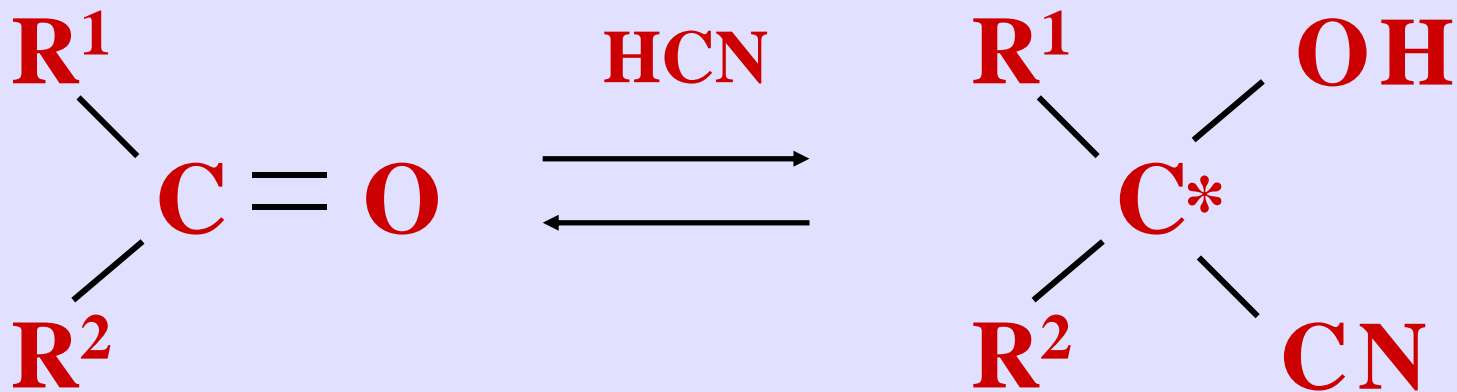


Hydroxynitrile lyase (Hnl)



S-selective: *Hevea brasiliensis*
R-selective: *Prunus spp.*

(S)-Hnl of *Hevea brasiliensis* and (R)-Hnl of *Prunus amygdalus*

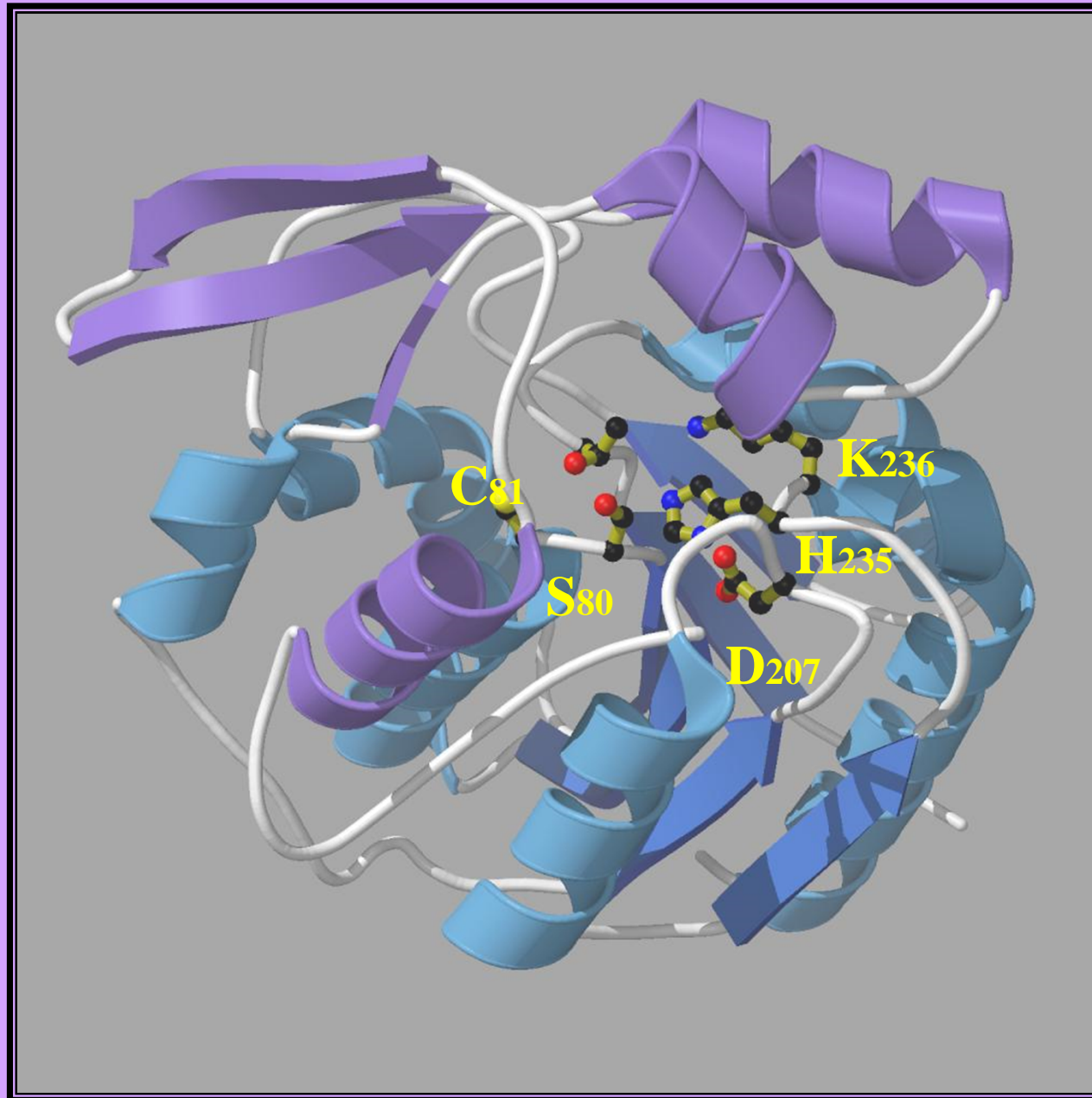
Hb_Hnl

- Type II Hnl
- intracellular protein
- 29.2 kDa
- homodimer
- α/β hydrolase fold protein
- catalytic triad
- (S)-selektive

Pam_Hnl

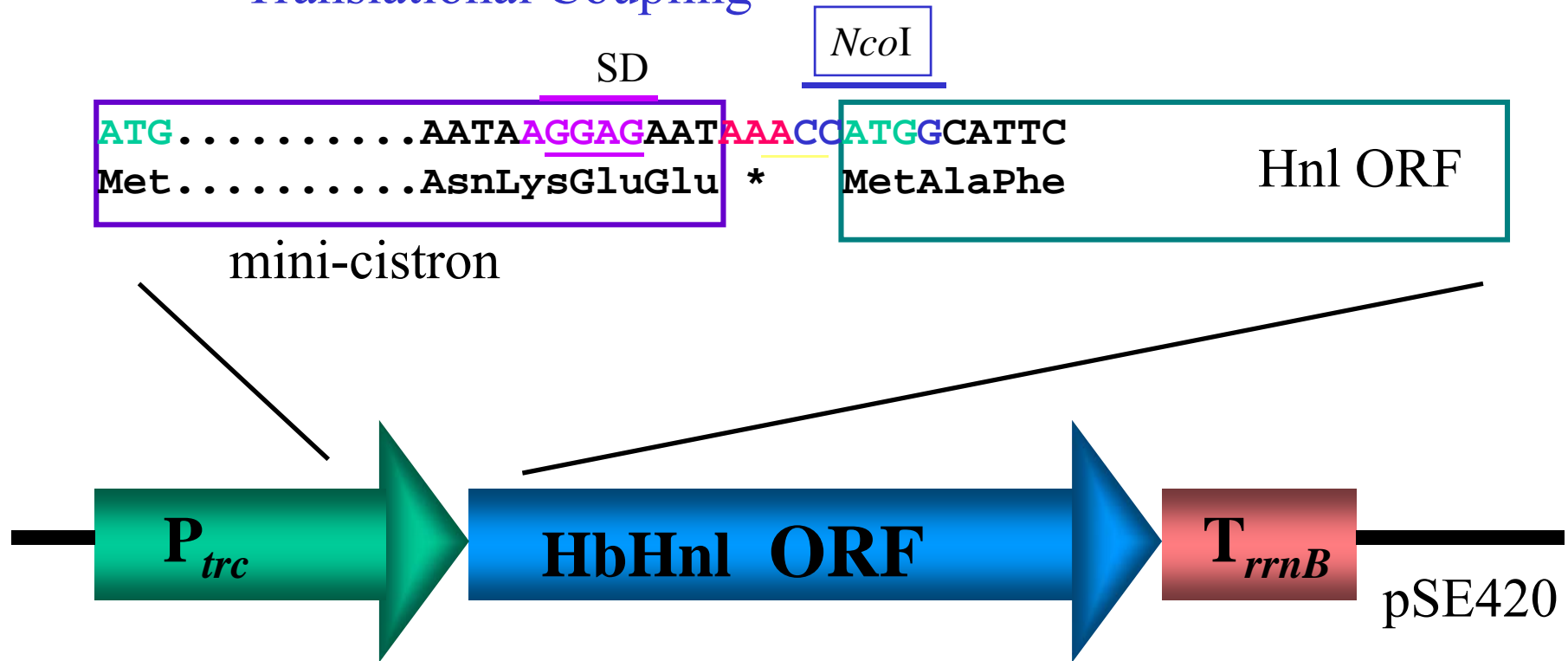
- Type I Hnl
- secretory protein
- 61 kDa (57.9 kDa)
- Homology to oxidases
- FAD
- N-glycosylated
- isoenzymes
- (R)-selektive

