Mining endophytic bacteria and fungi for polyketide biosynthetic genes

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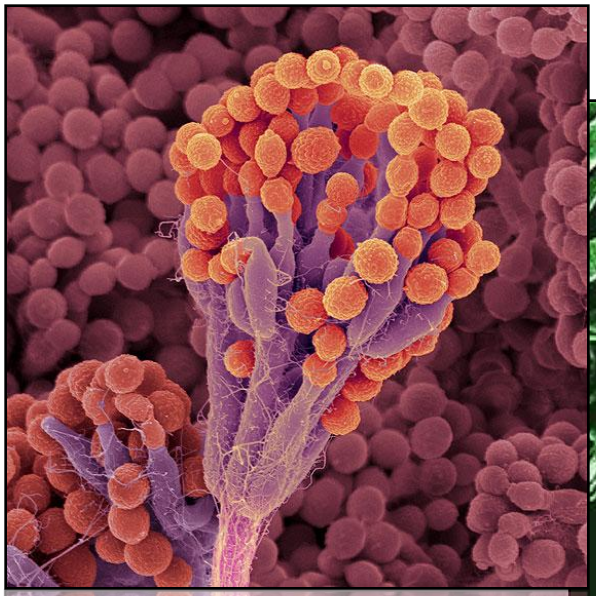
Topics

- Endophytic microorganisms
- Prospecting a culture collection
- Polyketide genes
- PKS I Fungi
- PKS II Actinobacteria
- PKS III Actinobacteria



- 700 strains
- 65% bacteria
- 30% fungi
- 5% archaea

Culture Collection

LGM ESALQ **LGS ICB** **NIB** UMC

Bioprospection of endophytic microorganisms

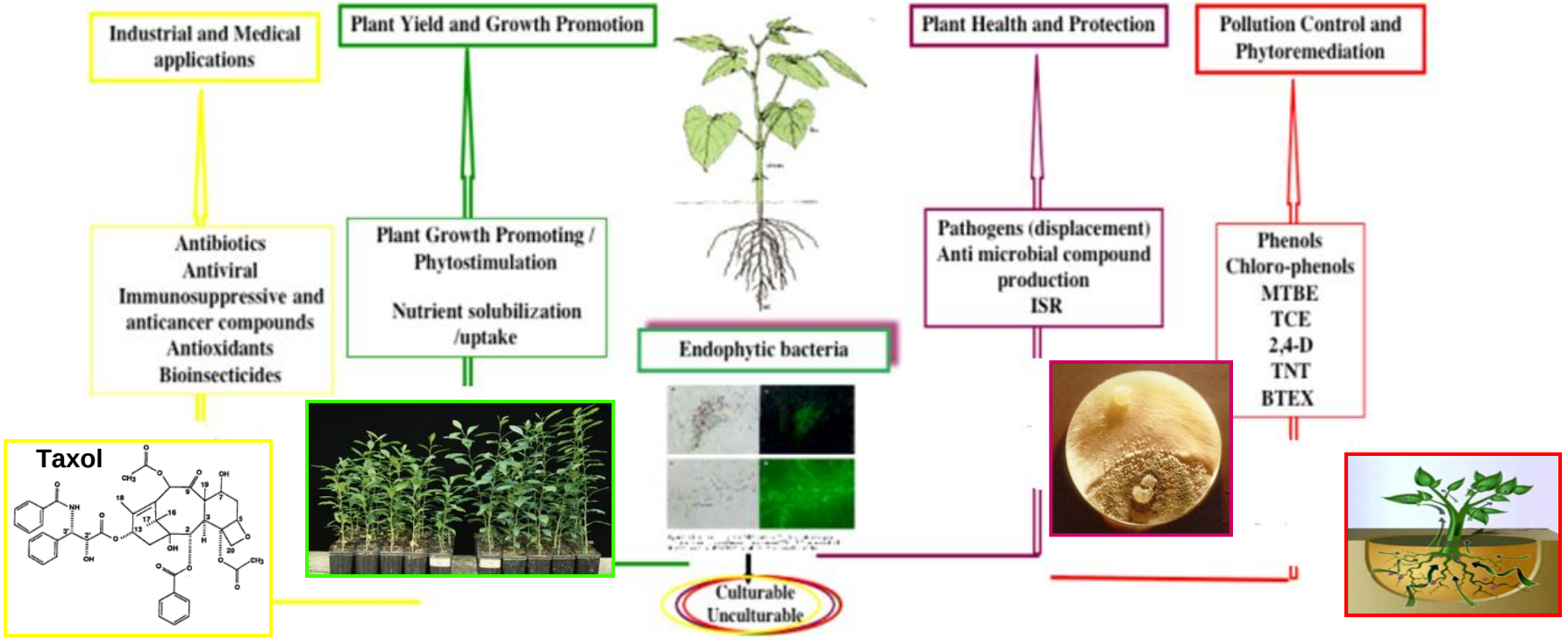
Endophytics are bacteria and fungi which live within plant tissues for all or part of their life cycle and cause no apparent infection

Curr. Sciences 90:1309 (2006)

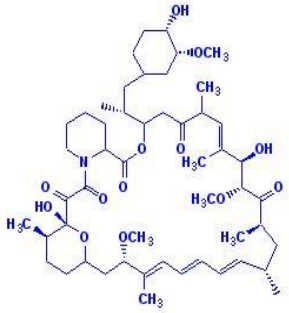
WHY TO STUDY ENDOPHYTIC MICROORGANISMS?

- **Poorly biotechnologically explored**
- **They form a significant part of the microbiota**
- **Compounds with low toxicity**
- **Diverse biological activity** (antibacterial, antifungic, antitumoral, antiviral, antioxidant, imunossupressor, inseticide and control of diabetes and malaria).
- **Taxol (Plaxitaxel):** produced byr *Taxomyces andreanae*, endophytic fungus from *Taxus brevifolia.*

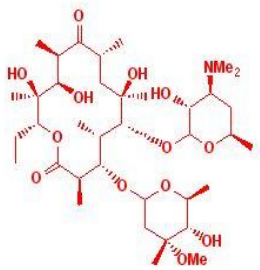
Biotechnological applications of endophytic microorganisms



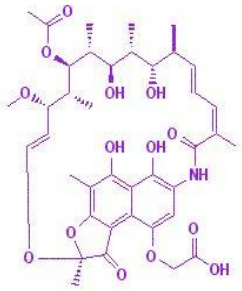
Polyketides



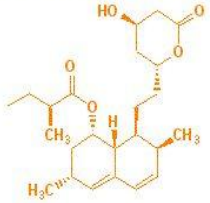
rapamycin
(immunosuppressant)



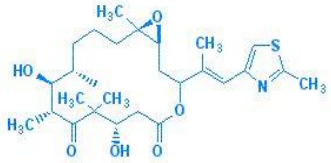
erythromycin A
(antibacterial)



rifamycin B
(antituberculosis)



lovastatin
(anticholesterol)

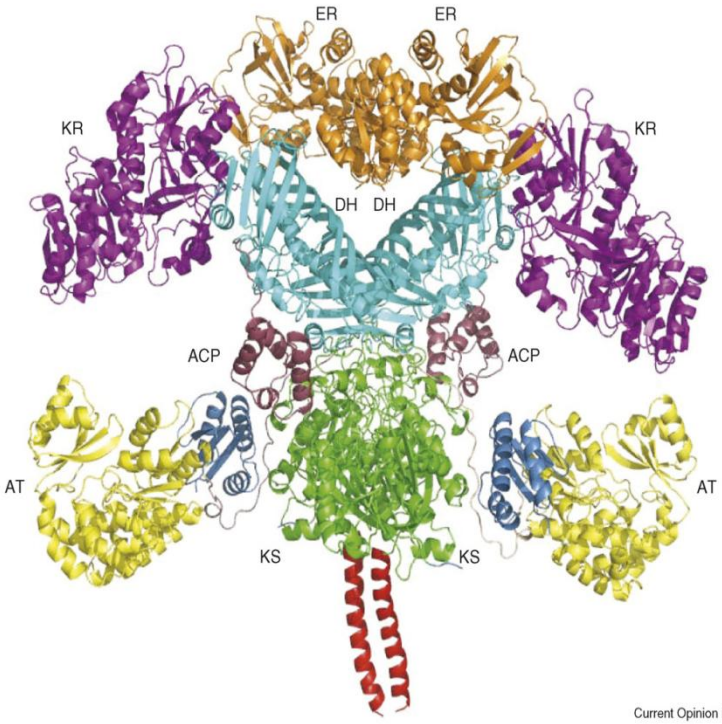


epothilone B
(anticancer)

(E)-resveratrol
(chemopreventive)

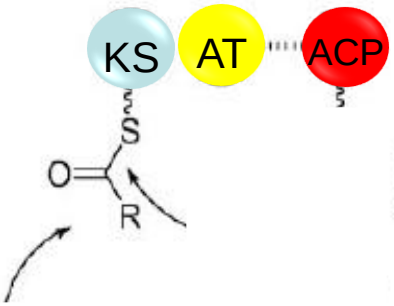
Structure Function

PKS



Current Opinion in Structural Biology

Biosyntesis of polyketides

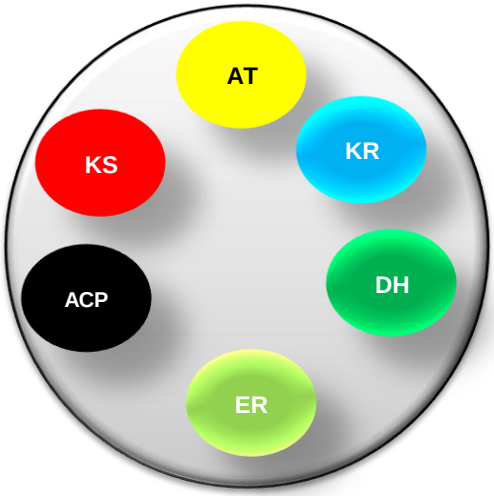


KS ketosynthase
AT acyl transferase,
ACP acyl carrier protein
DH dehydratase
ER enoylreductase,
KR ketoreductase
TE thiosesterase
MeT metyltransferase

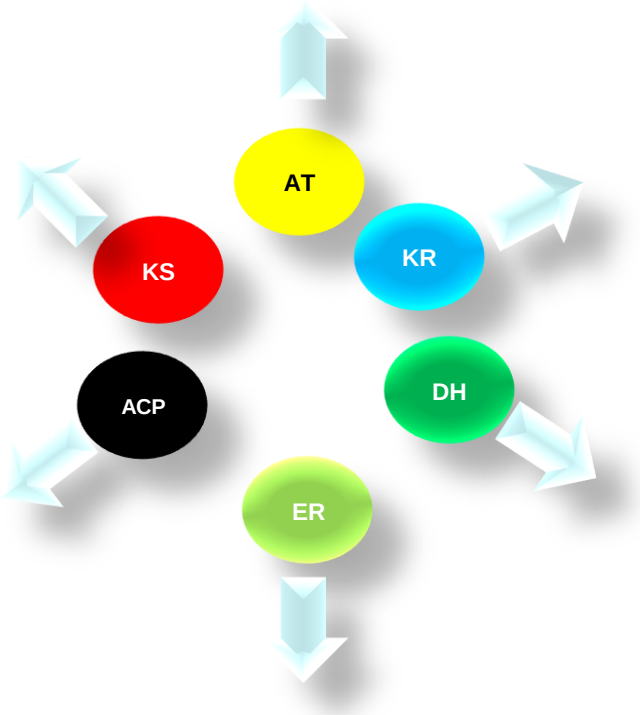
PKSs classification

Depending of the kind of organism and FAS structural organization (analogy)

Type I
Mammals

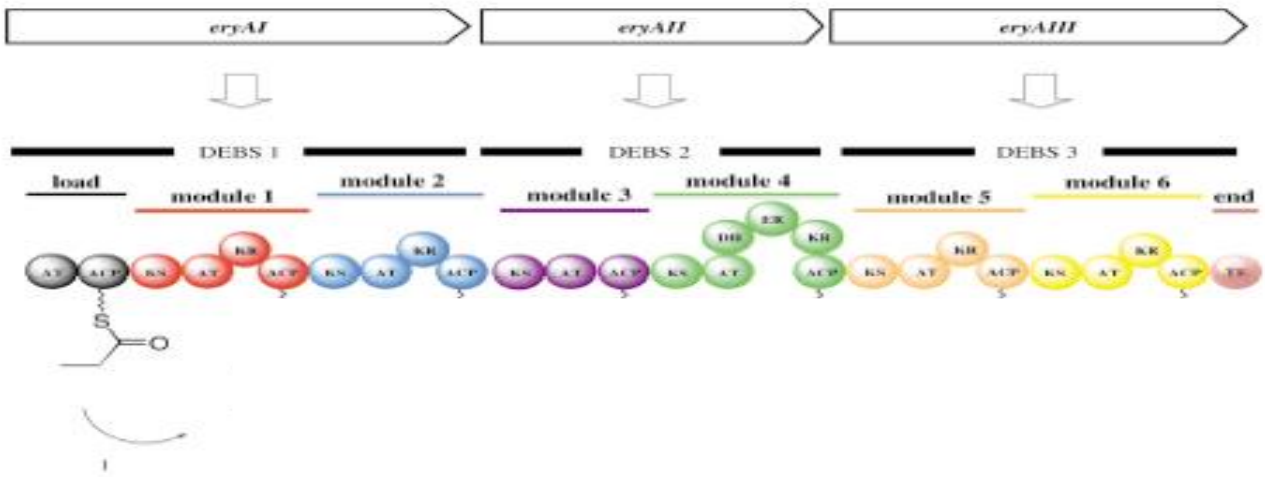


Type II
Bacteria



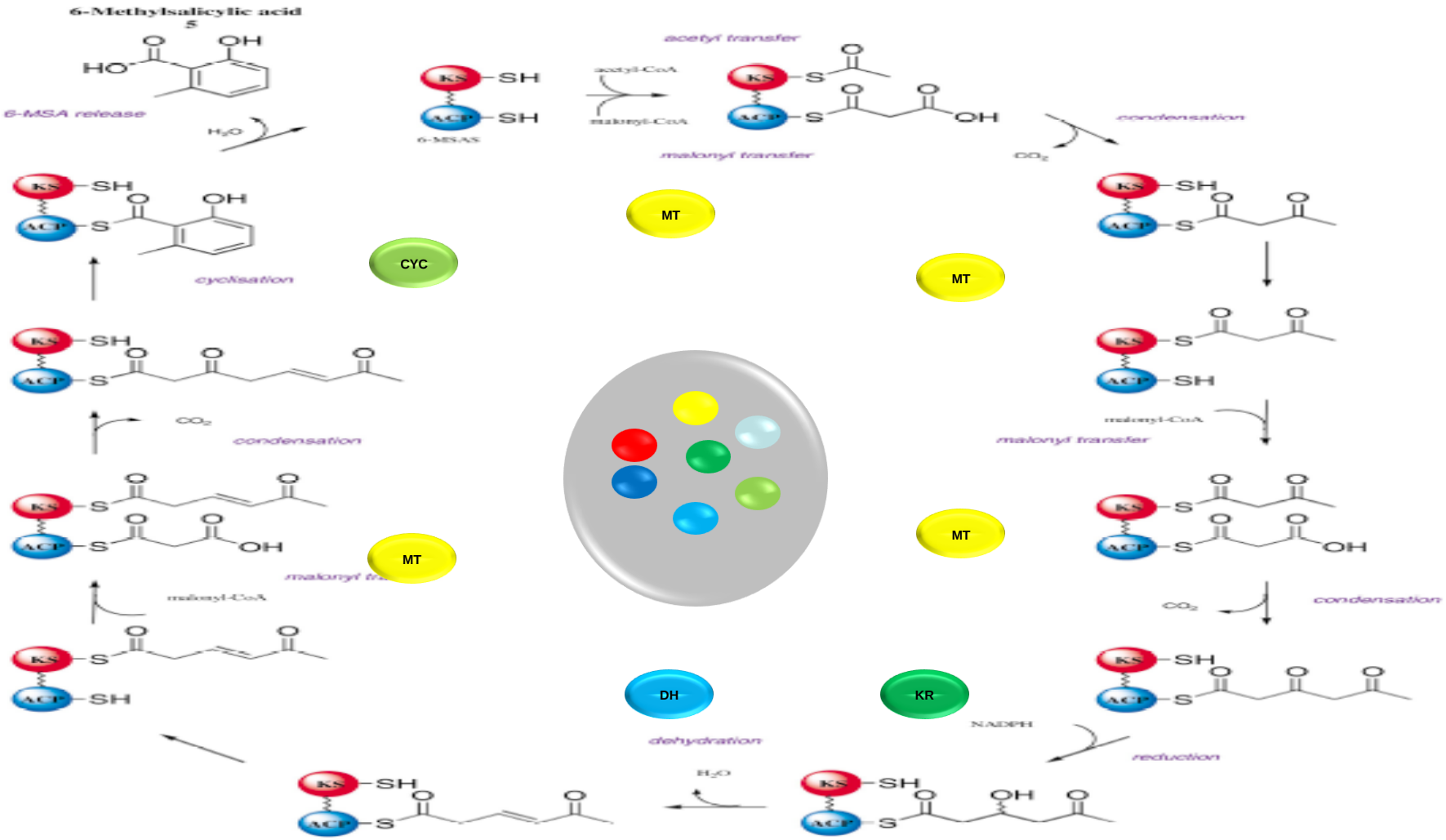
Type III
Actinobacteria

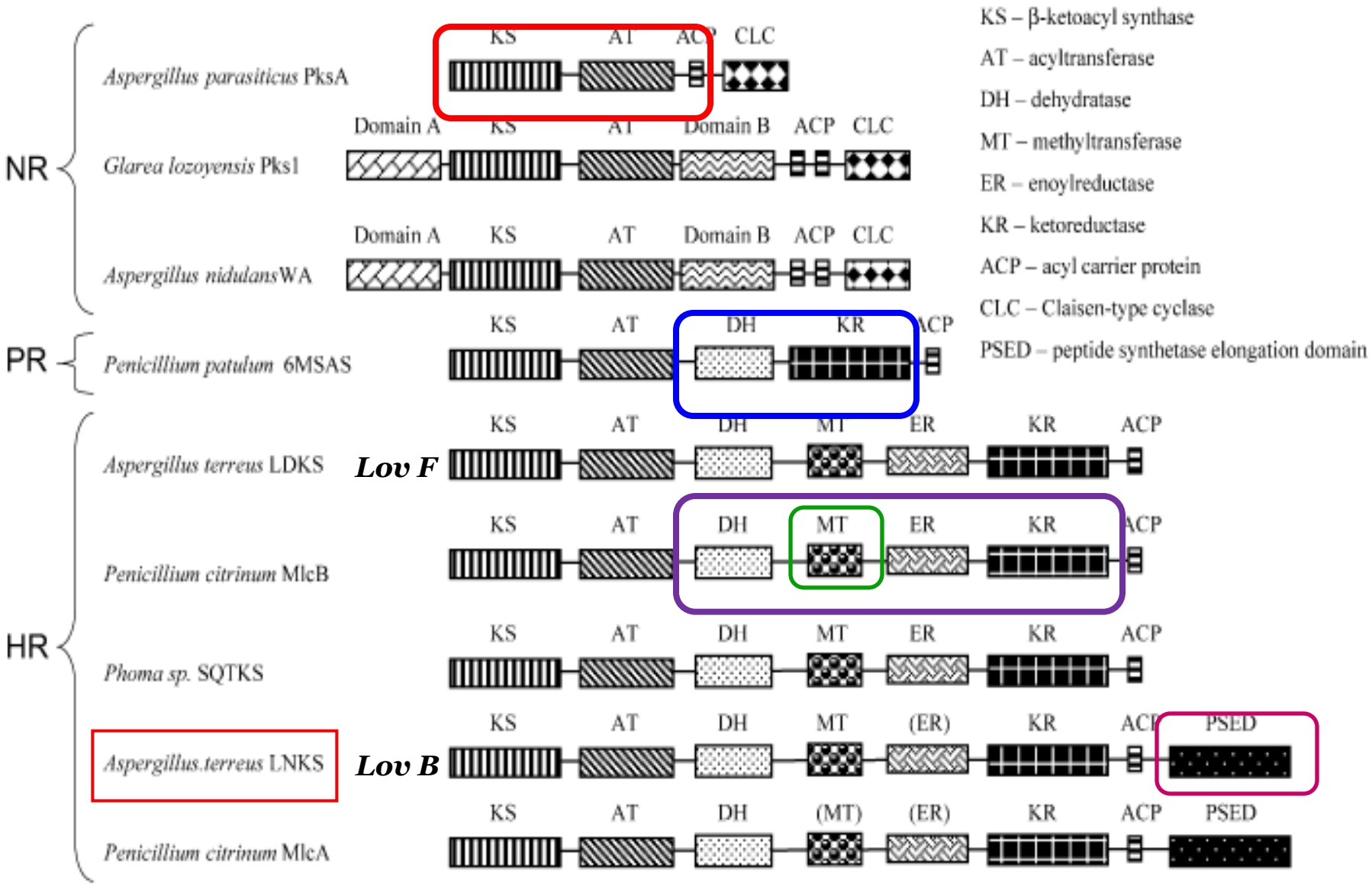
PKS type I Modular



AT = Acyltransferase
ACP = Acyl carrier pro
KS = Ketosynthase
KR = Ketoreductase
ER = Enoyl reductase
DH = Dehydratase
TE = Thioesterase

