Bacillus, Enzim, Spora: *Sebuah Kajian Bioteknologi*

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Outline

- What is *Bacillus*
- The anvantage of using Bacilli as host in Industrial Biotechnology
- Screening/Bioprospecting for Enzymes Producing Bacilli
- Problem in using Bacilli related to endospore formation
- Sporulation and Germination
- Polysaccharide deacetylases and cortex layer synthesis
- Bioinformatics Study
- Gene Techniques
- The Result and Discussion
- Conclusion

What is *Bacillus* ?

Bacillus spp.

- Gram-positive bacteria
- High content of G+C
- Endospore forming bacteria
- Almost all of them Catalase-positive

Systematics:

Domain Bacteria, kingdom Bacteria, subkingdom Bacteria Gram-positive, phylum Bacillota (Firmicutes), class Bacilli, ordo Bacillales, family Bacillaceae.

Figure _**Phylogeny of *Bacillus* sensu lato** from 16S rRNA gene sequences. GenBank DNA sequences for species type strains were aligned with ClustalW. An unrooted phylogenetic tree was constructed from the ClustalW distance matrix with the PHYLIP Neighbour application and visualized with PhyloDraw. Dashed lines group sequences that have been assigned to the same taxon. The rather close "*B. subtilis* group" consists of 14 species, including *B. licheniformis*. The "*B. cereus* group" consists of six species, including *B. anthracis* and *B. thuringiensis* (taken from Zeigler and Perkins 2009)

The anvantage of using Bacilli as host in Industrial Biotechnology

- *Bacillus* and the relatives so call "Bacilli" are one the best "**workhorses**" in Industrial Biotechnology.
- The other *Bacillus* has important in their biome and industry such as *Paenibacillus*, *Brevibacillus*, and *Lysinibacillus*.
- Their products and its function related to high valuable functional molecules that use in **medicinal**, **agricultural**, **pharmaceutical**, and **industrial**

Example:

- **Many industrial enzymes** that use in the detergent industry, food, the environment, etc.
- **Primary metabolites** such as vitamins and ribonucleotides
- **Secondary metabolites** i.e. bacteriocin, surfactin, fusaricidin (as **antibiotics**), and biosurfactants, etc.
- **Biomaterial** such as **2,3-butanediol** and derivatives, **bioplastics PHA/PHB/PHV** and derivatives;
- **Plant growth promoting** chemicals.
- **Fine chemicals** such as carotenoid pigment, **biopolymers** i.e exopolysaccharide (EPS), poly-γ-glutamic and poly lactic acids (PLA), etc.

Lichenase (endo-1,3:1,4-β-D-Glucanase) (*Bacillus licheniformis*, *B subtilis*)

Product code: **E-LICHN**

€259.00

5,000 Units

Prices exclude VAT

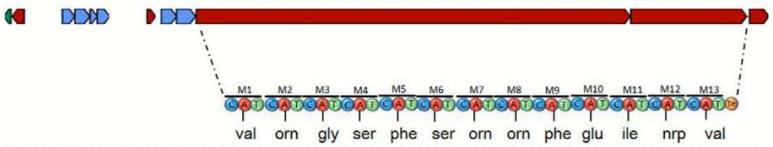
✔ Available for shipping

Enzyme Activity:	β-Glucanase/Lichenase
EC Number:	3.2.1.73
CAZy Family:	GH16
CAS Number:	37288-51-0
Synonyms:	licheninase; (1→3)-(1→4)-beta-D-glucan 4-glucanohydrolase
Source:	*Bacillus subtilis*
Molecular Weight:	26,750
Concentration:	Supplied at ~ 1,000 U/mL
Expression:	Purified from *Bacillus subtilis*
Specificity:	Hydrolysis of (1,4)-β-D-glucosidic linkages in β-D-glucans containing (1,3)- and (1,4)-bonds.
Specific Activity:	~ 230 U/mg (40°C, pH 6.5 on barley β-glucan)
Unit Definition:	One Unit of lichenase activity is defined as the amount of enzyme required to release one µmole of glucose reducing-sugar equivalents per minute from barley β-glucan (10 mg/mL) in sodium phosphate buffer (100mM), pH 6.5 at 40°C.

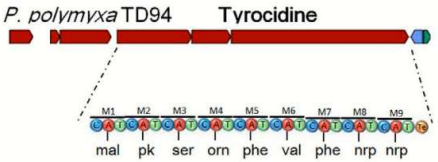
Paenibacillus spp.

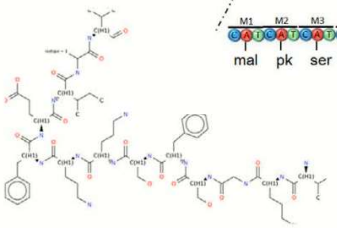
Nonribosomal peptide synthetase (Nrps)