3-D structure of *Hb_HNL*



Intracellular Hnl Expression in *Escherichia coli*

Translational Coupling



pHNL-200

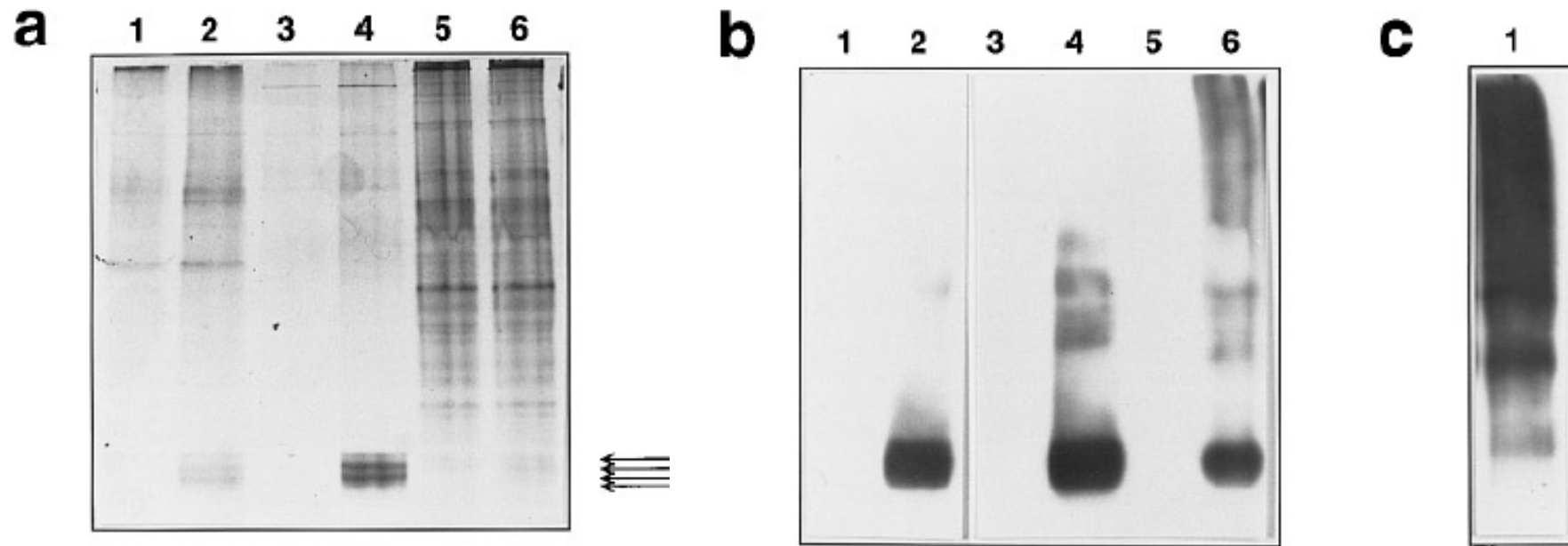
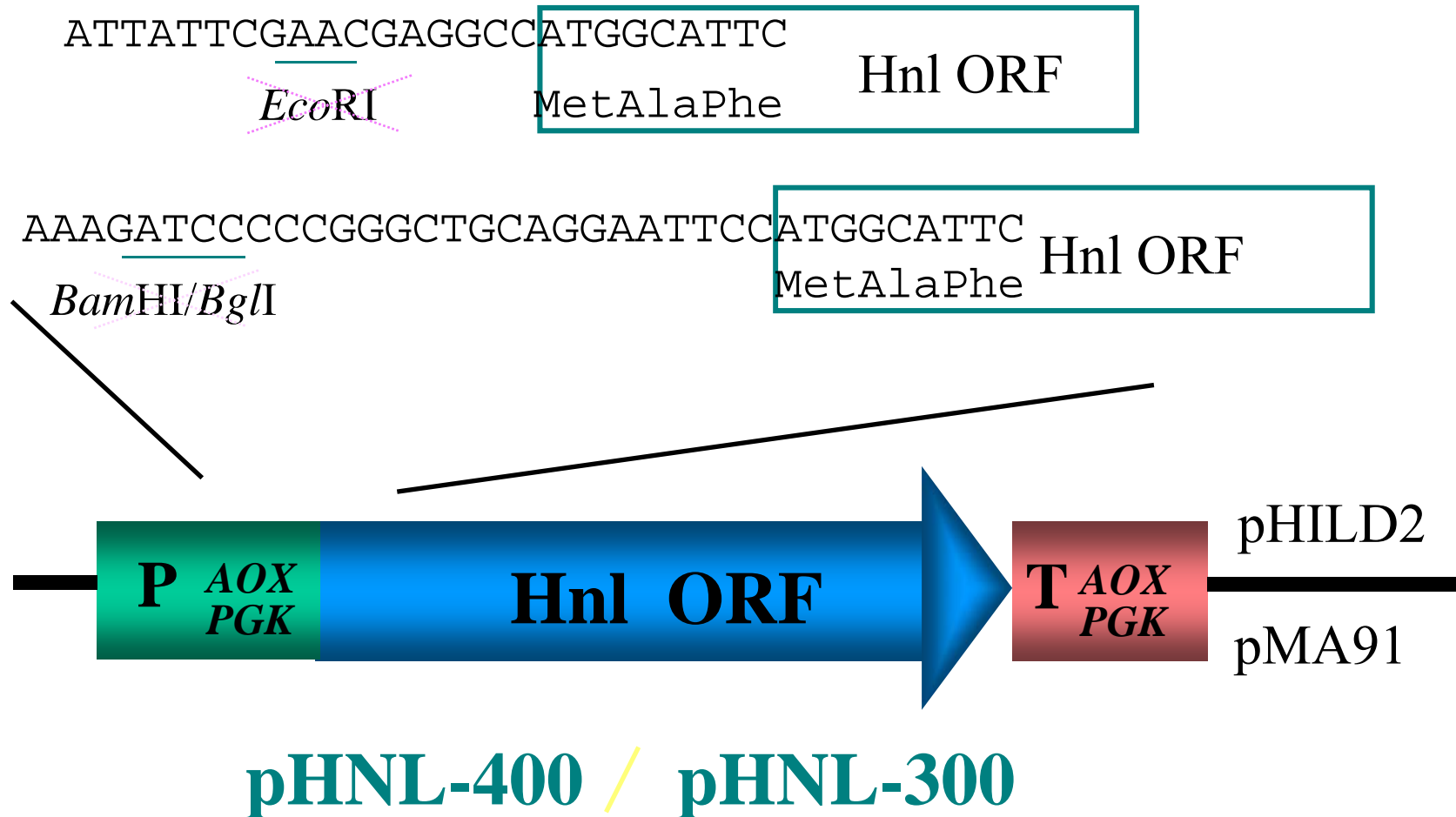


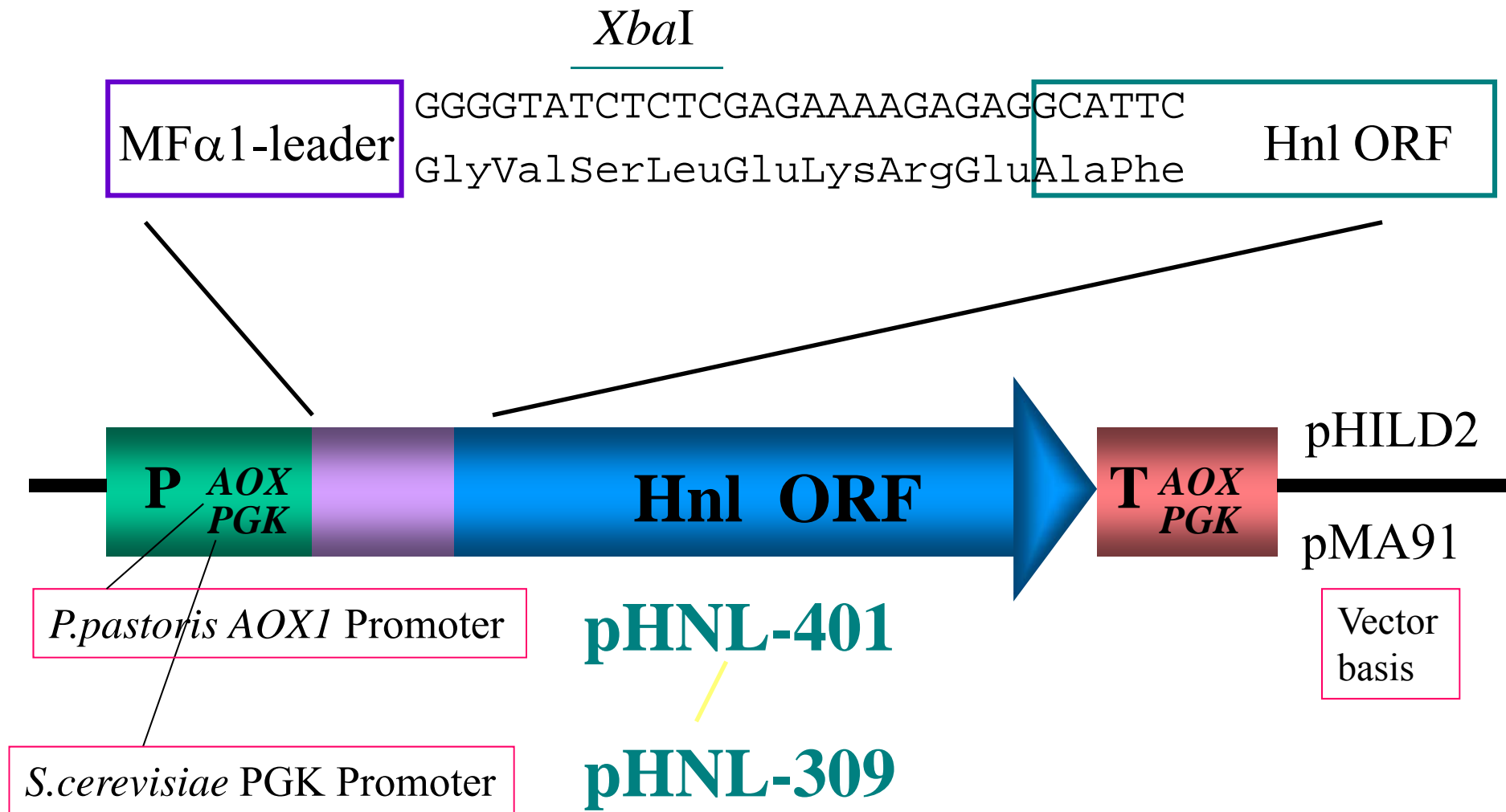
FIG. 2. Native polyacrylamide gel electrophoresis of crude soluble fractions of cell lysates of Hnl expressing transformants. Proteins were electrophoresed in 7.5% gels and stained with Coomassie blue (a) or transferred to membranes for immunostaining using polyclonal anti-Hnl antiserum (b, c). Lanes 1a, b: *S. cerevisiae* W303D/pMA91; lanes 2a, b: *S. cerevisiae* W303D(pHNL-300) (transformant ScW303D-HNL2); lanes 3a, b: *P. pastoris* GS115 transformed with pHIL-D2; lanes 4a, b: *P. pastoris* GS115 transformed with pHNL-400 (PpD1-17); lanes 5a, b: *E. coli* XL1-Blue (pSE420); lanes 6a, b: *E. coli* XL1-Blue (pHNL-200); lane 1c: soluble fraction of refolded inclusion bodies of *E. coli* XL1-Blue/pHNL-200. Hnl-specific bands in (a) are indicated by arrows.

Soluble

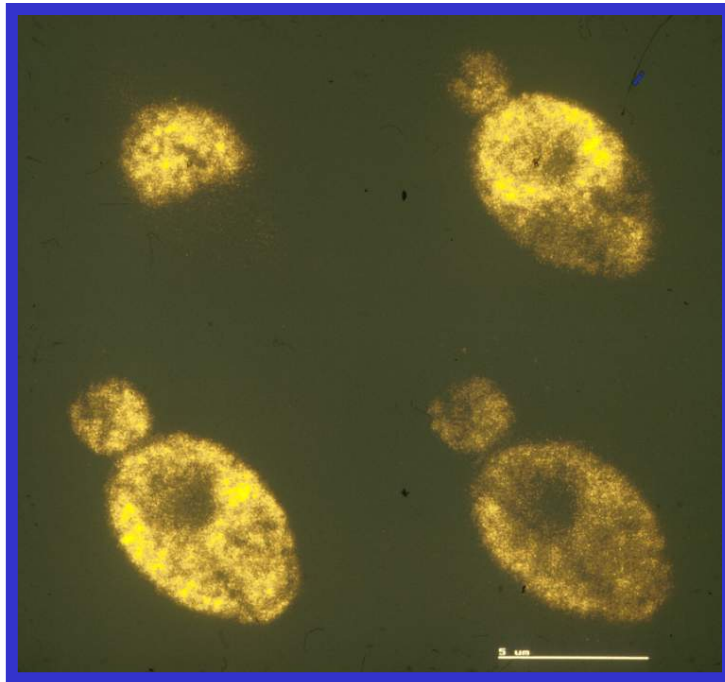
Intracellular Hnl Expression in *Saccharomyces cerevisiae* and *Pichia pastoris*



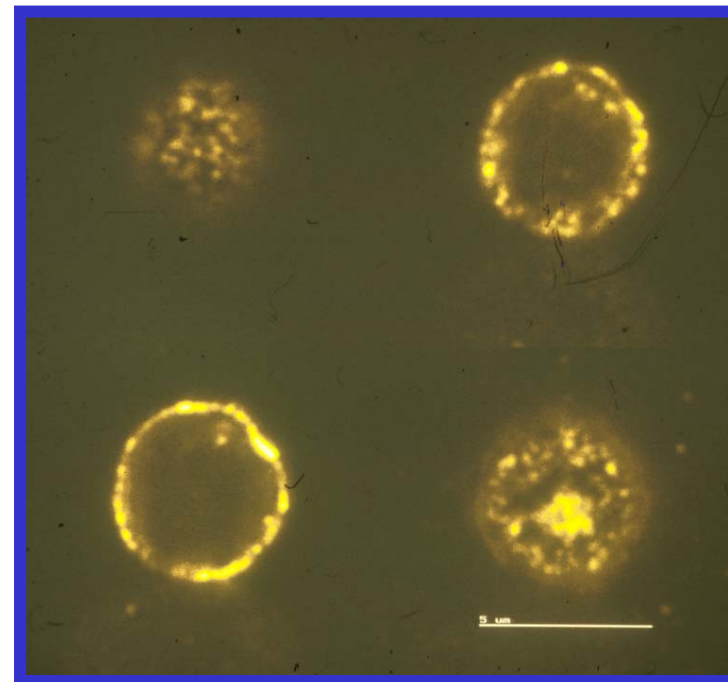
Secretory Hnl Expression in *Saccharomyces cerevisiae* and *Pichia pastoris*



