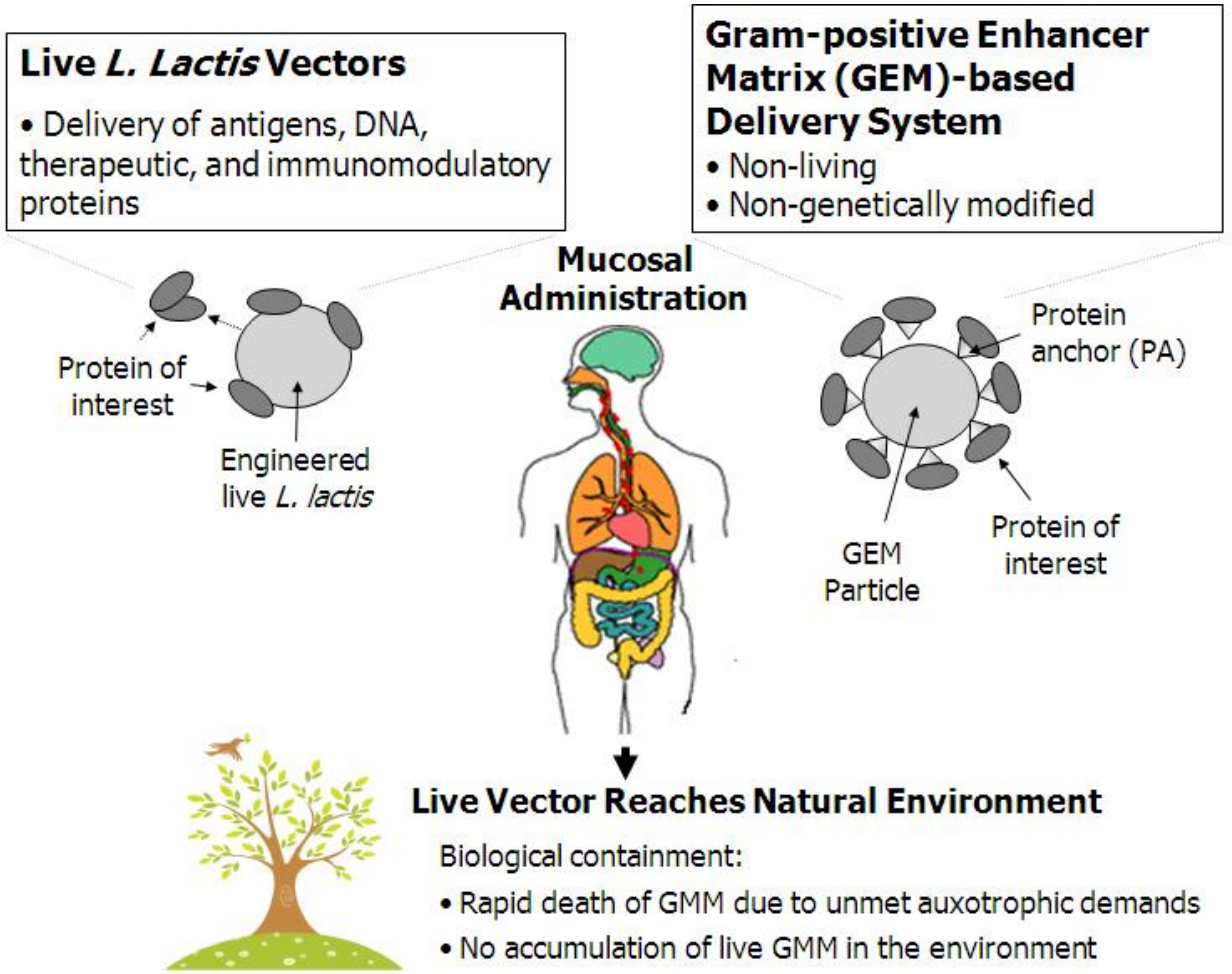
Table 2 Recombinant therapeutics produced from various *Lactococcus lactis* strains

Therapeutic type & products	Disorder/disease	*Lactococcus lactis* strain
Cytokines/ligands		
Interleukin-6 (IL-6)	Adjuvant	IL1403
Interleukin-10 (IL-10)	Adjuvant, hypersensitivity type I, inflammatory bowel disease (IBD)	*N/S*
Interleukin-12 (IL-12)	Adjuvant; hypersensitivity type I; asthma	NZ9000
Interleukin-18 (IL-18)	Adjuvant, immunomodulatory,	MG1363
Hemagglutinin-neuraminidase (HN) protein of NDV	Breast cancer	NZ9000
RANKL	Cancer vaccine adjuvant	IL1403
Transforming growth factor beta 1 (TGF-β1)	IBD	NZ9000
Epidermal growth factor (EGF) Trefoil factor 3 (TFF3)	Wound healing	NZ9000
Kisspeptin (KiSS 1)	Colorectal cancer	NZ9000
Insulin-like growth factor I (IGF-I)	Colitis	NZ9000
Allergens		
Peanut allergen (Ara 2)	Hypersensitivity type I	CHW9
Birch allergen (Bet v1)	Hypersensitivity type I	NZ9800
House dust mite allergen (Der p2)	Hypersensitivity type I	NZ9000
Enzymes		
Subtilisin QK-2	Thrombosis	NZ9000 & NZ3900
Heme oxygenase-1 (rmHO-1)	Acute colitis	NZ9000
Vaccines/antigens		
Tetanus toxin fragment C (TTFC)	Tetanus	UCP1054
HPV-16-E7	HPV-16 induced cancers	NZ9000
Pneumoccal antigen	Pneumococcal infections, meningitis	*N/S*
Listeriolysin O & mt Internalin A	Listeriosis	NZ9000
Glycosylated tyrosinase related protein-2 (TRP-2)	Skin cancer	MG1363
Carcinoembryonic antigen (CEA)	Colon cancer	NZ9000
Plasmodium falciparum recombinant antigen (R0.10C)	Malaria	*N/S*
Influenza virus nucleoprotein (NP)	Influenza	NZ9000
Shigella IpaB and IpaD	Shigellosis	PA1001
Neuraminidase (NA1)	Avian influenza H5N1	NZ3000
Hemagglutinin (HA1)	Avian influenza H5N1	NZ9000
Hemagglutinin (HA1)	Avian influenza H1N1	NZ9000
M2e antigen	Avian influenza H5N2	LM2301
IBV multi-epitope gene*EpiC*	Avian bronchitis	NZ3900
Campylobacter rCjaAD antigen	Avian gastroenteritis	IL1403
GroEL, heat-shock protein	Brucelosis	NZ9000
Cu–Zn SOD of *Brucella abortus*	Brucelosis	NZ9000
Mycobacterial ESAT-6 antigen	Tubercolosis	*N/S*
D1 and D4 aerolysin	*Aeromonas* spp. infection	Lac-D1ae
SiMA antigen	*Streptococcal* infection	BFE920
Myelin epitopes	Multiple sclerosis, encephalomyelitis	IBB360
T1D autoantigens	Type-1 diabetes mellitus	*N/S*
Enterohemorrhagic *Escherichia coli* (EHEC) antigen (EspB)	EHEC infection	*N/S*
Multi-urease epitopes (CTB-UE)	*Helicobacter pylori* infection	NZ9000
Helicobacter pylori hspA	*Helicobacter pylori* infection	NZ3900
HIV-1 Gag-p24	Human immunodeficiency virus (HIV) infection	*N/S*
Capsid protein of porcine circovirus type 2 (PCV2)	Swine circovirus associated disease	*N/S*