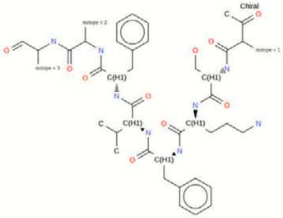
P. polymyxa ATCC 842 **Nrps**



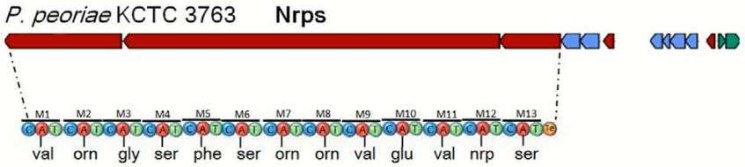
P. polymyxa TD94 **Tyrocidine**

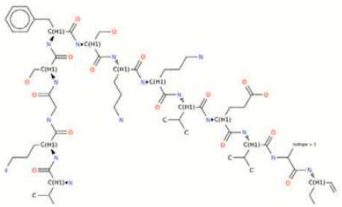




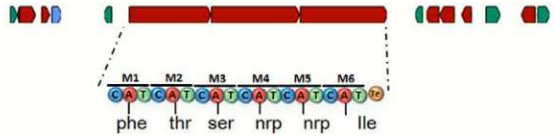


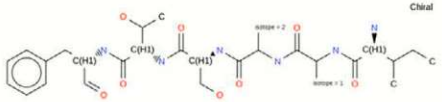
P. peoriae KCTC 3763 **Nrps**



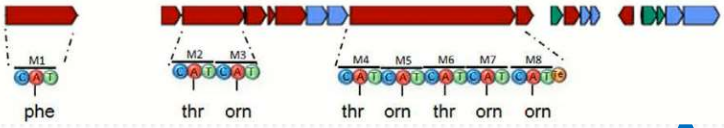


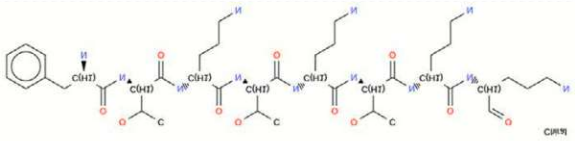
P. beijingensis 1-18 **Surfactin**





P. polymyxa 1-43 **Fusaricidin**



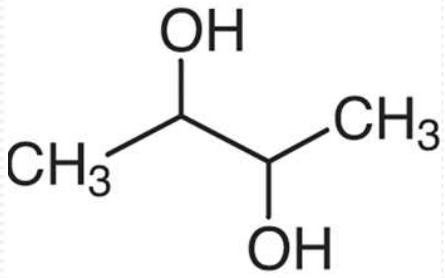


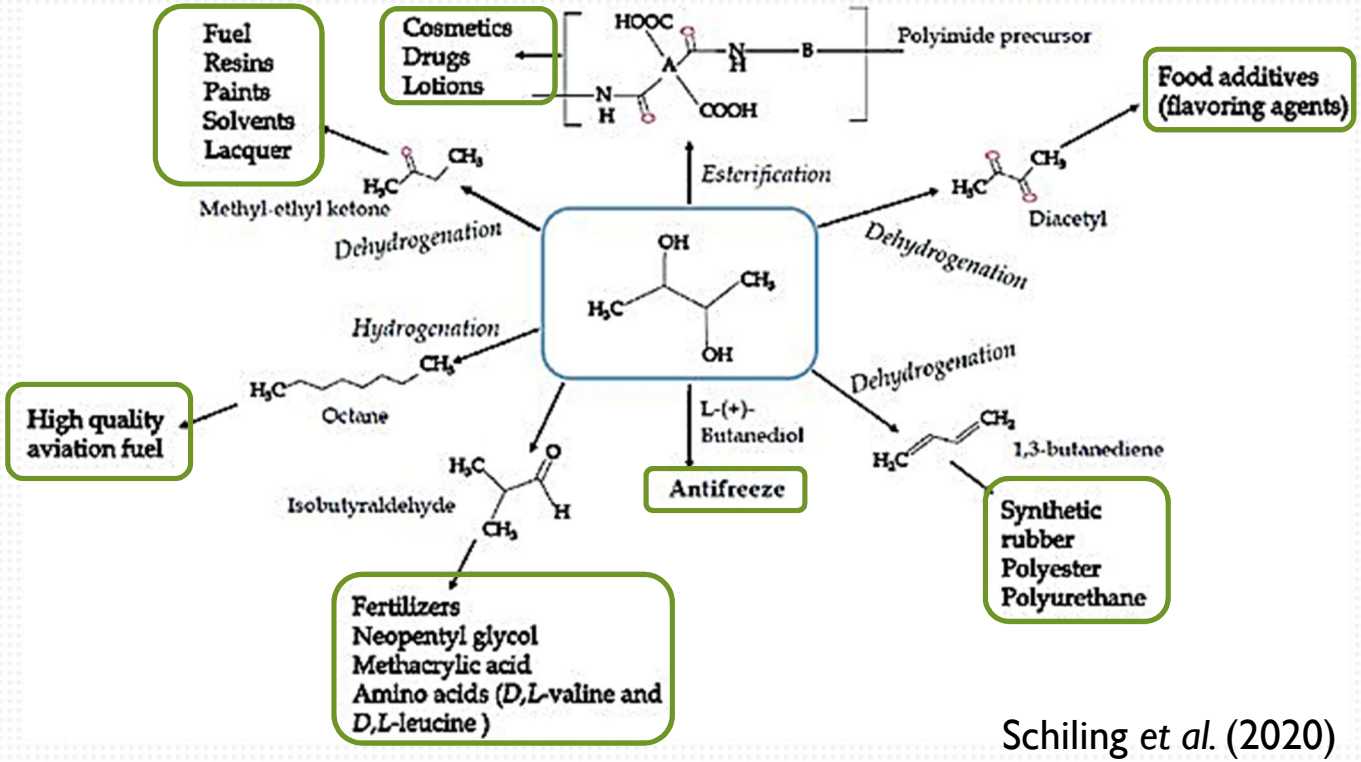
Antimicrobial Peptides (AMPs)

Xie *et al.* (2016)

P. polymyxa, *Bacillus licheniformis* and *B. subtilis*

2,3-butanediol



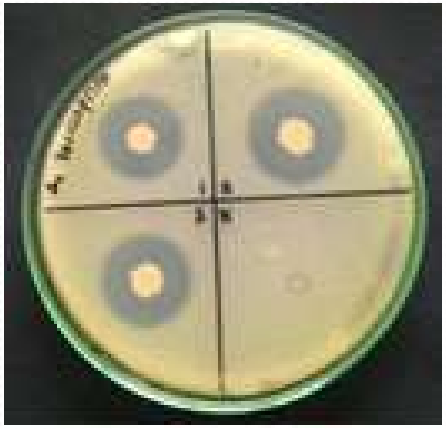


Schiling *et al.* (2020)
Hakizimana *et al.* (2020)

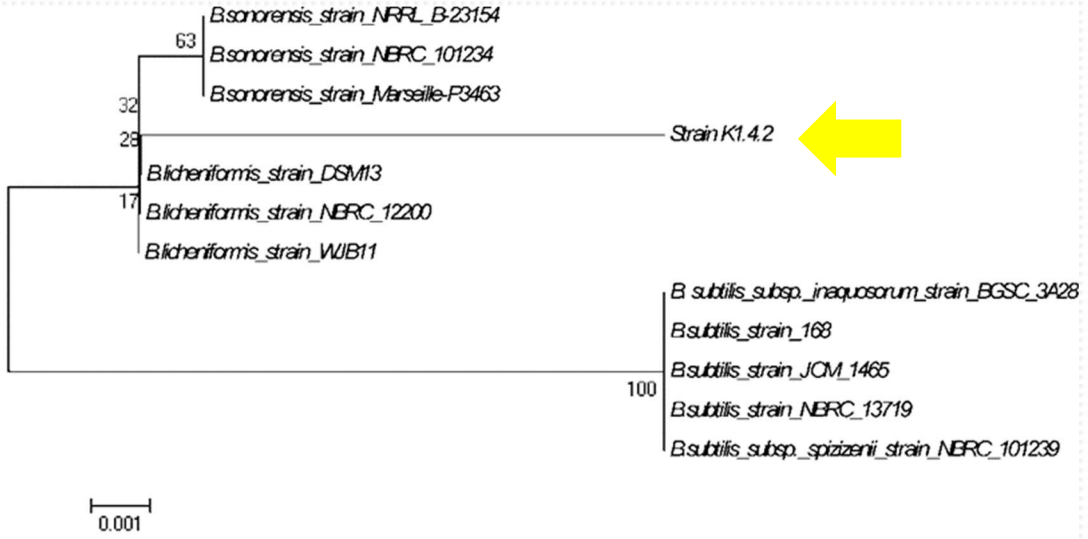
Screening/Bioprospecting for Enzymes Producing Bacilli

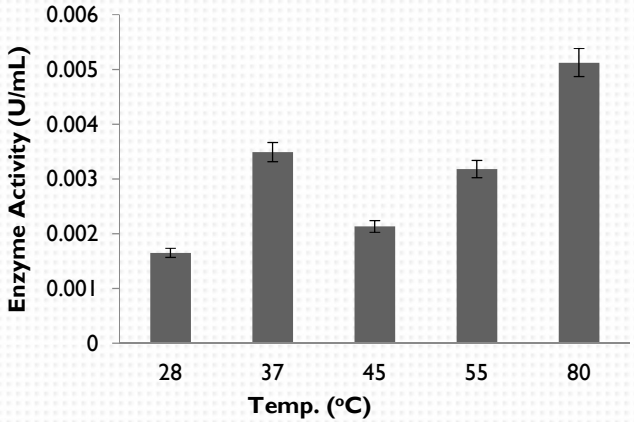
Thermotolerant *B.licheniformis* K1.4.2

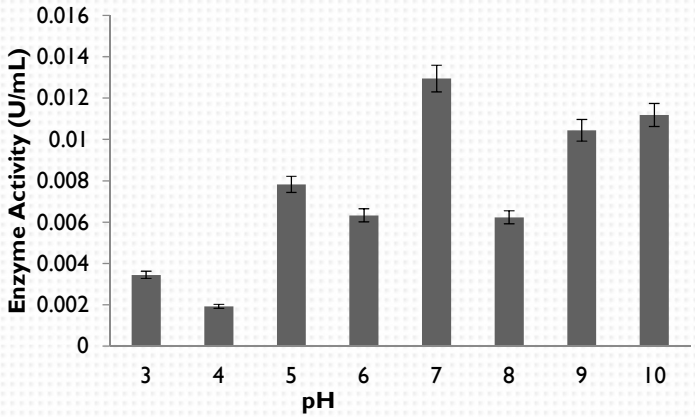
Maulida, (2015)
Dani, (2017)