Secretion-targeted *Hb_Hnl* accumulates in the cell periphery

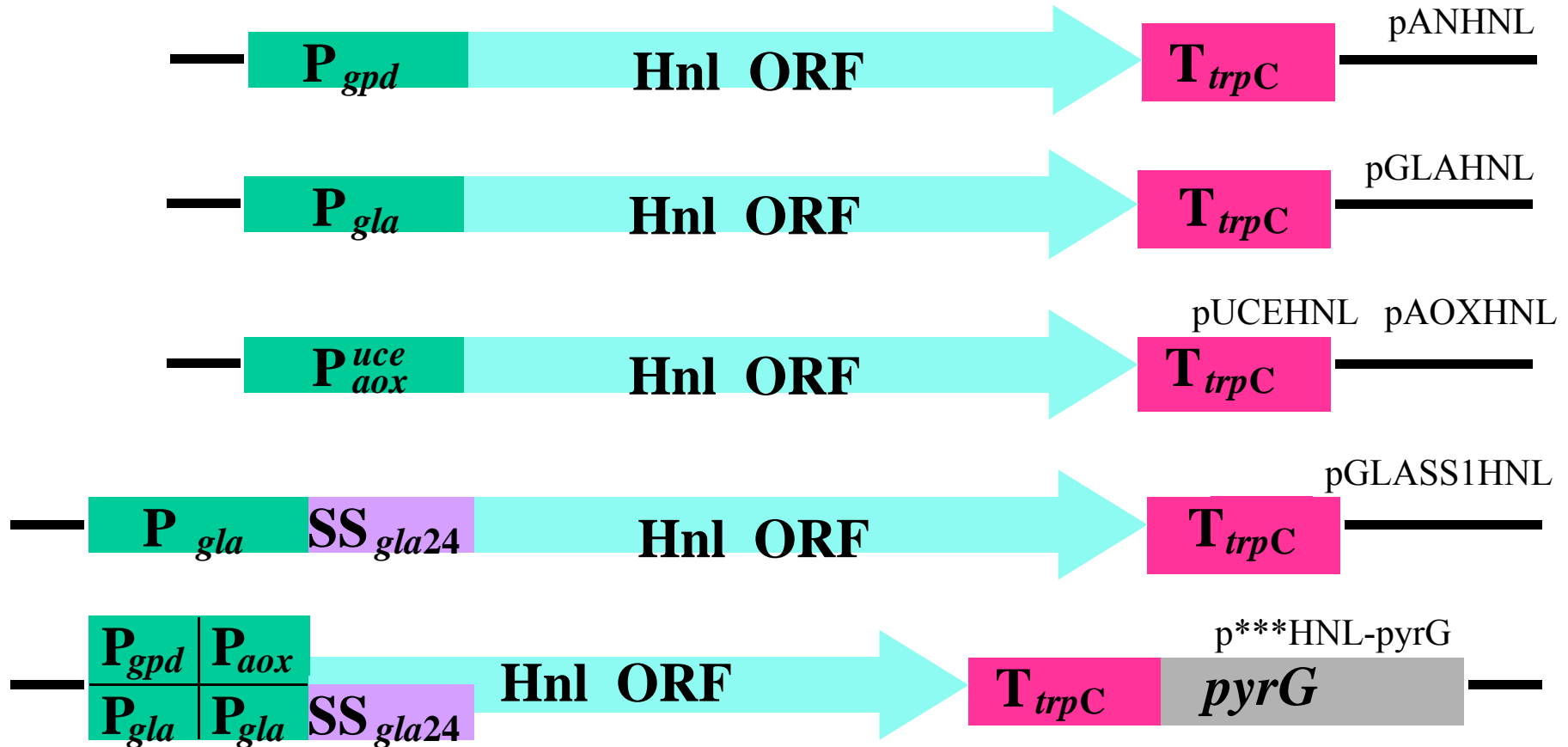


intracellular



secretory

Hb-Hnl expression in filamentous fungi



gpd: *A.niger* glyceraldehyde phosphate dehydrogenase

gla: *A.awamori* glucoamylase

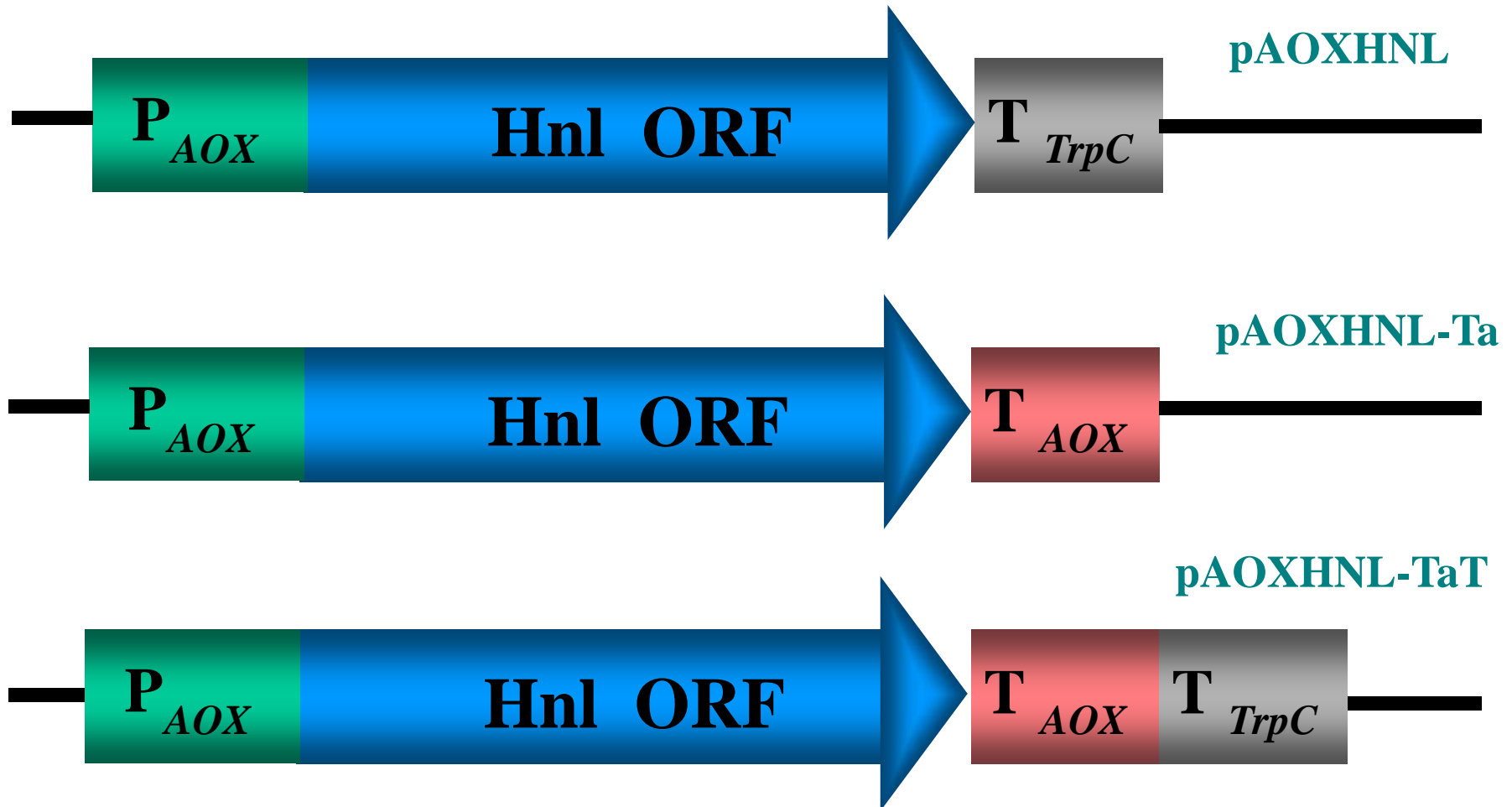
uce: unknown constitutively expressed gene, *P.chrysogenum*

aox: *P.chrysogenum* alcohol oxidase

Intracellular Hnl Expression in *Penicillium chrysogenum* under control of P_{AOX}

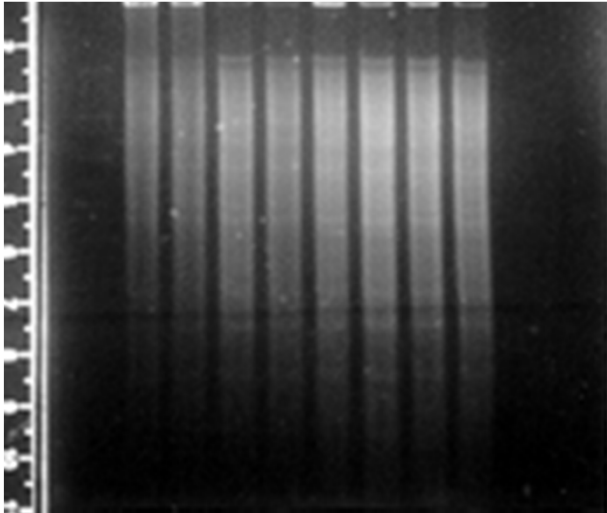
Termination Problem

AOX: *Penicillium chrysogenum* Alcohol Oxidase

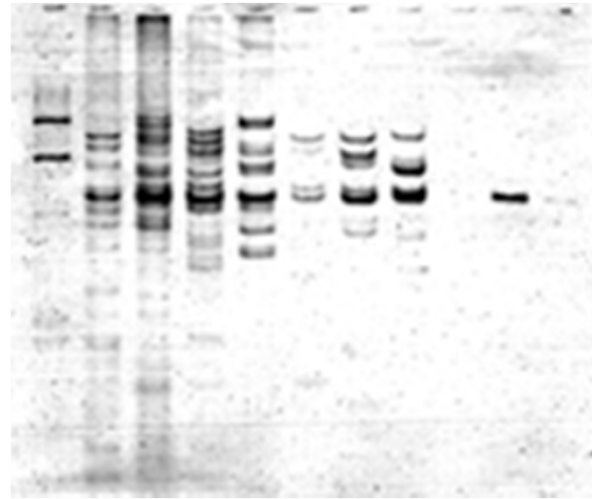


Southern analysis of *Penicillium chrysogenum* P_{AOX} transformants

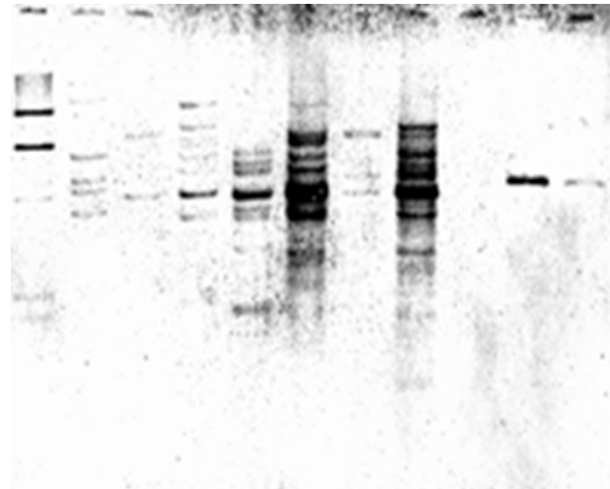
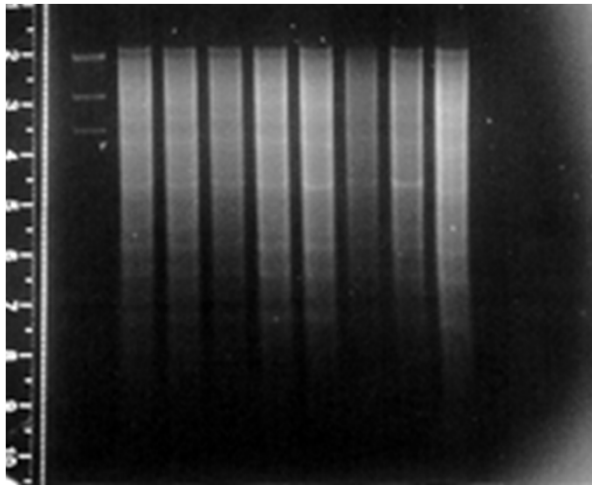
1 2 3 4 5 6 7 8 9 10 11