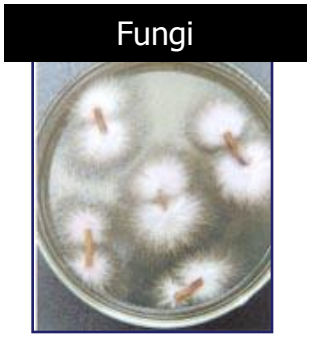
PKS type I Interactive

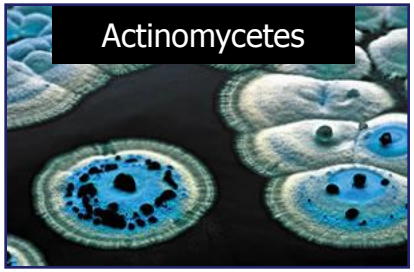




NR : Non- reduced PR: Parcial reduced HR: Highly reduced

Bioprospection of polyketides in endophytics





Endophytic bacteria and fungi

(ESALQ/Piracicaba – isolates from plants with economical importance)

PKS I

Juan Diego Rojas
Lovastatins X
Filaments fungi

PKS II

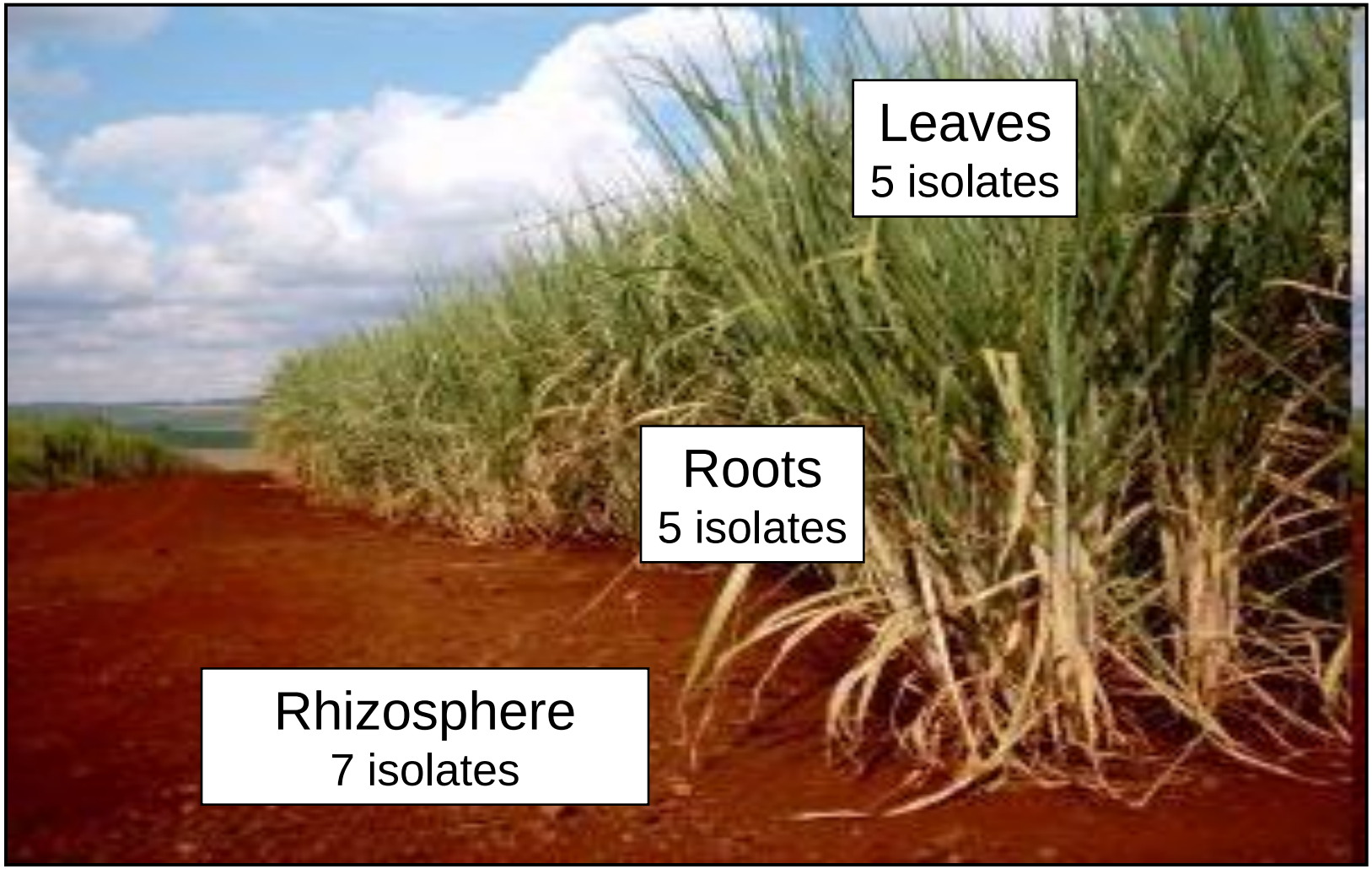
Erik Saenz
Antracyclines X
Actinomycetes

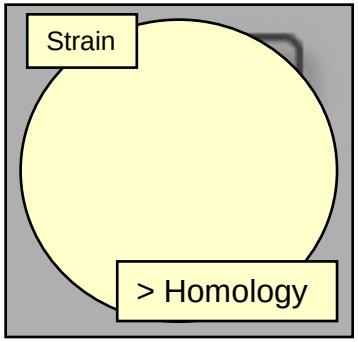
PKS III

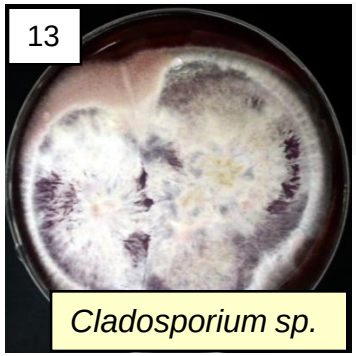
Fabiana Andrieli
Flavonoids X
Actinomycetes

1. Isolation

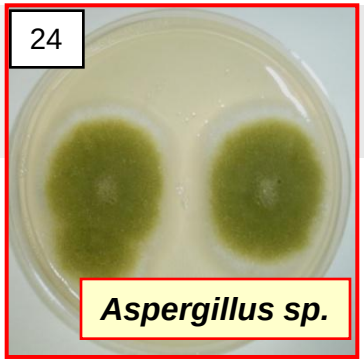
Brazilian Sugar Cane

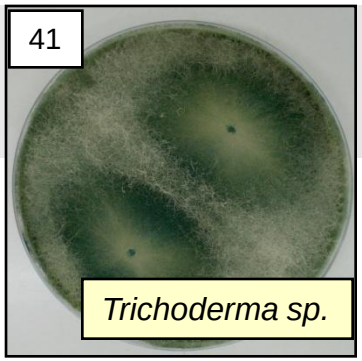


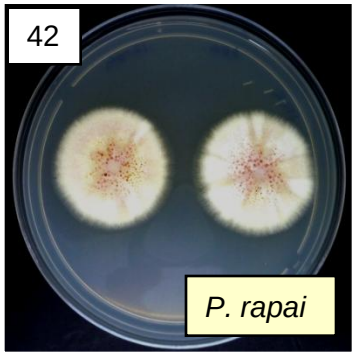

Strain
> Homology

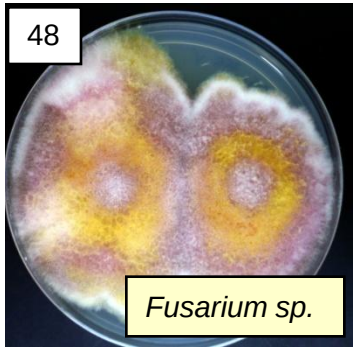

13
Cladosporium sp.

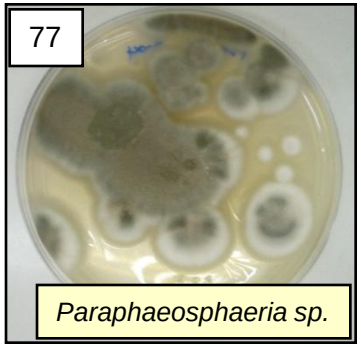

23
Saccharicola bicolor


24
Aspergillus sp.


41
Trichoderma sp.


42
P. rapai


48
Fusarium sp.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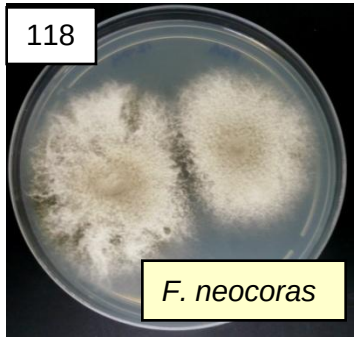
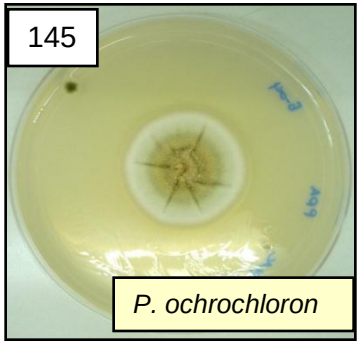
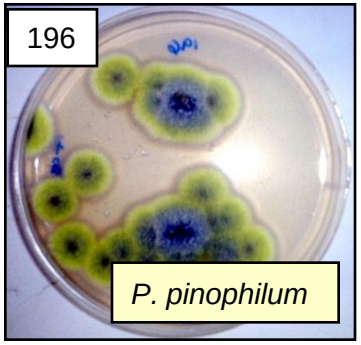
Paraphaeosphaeria sp.


118
F. neocoras


145
P. ochrochloron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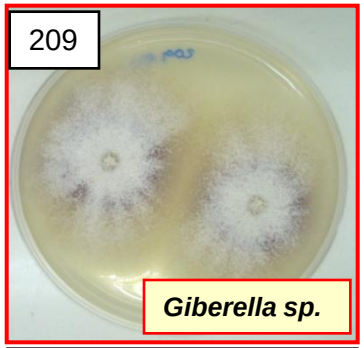
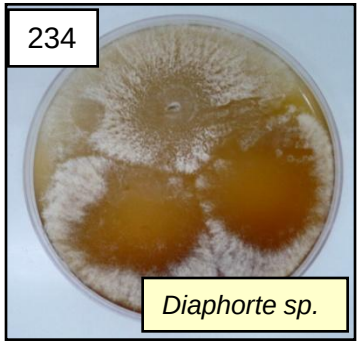
P. pinophilum


209
Giberella sp.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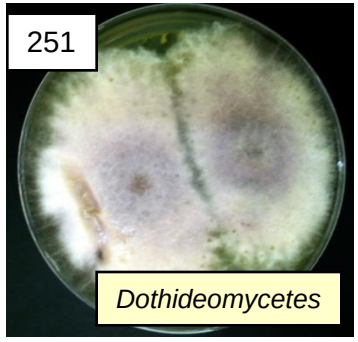
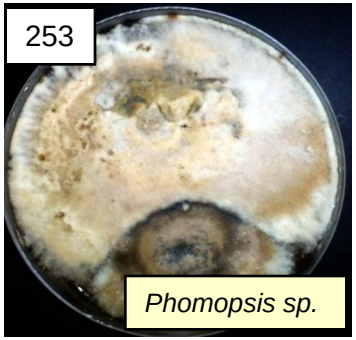
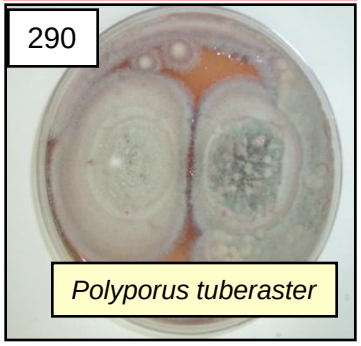
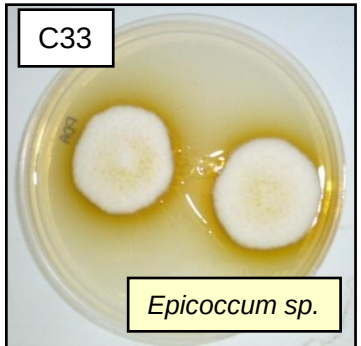
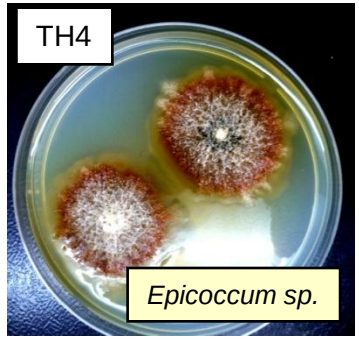
Diaphorte sp.


251
Dothideomycetes

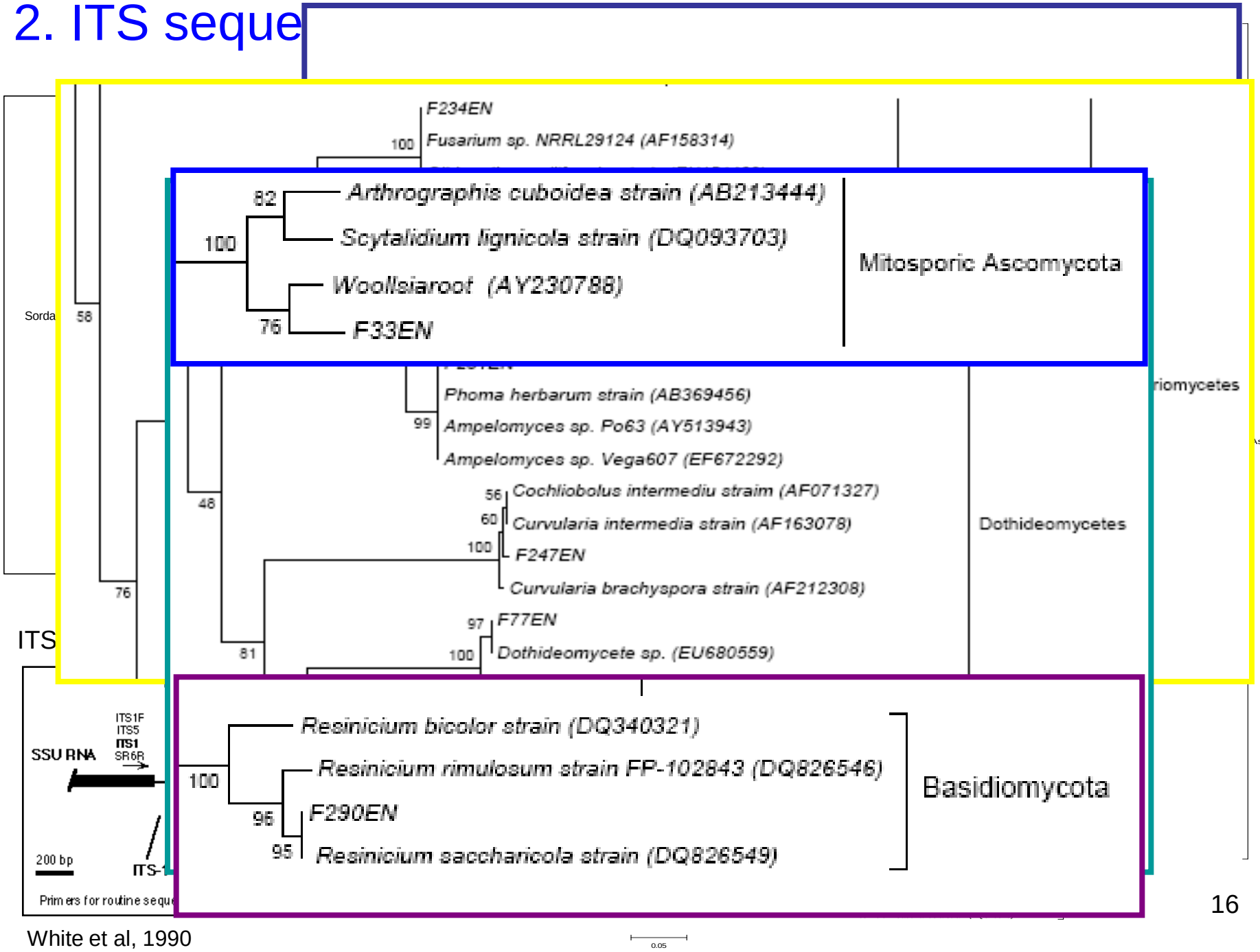

253
Phomopsis sp.


290
Polyporus tuberaster


C33
Epicoccum sp.