femto-st SCIENCES & TECHNOLOGIES

Song et al., 2017, MCF

Production of therapeutic proteins



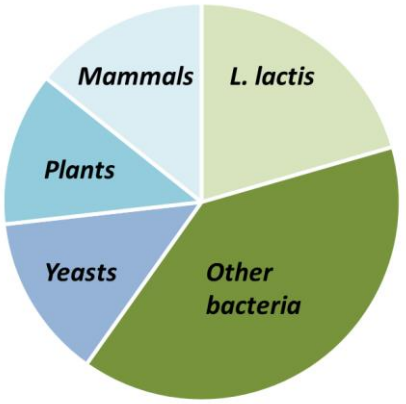
Production of viral and bacterial antigens, immunomodular & therapeutic proteins (interleukines, interferon, growth factors)

Bahey El Din, 2010, Discovery Medecine

L. lactis to overexpress membrane proteins (MP) (1)

In 2022,
115 MPs
expressed

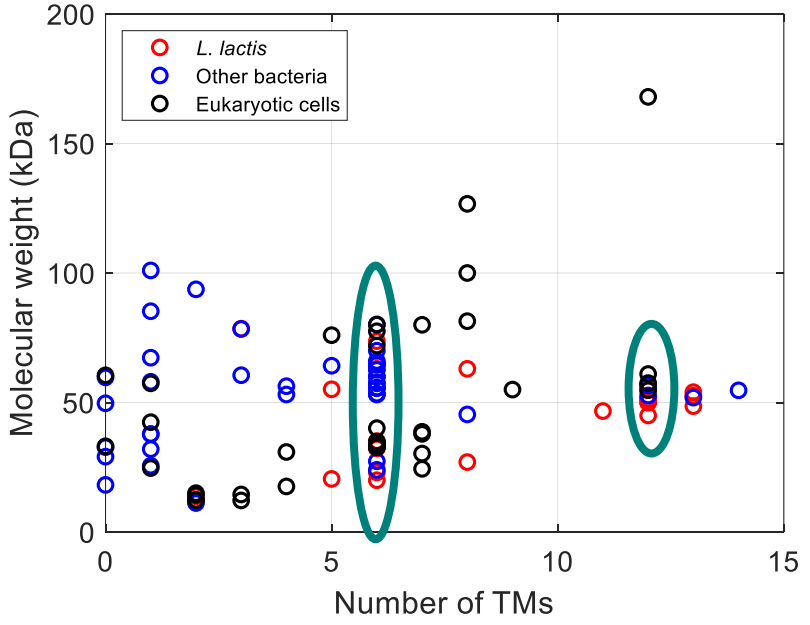
MPs expressed in *L. lactis* since 2003		
23	prokaryotic	*L. lactis*
45	prokaryotic	Other bacteria
47	eukaryotic	Yeast/plant/ human



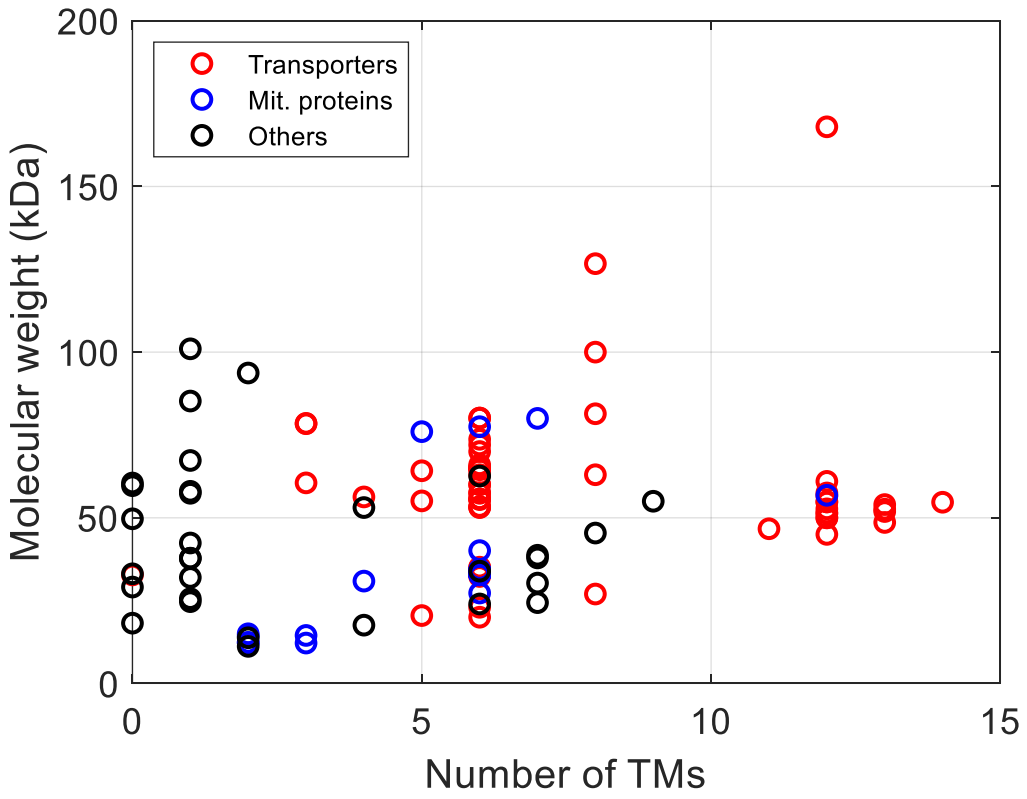
From 0 to 14 TM helices
From 12 to 170 kDa