1 2 3 4 5 6 7 8 9 10 11



pAOXHNL-TaT

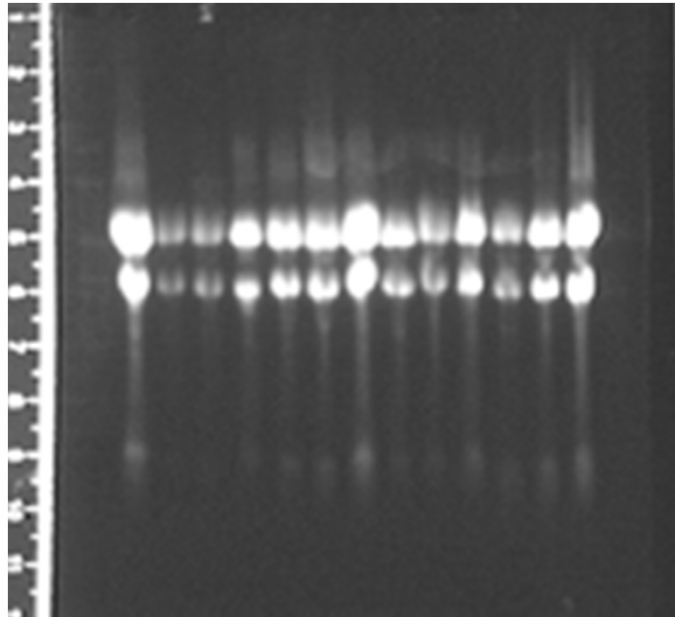


pAOXHNL-Ta

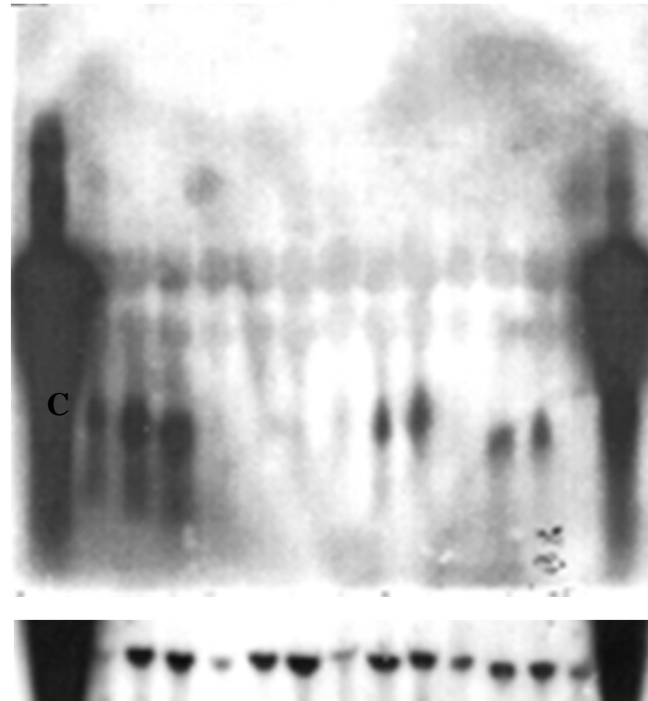
Northern blot analysis of *Penicillium chrysogenum* P_{AOX} transformants

pAOXHNL-TaT

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
C



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15



Hnl probe

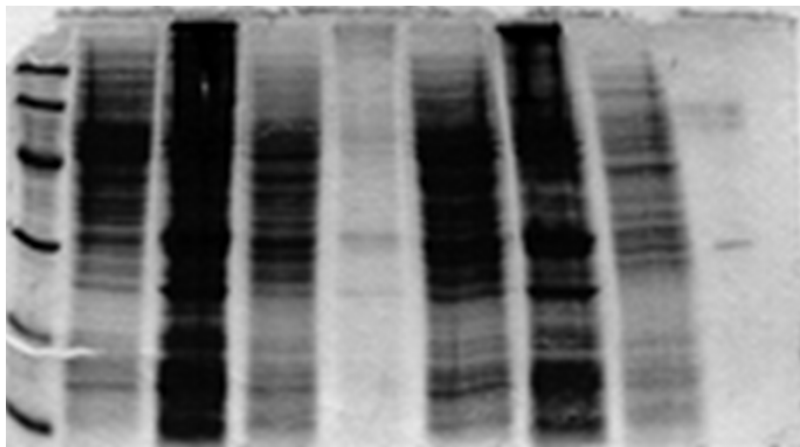
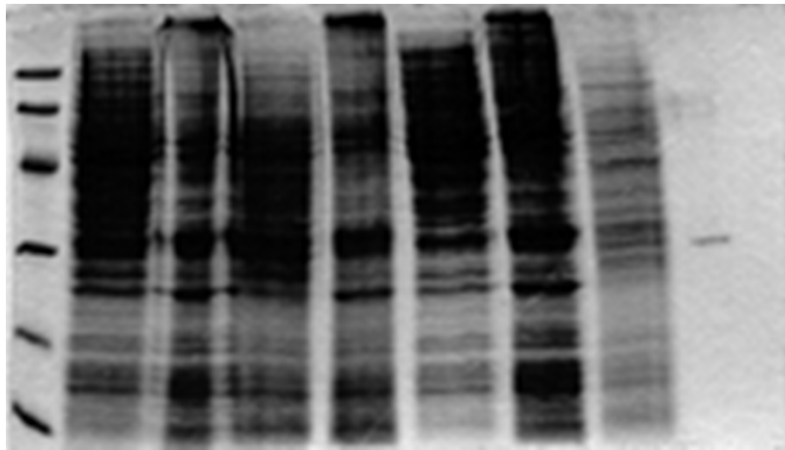
Actin probe

Western blot analysis of *Penicillium chrysogenum* P_{AOX} transformants

→ Protease Problems

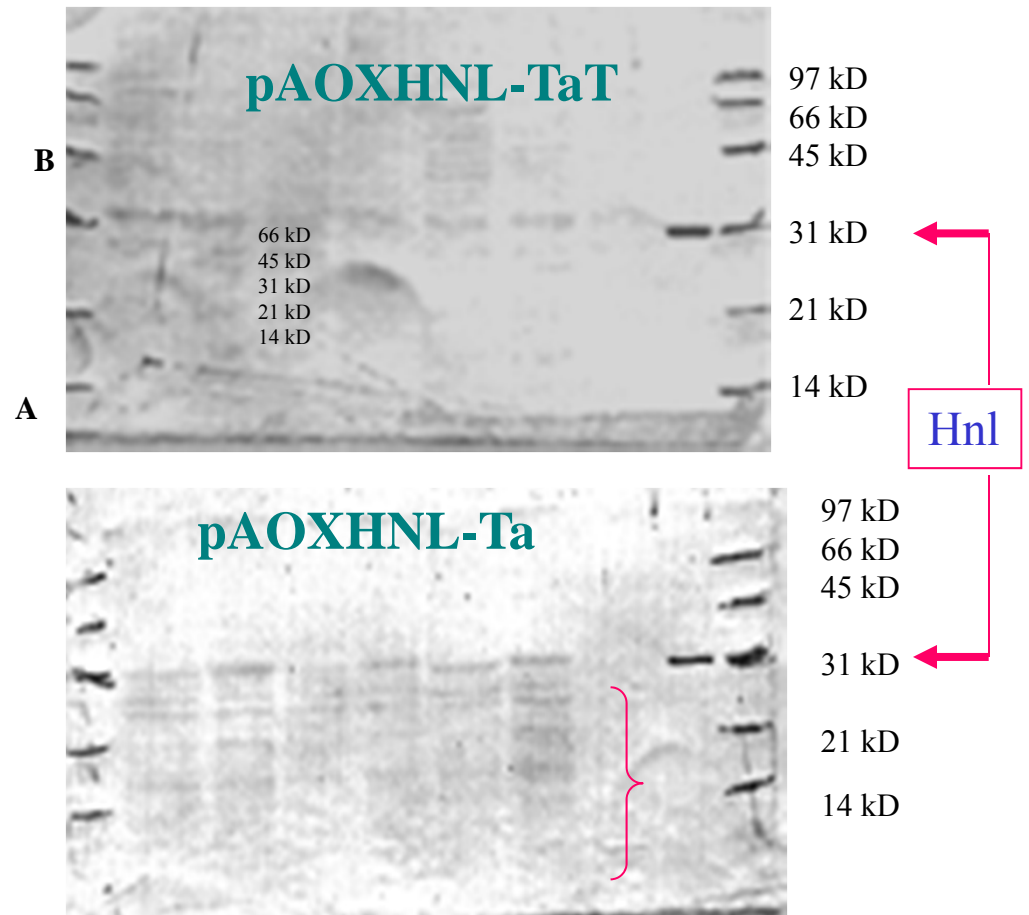
Total cellular proteins - SDS PAGE

1 2 3 4 5 6 7 8 9



Western blot with Hnl Antibody

1 2 3 4 5 6 7 8 9 10



Degradation!