Proteases

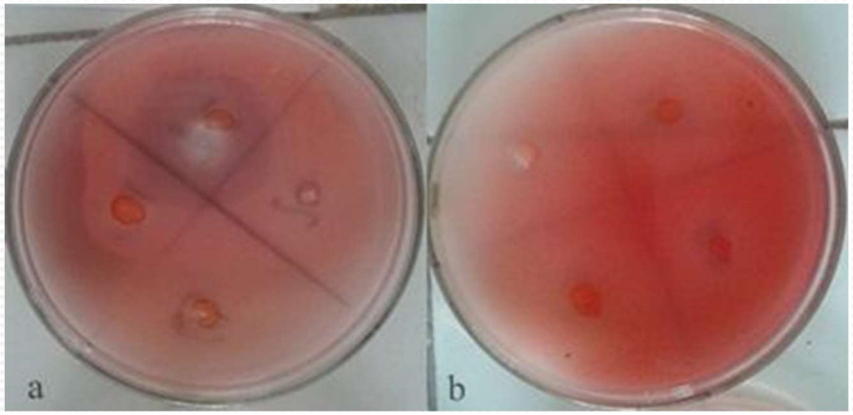






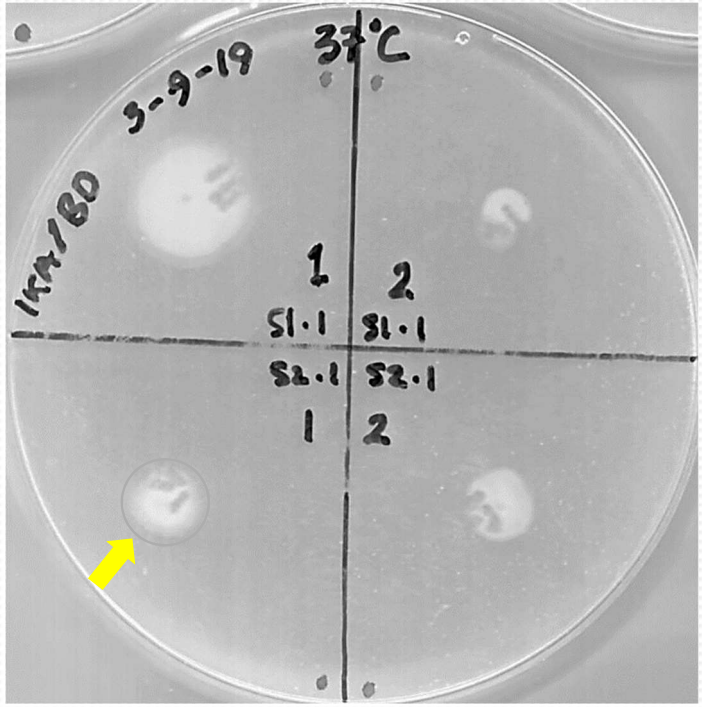
Chitin deacetylases

Fitriani (2016)
Nailufar (2017)



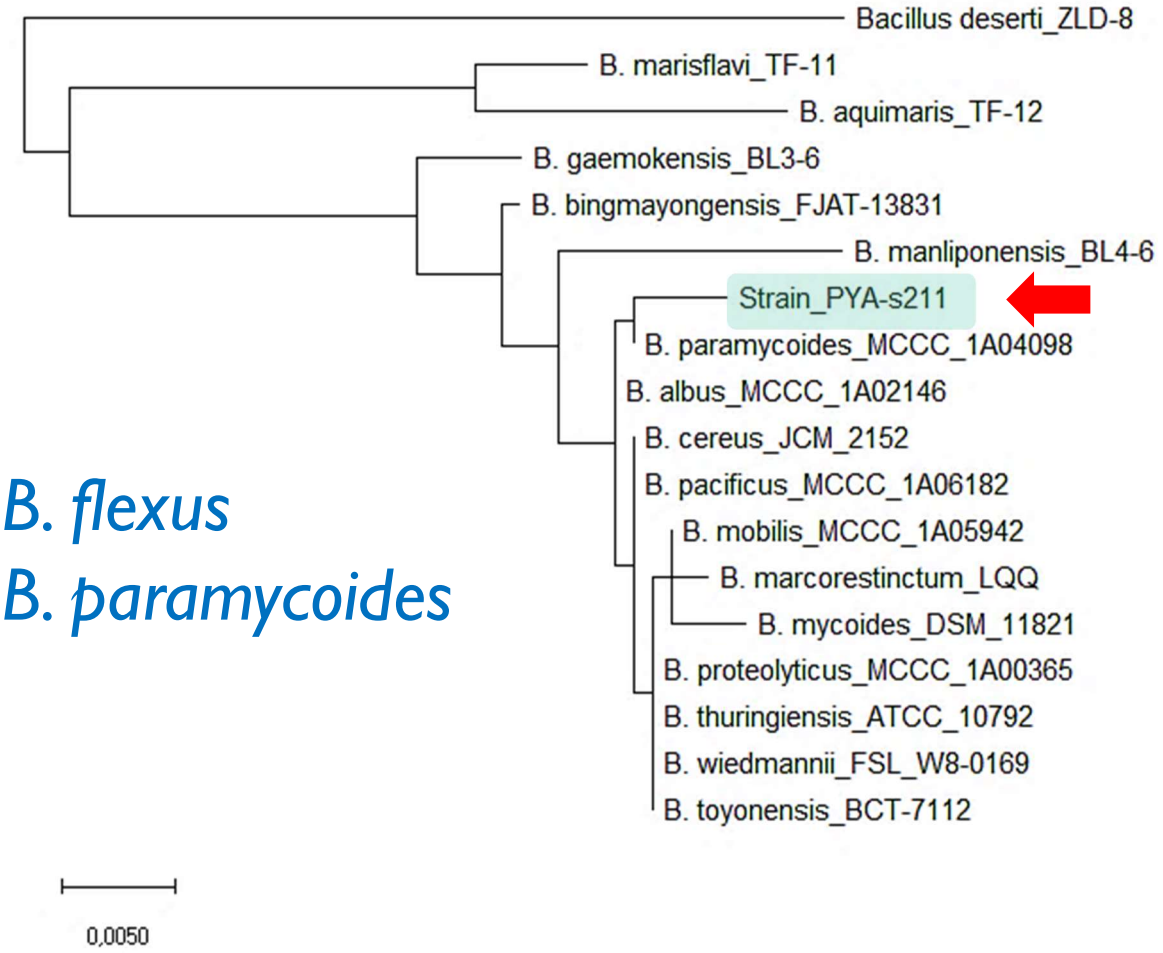
Strain	Kingdom	Filum	Kelas	Ordo	Famili	Genus	Spesies
P.A1.6	Bacteria	Firmicutes	Bacilli	Bacillales	Stapylococcaceae	*Staphylococcus*	*Staphylococcus aureus*
P.A2.5	Bacteria	Firmicutes	Bacilli	Bacillales	Bacillaceae	*Bacillus*	*Bacillus* spp
A.B2.7	Bacteria	Firmicutes	Bacilli	Bacillales	Stapylococcaceae	*Staphylococcus*	*Staphylococcus aureus*
A.B2.8	Bacteria	Firmicutes	Bacilli	Bacillales	Paenibacillaceae	*Paenibacillus*	*Paenibacillus polymyxa*
A.B2.10	Bacteria	Firmicutes	Bacilli	Bacillales	Bacillaceae	*Bacillus*	*Bacillus* spp
A.B3.4	Bacteria	Firmicutes	Bacilli	Bacillales	Stapylococcaceae	*Staphylococcus*	*Staphylococcus aureus*
A.C1.2	Bacteria	Firmicutes	Bacilli	Bacillales	Bacillaceae	*Bacillus*	*Bacillus subtilis*
P.B3.4	Bacteria	Firmicutes	Bacilli	Bacillales	Paenibacillaceae	*Brevibacillus*	*Brevibacillus brevis*
P.B3.5	Bacteria	Firmicutes	Bacilli	Bacillales	Stapylococcaceae	*Staphylococcus*	*Staphylococcus aureus*
P.C1.1	Bacteria	Firmicutes	Bacilli	Bacillales	Stapylococcaceae	*Staphylococcus*	*Staphylococcus aureus*
P.C3.1	Bacteria	Firmicutes	Bacilli	Bacillales	Stapylococcaceae	*Staphylococcus*	*Staphylococcus aureus*
P.C3.2	Bacteria	Firmicutes	Bacilli	Bacillales	Stapylococcaceae	*Staphylococcus*	*Staphylococcus aureus*