2 main clusters

In their native oligomeric state



L. lactis to overexpress MPs (2)



Mainly transporters including the large family of known ABC transporters

Some mitochondrial proteins, mainly from *S. cerevisiae*

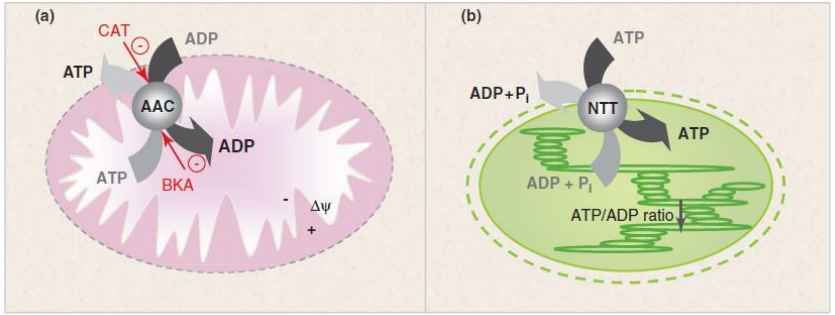
Linked to the bacteria lipid composition

- Yields : 0.1 to 30% of Total Membrane Proteins
- Yield = 10 times less than *E. coli*

Frelet-Barrand, 2022, MDPI Biomolecules; Bernaudat *et al.*, *Plos One*, 2011; Marreddy *et al.*, *Plos One*, 2010

Chloroplast ATP/ADP translocator (NTT1)

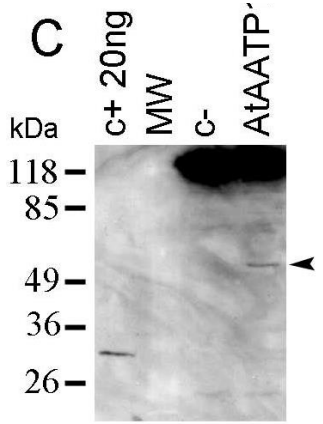
- 11-12 TMD, 60 kDa, chloroplast transit sequence (79 aa) in plants and bacteria (rickettsia, chlamydia)
- Exchange ATP against ADP and Pi (Pi : co-substrate of ADP)
  - ► import of ATP into chloroplast (b) versus AAC and mitochondria (a)

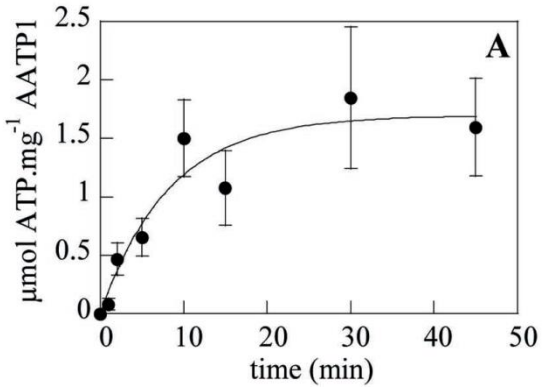




Functional tests in *L. lactis*

Even produced at 0.2% TMP, NTT1 is active and transports ATP

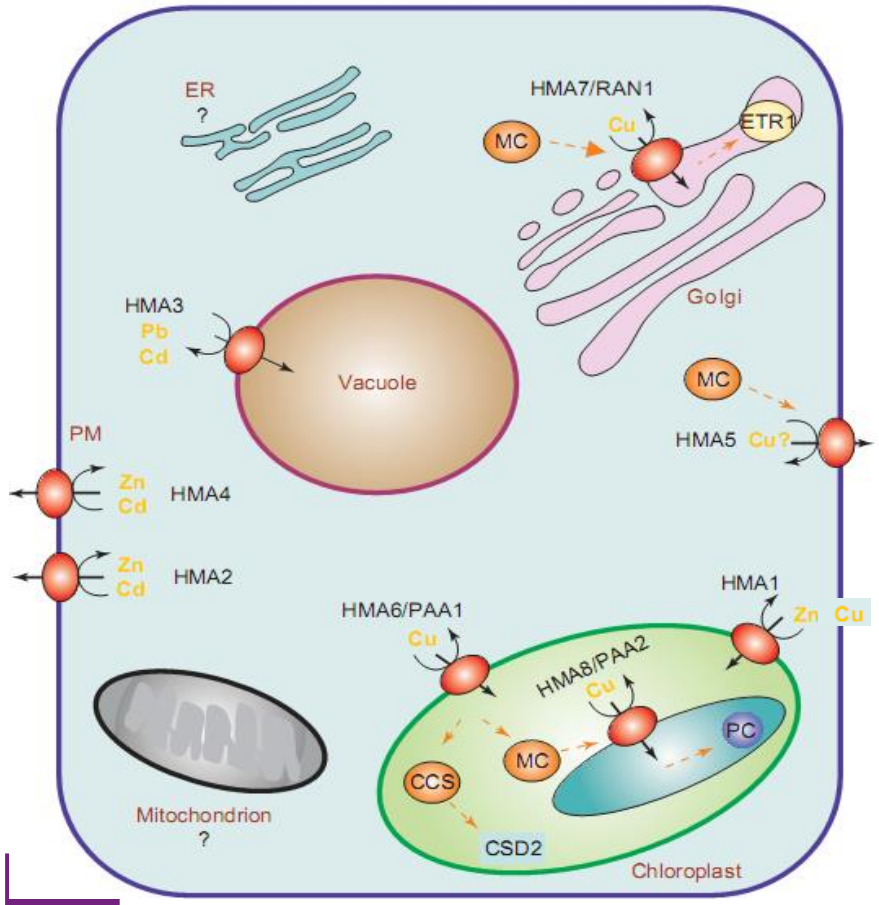




Haferkamp et al., Trends Biochem Sci, 2011; Frelet-Barrand et al., Plos One, 2010

Plant P1B-ATPases

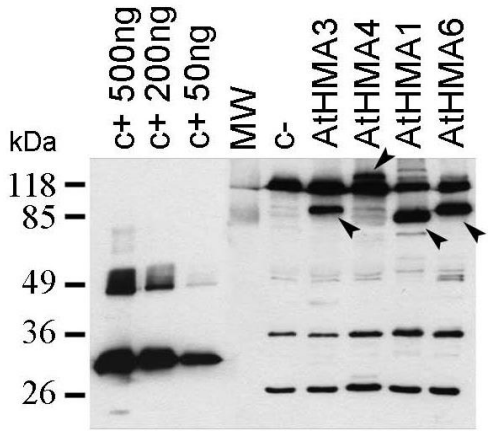
Arabidopsis thaliana possesses 8 P_{1B}-ATPases involved in heavy metal transport (Cu, Zn, Co, Ag, Pb, Cd).