Expression analysis of *Penicillium chrysogenum* P_{AOX} transformants

Hnl Expression

AOX Promoter Activity Test

uidA: Reporter gene,
 β -glucuronidase

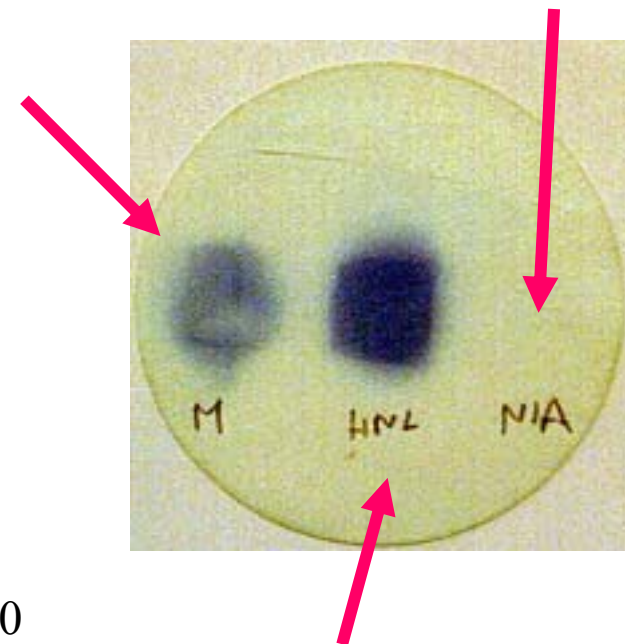
***AOX::uidA* fusion**

2% lactose pH 8

**Blue colour = cleavage of X-Glucose
by β -glucuronidase**

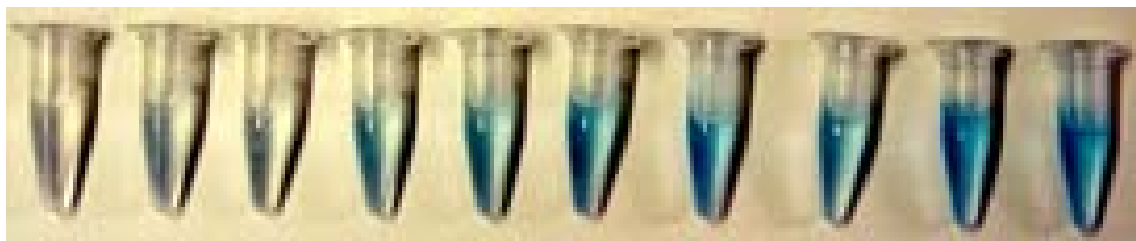
pAOXHNL-TaT

negative control



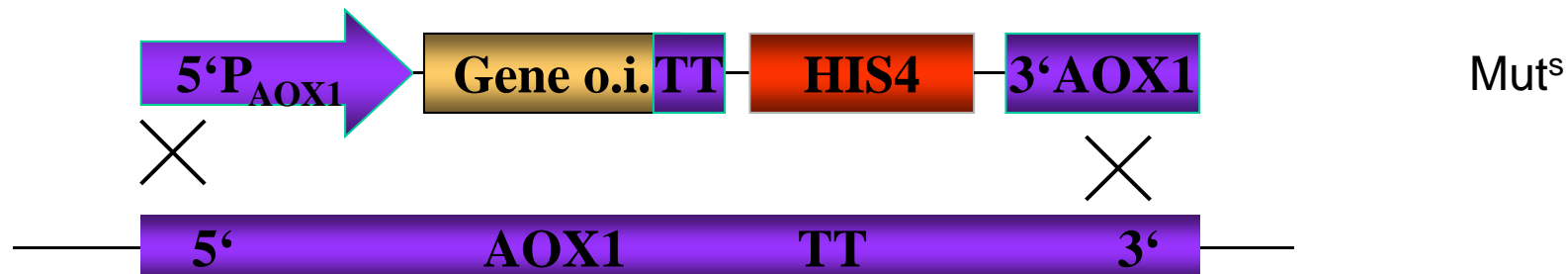
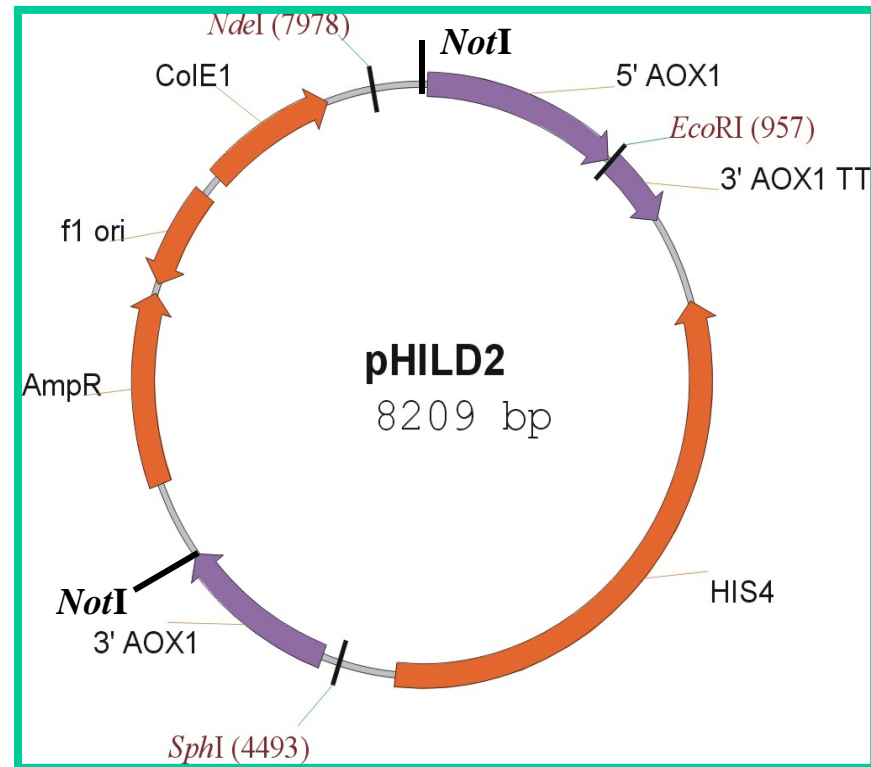
purified HNL

v.I. 2 3 4 5 6 7 8 9 10



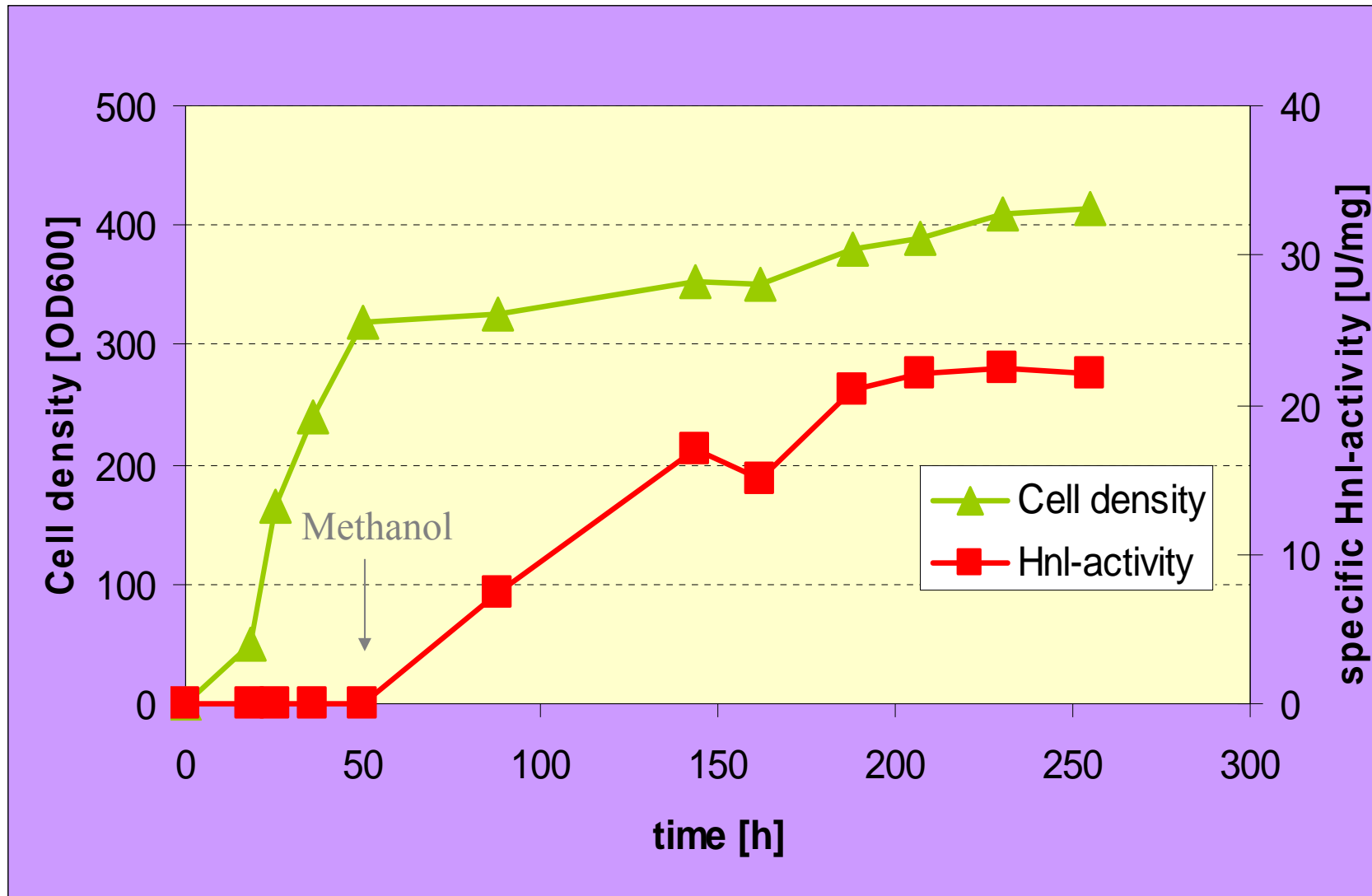
Gene replacement in *Pichia pastoris* at *AOX1*

- *AOX1* promoter
- *HIS4* selection marker
- *AOX1* transcription termination signal
- 3' *AOX1* region
- Amp^R
- F1 ori
- ColE1 replicon



Pichia pastoris Hb_Hnl expression strain 1-17

Fed-batch fermentation

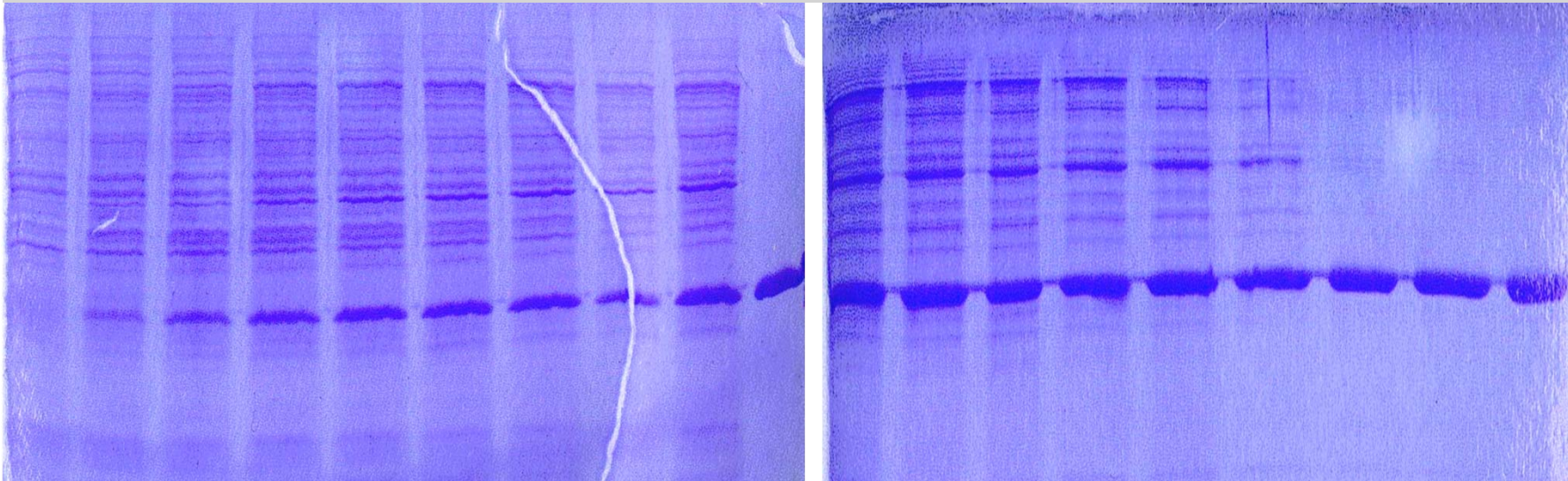


Pichia pastoris Hb_Hnl expression strain 1-17

Fed-batch fermentation

Fermentation time (hours after induction)

0 15 27 29 49 63 79 87 97 ST 111 119 135 145 151 159 169 194 ST



Soluble proteins in cell extracts

Heterologous Hnl Expression (shake flask experiments)

Construct	Host	cytosol (U/mg)	purified enzyme (U/mg)	total per culture
<i>Hevea brasiliensis</i>		0.42	15-20	0.5 U/g (leaves)
pHNL-200	<i>E. coli</i>	0.6	-	0.1 U/ml ^{OD=4}
pHNL-300	<i>S. cerevisiae</i>	4.6	20	1.2 U/ml ^{OD=4}
pHNL-400	<i>P. pastoris</i>	15.7	40	6.2 U/ml ^{OD=4}
pANHNL	<i>A. niger</i>	0.6	-	~ 0.1 U/ml nd

**Production of *Hb_Hnl*
with *Pichia pastoris* Expression System
(Fed-Batch Fermentation)**

Cell wet weight	400 g / l
Cell dry weight	100 g / l
Total protein	56 g / l
Hnl protein	23 g / l
Hnl units	1.4 x 10⁶ / l

(S)-Hnl of *Hevea brasiliensis* and (R)-Hnl of *Prunus amygdalus*

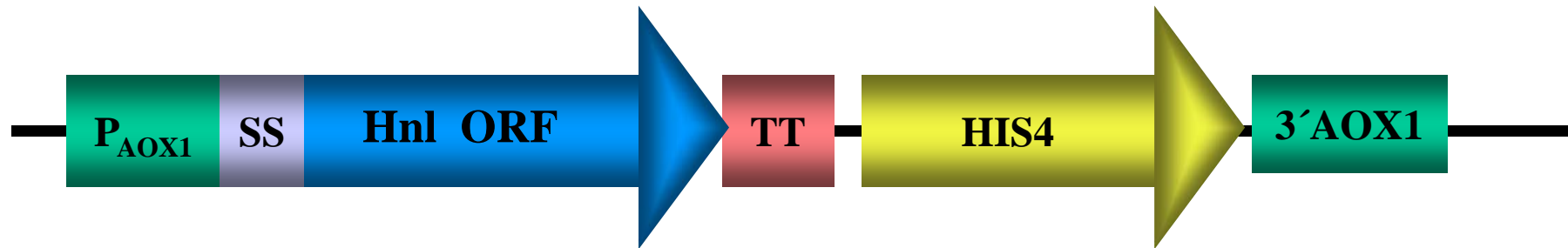
Hb_Hnl

- Type II Hnl
- intracellular protein
- 29.2 kDa
- homodimer
- α/β hydrolase fold protein
- catalytic triad
- (S)-selektive