Sumber: www.ncbi.nlm.nih.gov/taxonomy

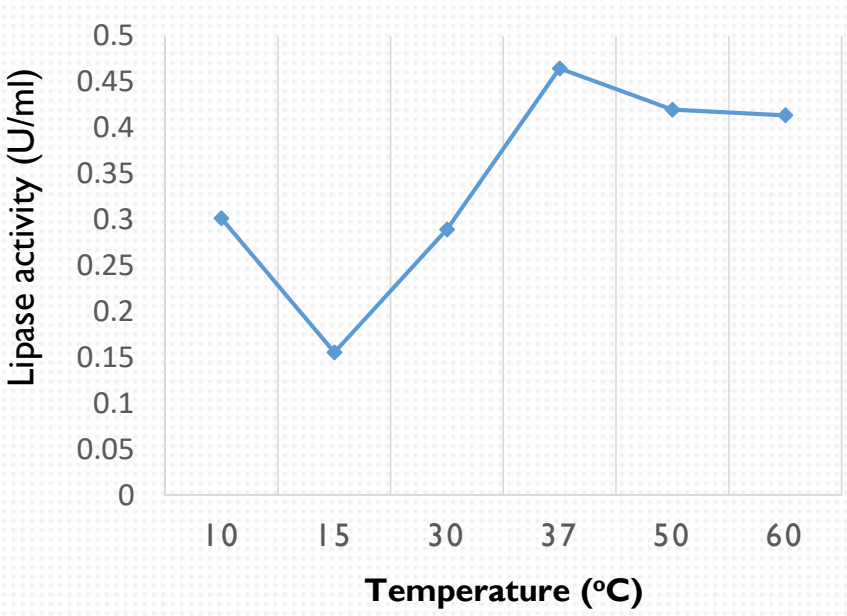


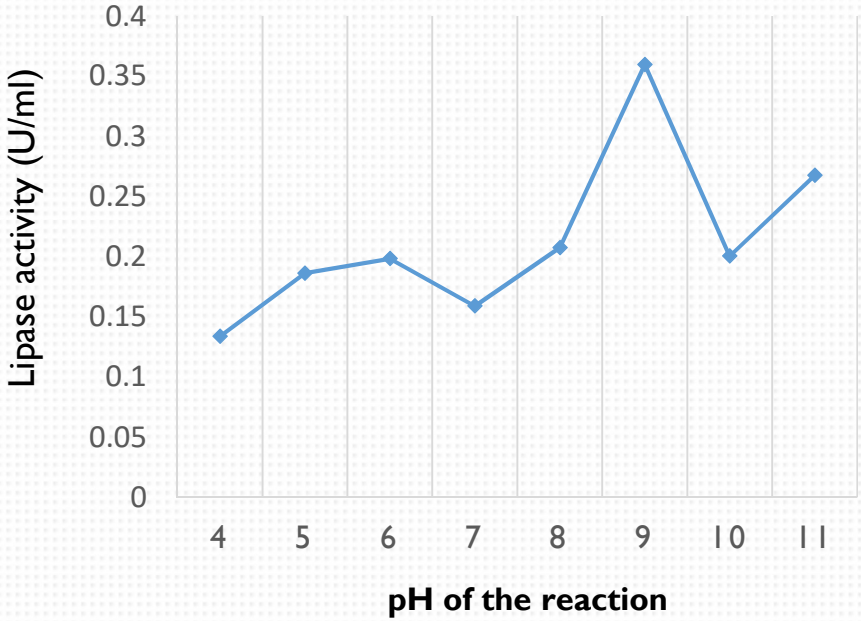
Lipases

Dharma *et al.*, (2019)
Hastri, (2021)



B. flexus
B. paramycoides





Alkaline Lipases

Problem in using Bacilli related to endospore formation

In industrial processes, however, spore formation is afflicted with a number of problems:

- As spores represent a frequent **source of contamination** and –
- When released into the environment – **endure long periods of time without losing viability**,
- **Harsh sterilization procedures** have to be employed to ensure that waste biomass is free from spores.
- **The production** of chemicals, enzymes, etc. **will reduces** since the cells switch their energy to build the endospore.

Nahrstedt et al. / Journal of Biotechnology 119 (2005) 245–254

Sporulation & EndoSpore

- The formation of highly resistant, non-reproductive, dormant structures, so called endospores,
- Sporulation is a unique feature of the Firmicutes genera *Bacillus and Clostridium*.
- *Bacillus spores* have survived for **approximately 250 million years** in **a salt crystal** (Vreeland *et al.*, 2000).
- The spore's potential to outlast extremely long periods of time under harsh conditions.

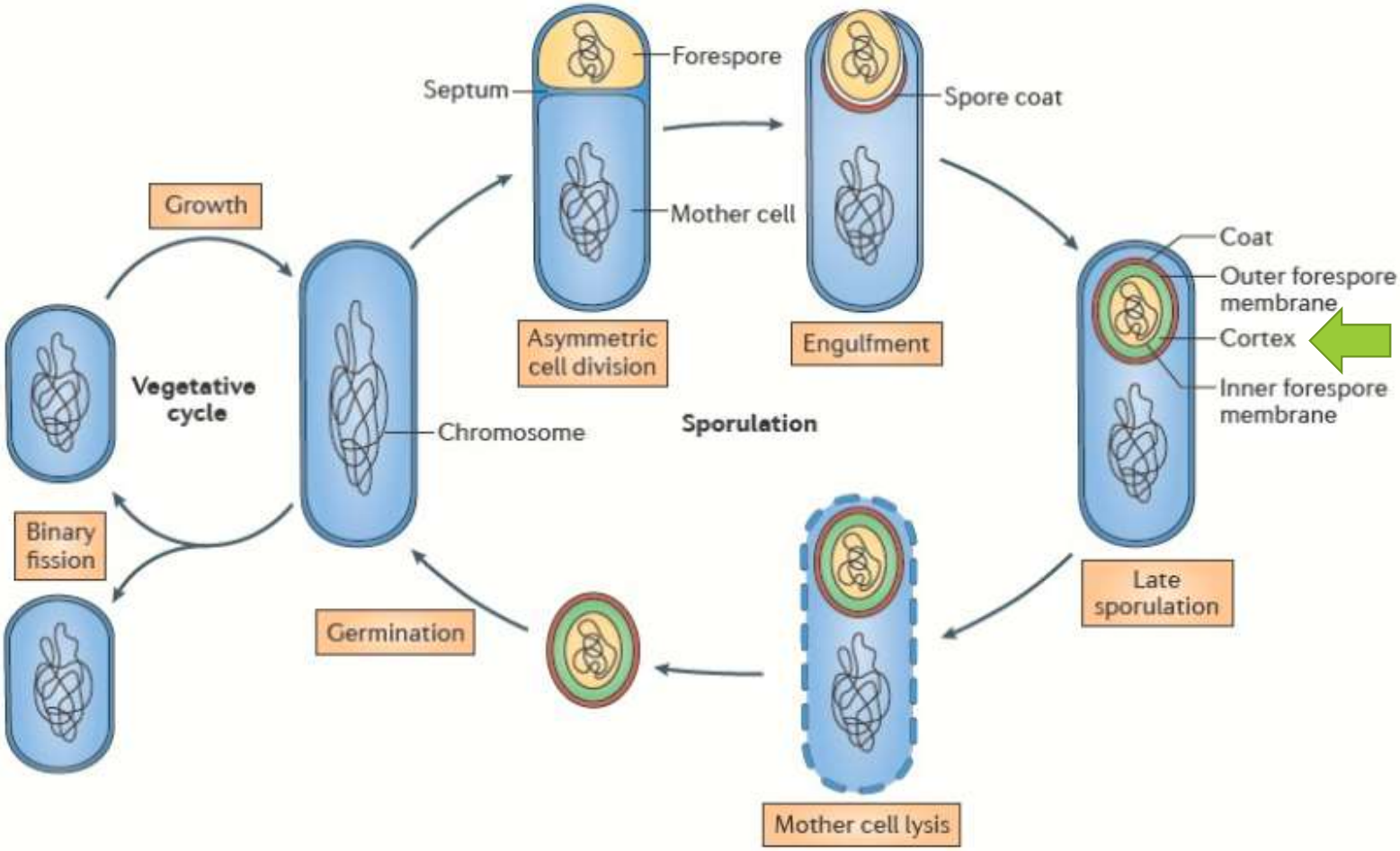


Figure _ The sporulation and germination cycle in *B. subtilis* (Errington, 2003)

Endospore Structure in *Bacillus* spp.