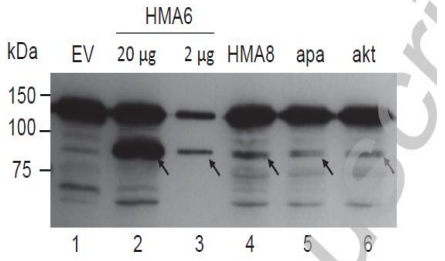


→ Source of energy : ATP.

→ 6-10 predicted helices / 80-130 kDa

Expression yields : <0,5-3% TMP







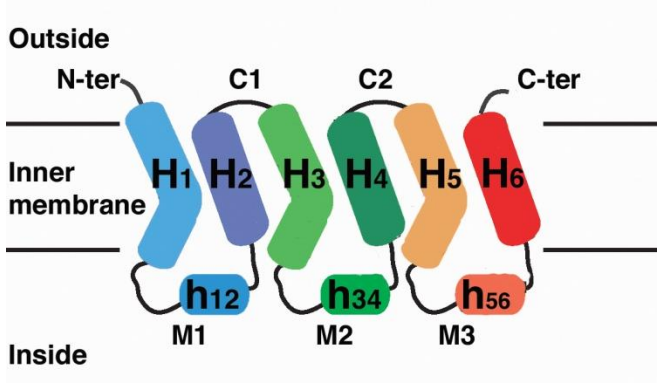
Functional tests

- AtHMA6 : high affinity Cu(I) transporter
- AtHMA8 : higher affinity transporter for Cu (vs AtHMA6) + PC-dependent turnover rate

Frelet-Barrand et al., Plos One, 2010; Catty et al., 2011; Sautron et al., 2015

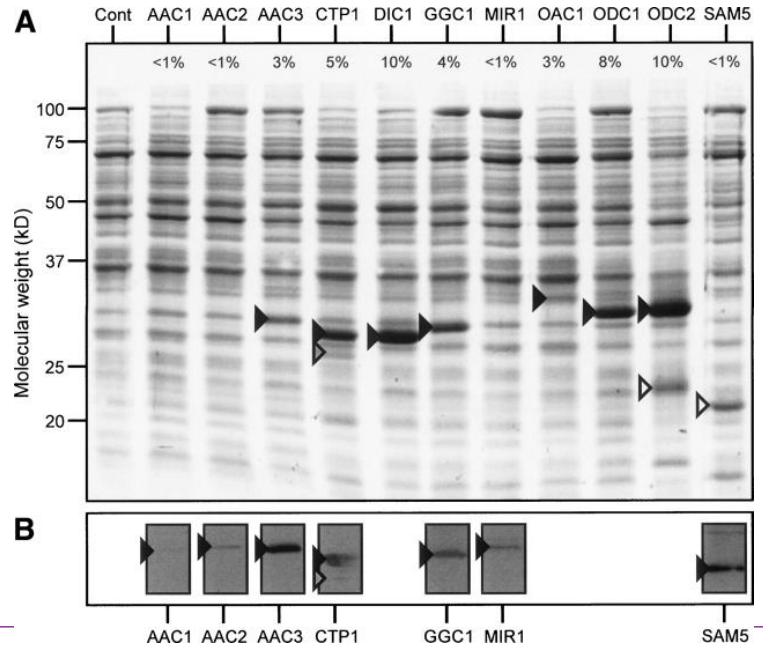
Mitochondrial carriers

- 6 TMD, 30 kDa
- Eukaryotes exclusively
- Different substrates (nucleotides, pyruvate…)



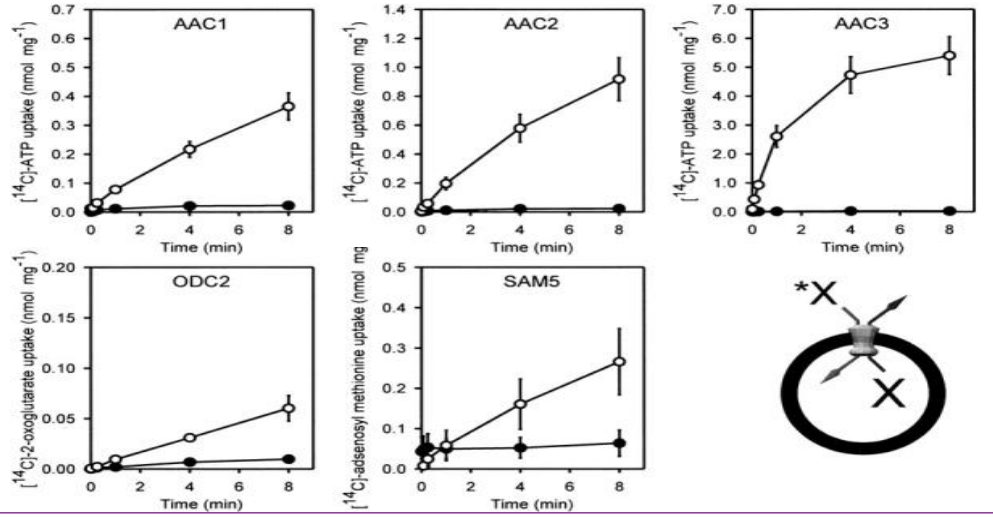
Expression

From 1 to 10% TMP (monomer/heterodimer)