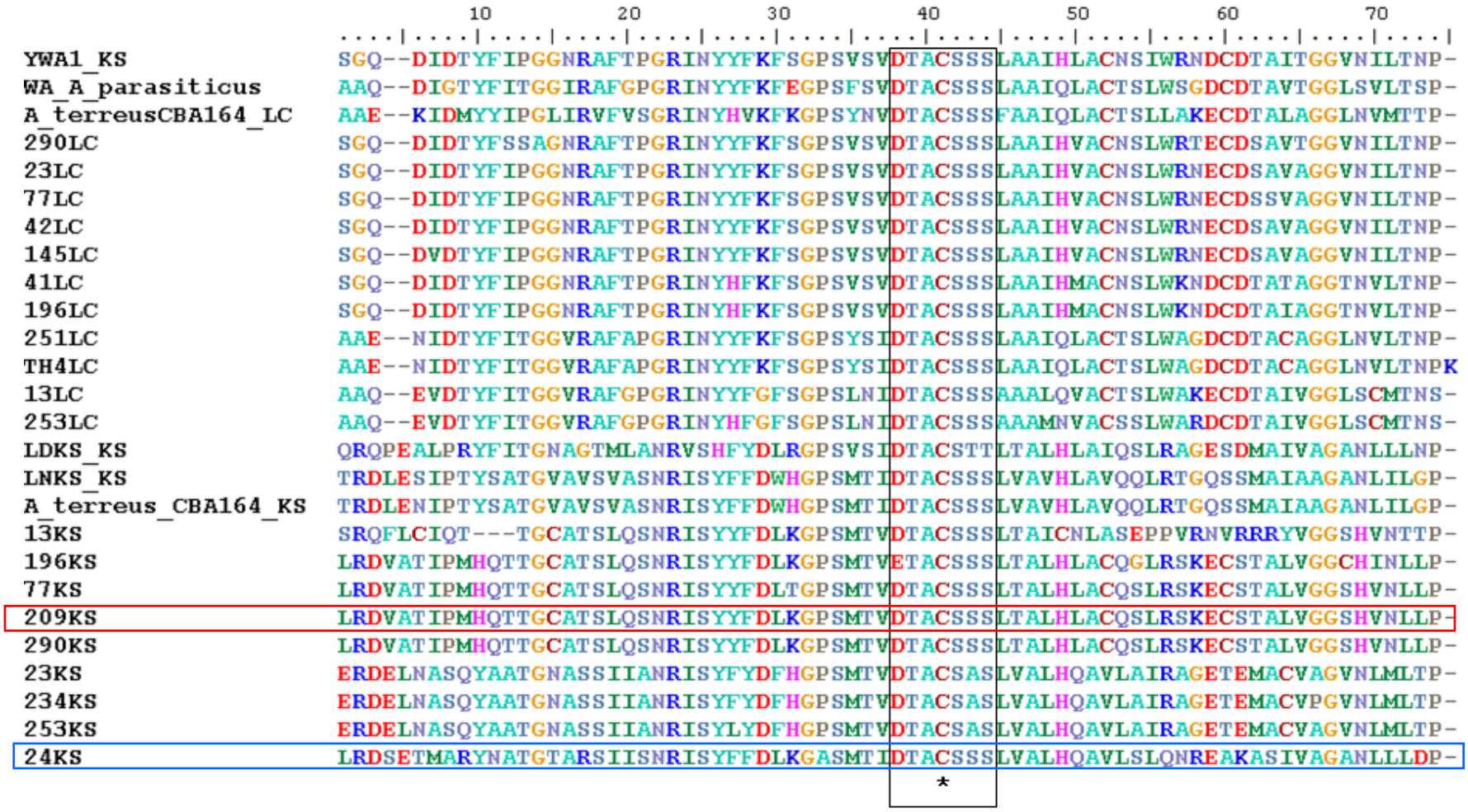

TH4
Epicoccum sp.

2. ITS seque

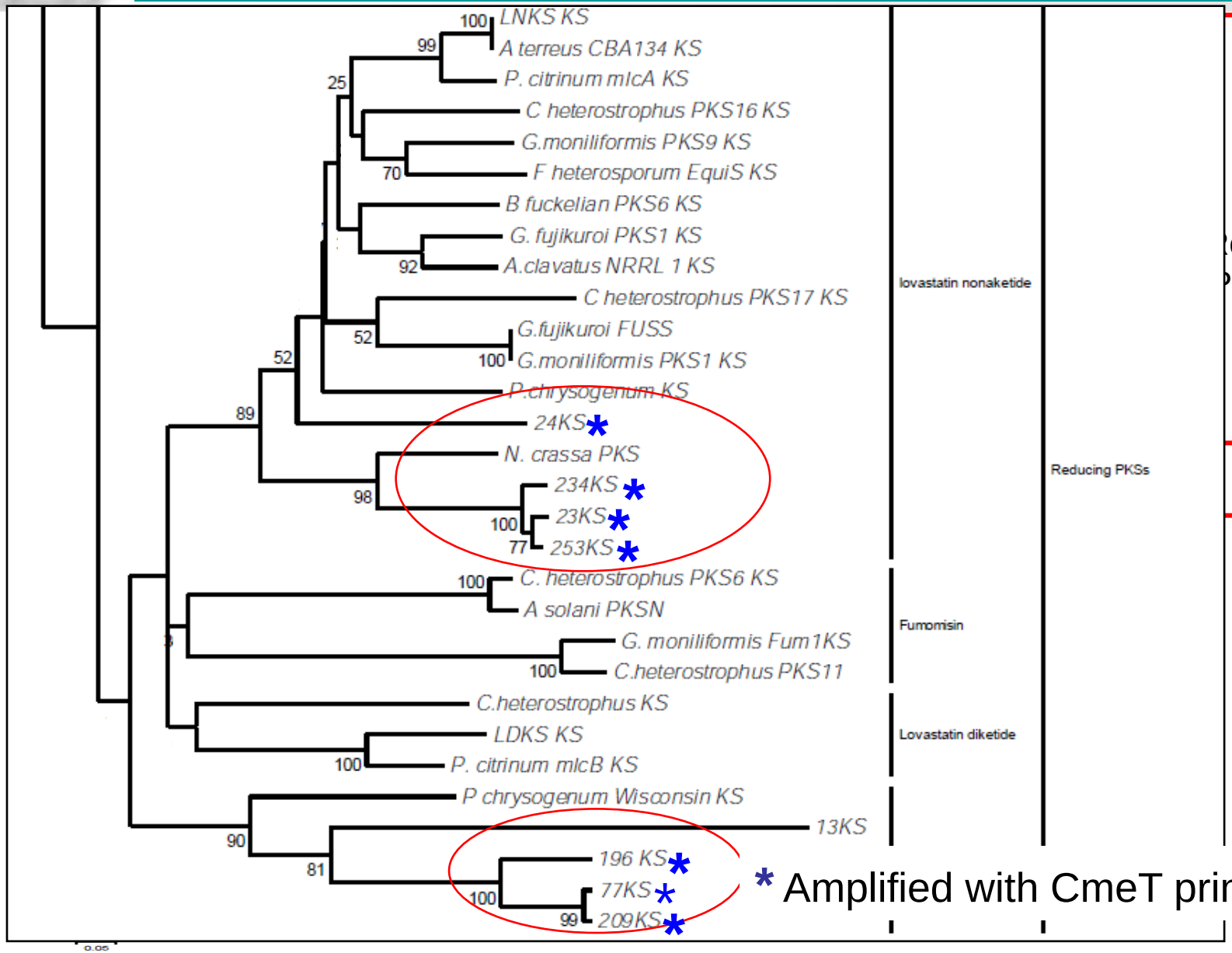


White et al, 1990

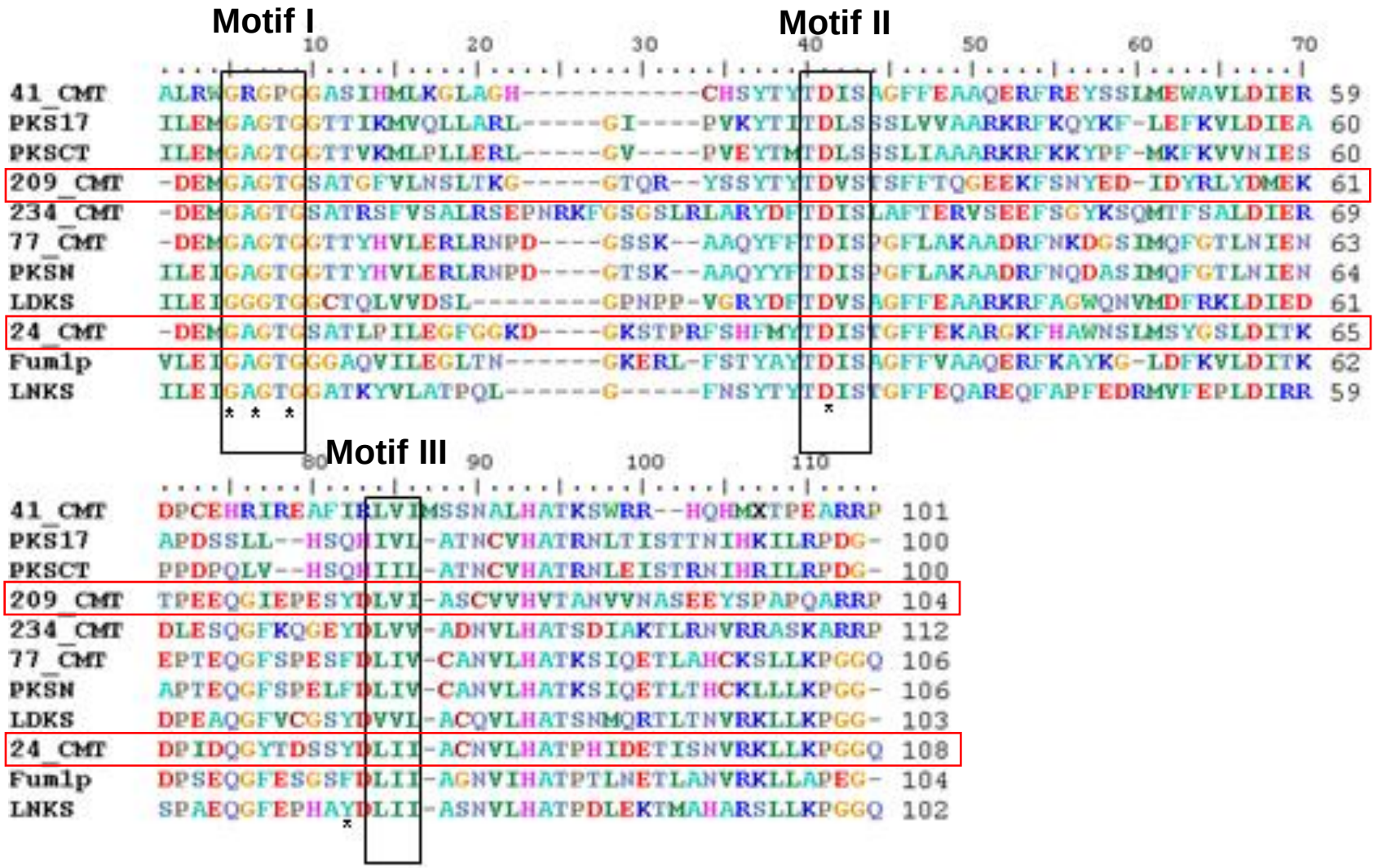
Multiple alignment of the Keto- synthase sequences