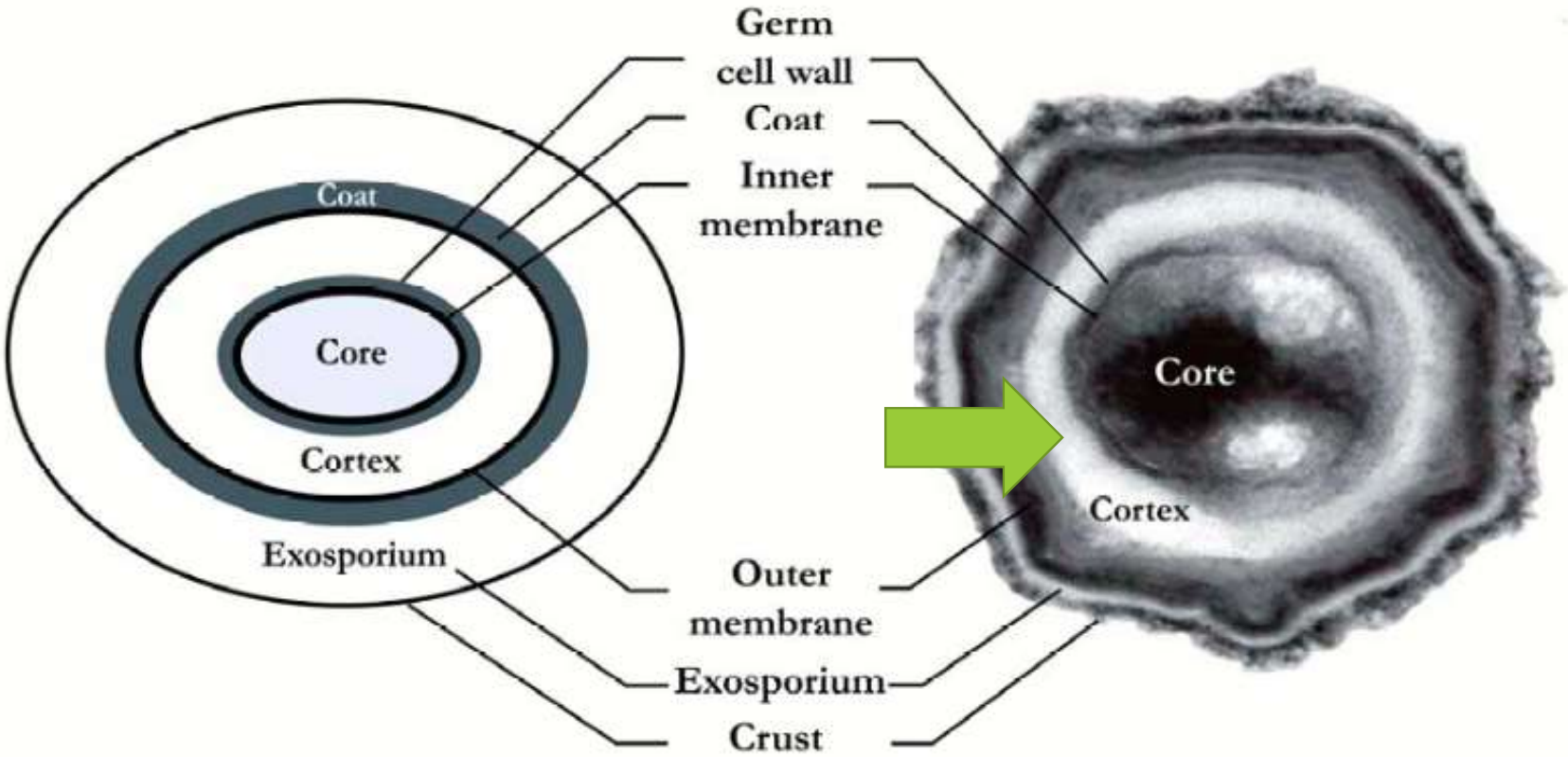
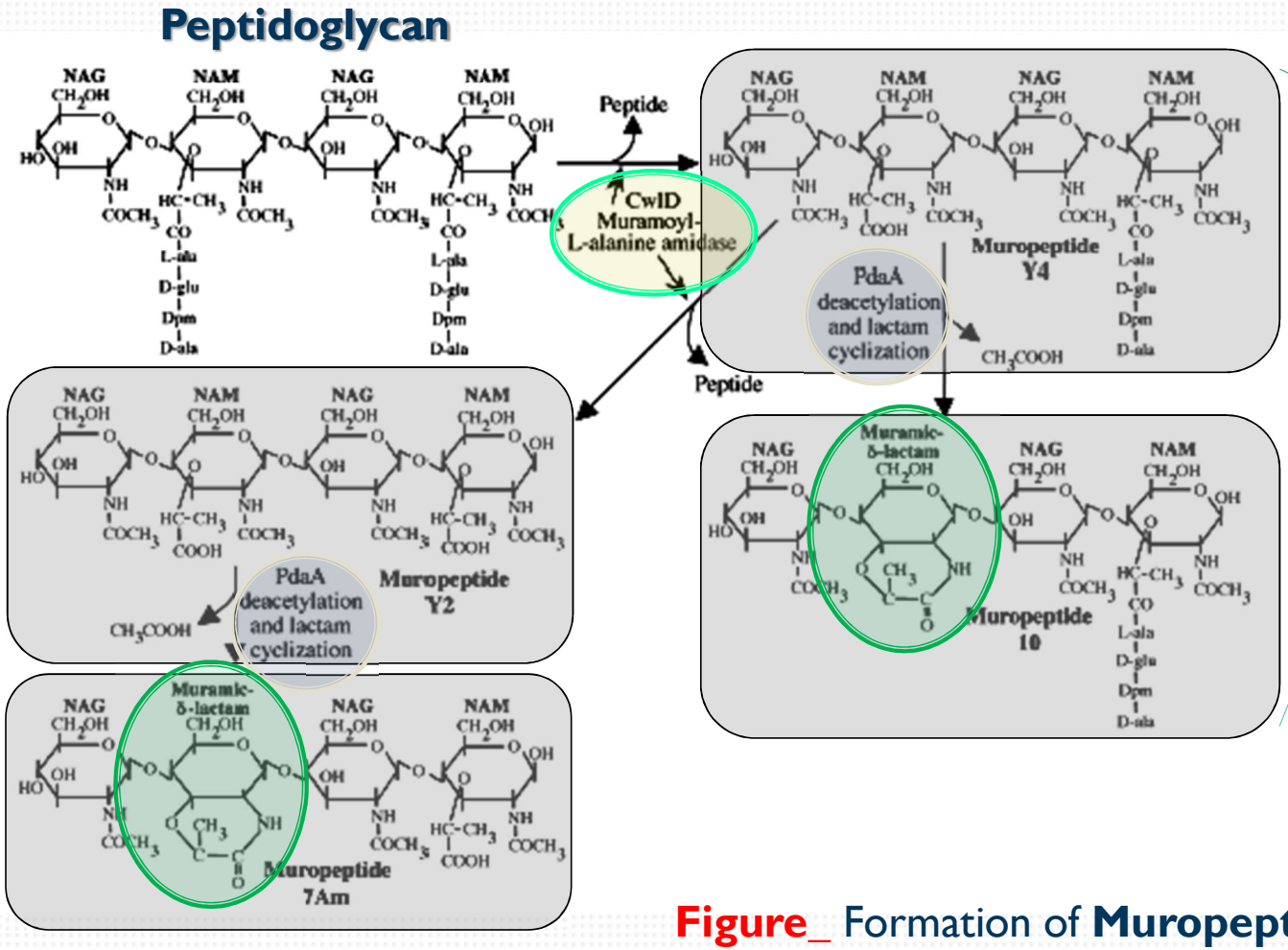
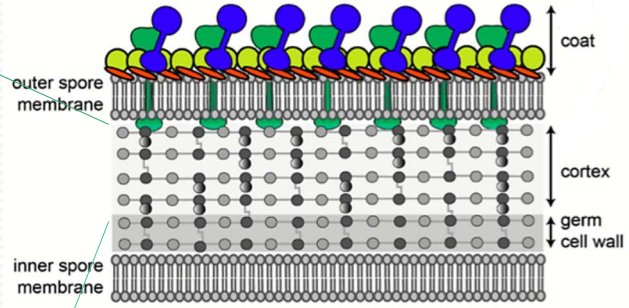


Figure _ The main structures of the *Bacillus endospore*





doi: https://doi.org/10.1371/journal.pgen.1009246.g008

Figure_ Formation of **Muropeptides** in the Spore cortex

Germination

- Germination is a series of successive and degradative events **triggered by specific germinants**, which leads to the loss of the typical spore properties.
- The germinants comprise nutrient molecules with a **low molecular weight**, mainly **amino acids**, **purine derivatives**, and **sugars**.
- The signalling process, which occurs when **the nutrient germinant** binds to **a receptor complex** and subsequently **activates the spore germination-specific cortex lytic enzyme (SLEs).**
- The heat, combined with additional controlling factors (pH, organic acids, preservatives) affect *Bacillus* species spore viability, germination and outgrowth.

- The combinations of germinant can trigger spore germination, e.g. a mixture of asparagine, glucose, fructose and K^+ (AGFK) *triggers B. subtilis* germination.