Functional tests in *L. lactis*

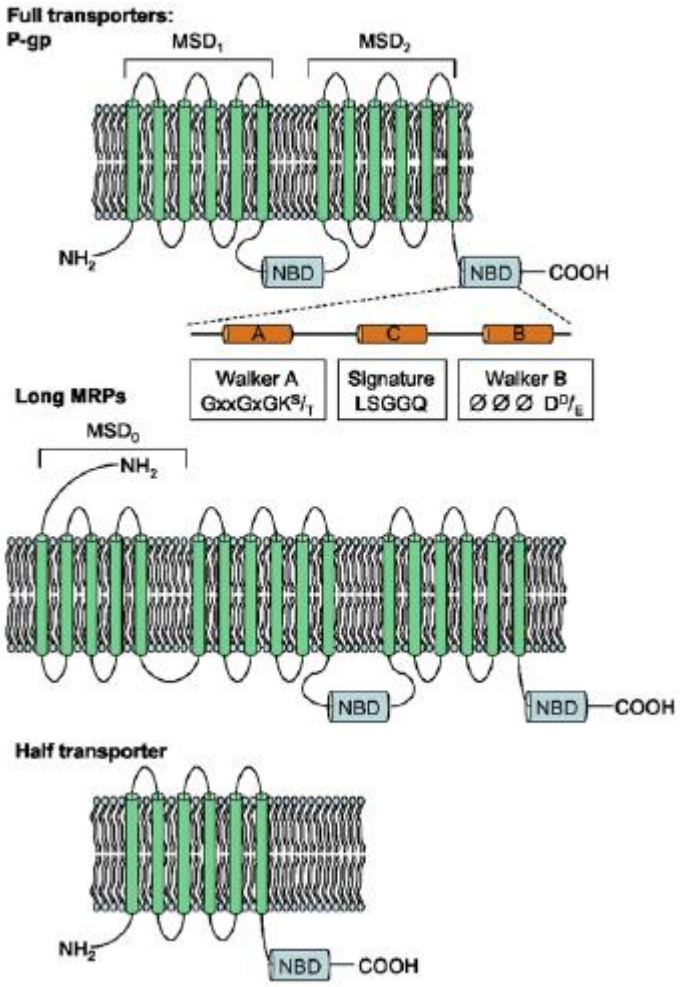
On whole cells/proteoliposomes
with specific substrates (non)-radioactive



Monné et al., 2005; Herzig et al., 2012; Vest et al., 2013

ABC transporters: characteristics

- ✓ ABC – A to G
- ✓ Half/full-transporters
- ✓ Homo or hetero-dimeric proteins
- ✓ 1 ATP-binding cassette: ATP binding and hydrolysis
- ✓ Present in all studied organisms
- ✓ Many different transport systems described
- ✓ ABCB/C:
  - responsible for drug resistance
  - involved in catabolite and toxic xenobiotics in mammal liver
- ✓ In human, 2 well-known ABC transporters : CFTR and SUR (ionic channels)



Fukuda and Schuetz, Biochem Pharmacol, 2012

Functional characterization of ABC transporters

- ✓ ABC transporters :
  - ✓ LmrA (Hellmich *et al.*, 2012) – EPR + NMR
  - ✓ MsbA and Sav1866 (Doshi *et al.*, 2013; Velamakanni et al., 2008)
  - ✓ Human ABCG2 (Janvilisri *et al.*, 2005)
  - ✓ Heterodimer TM287/288 (Hohl *et al.*, 2012)

- ✓ Strain NZ9000 ΔLmrA ΔLmrCD (deletion of homologues of ABC transporters)

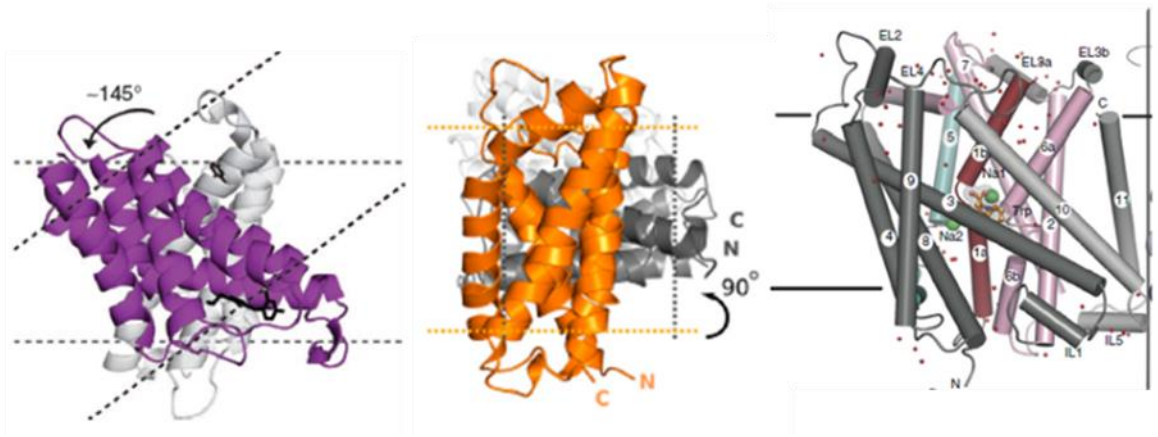
- ✓ Measurements:
  - Transport /ATPase activity/ATP binding
  - In bacteria, vesicles and proteoliposomes

- ✓ Reconstitution of homo/hetero-dimers after co-expression of proteins

→ Advantage over *E. coli* for functional characterization

Lactococcus lactis for 3D structures of MPs

28



ABC transporters

Secondary transporters (folate; thiamine; glutamine)

ATP Mg/Pi carrier

Lactococcus lactis vs *E. coli*

- A tightly regulated system = NICE → expression of toxic gene products
- Functional expression and right localization of soluble and MPs without leader sequence
- Only 1 glycolipid-rich membrane ↔ integration of functional proteins → functional studies (whole-cell/membrane vesicles)
- Easy labeling of proteins (auxotrophic metabolism); incorporation of selenomethionine
- No extracellular prote(in)ases
- Growth under fermentation or respiration
- Production of proteins in their native oligomeric state
- No endotoxins
- No formation of inclusion bodies

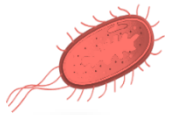
Superior to E. coli for :

- ✓ Production of proteins for food, feed and pharma (oral or topical use) applications
- ✓ Production of (food) ingredients, amino acids, poly-saccharides, flavours
- ✓ *In situ* production (food, GI tract [bioactive peptides and antigens])
- ✓ Co-factor independent biocatalysis