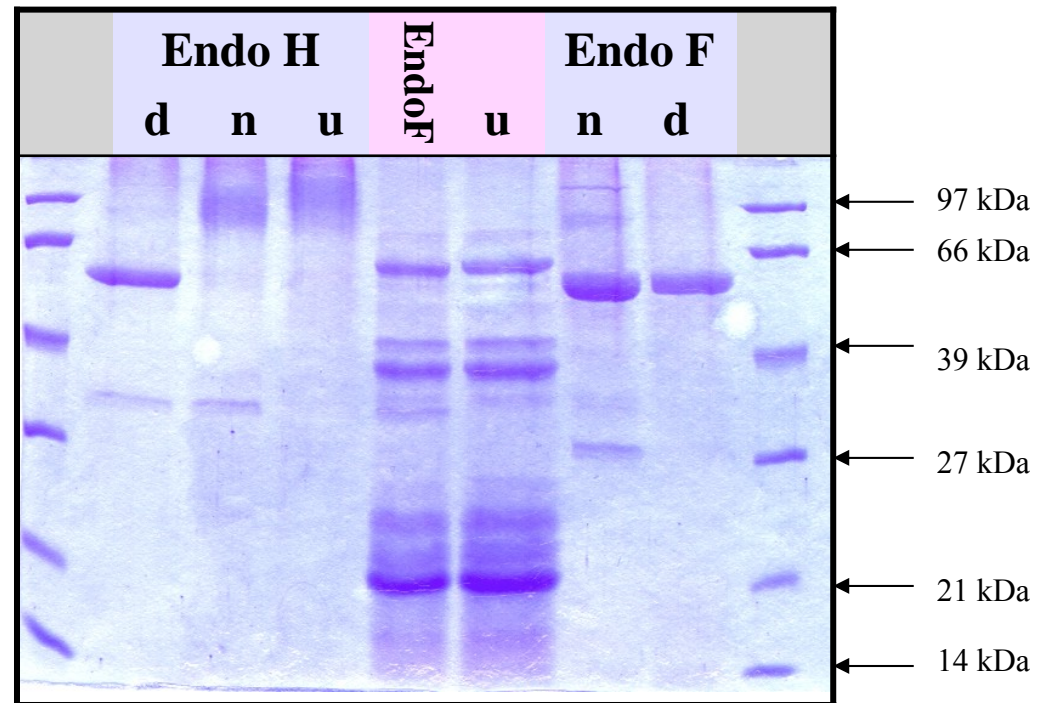
Pam_Hnl

- Type I Hnl
- secretory protein
- 61 kDa (57.9 kDa)
- Homology to oxidases
- FAD
- N-glycosylated
- isoenzymes
- (R)-selektive

Pam_Hnl5: Secretory Expression of Prunus amygdalus (R)- Hnl in Pichia pastoris



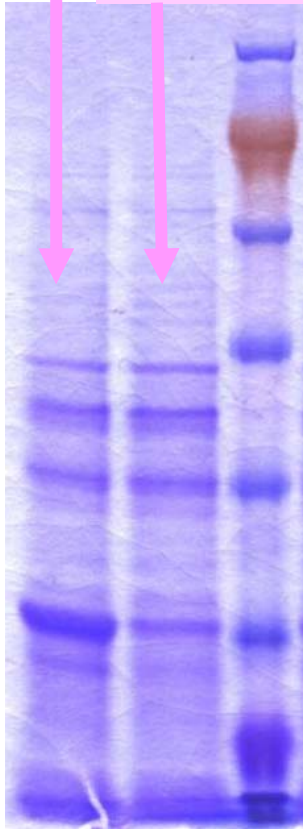
- Host: *Pichia pastoris* GS115
- Alpha factor signal sequence
- Mut^s and Mut⁺ Transformants
- Functional secretory expression
- highly glycosylated



High-level Expression Clone D1-17 → ???

Super expression strain D1-17

Standard expression clone



Hnl wt

→ great success with expression

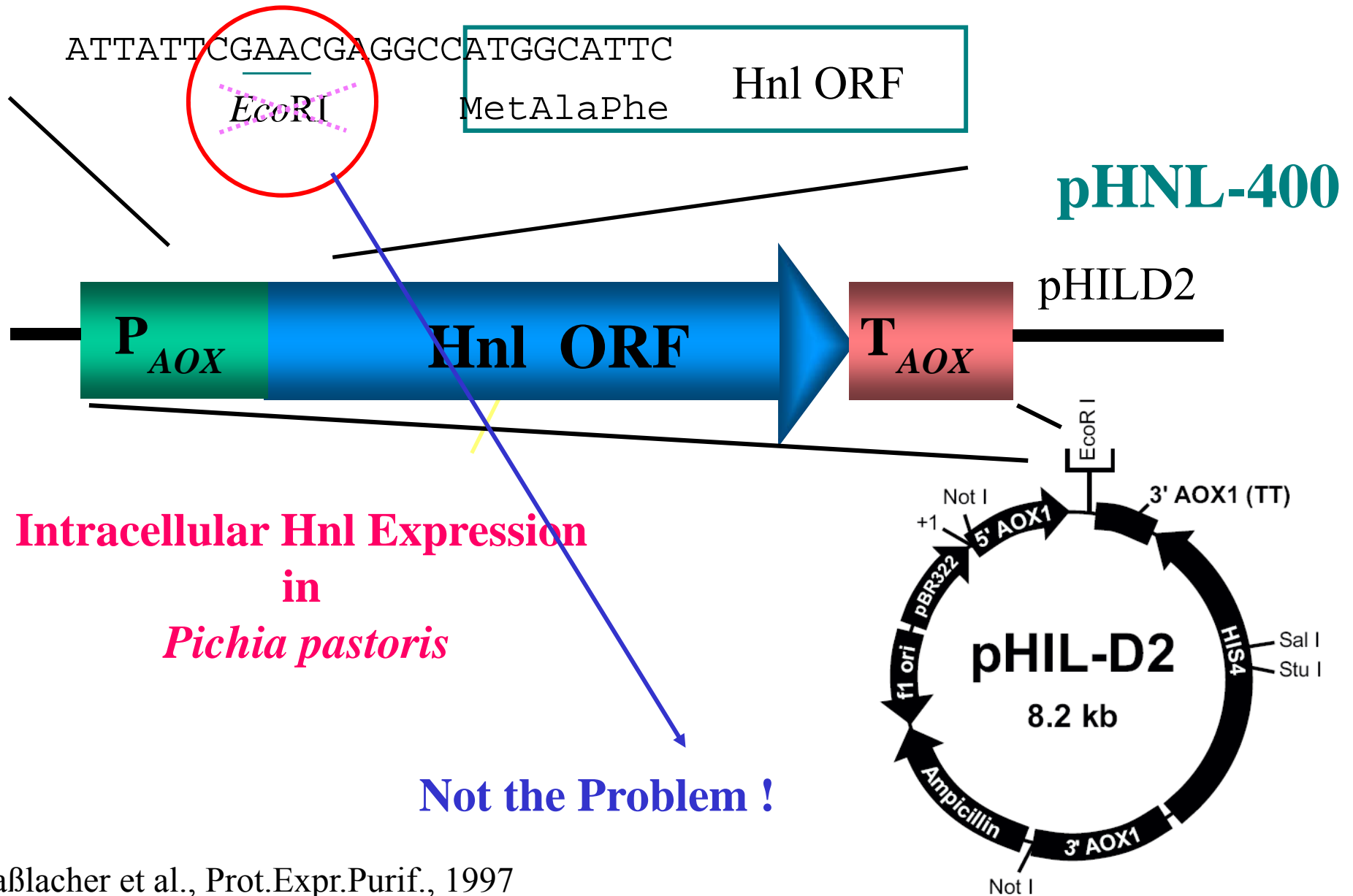
Engineered Hnl Proteins

→ do it the same way !

High expression levels were

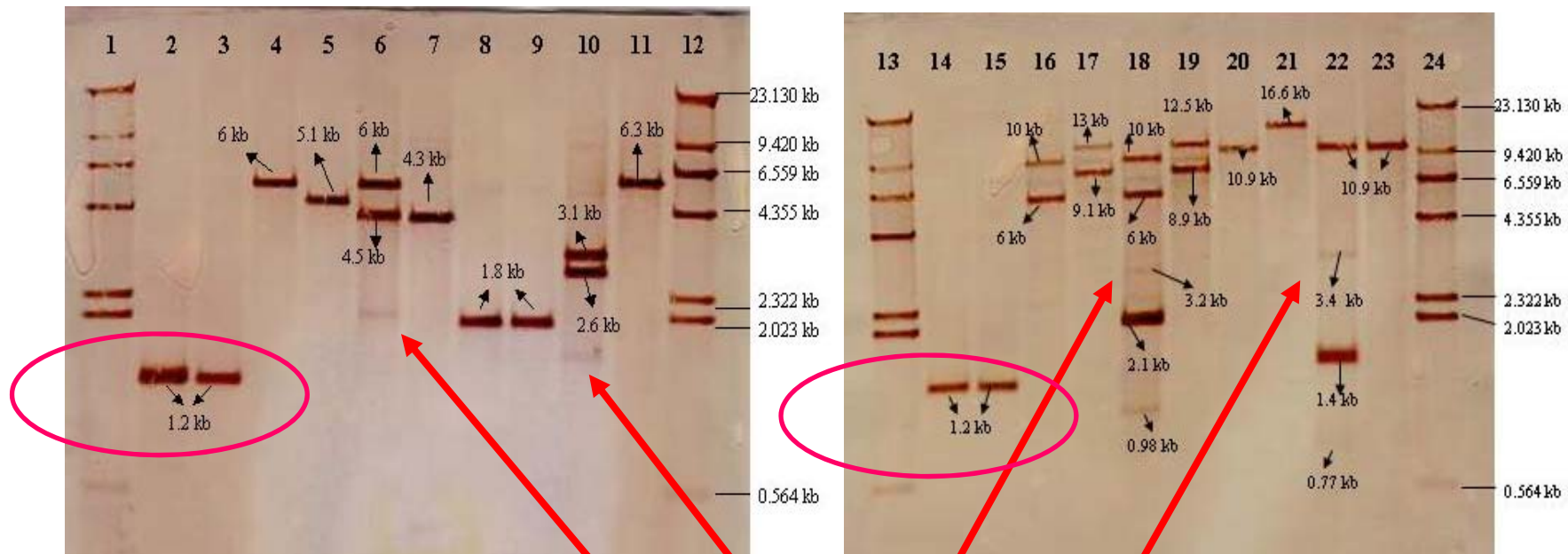
Not Reproducible !!

High-level Expression Clone D1-17 → ???