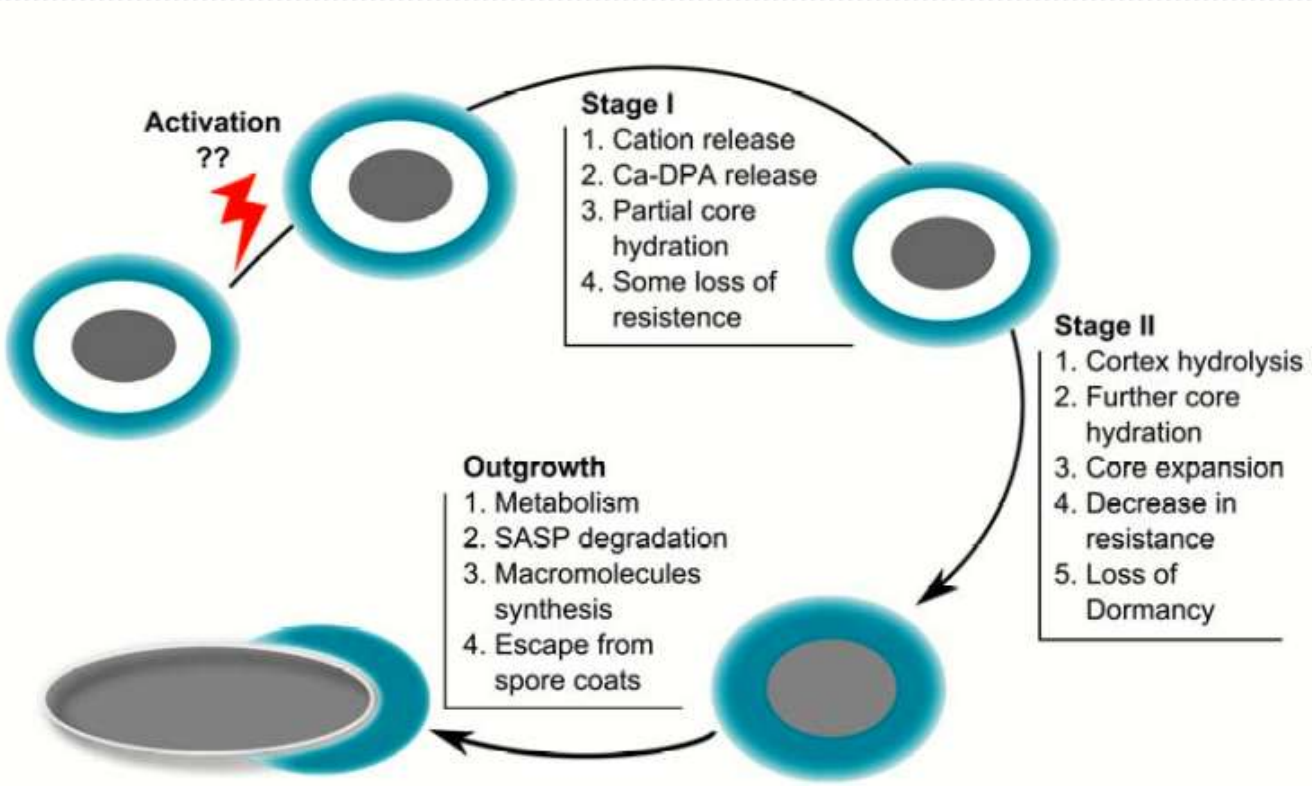


Figure _ The stages in spore germination (adapted from Setlow 2003)