Expression of an eukaryotic MP = caveolin

ANR
CAVEOTANK
(2017-2021)





Escherichia coli

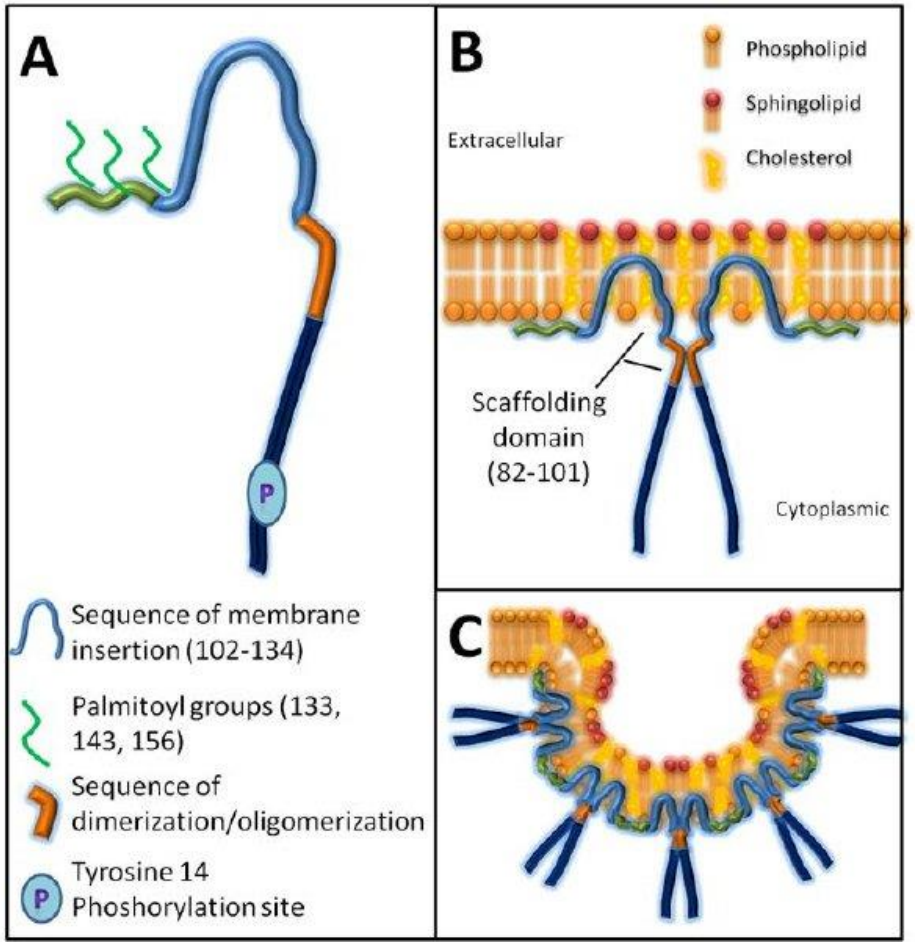


Insect cells S*f*21

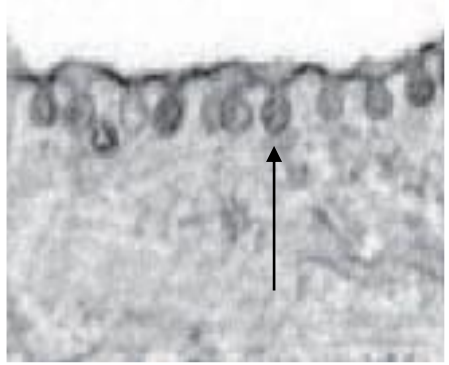


Lactococcus lactis

Context of the study – Expression of caveolin



- Caveolin (23 kDa)
- Functional oligomeric state
- Essential for caveolae formation

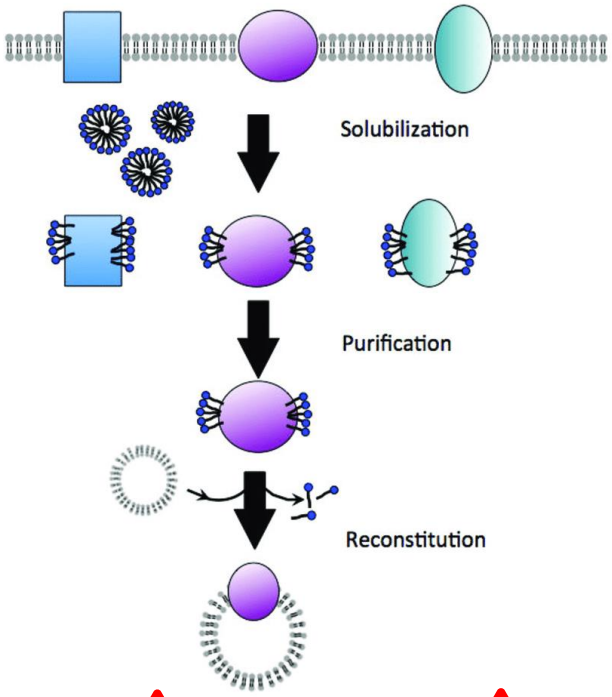


Caveolae in adipocytes

Lobos et al., 2011, DOI: 10.5772/21829/InTech

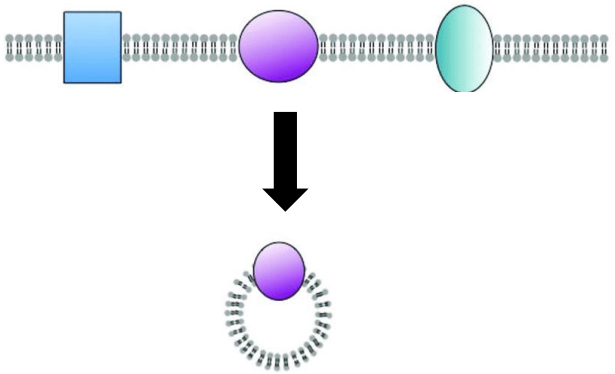
Functional characterizations of membrane proteins