Molecular Analysis of Expression Strain

Southern blotting



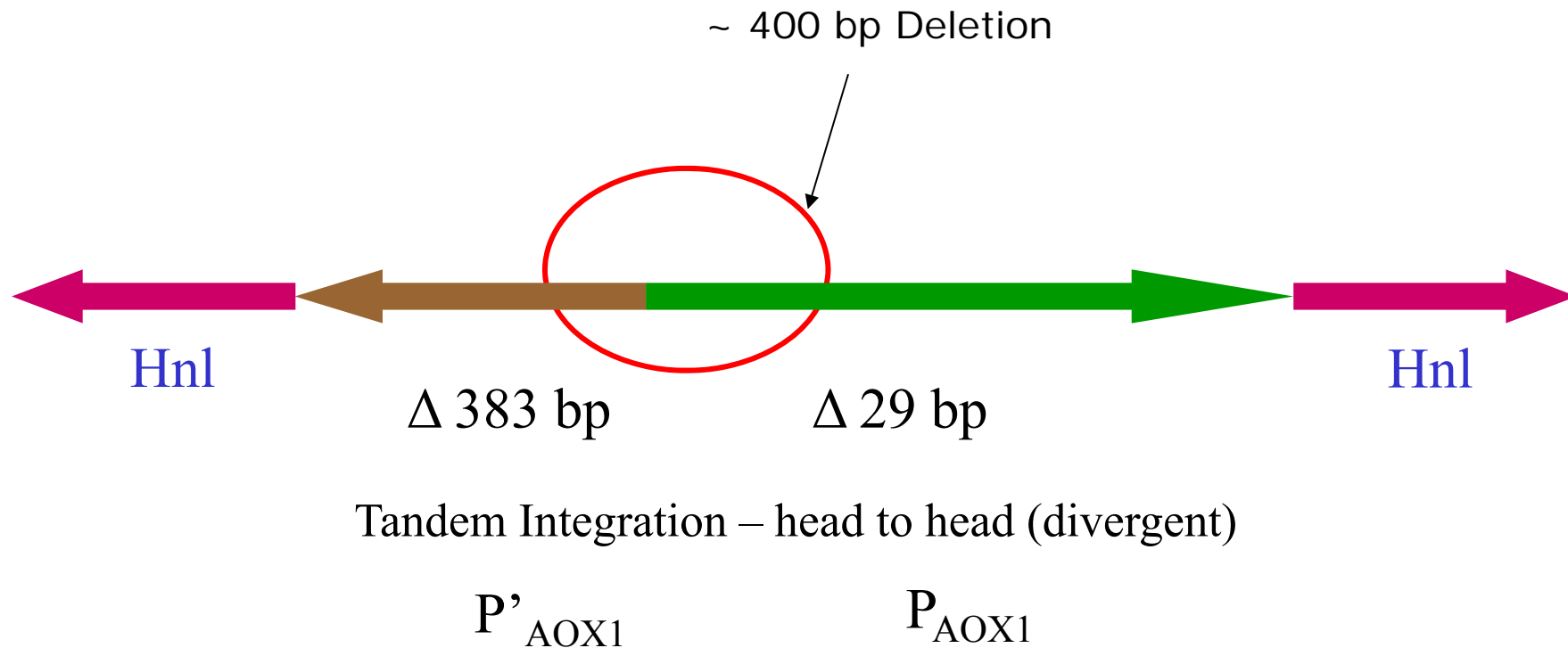
HNL probe

AOX1 Probe

Strange Fragments

→ More than one Copy Integrated → How ??

Molecular Analysis of Expression Strain


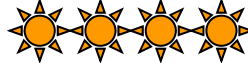











3 copies of Hnl,

→ 1 standard Integration in AOX1 Locus

→ 2 truncated, in a head to head oriented AOX1 Promoter Fragments

Expression analysis

	Expression
phhAOX561(-HbHNLwt) 	
phhAOX915(-HbHNLwt) 	
pAOXGRAZlang(-HbHNLwt) 	
pAOXGRAZshort(-HbHNLwt) 	
pAOXGRAZtotal(-HbHNLwt) → single copy 	
D1.17	

Specific genomic setup

A

```

-960                                     -881
AGATCTAACA TCCAAAGACG AAAGGTTGAA TGAAACCTTT TTGCCATCCG ACATCCACAG
TCTAGATTGT AGGTTTCGTC TTTCCAACCTT ACTTTGGAAA AACGGTAGGC TGIAGGIGTC
                                     Hap1

-880                                     -801
GTCCATTCTC ACACATAAGT GCCAAACGCA ACAGGAGGGG ATACACTAGC AGCAGACCGT TGCAAACGCA GGACCTCCAC
CAGGTAAGAG TGTGTATTCA CGGTTTGCGT TGTCCCTCCC TATGTGATCG TCGTCTGGCA ACGTTTGCGT CCIIGGAGGIG
                                     HSF

-800                                     -721
TCCTCTTCTC CTCAACACCC ACTTTTGCCA TCGAAAAACC AGCCCAGTTA TTGGGCTTGA TTGGAGCTCG CTCAITCCAA
AGGAGAAGAG GAGTTGTGGG TGAAAACGGT AGCTTTTTTGG TCGGGTCAAT AACCCGAAC AACCTCGAGC GAGTAAGGTT
HSF                                     Hap2/3/4/5 (2x) abmA

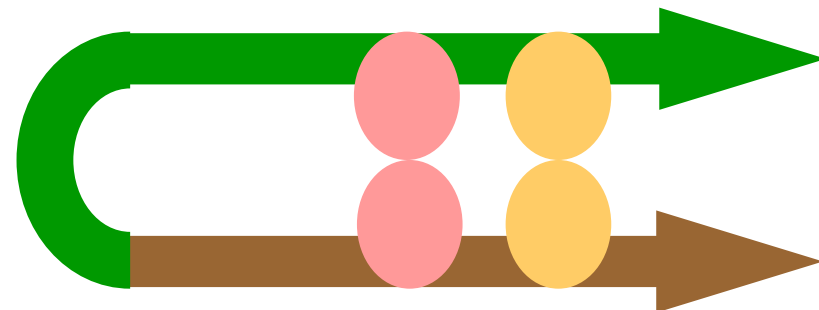
-720                                     -641
ITCCTTCTAT TAGGCTACTA ACACCATGAC TTTATTAGCC TGTCTATCCT GGCCCCCCTG GCGAGGTTCA TGTITGTTA
AAGGAAGATA ATCCGATGAT TGTGGTACTG AAATAATCGG ACAGATAGGA CCGGGGGGGAC CGCTCCAAGT ACAAACAAAT
                                     STRE

-640                                     -561
TTTCCGAATG CAACAAGCTC CGCATTACAC CCGAACAICA CTCCAGATGA GGGCTTTCTG AGTGIGGGGT CAAAATAGTTT
AAAGGCTTAC GTTGTTGAG GCGTAATGTG GGCTTGTAGT GAGGTCTACT CCCGAAAGAC TCACACCCCA GTTTATCAAA
                                     Rap1 Adr1

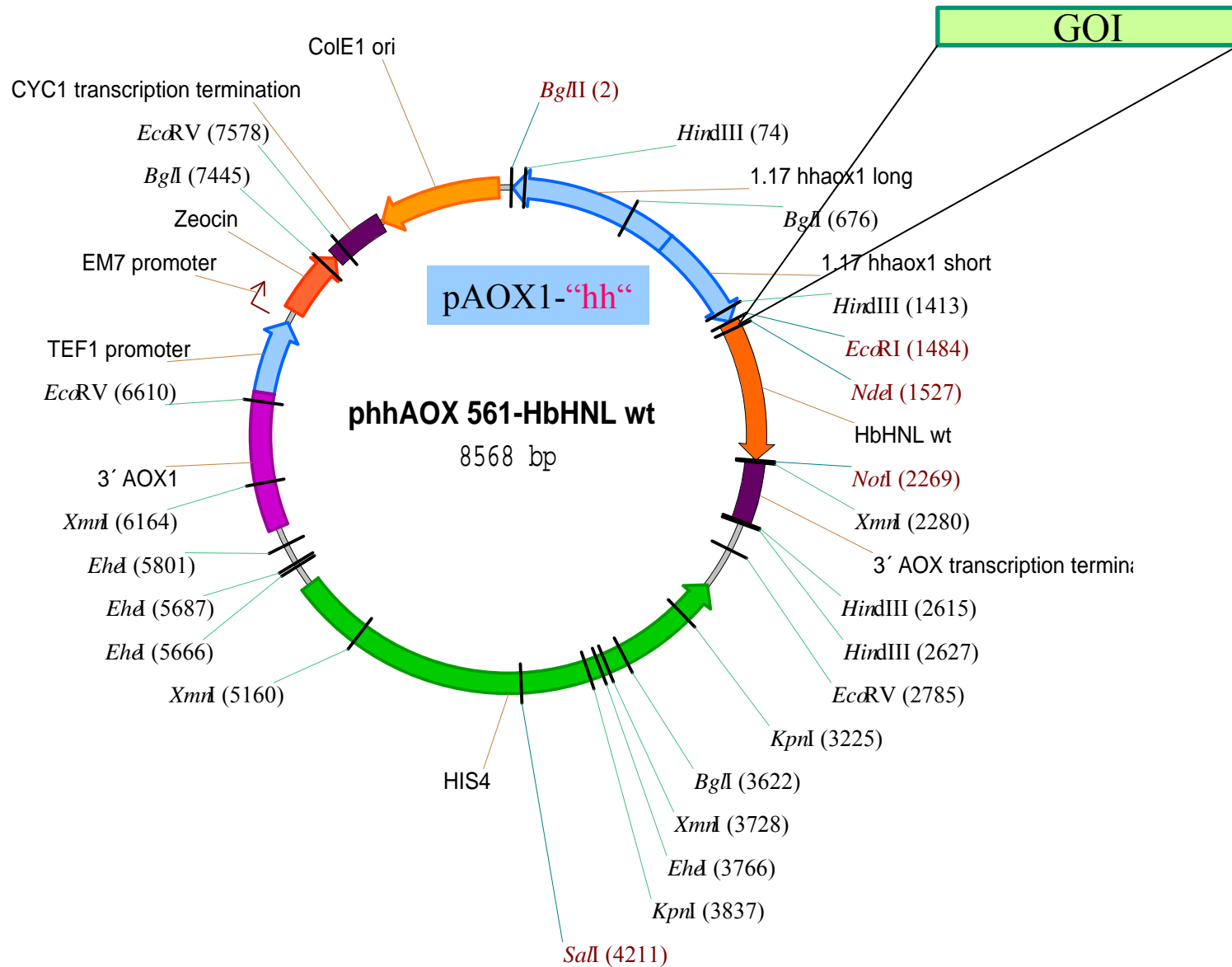
```

Hartner et al., Nucleic Acids Research, 2008, Vol. 36,

- Specific complex ??
- Concerted action of activators ??
- Repression-active site deleted ??