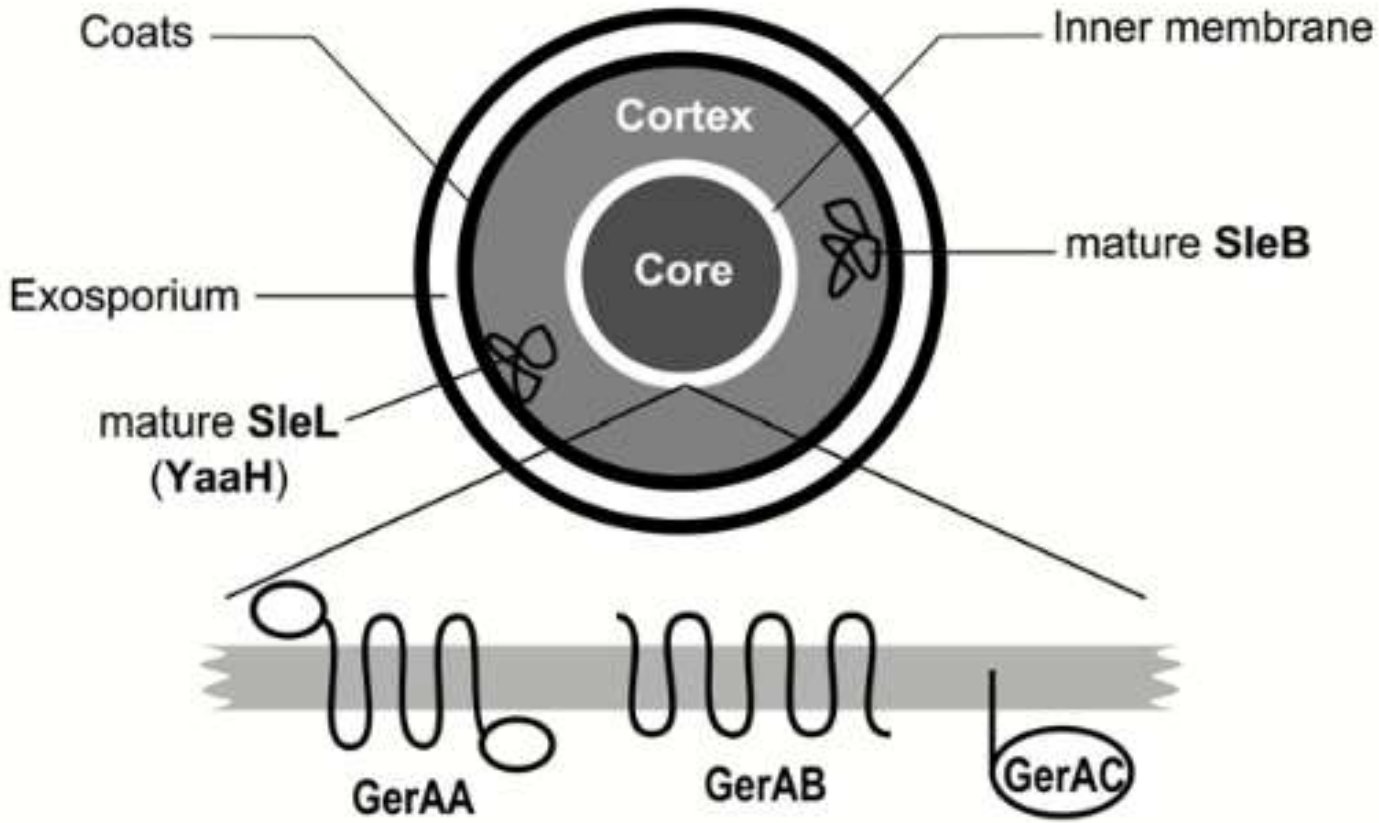


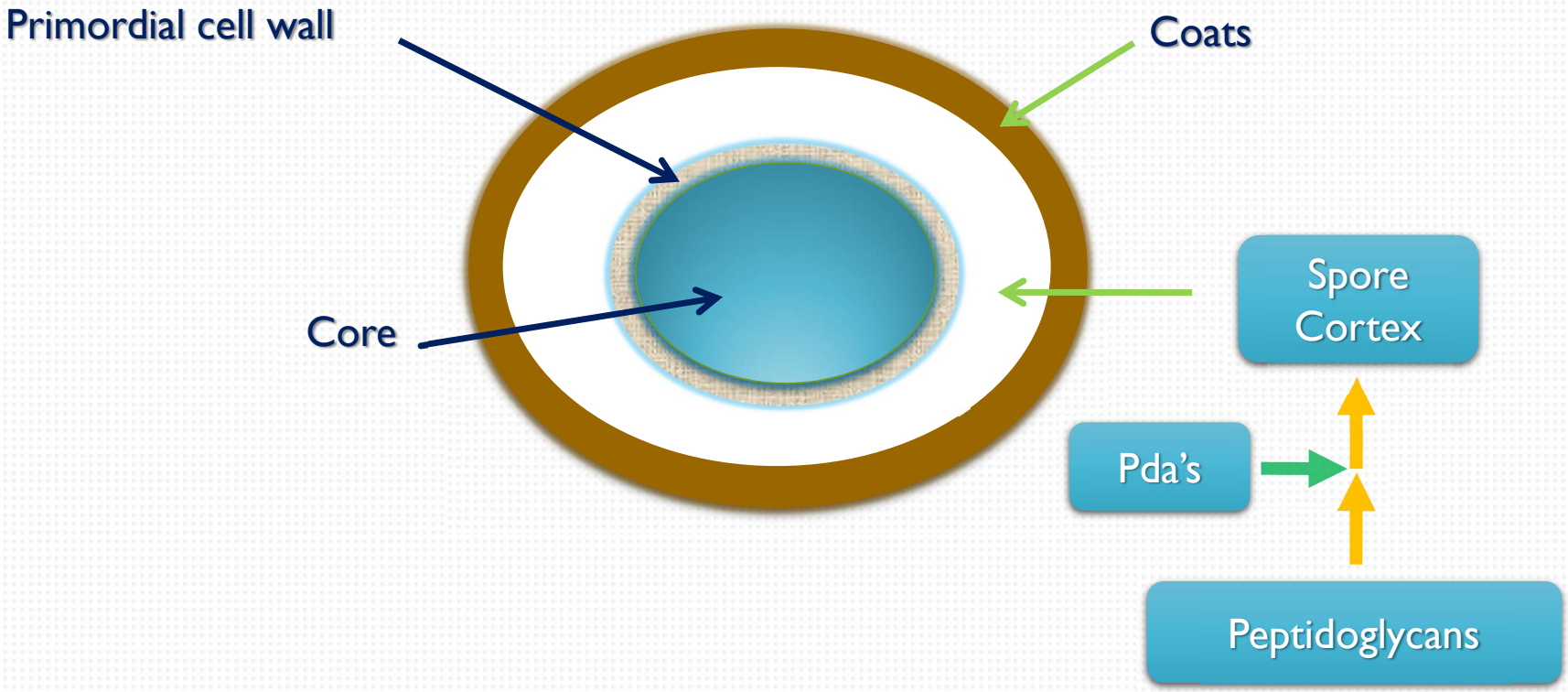
Figure _Model of the germination receptor GerA consisting of GerAA, GerAB, and GerAC from *B. subtilis* (adapted from Moir et al. 2002, Makino and Moriyama 2002, Ross and Abel-Santos 2010, Wilson et al. 2011)

Polysaccharide deacetylases (PDAs) and Cortex layer synthesis

- Polysaccharide deacetylases (PDAs) play important roles in endospore formation and germination processes, especially in the formation of **muramic δ–lactam** of the spore peptidoglycan (Atrih et al. 1996, Fukushima et al. 2002, Gilmore et al. 2004)
- Polysaccharide deacetylases belong to **family 4 Carbohydrate Esterases** (Blair,*et al.*, 2004).
- PDAs are associated with **biosynthesis** of **muramic-δ-lactam** in cell wall peptidoglycan.

- In ***Bacillus subtilis***, there are **6-7 genes paralogous of PDAs** e.g. ***pdaA (yfjS), pdaB (ybaN), cda1, yjeA, yheN, yxkH*** **and** ***ylxY*** (Fukushima, *et.al.*, 2004).
- However, from a bioinformatics analysis, in ***B. licheniformis***, there are **7 genes** paralogous **of PDAs.**
- **The cortex consists** of **specifically modified peptidoglycan**, notably **the complete absence of teichoic acids from the N-acetylmuramic acid (NAM) residues**.
- Cortex synthesis, unlike vegetative cell wall synthesis, is not essential for cell viability and growth (Bukowska-Faniband and Hederstedt, 2013)
- Peptidoglycan synthesis during sporulation enables analysis of mutants defective in enzymes that otherwise are essential for growth (Todd et al., 1986).

Putative location of PDAs activitity



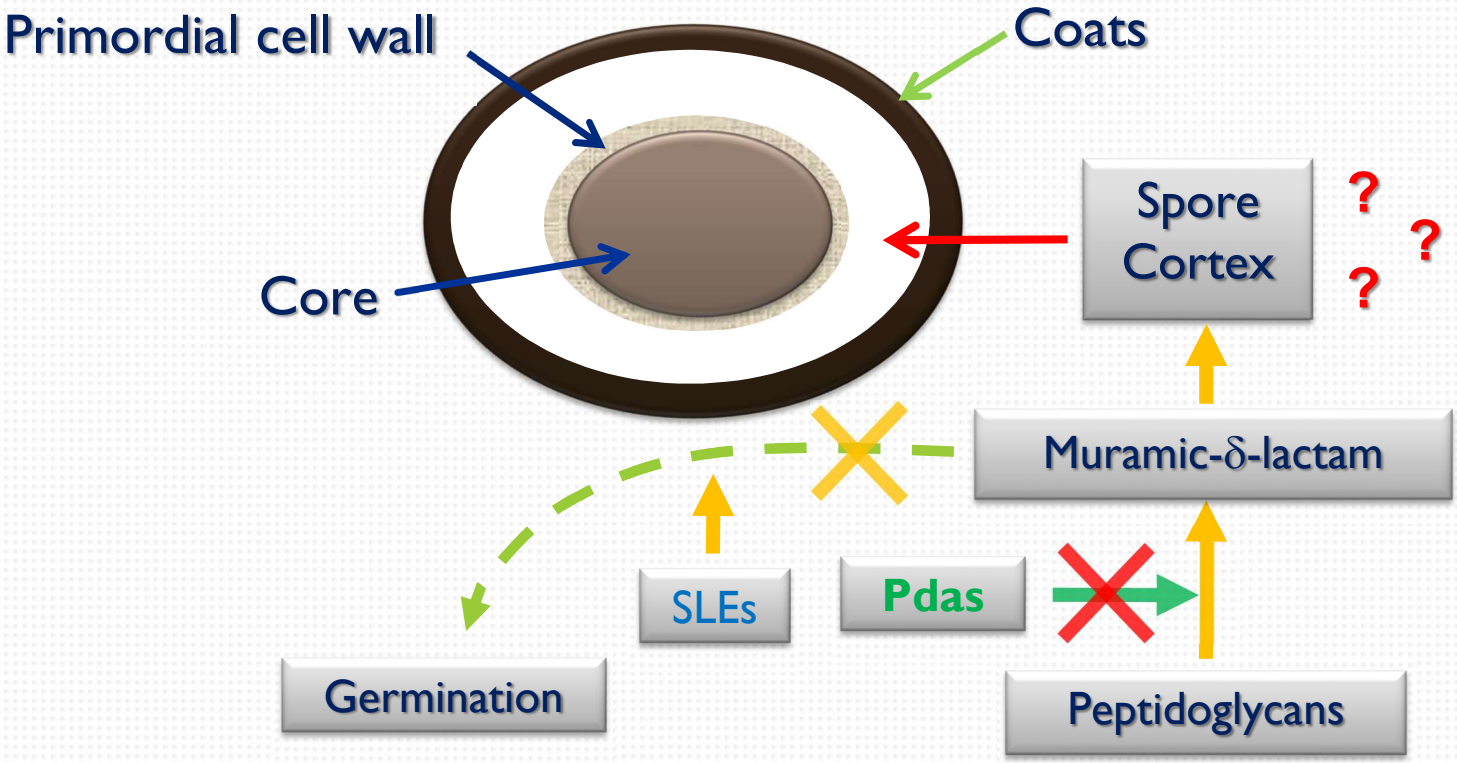
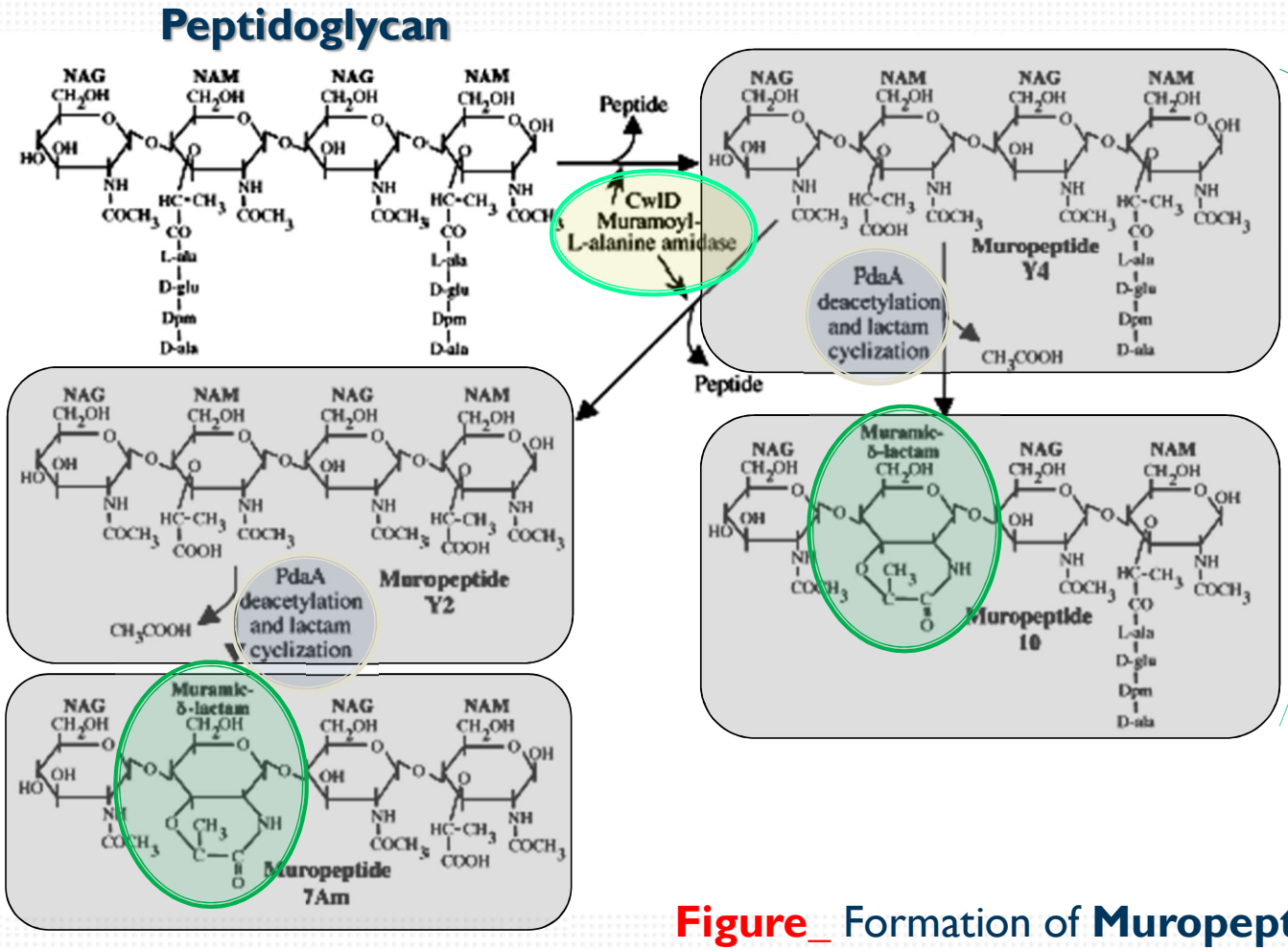
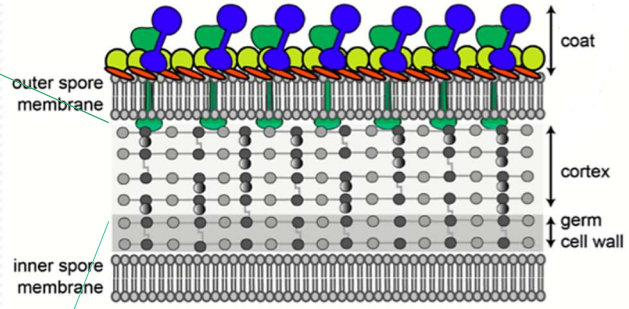