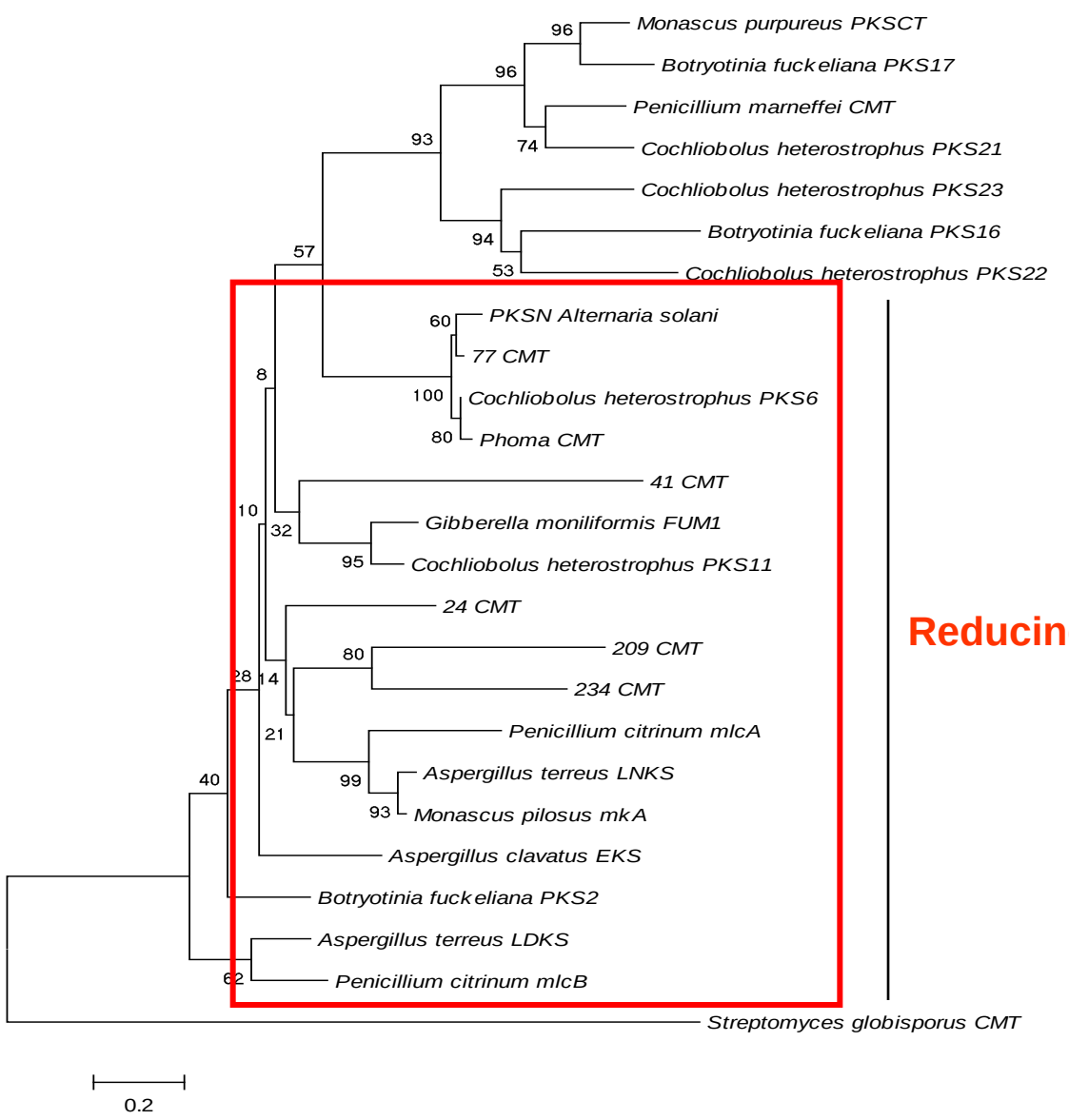
Phylogenetic analysis of KS sequences

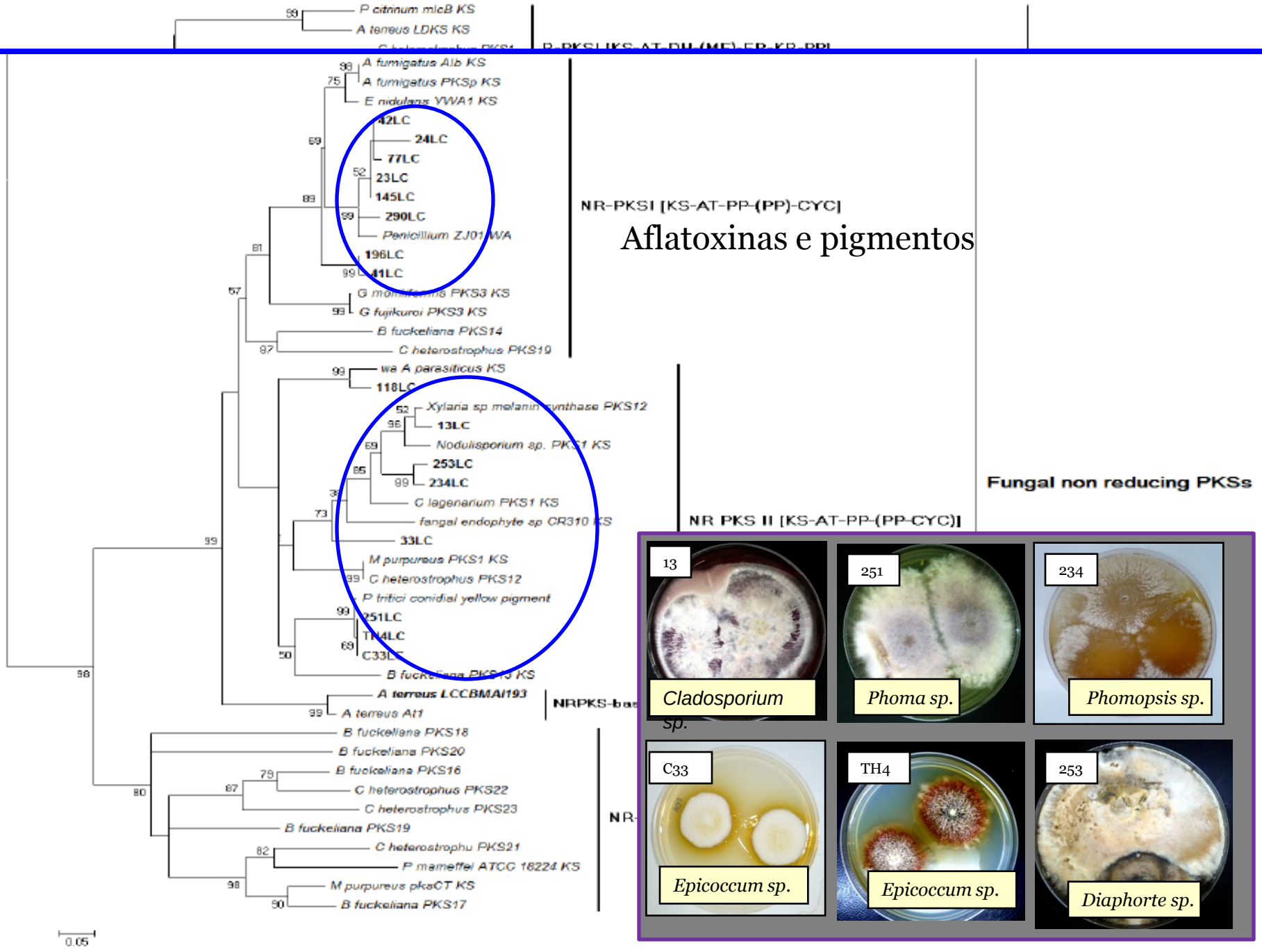


Multiplce Alignment of C- methyl transferase sequences

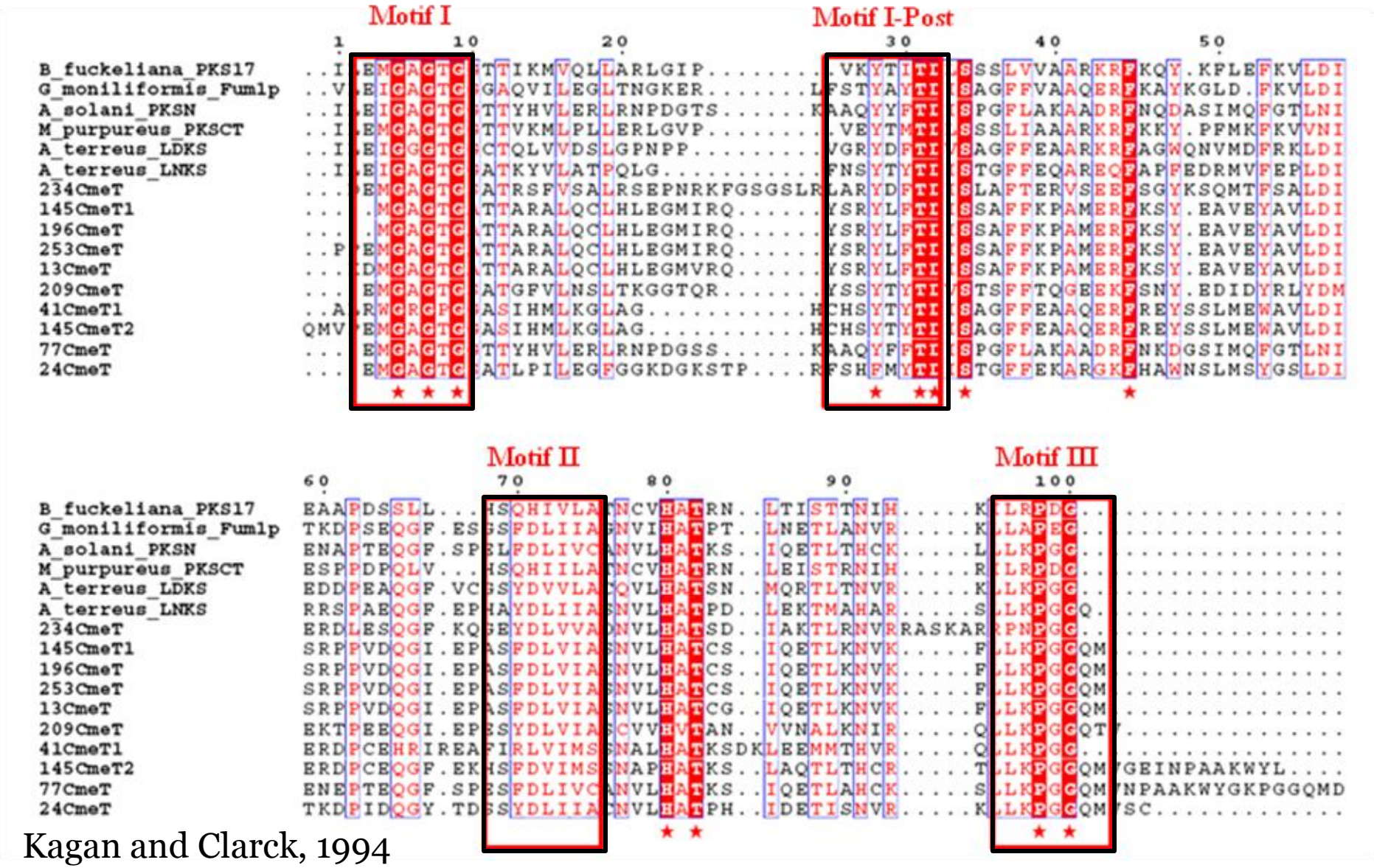


Phylogenetic analysis of CmeT sequences

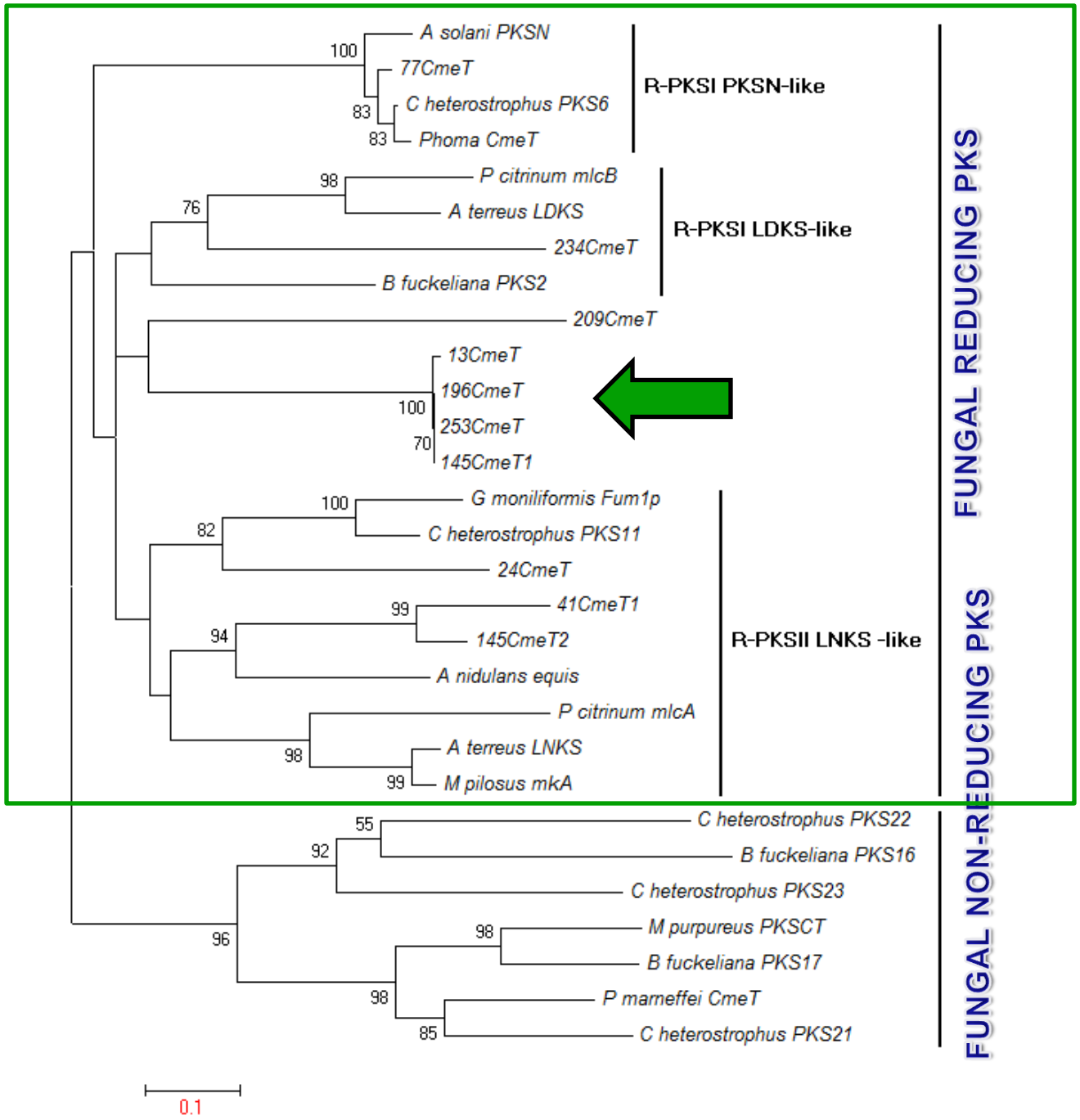



P citrinum mlcB KS
A terreus LDKS KS
A fumigatus Alb KS
A fumigatus PKSp KS
E nidulans YWA1 KS
42LC
24LC
77LC
23LC
145LC
290LC
Penicillium ZJ01 WA
196LC
41LC
G moniliformis PKS3 KS
G fujikuroi PKS3 KS
B fuckeliana PKS14
C heterostrophus PKS19
NR-PKSI [KS-AT-PP-(PP)-CYC]
Aflatoxinas e pigmentos
we A parasiticus KS
118LC
Xylaria sp melanin synthase PKS12
13LC
Nodulisporium sp. PKS1 KS
253LC
234LC
C lagenarium PKS1 KS
fangal endophyte sp CR310 KS
33LC
M purpureus PKS1 KS
C heterostrophus PKS12
P tritici conidial yellow pigment
251LC
TH4LC
C33LC
B fuckeliana PKS15 KS
A terreus LCCBMAI193
A terreus At1
NRPKS-bas
NR PKS II [KS-AT-PP-(PP-CYC)]
Fungal non reducing PKSs
B fuckeliana PKS18
B fuckeliana PKS20
B fuckeliana PKS16
C heterostrophus PKS22
C heterostrophus PKS23
B fuckeliana PKS19
C heterostrophu PKS21
P mameffei ATCC 18224 KS
M purpureus pksCT KS
B fuckeliana PKS17
NR-
0.05
13
Cladosporium sp.
251
Phoma sp.
234
Phomopsis sp.
C33
Epicoccum sp.
TH4
Epicoccum sp.
253
Diaphorte sp.

Aligment of Cmet SAM dependents

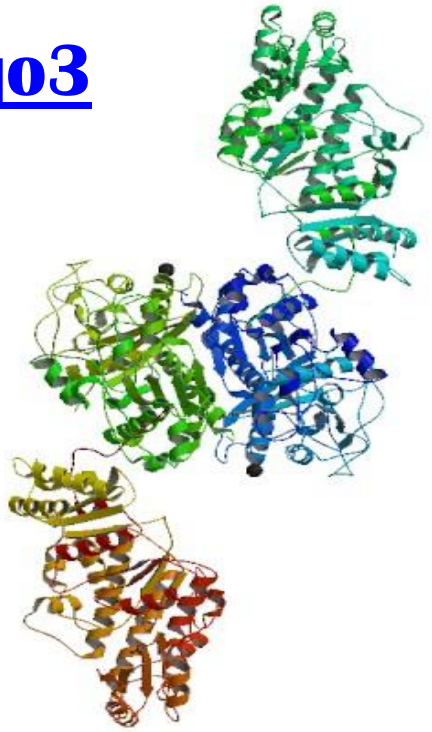


Kagan and Clarck, 1994


A solani PKSN
77CmeT
C heterostrophus PKS6
Phoma CmeT
R-PKSI PKSN-like
P citrinum mlcB
A terreus LDKS
234CmeT
B fuckeliana PKS2
R-PKSI LDKS-like
209CmeT
13CmeT
196CmeT
253CmeT
145CmeT1
G moniliformis Fum1p
C heterostrophus PKS11
24CmeT
41CmeT1
145CmeT2
A nidulans equis
P citrinum mlcA
A terreus LNKS
M pilosus mkA
R-PKSII LNKS -like
FUNGAL REDUCING PKS
C heterostrophus PKS22
B fuckeliana PKS16
C heterostrophus PKS23
M purpureus PKSCT
B fuckeliana PKS17
P marneffei CmeT
C heterostrophus PKS21
FUNGAL NON-REDUCING PKS
0.1

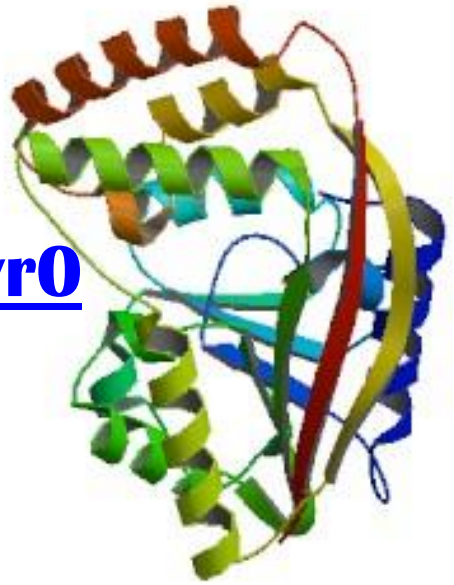
Comparative tridimensional modelling

2qo3

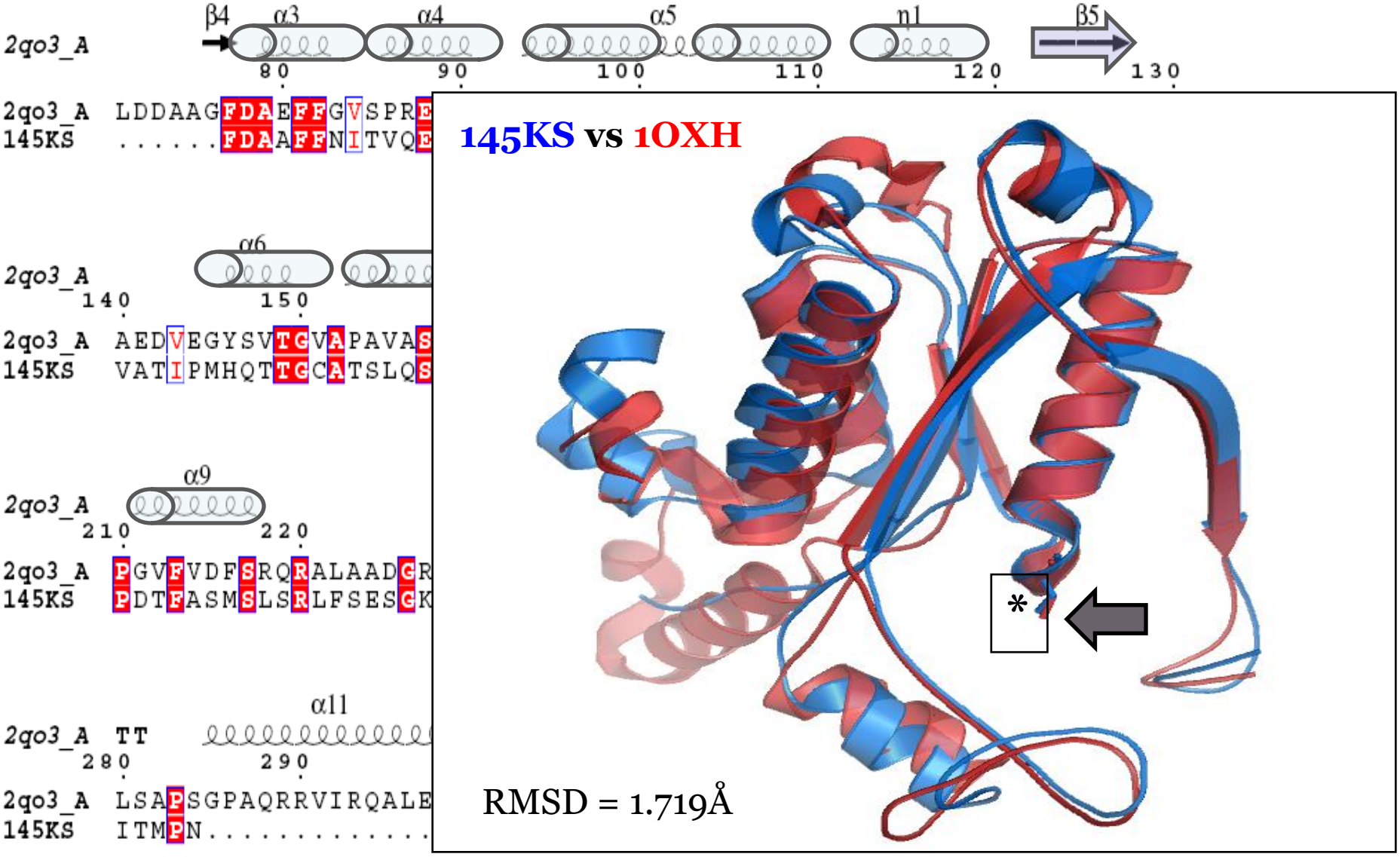


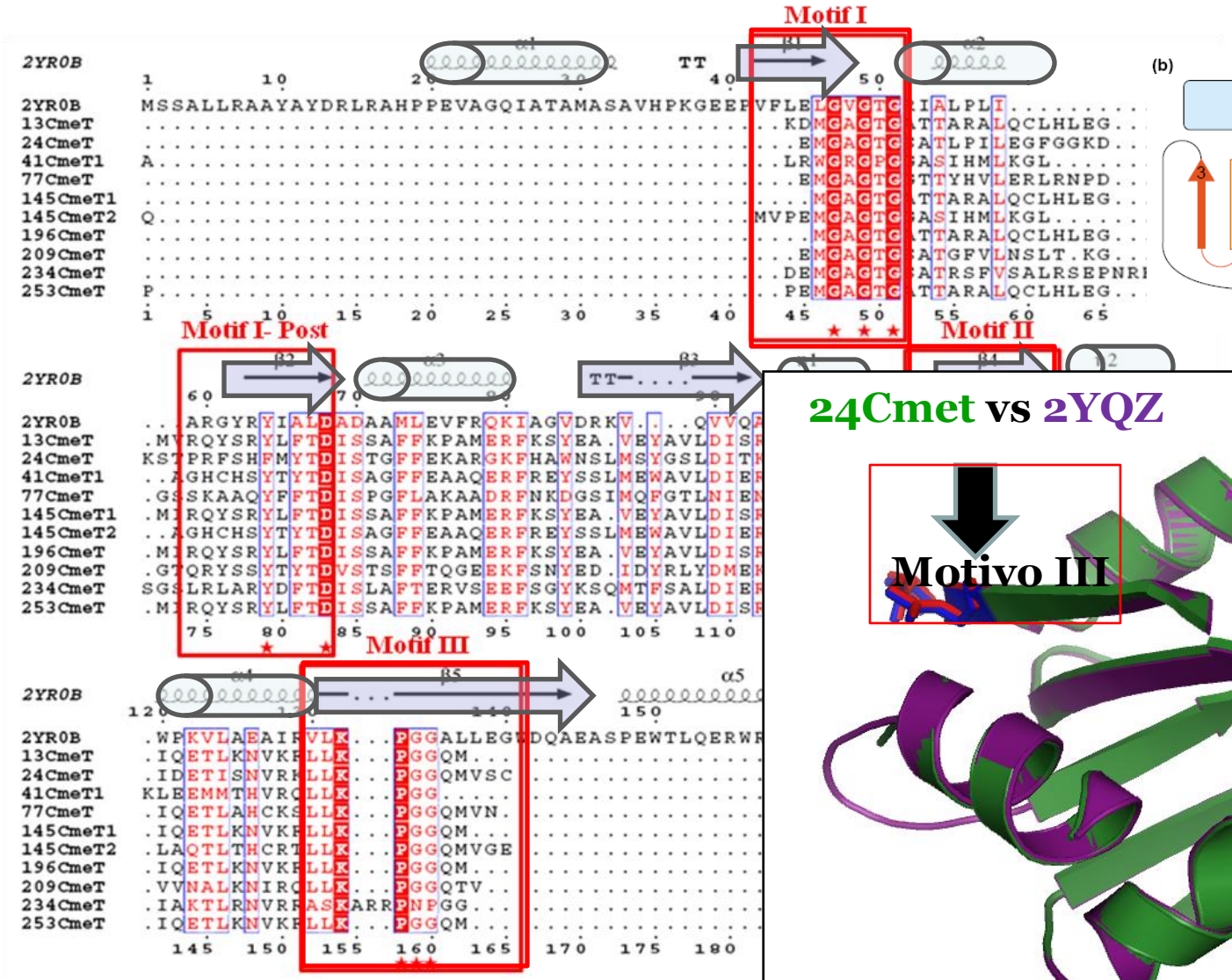
•Transferase
•B cetoacil-ACP synthase
•beta-ketoacyl-[acyl carrier protein] synthase II from *Streptococcus pneumoniae,* tricyclic form