Currently



caveole

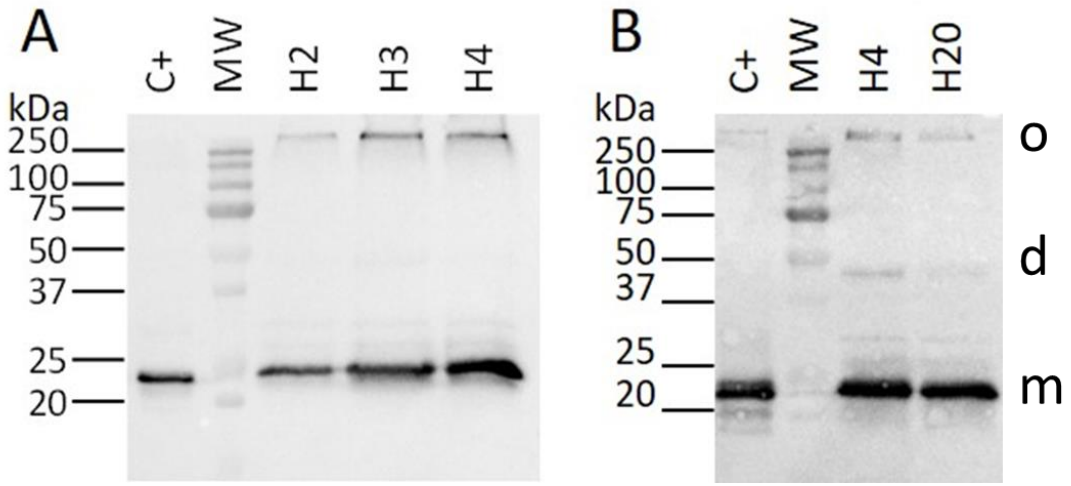
Simplification



MP within a more favorable native-like environment

Steps perturbing/denaturing protein = solubilisation/purification & proteoliposome reconstitution

Caveolin is expressed in *L. lactis* under its native form

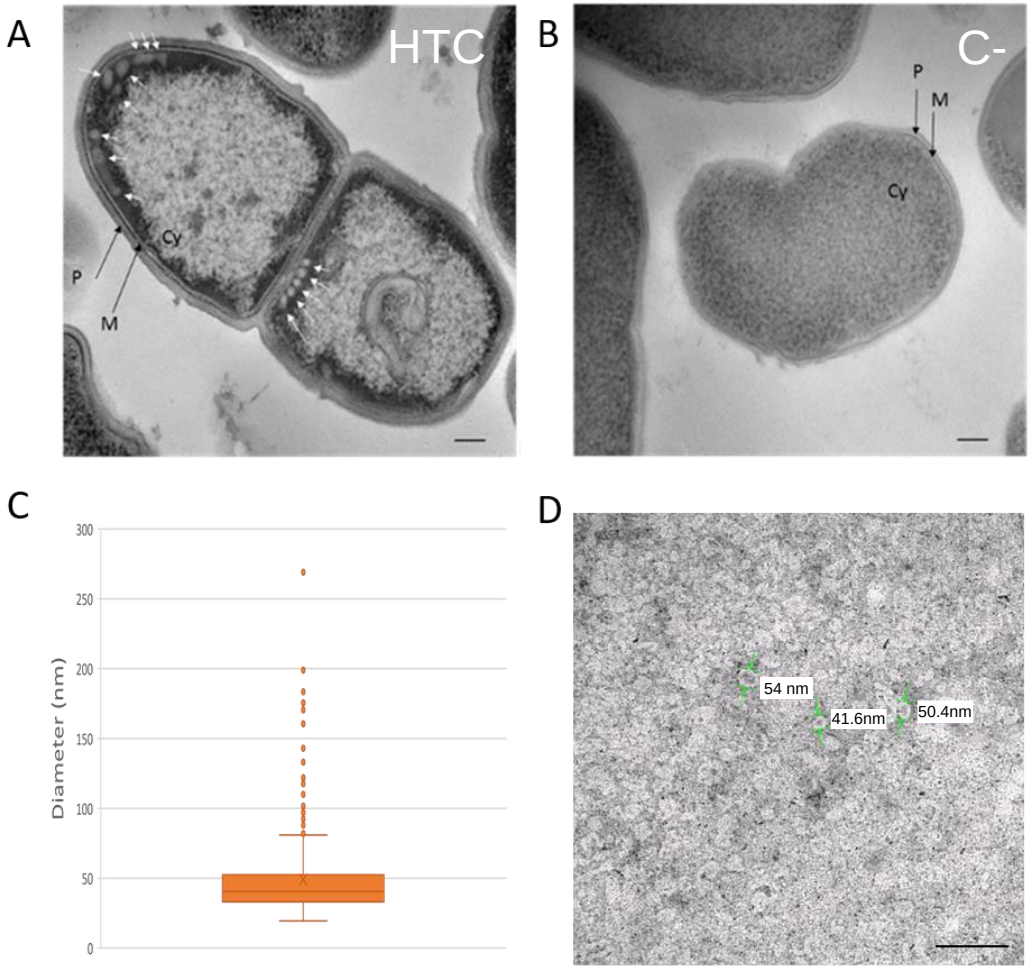


- Caveolin (23 kDa) expressed with maximum at 4h post induction
- At high yield = 25% of total membrane proteins
- Under its native oligomeric state
- Still present at relatively high yield after 20h

✓ *Lactococcus lactis* is able to express caveolin under its functional oligomeric state