“hh”- Expression vector

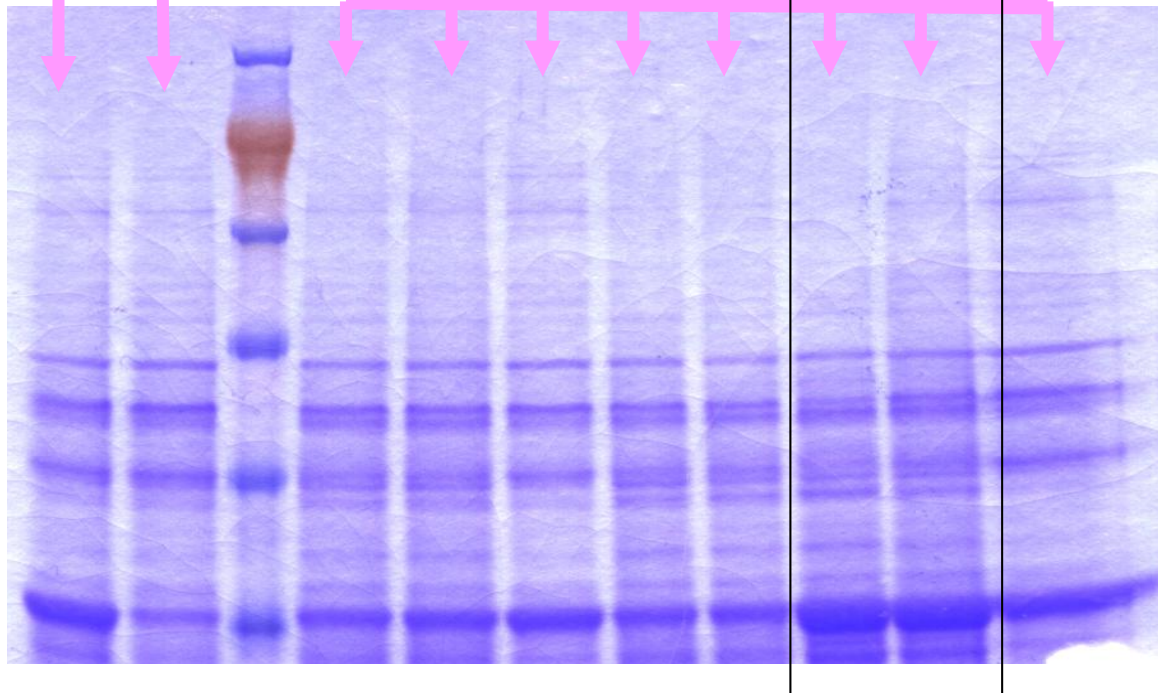


**Expression of *Hb_Hnl* mutants
in *Pichia pastoris***

Super expression strain D1-17

Standard expression clone

Novel expression clones



Modified
hh-AOX1-based promoter
System

Intracellular Expression

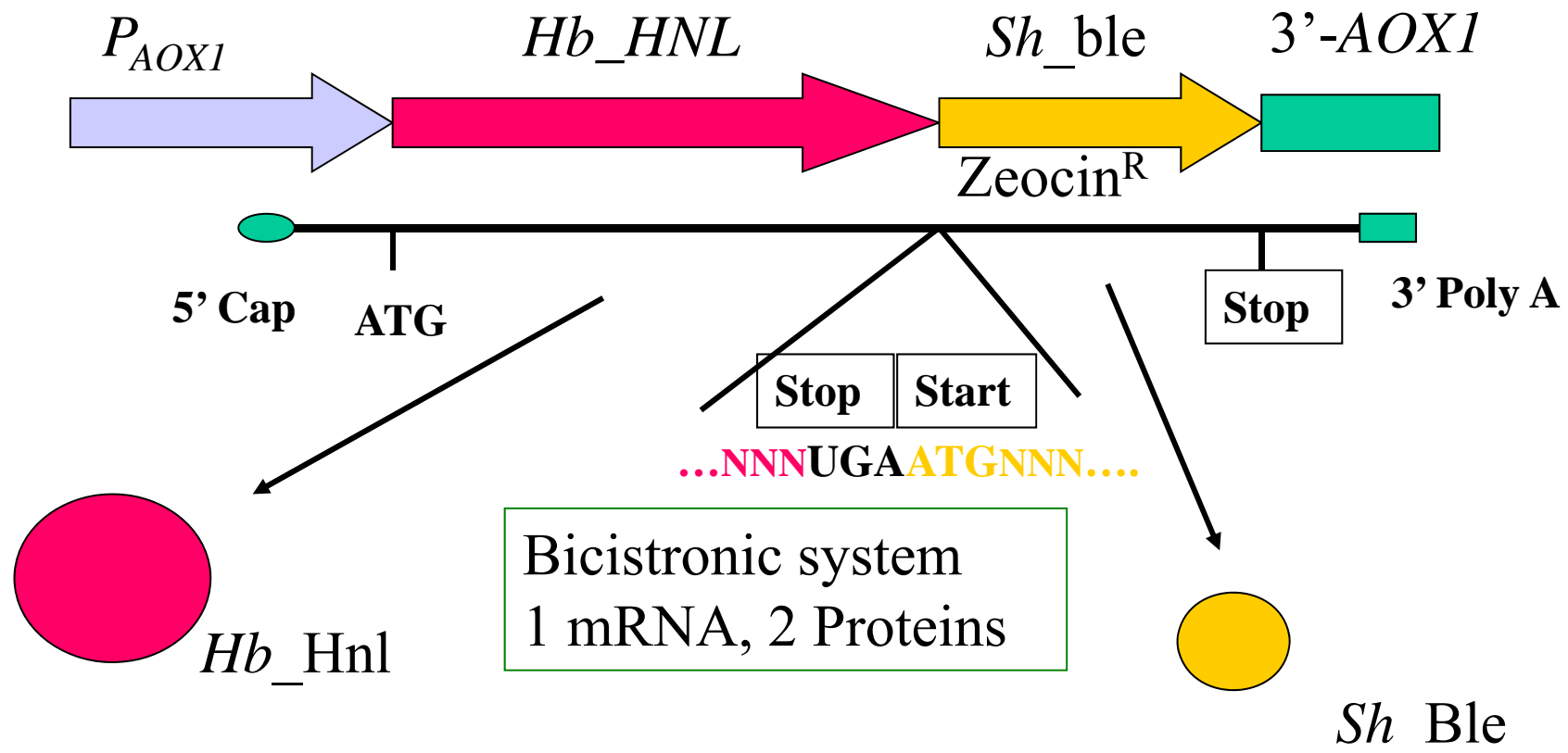
← **Hnl**

Screening for High-level Expression

Screening systems based on principle of **translational coupling**

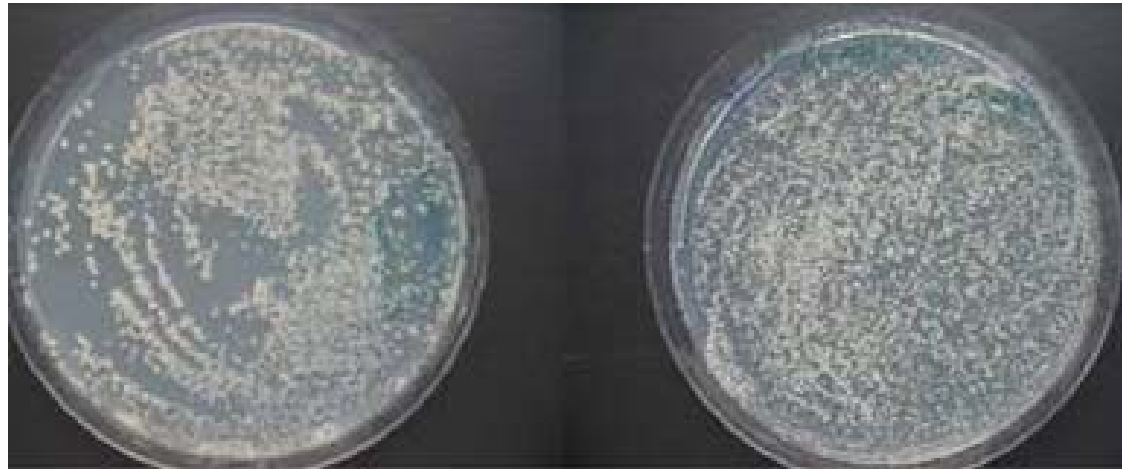
Well known in Prokaryotes

Does it work in Eukaryotes ???



Correlating Hnl expression to zeocin resistance

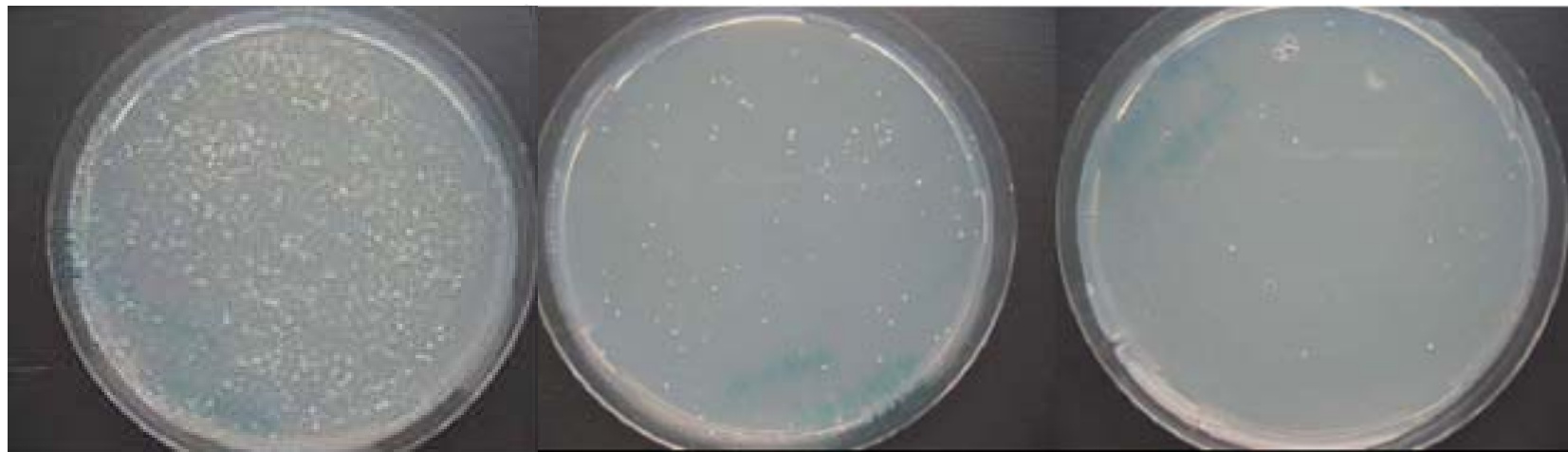
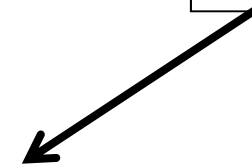
Veeresh Juturu,
PhD Thesis



MD 4.9×10^4

BMMS 4.9×10^4

Increasing
Zeocin
Concentration



BMMS 4.9×10^4
50 µg/ml

BMMS 4.9×10^4
100 µg/ml

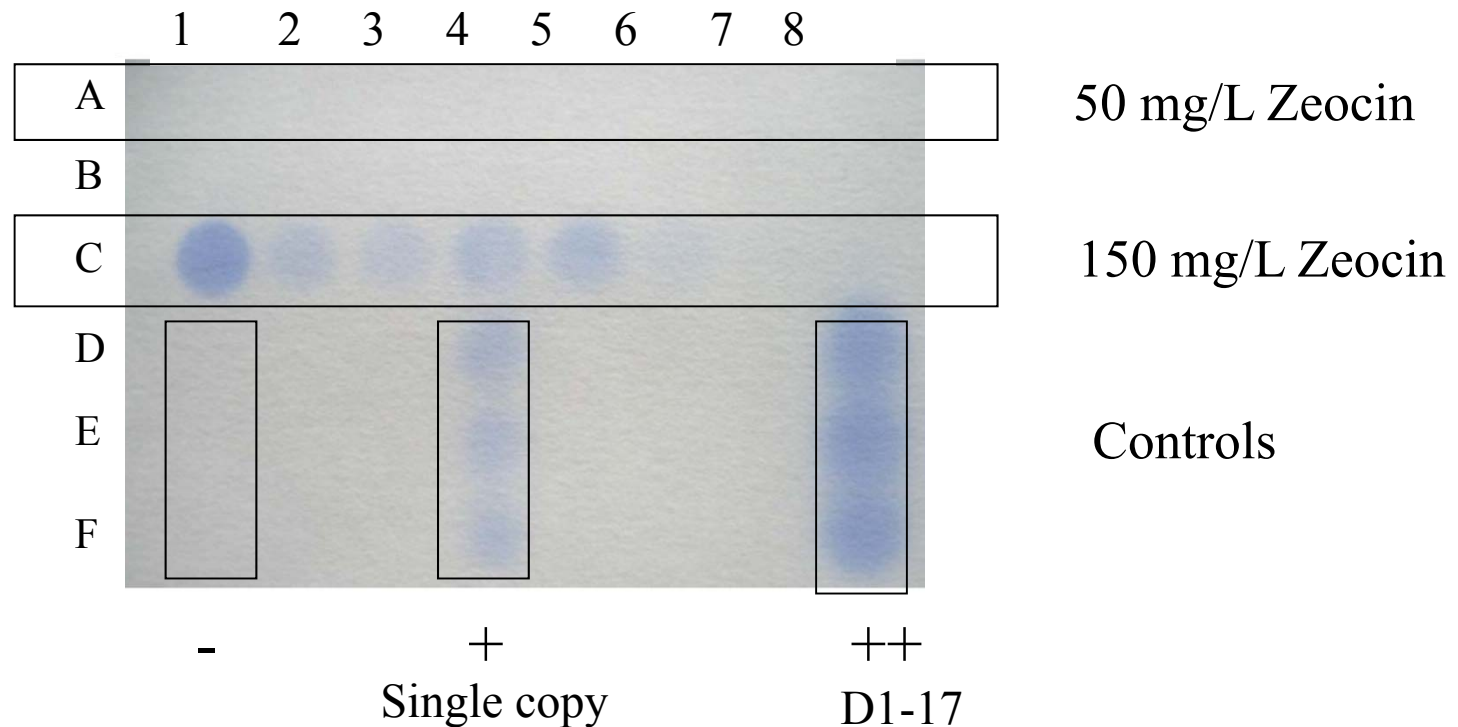
BMMS 4.9×10^4 ,
150 µg/ml

Hnl expression

Screen for High Resistance



Check for Expression Level



Row A → Clones from BMMS 50 µg/ml Zeo

Row C → Clones from BMMS 150 µg/ml Zeo

Lane 1D-1F → GFP expressing strain (- control)

Lane 4D-4F → *P. pastoris* HNL single copy strain (+ control)

Lane 8D-8F → *P. pastoris* HNL multi copy strain (+ control)

13.11.14