2yr0

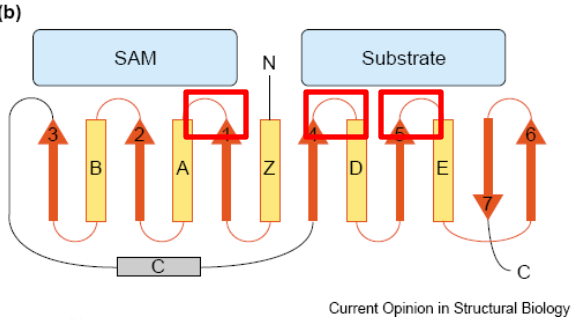


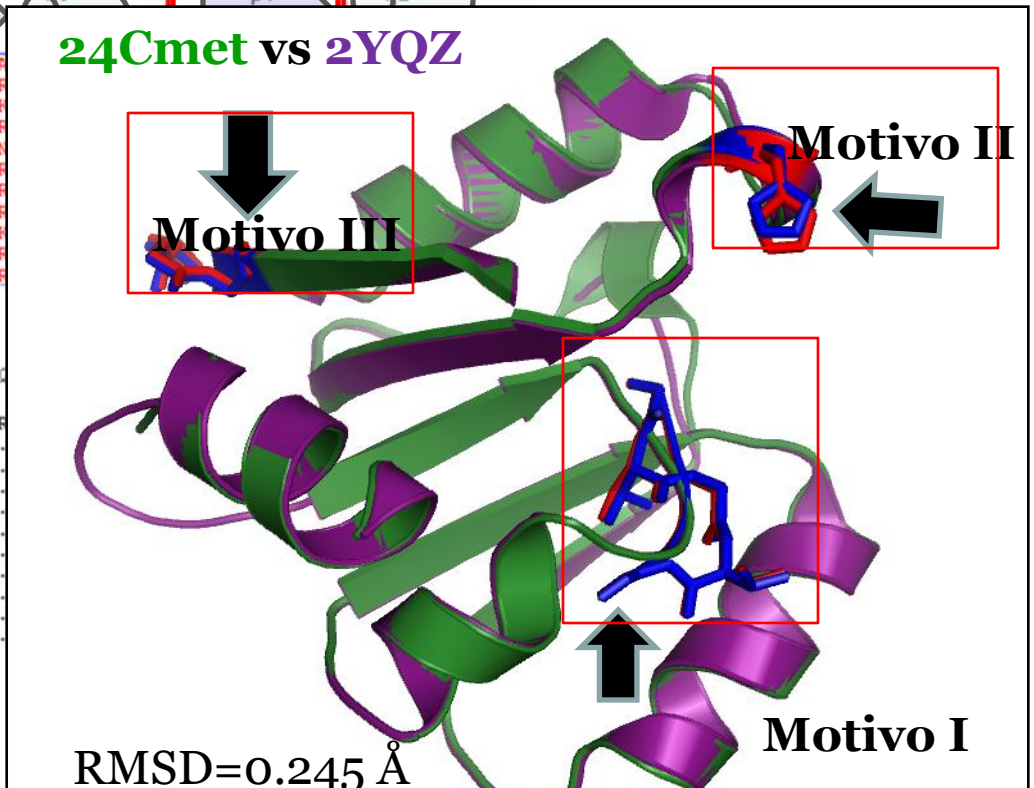
•Transferase
•Metil transferase SAM dependent
•Crystal structure of hypothetical methyltransferase2 ttha0223 from *Thermus thermophilus* hb8


β4 α3 α4 α5 η1 β5
2qo3_A
80 90 100 110 120 130
2qo3_A LDDAAGFDAEFFGVSPRE
145KS .....FDAAFFNITVQE
α6
2qo3_A
140 150
2qo3_A AEDVEGYSVTGVAPAVAS
145KS VATIPMHQTTGCATSLQS
α9
2qo3_A
210 220
2qo3_A PGVFVDFSRQRALAADGR
145KS PDTFASMSLSRLFSESGK
α11
2qo3_A TT
280 290
2qo3_A LSAPSGPAQRRVIRQALE
145KS ITMPN.....
145KS vs 1OXH
*
RMSD = 1.719Å

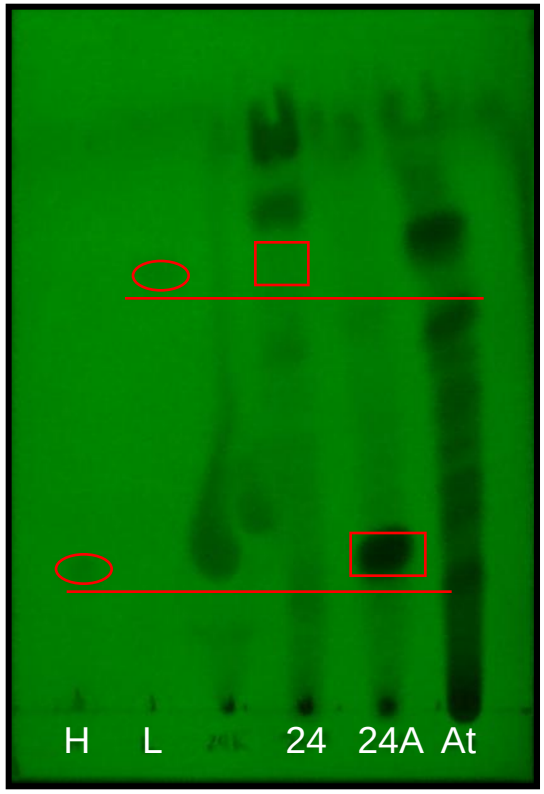

Motif I
Motif I- Post
Motif II
Motif III
2YR0B
13CmeT
24CmeT
41CmeT1
77CmeT
145CmeT1
145CmeT2
196CmeT
209CmeT
234CmeT
253CmeT

Martin 2002

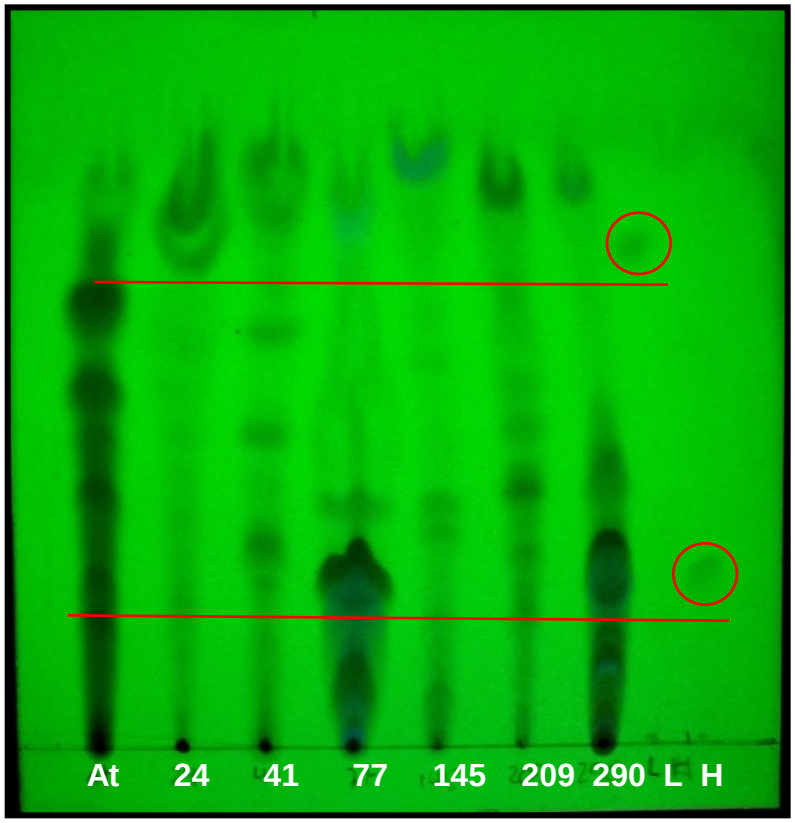

(b)
SAM
Substrate
N
C
Current Opinion in Structural Biology


24Cmet vs 2YQZ
Motivo II
Motivo III
Motivo I
RMSD=0.245 Å

4. Prospecting for Statins



TLC of extracts from strain 24

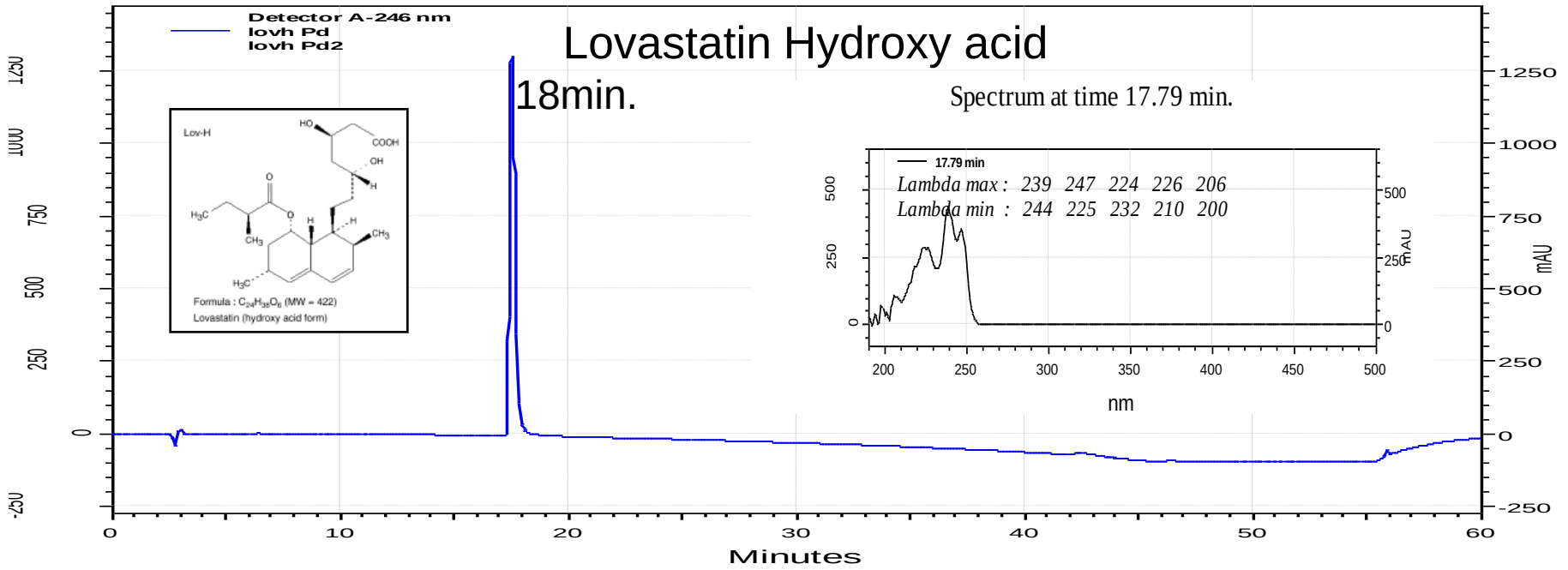


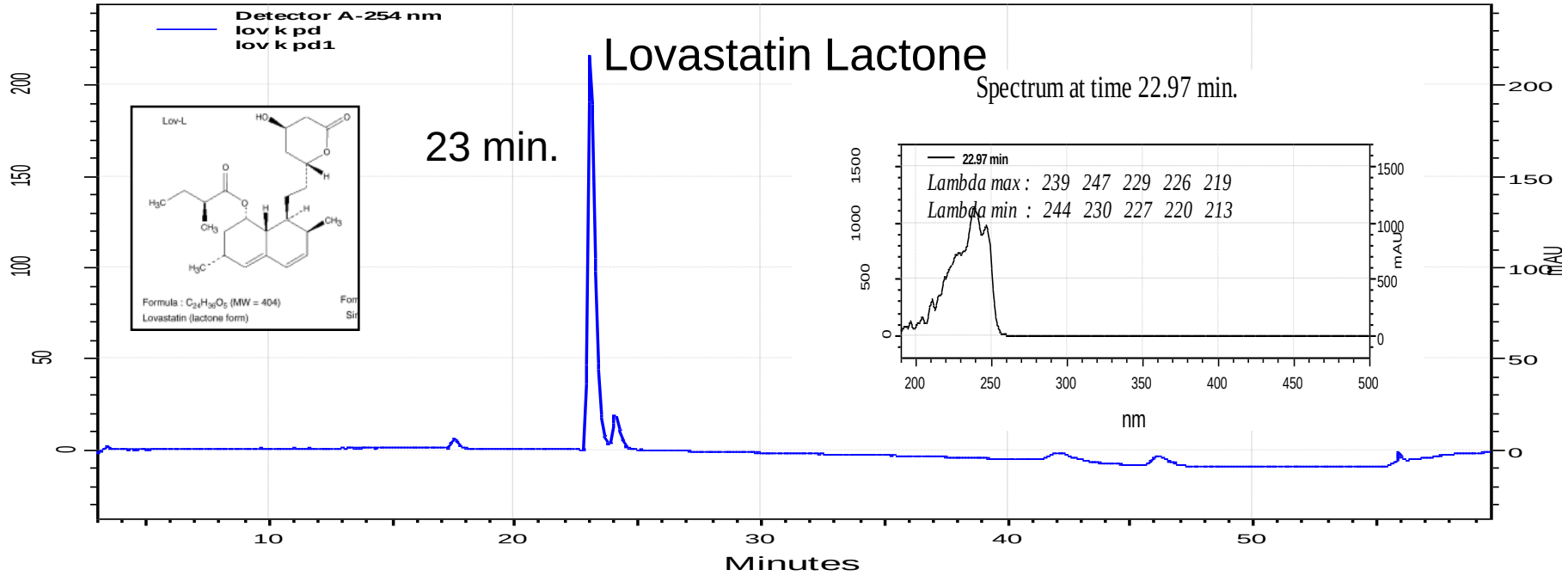
TLC of endophytic extracts

Dichlorometan: Etyl Acetate: Methanol
7:3:1

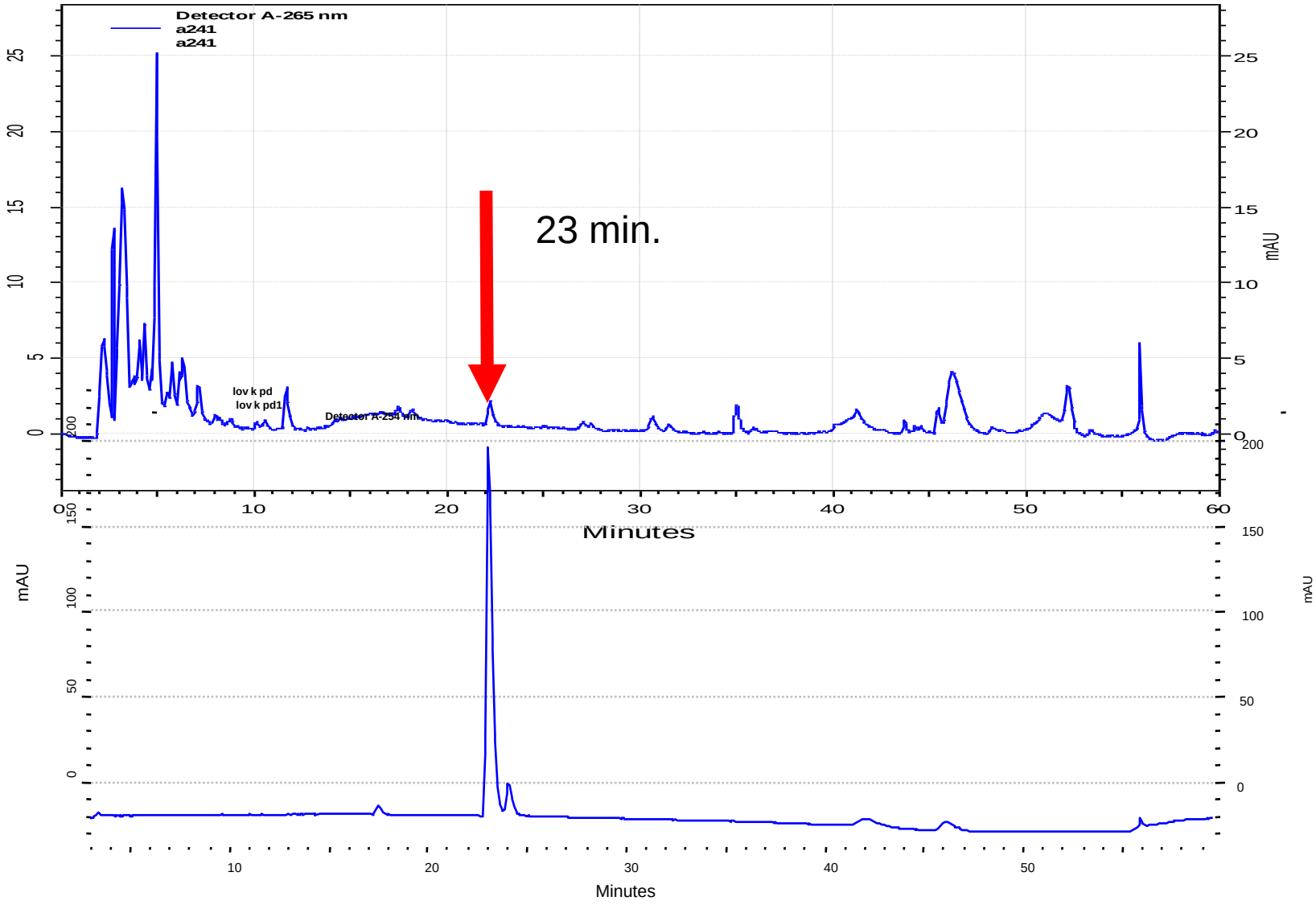
H: Lovastatin hydroxy acid

L: Lovastatin lactone


Detector A-246 nm
lovh Pd
lovh Pd2
Lovastatin Hydroxy acid
18min.
Spectrum at time 17.79 min.
Lov-H
Formula : C24H38O6 (MW = 422)
Lovastatin (hydroxy acid form)
17.79 min
Lambda max : 239 247 224 226 206
Lambda min : 244 225 232 210 200
nm
mAU
Minutes