Flourieusse et al., 2022, MCF

Microscopy revealed caveolae within *L. lactis* bacteria

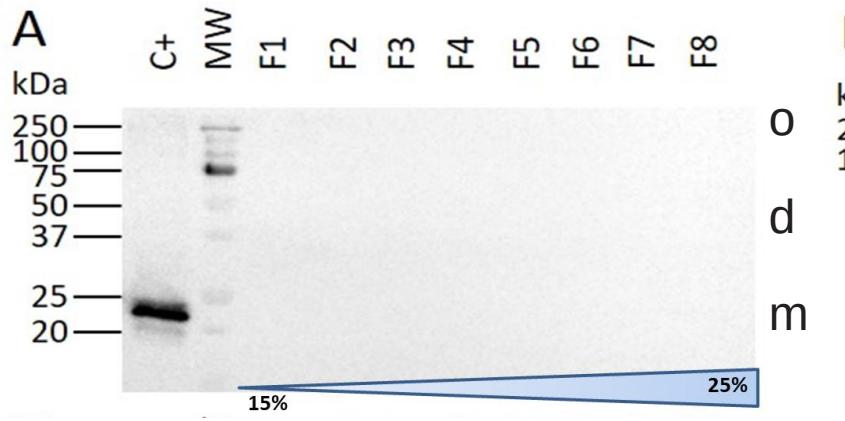


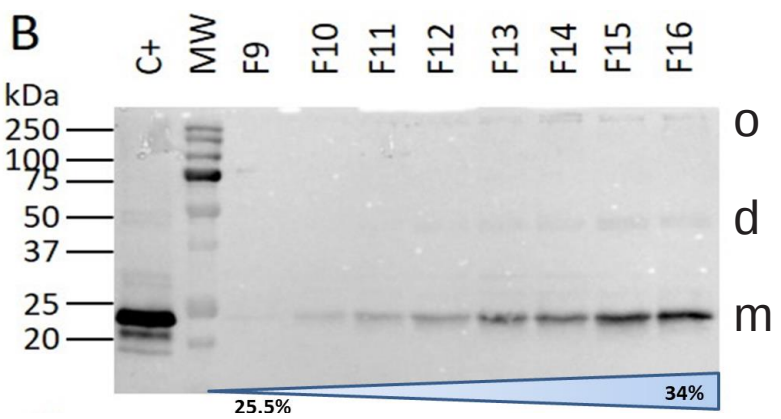
- Caveolae at the apex in HTC recombinant *L. lactis*
- *A*bsent in negative control
- Mean diameter = 40.2 nm (n=390)

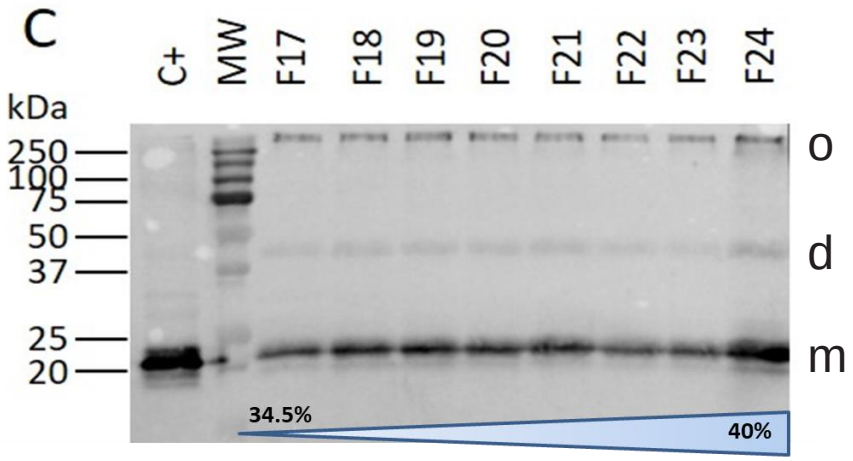
✓ *L. lactis* produce caveolae of 30 to 50 nm diameter

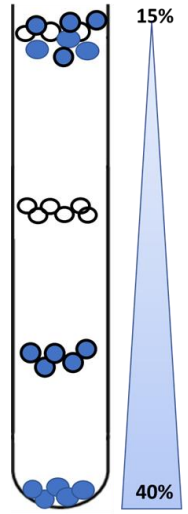
Flourieusse et al., 2022, MCF

Sucrose density gradient allowed caveolae isolation (1)







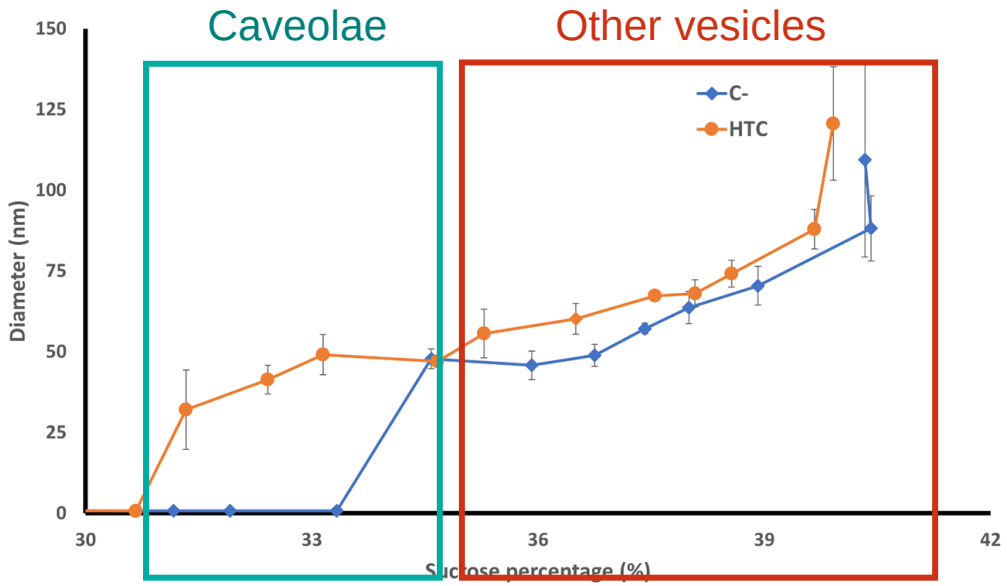


- Caveolin present in fractions 9 to 24
- Dimers and oligomers visible in fractions from 12 to 24

Flourieusse et al., 2022, MCF

Sucrose density gradient allowed caveolae isolation (2)

DLS analysis (N=3, n=9)



- No vesicles in fractions below 31%
- Light fractions (31 to 34.5%) : size comparable to caveolae, coïncidence of vesicles with appearance of caveolin oligomers on WB analysis
- Heavy fractions (> 35%): vesicles with higher diameter (> 50 nm)

- *Lactococcus lactis* is able to produce « heterologous caveolae » of 30-50 nm diameter

Flourieusse et al., 2022, MCF

In conclusion,

- ✓ *Lactococcus lactis* = promising, efficient host for expression of proteins including membrane proteins (of pharmaceutical interest)
- ✓ NICE system for tightly regulated expression system of proteins
- ✓ Functional and structural characterizations of proteins

Acknowledgements

CEA Grenoble

2006-2009

Post doctorate

iRTSV/LPCV

iBS/LPM & LIM





CEA Saclay

2009-2015

CNRS researcher

I2BC/B3S/LSOD

Institute FEMTO-ST (since 2015)

CNRS researcher

Dep. MN2S / Team BIND

Funding :
ANR Caveotank (2017-2021)