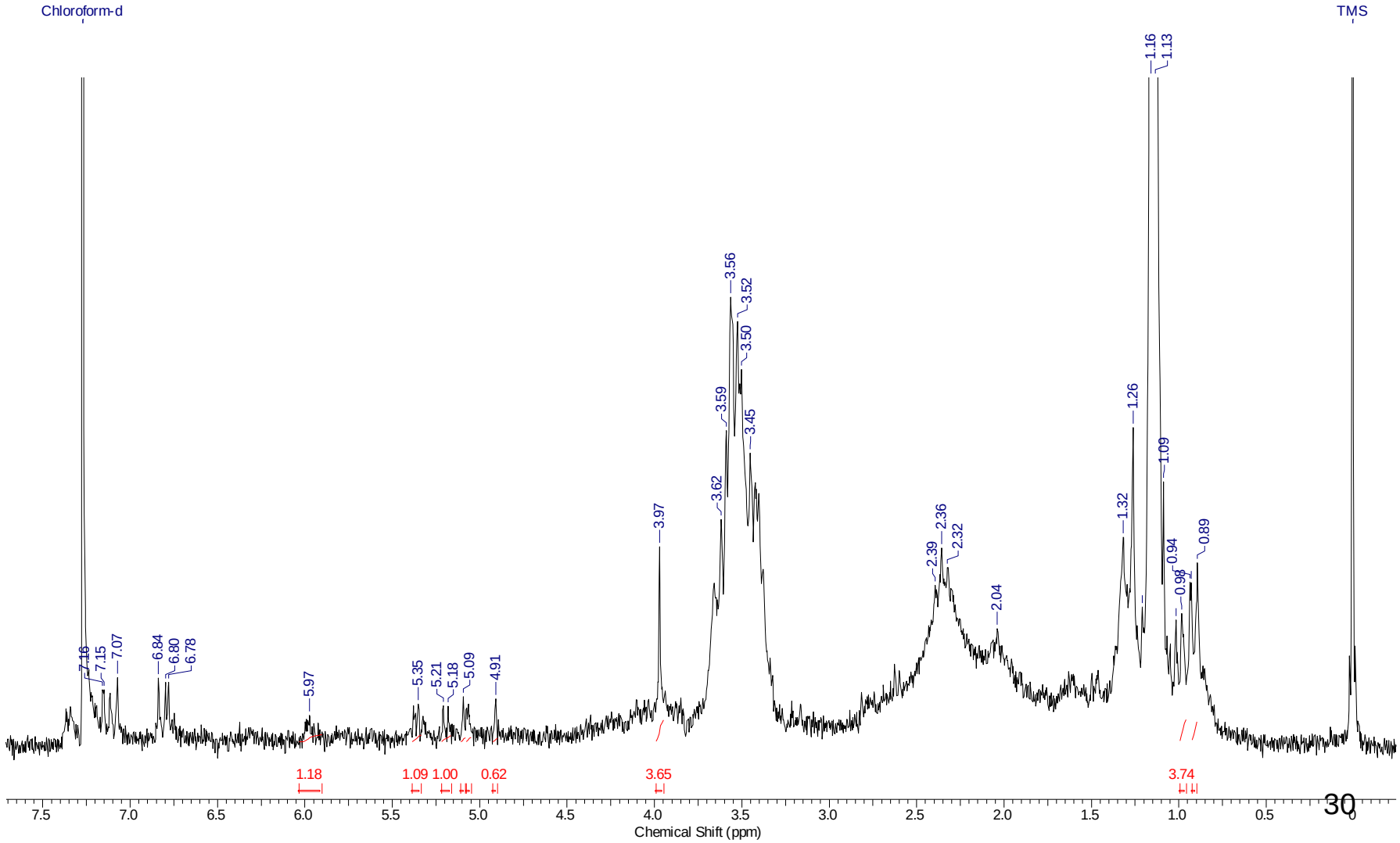

Detector A-254 nm
lov k pd
lov k pd1
Lovastatin Lactone
23 min.
Spectrum at time 22.97 min.
Lov-L
Formula : C24H36O5 (MW = 404)
Lovastatin (lactone form)
22.97 min
Lambda max : 239 247 229 226 219
Lambda min : 244 230 227 220 213
nm
mAU
Minutes

Chromatogram Strain 24 (extract)



NMR ^{1}H from extract strain 24

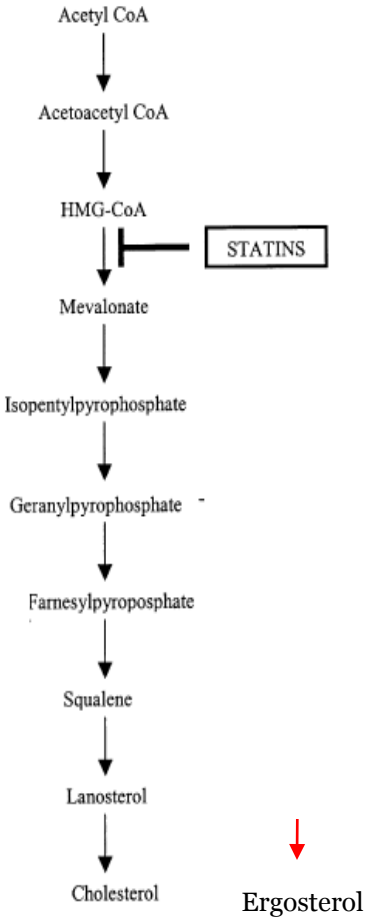
Acquisition Time (sec)	2.2774	***Frequency (MHz)***	200.13	***Nucleus***	1H	***Temperature (degree C)***	24.000
Number of Transients	128	***Original Points Count***	8192	***Points Count***	8192	***Sweep Width (Hz)***	3597.12



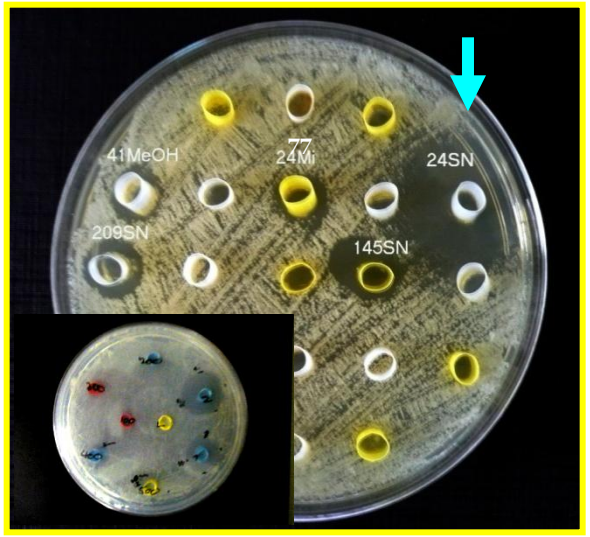
Biological Activity of extracts strain 24

Mucor sp.

Roze *et al*, 1998
Chamilos *et al*, 2006



Candida albicans



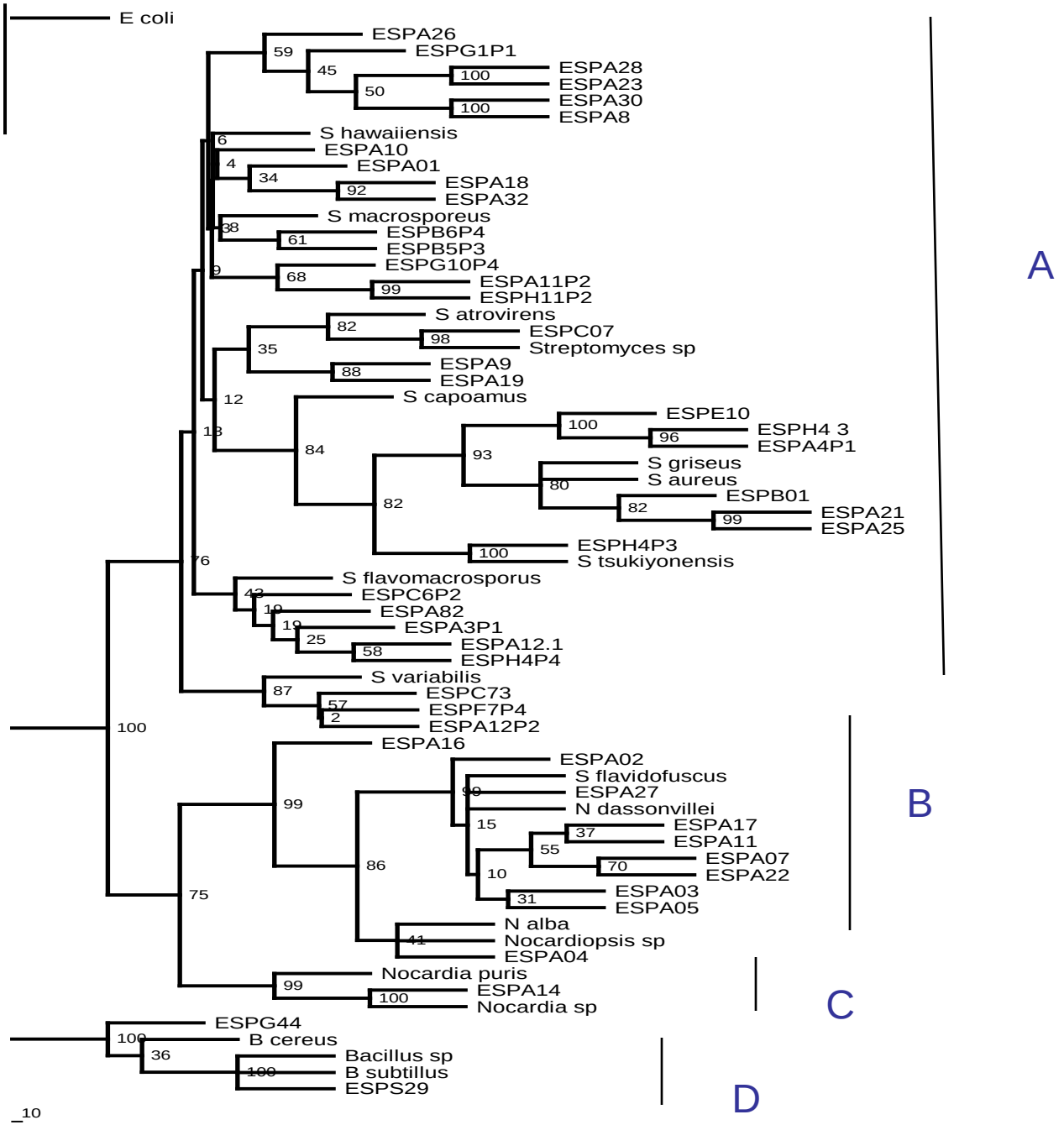
Chin *et al*, 1997
Lorenz *et al*, 1990

Kelvin, 2003

PKS Type II in Actinobacteria

Phylogenetic analysis of 16SrRNA DNA

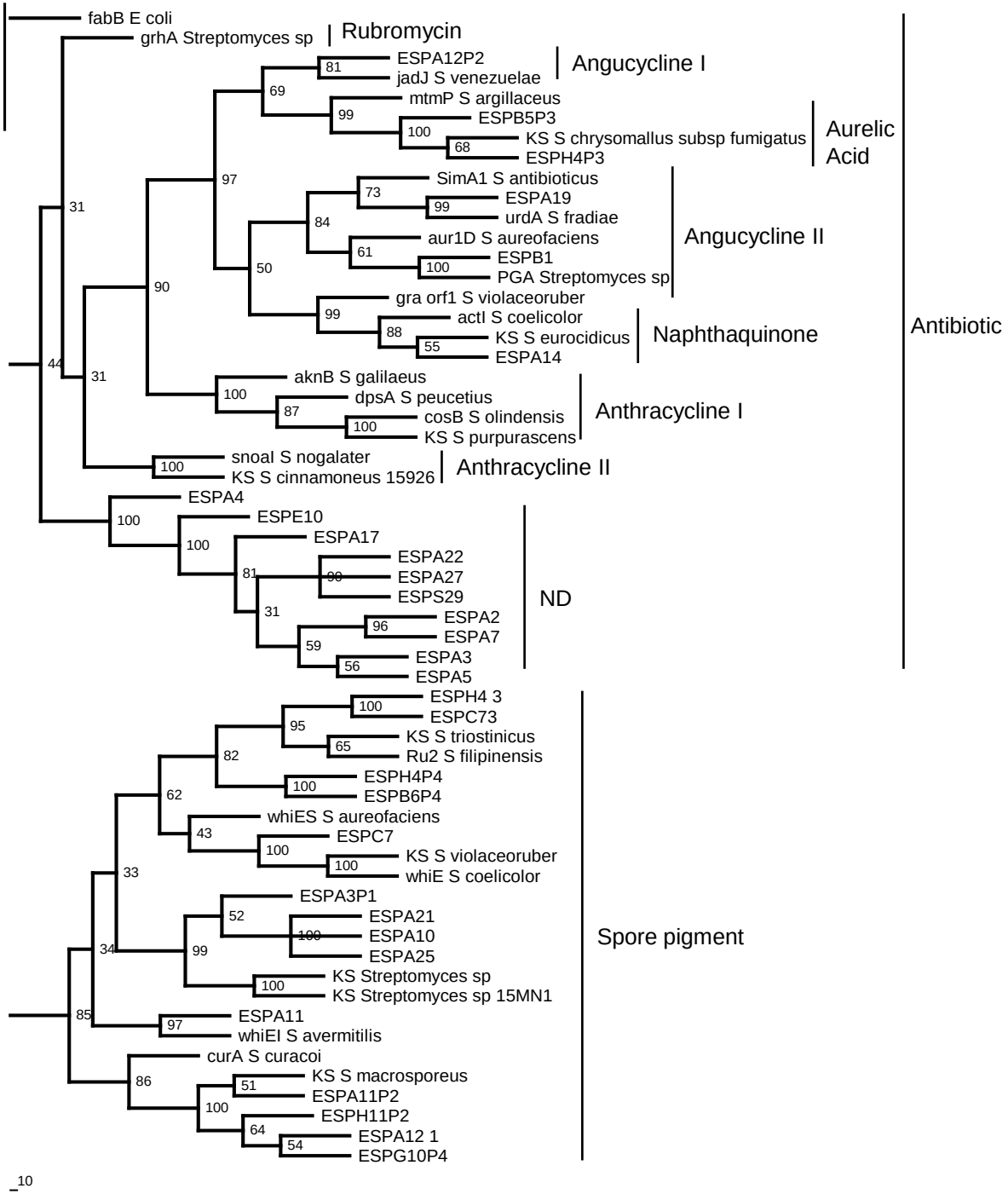
A. *Streptomyces 74,5%*. B. *Nocardiopsis 19,1%* C. *Nocardia 4,3%* D. *Bacillus 2,1%*



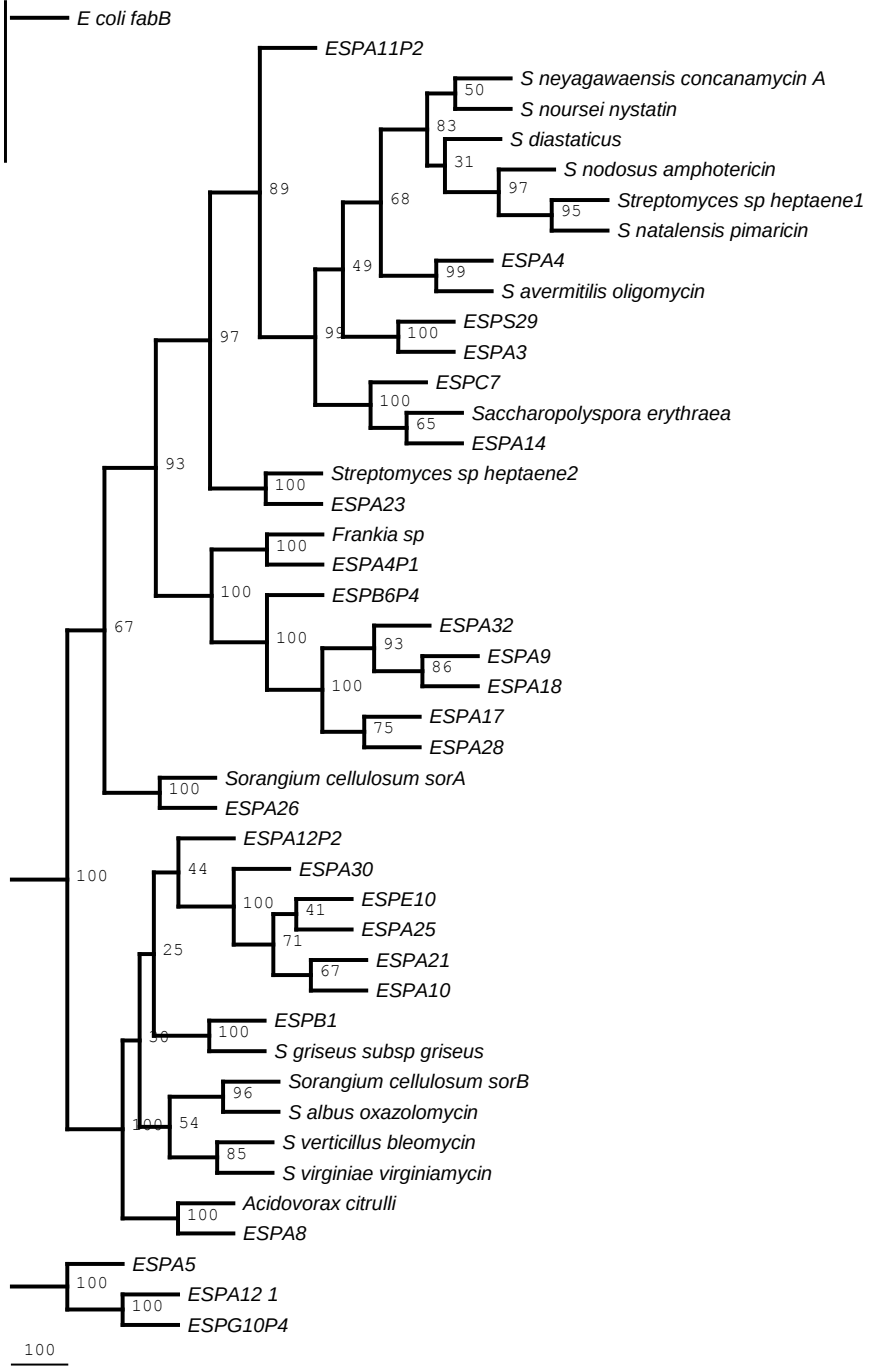
KS gene phylogenetic tree

Controls: KS gene (2) *whiE* (1)

(ND) No determined

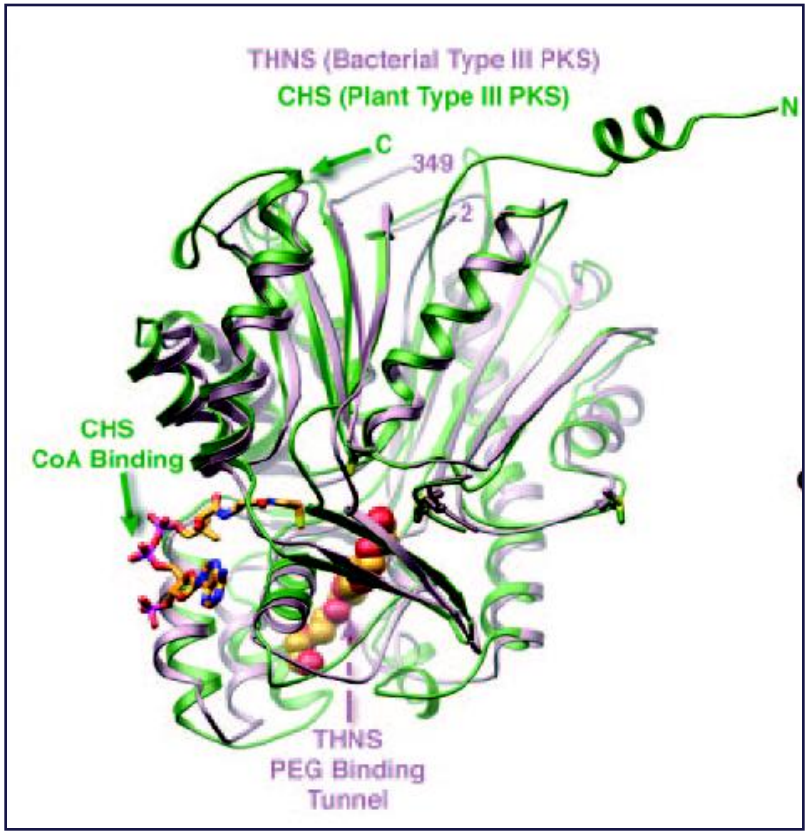


KS type I


E coli fabB
ESPA11P2
S neyagawaensis concanamycin A
S noursei nystatin
S diastaticus
S nodosus amphotericin
Streptomyces sp heptaene1
S natalensis pimaricin
ESPA4
S avermitilis oligomycin
ESPS29
ESPA3
ESPC7
Saccharopolyspora erythraea
ESPA14
Streptomyces sp heptaene2
ESPA23
Frankia sp
ESPA4P1
ESPB6P4
ESPA32
ESPA9
ESPA18
ESPA17
ESPA28
Sorangium cellulosum sorA
ESPA26
ESPA12P2
ESPA30
ESPE10
ESPA25
ESPA21
ESPA10
ESPB1
S griseus subsp griseus
Sorangium cellulosum sorB
S albus oxazolomycin
S verticillus bleomycin
S virginiae virginiamycin
Acidovorax citrulli
ESPA8
ESPA5
ESPA12 1
ESPG10P4
100

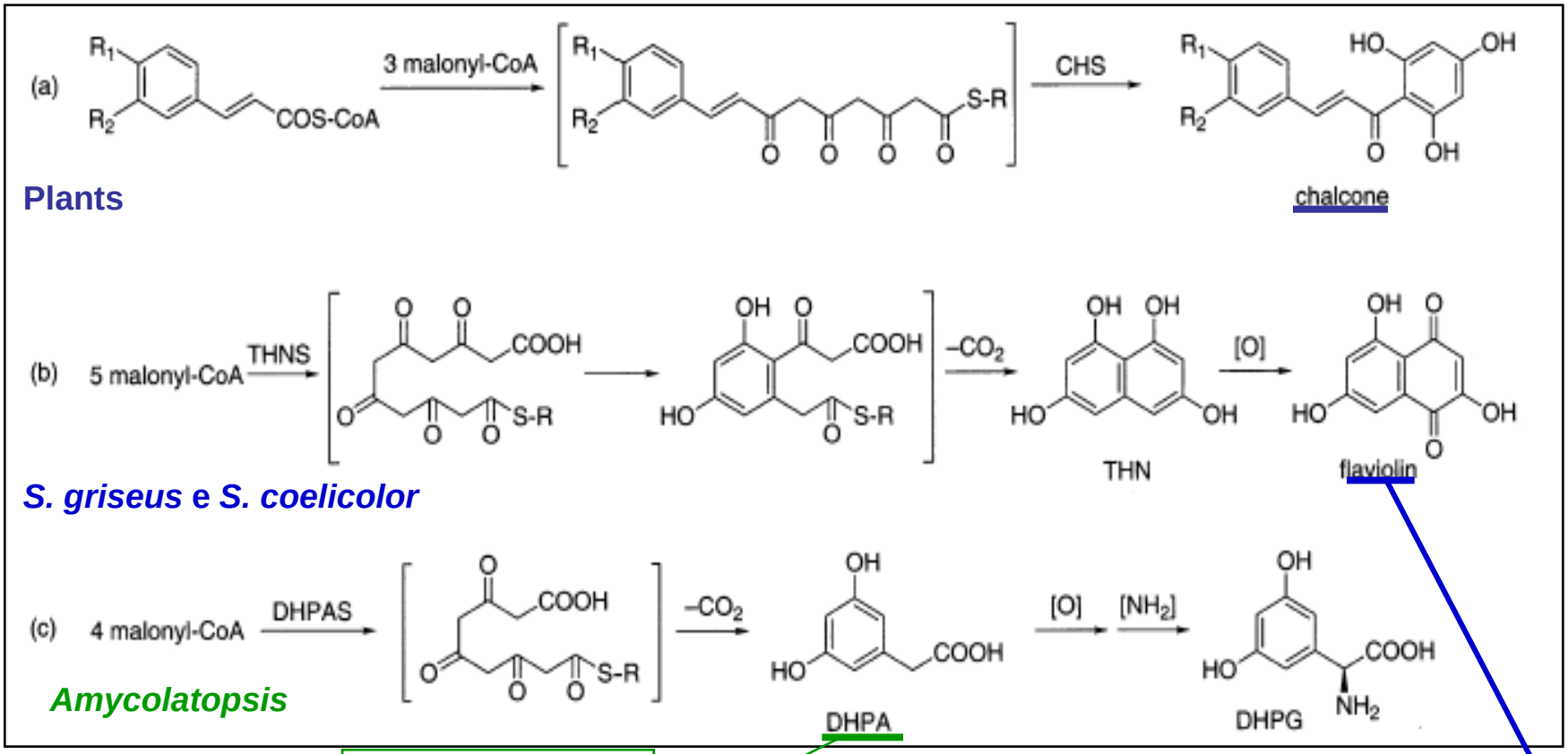
Polyketide synthase type III

- **Biosynthetic superfamily enzymes (15 types) all with homology with** CHS;
- **Superior plants** (1970): Chalcona synthase (CHS) catalise the flavonoids biosynthesis.



CHS structure alfafla (green).
THNS structure from de *S. coelicolor (red).*
Cysteine catalytic site (bars and spheres).

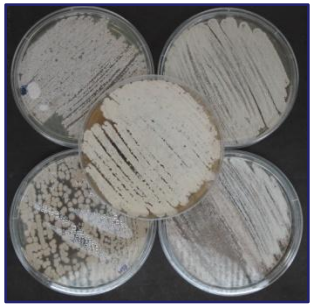
Metabolites synthesized by PKS type III



Biosynthesis of antimicrobials of vancomycin group

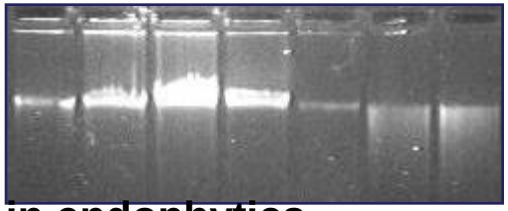
Biosynthesis of melanine and other metabolites

Bioprospection of endophytic *Actinomycetes*

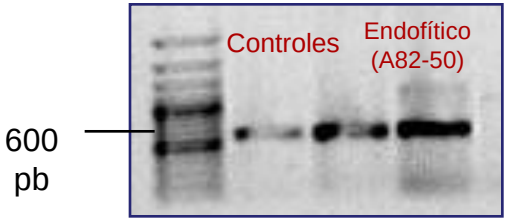


- **46 endophytic actinomycetes + 4 *Streptomyces* control**
  (*Streptomyces* sp, *Nocardia* sp e *Nocardiopsis* sp)

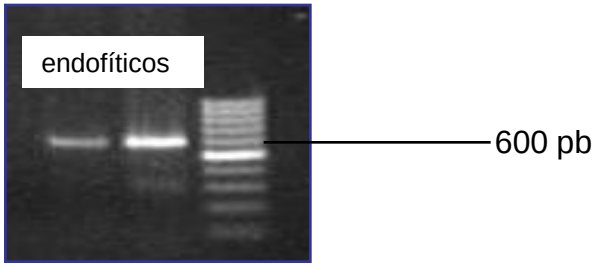
- **Extraction of genomic DNA**
  (Method Salting out: Prospiech & Newmann, 1995)



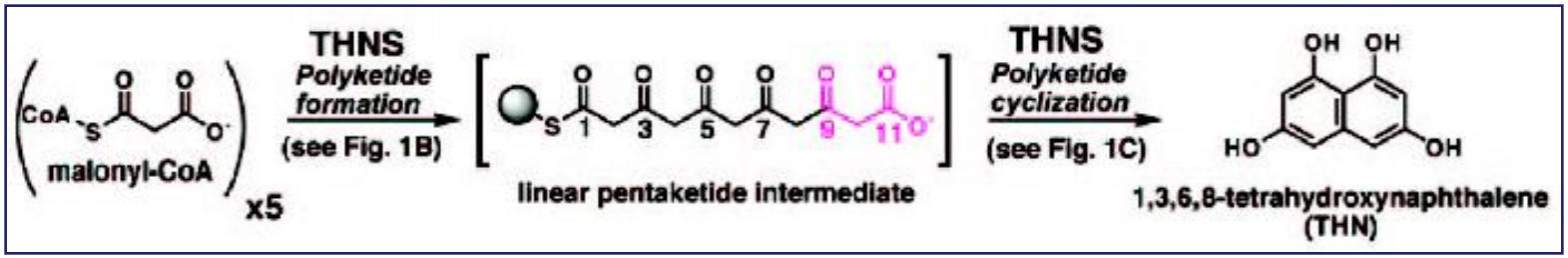
- **Detection of rppA-like (PKS III) genes in endophytics**



- **Transcription analysis by RT-PCR**



THNS in *Streptomyces*



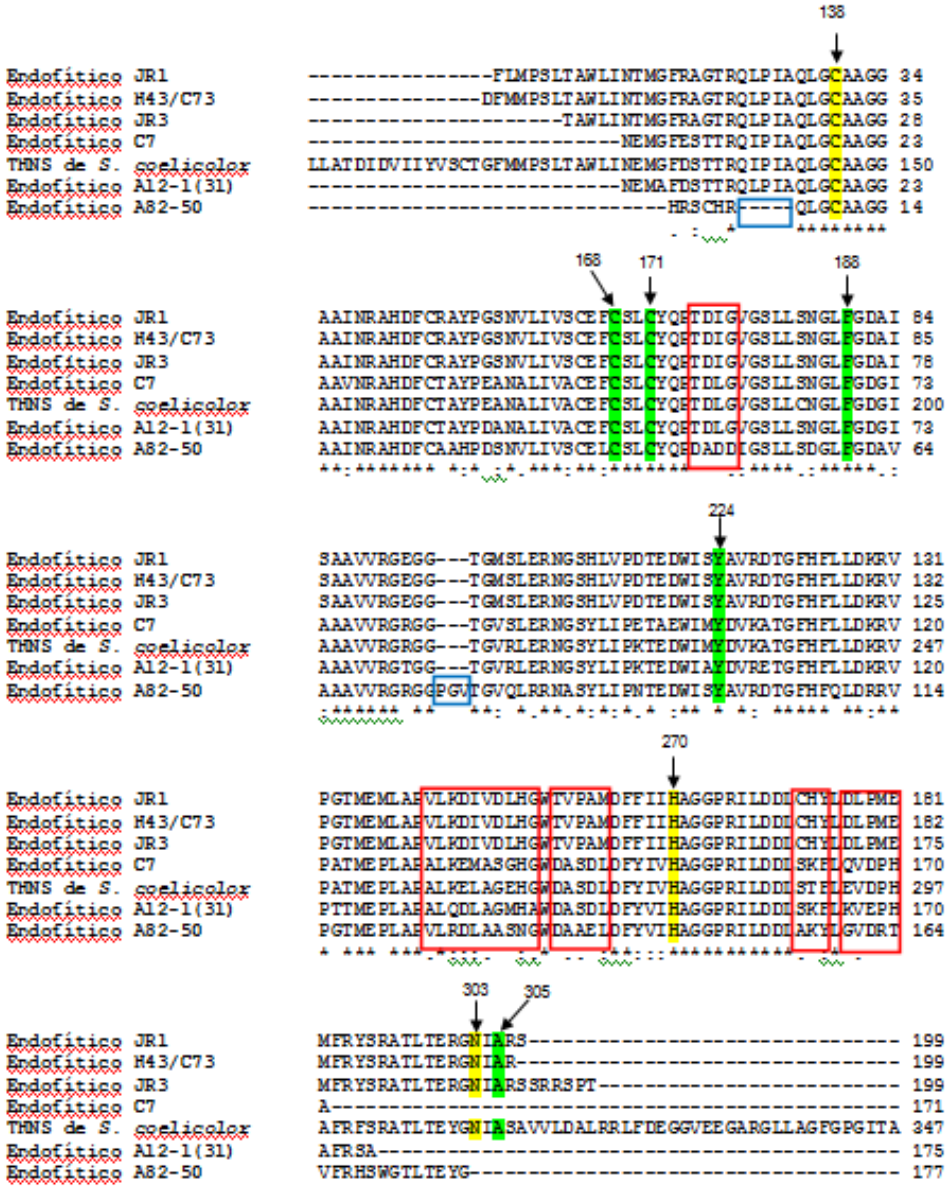
- **THNS enzyme** (1,3,6,8-tetrahidroxynaphtalene synthase) ou **RppA** = PKS III (*Streptomyces*) (Li et al, 2007).
- **Biosynthesis**:
  - 1° part:: THNS catalize malonil-CoA condensation to form na intermediary polyketide.
  - 2° part: THNS catalize two condensations (Claisen and Aldol) to form the metabolite **THN**.
- **THN**: melanin metabolism and backbone of metabolites (flavioline).
- Biological activity (antibiotic, antitumoral)

rppA-like genes between the endophytics

BLAST p - GenBank

Endophytic	**Enzyme**	**Organism/Cód.**	**Funçtion**	**% identid./similarid.**
C7	THNS	*S. coelicolor* (pdb1U0M)	Condensation enzyme	92 %
A12-1(31)	RppA	*S. lividans* (BAB91445.1)	"	89%
A82(50)	RppA	*S. antibioticus* (BAB94443.1)	"	84%
JR1	STS e CHS	*Streptomyces* sp (ZP06276870.1)	"	90%
JR3	CHS	*S. griseus* (BAA33495.1)	"	90%
H43/C73	STS e CHS	*Streptomyces* sp (ZP06276870.1)	""	91%

Endophytic a.a. sequences aligment with THNS of *S. coelicolor*



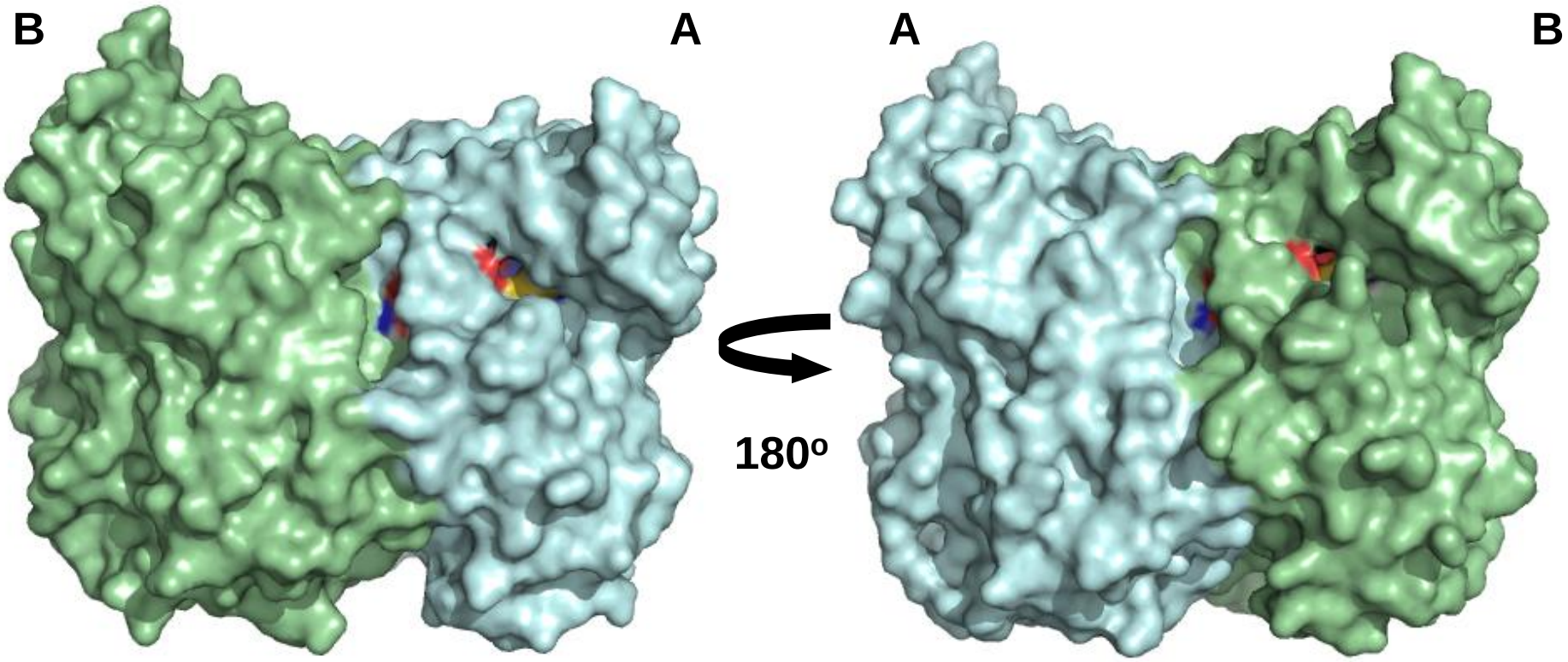
Catalytic triade residues

Othe catalytic residues

High variability regions

Insertions or delitions

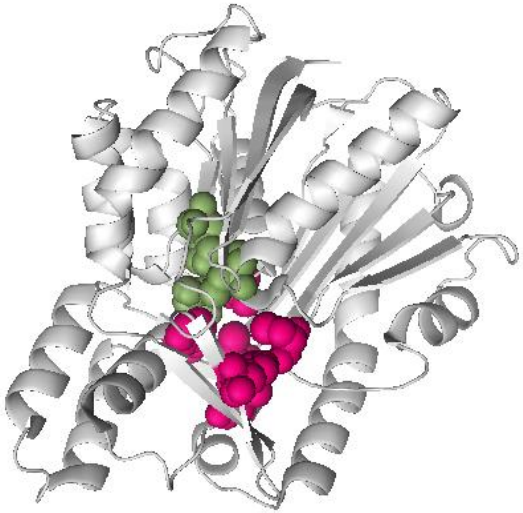
S. coelicolor *THNS structure*



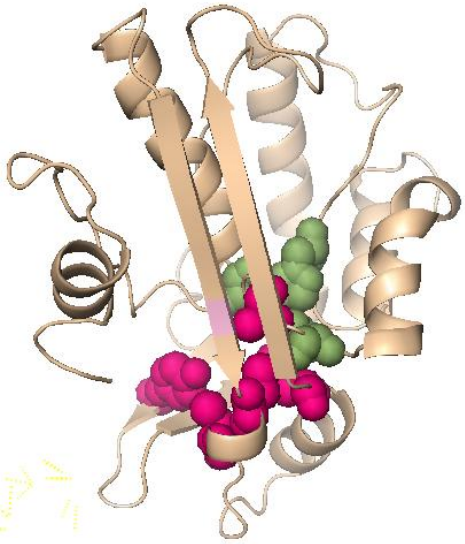
THNS Homodímers (KS monomers) A and B chains; catalytic site in colours

Structural model

S. coelicolor THNS



Hypothetic JR1 THNS



Catalytic residues (Cys, His, Asn)

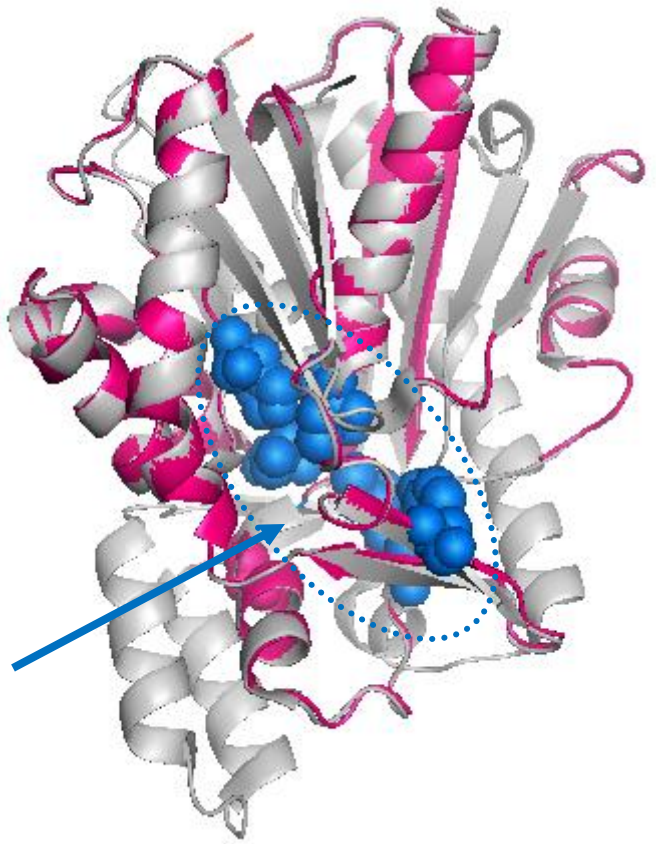
Other catalytic residues

Structural analysis

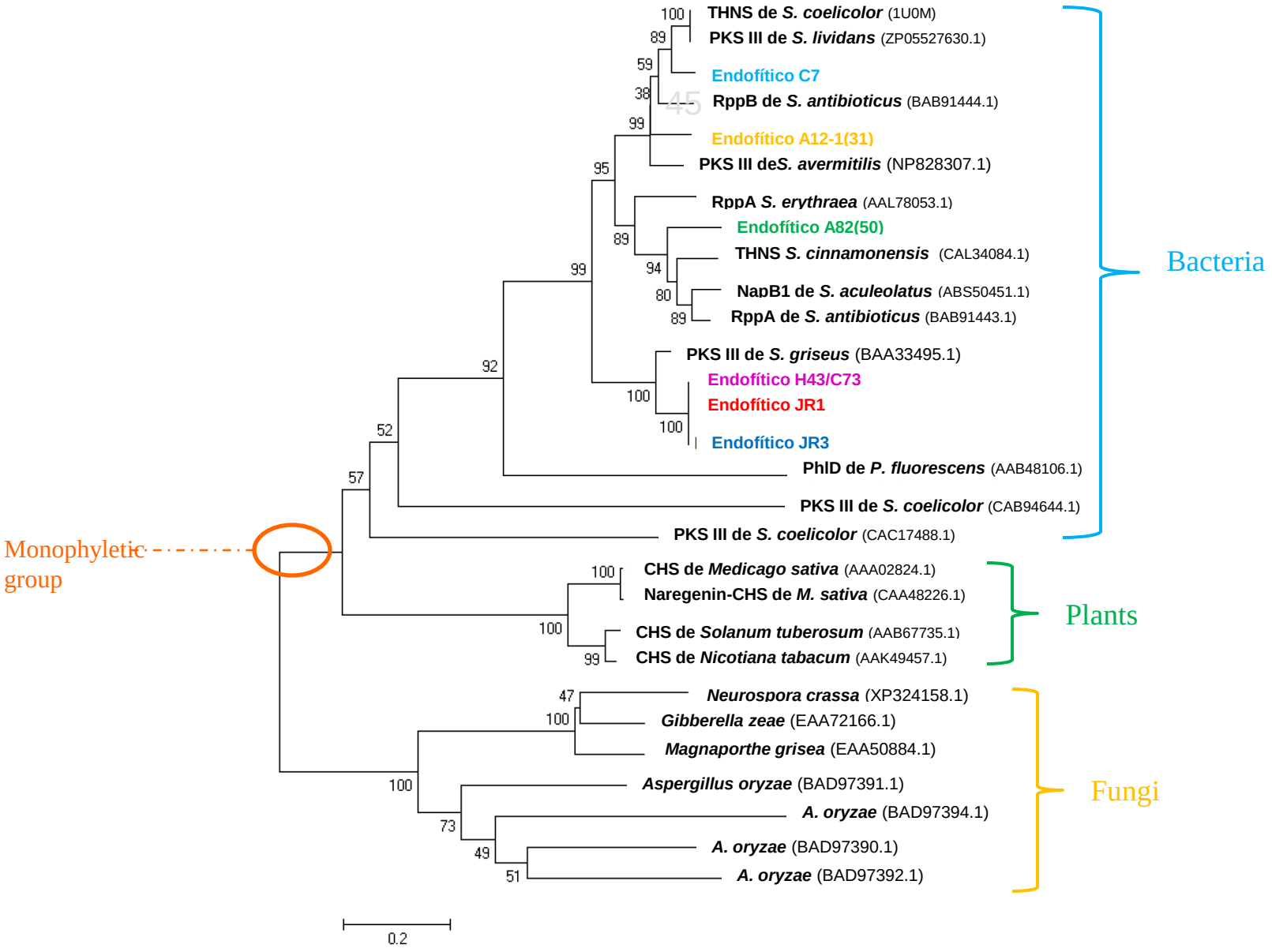

```
THNS_S.coel      PGFEDRNKVYEREAKSRVPAVIQRALDDAELLATDIDVIIYVSCTGFMMP 50
Endofítico JR1   ----------------------------------------------FLMP 4
THNS_S.coel      SLTAWLINEMGFDSTTRQIPIAQLGCAAGGAAINRAHDFCTAYPEANALI 100
Endofítico JR1   SLTAWLINTMGFRAGTRQLPIAQLGCAAGGAAINRAHDFCRAYPGSNVLI 54
                                         *
THNS_S.coel      VACEFCSLCYQPTDLGVGSLLCNGLFGDGIAAAVVRGRGGTGVRLERNGS 150
Endofítico JR1   VSCEFCSLCYQPTDIGVGSLLSNGLFGDAISAAVVRGEGGTGMSLERNGS 104
                      *  *                  *
THNS_S.coel      YLIPKTEDWIMYDVKATGFHFLLDKRVPATMEPLAPALKELAGEHGWDAS 200
Endofítico JR1   HLVPDTEDWISYAVRDTGFHFLLDKRVPGTMEMLAPVLKDIVDLHGWTVP 154
                            *
THNS_S.coel      DLDFYIVHAGGPRILDDLSTFLEVDPHAFRFSRATLTEYGNIASAVVLDA 250
Endofítico JR1   AMDFFIIHAGGPRILDDLCHYLDLPMEMFRYSRATLTERGNIARS----- 199
                       *                                  * *
THNS_S.coel      LRRLFDEGGVEEGARGLLAGFGPGITAEMSLGCWQTADVRRGIRQDVTRT 300
Endofítico JR1   --------------------------------------------------

THNS_S. coel     AARGVSRRVRQA 312
Endofítico JR1   ------------
```

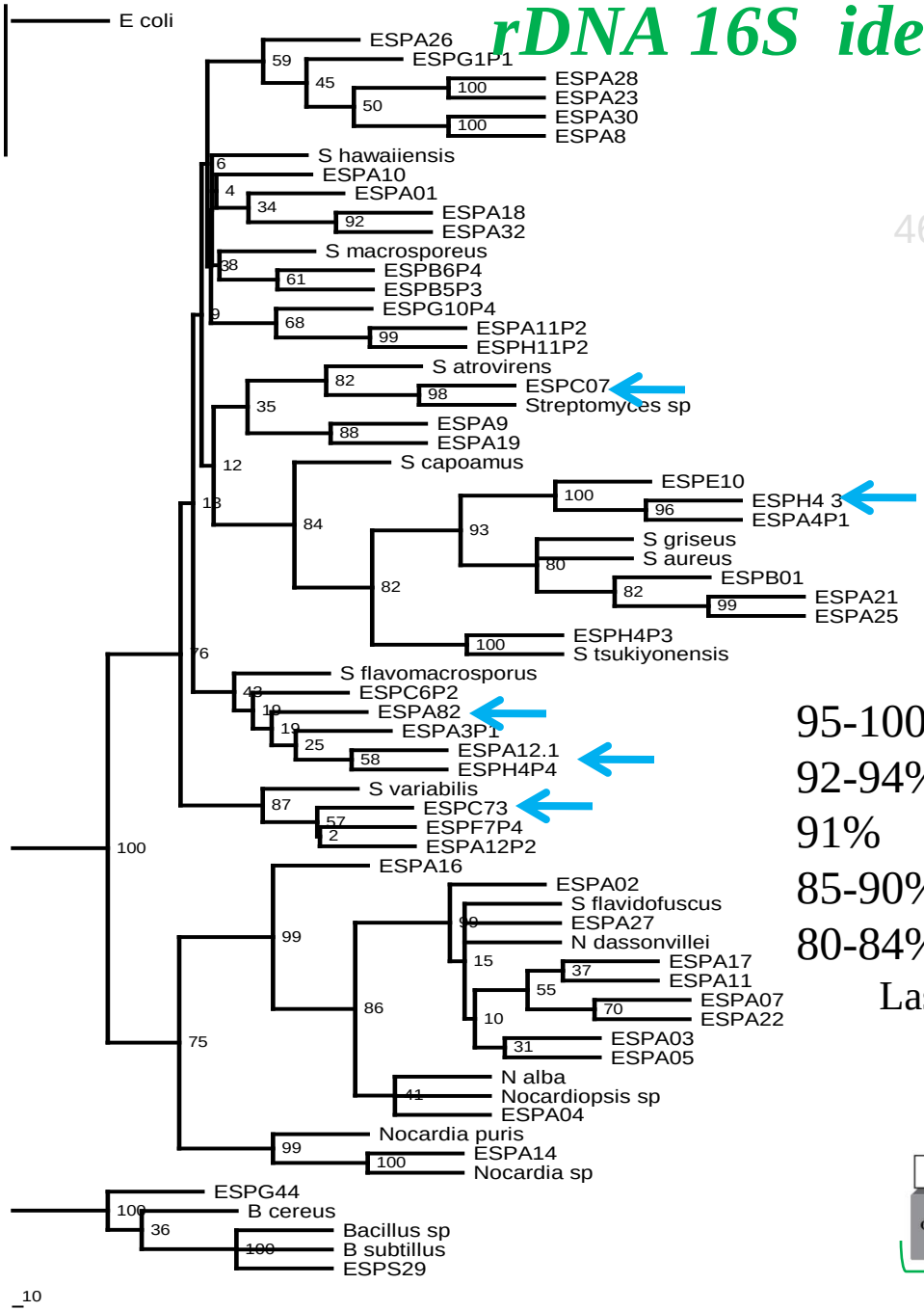
THNS *S. coelicolor*
Hypothetic JR1 THNS



Catakytic site conserved in both structures


THNS de S. coelicolor (1U0M)
PKS III de S. lividans (ZP05527630.1)
Endofítico C7
RppB de S. antibioticus (BAB91444.1)
Endofítico A12-1(31)
PKS III deS. avermitilis (NP828307.1)
RppA S. erythraea (AAL78053.1)
Endofítico A82(50)
THNS S. cinnamonensis (CAL34084.1)
NapB1 de S. aculeolatus (ABS50451.1)
RppA de S. antibioticus (BAB91443.1)
PKS III de S. griseus (BAA33495.1)
Endofítico H43/C73
Endofítico JR1
Endofítico JR3
PhlD de P. fluorescens (AAB48106.1)
PKS III de S. coelicolor (CAB94644.1)
PKS III de S. coelicolor (CAC17488.1)
Bacteria
Monophyletic group
CHS de Medicago sativa (AAA02824.1)
Naregenin-CHS de M. sativa (CAA48226.1)
CHS de Solanum tuberosum (AAB67735.1)
CHS de Nicotiana tabacum (AAK49457.1)
Plants
Neurospora crassa (XP324158.1)
Gibberella zeae (EAA72166.1)
Magnaporthe grisea (EAA50884.1)
Aspergillus oryzae (BAD97391.1)
A. oryzae (BAD97394.1)
A. oryzae (BAD97390.1)
A. oryzae (BAD97392.1)
Fungi
0.2

rDNA 16S identification



95-100% --- **specie**

92-94% --- **genus**

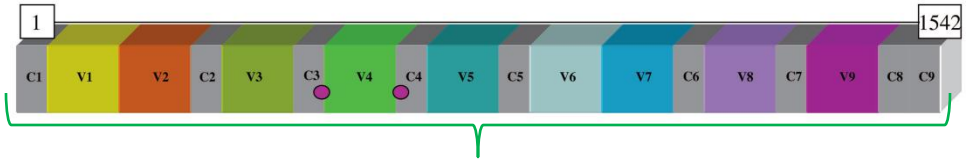
91% --- **order**

85-90% --- **class**

80-84% --- **phylo**

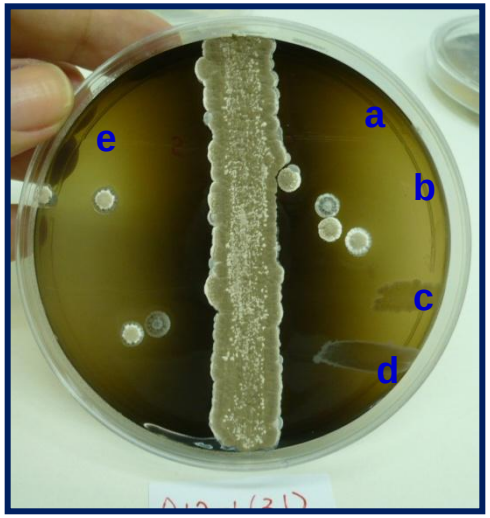
Lasher et al., 2009

Endo phytic	Sequências mais similares do NCBI (BLASTn)	% ident.
C7	*Streptomyces* sp. 102111	99 %
H43/C 7-3	*S. costaricanus*	83%
A12-1 (31)	*S. fumanus*	97%
A82 (50)	*S. spiralis* *	99%
B1	*Streptomyces* sp. BM-2	98%
JR1	*Streptomyces* sp. MB-D-1	98%
JR3	*Streptomyces* sp. MB-D-1	96%

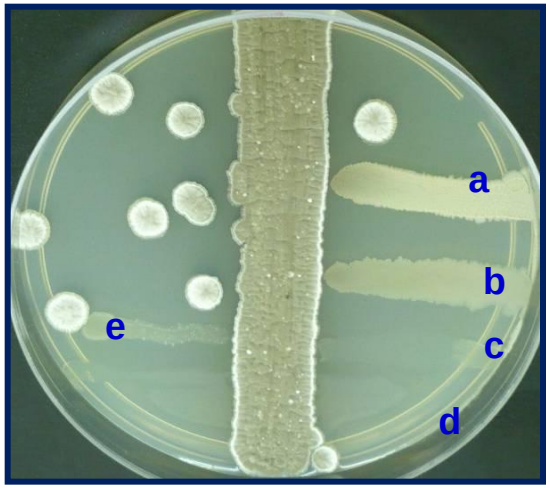


rDNA 16S

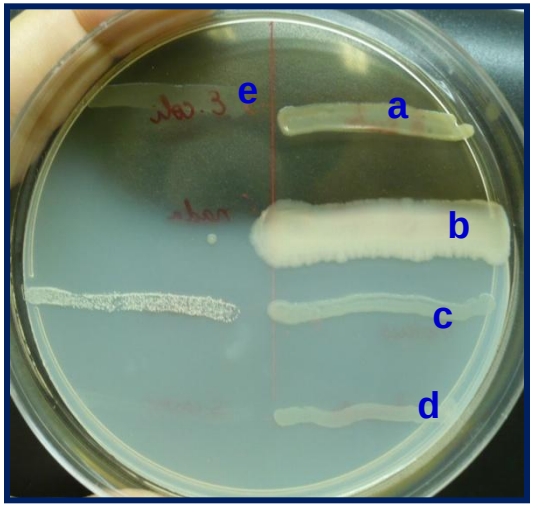
Antimicrobial activity



Endofphytic A12-1 (31)
semeada no centro em meio N.A (4 dias/28°C).



Endophytic A82 (50)
semeada no centro em meio N.A (4 dias/28°C).



Control
Meio NA, Sem endofítico, 37°C/24h.

a e b= *B. subtilis,* c= *Proteus* sp, d= *Salmonella,* e= *E. coli*
Culture conditions 37°C/24h

Research Team

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- Karen Massini
- Fabiana Andrieli
- Erik Saenz
- Renata Furlan
- Welington Araújo
- Lara Duraes Sette
- Massuo Jorge Kato
- Nidia Yoshida
- CNPq, CAPES, FAPESP