Figure _. **Possible Consequences of *pda* genes distruption:**
1. Spore forming deficient mutant
2. Germination defective mutant



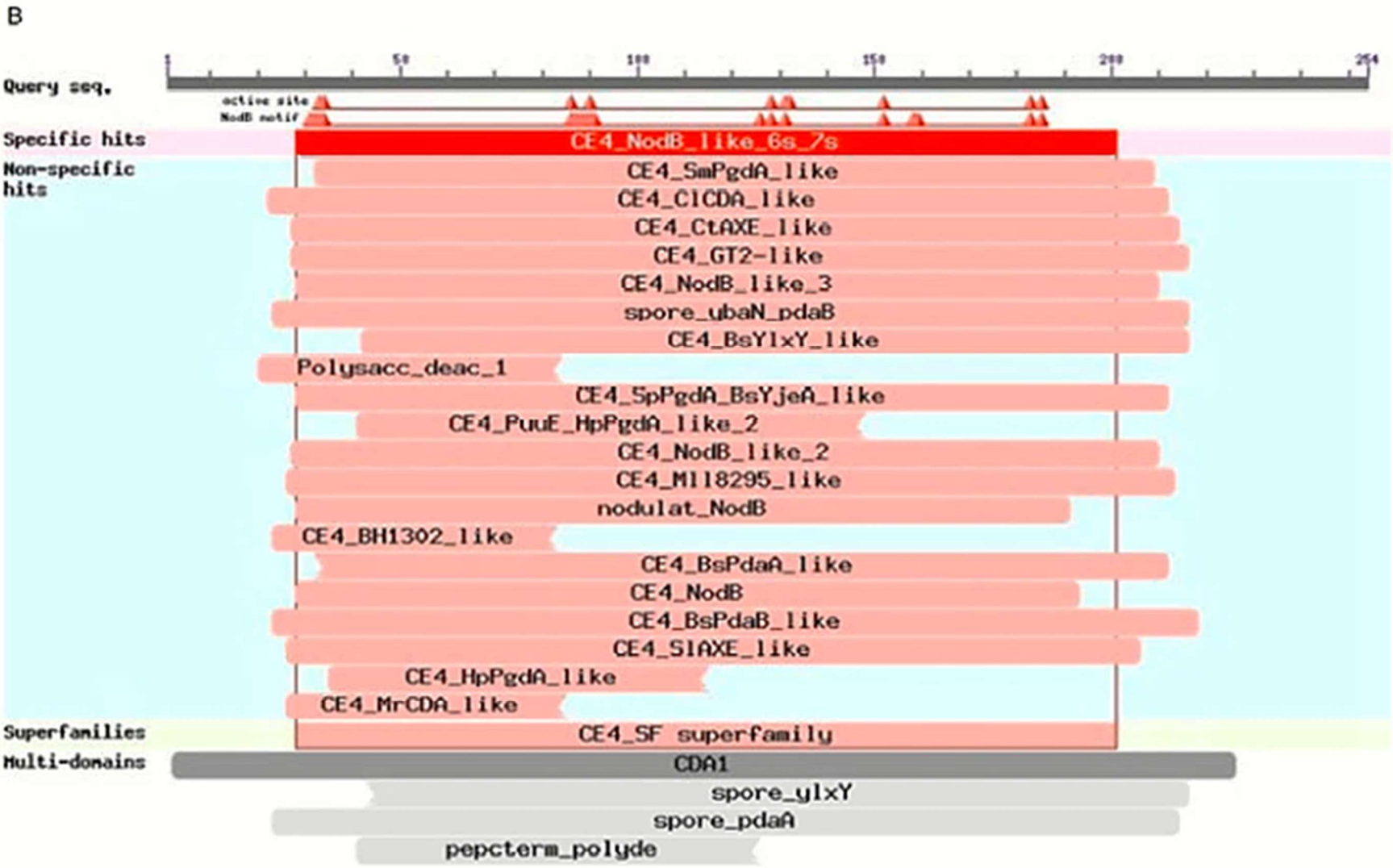


doi: https://doi.org/10.1371/journal.pgen.1009246.g008

Figure_ Formation of **Muropeptides** in the Spore cortex

Bioinformatics Study

- The CAZY database (http://www.cazy.org/CE4_bacteria.html)
  → carbohydrate enzymes hydrolysis database
- The Combrex database (http: //combrex.bu.edu/)
- The NCBI database (http://www.ncbinlm.nih.gov/)
- The Signal peptide and cleavage sites (https://services.healthtech.dtu.dk/service.php?SignalP-5.0)
- The SOSUI (http://bp.nuap.nagoya-u.ac.jp/sosui/sosui_submit.html)
  → soluble proteins or membrane proteins


B
Query seq.
active site
NodB motif
Specific hits
CE4_NodB_like_6s_7s
Non-specific hits
CE4_SmPgdA_like
CE4_ClCDA_like
CE4_CtAXE_like
CE4_GT2-like
CE4_NodB_like_3
spore_ybaN_pdaB
CE4_BsYlxY_like
Polysacc_deac_1
CE4_SpPgdA_BsYjeA_like
CE4_PuuE_HpPgdA_like_2
CE4_NodB_like_2
CE4_M118295_like
nodulat_NodB
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CE4_NodB
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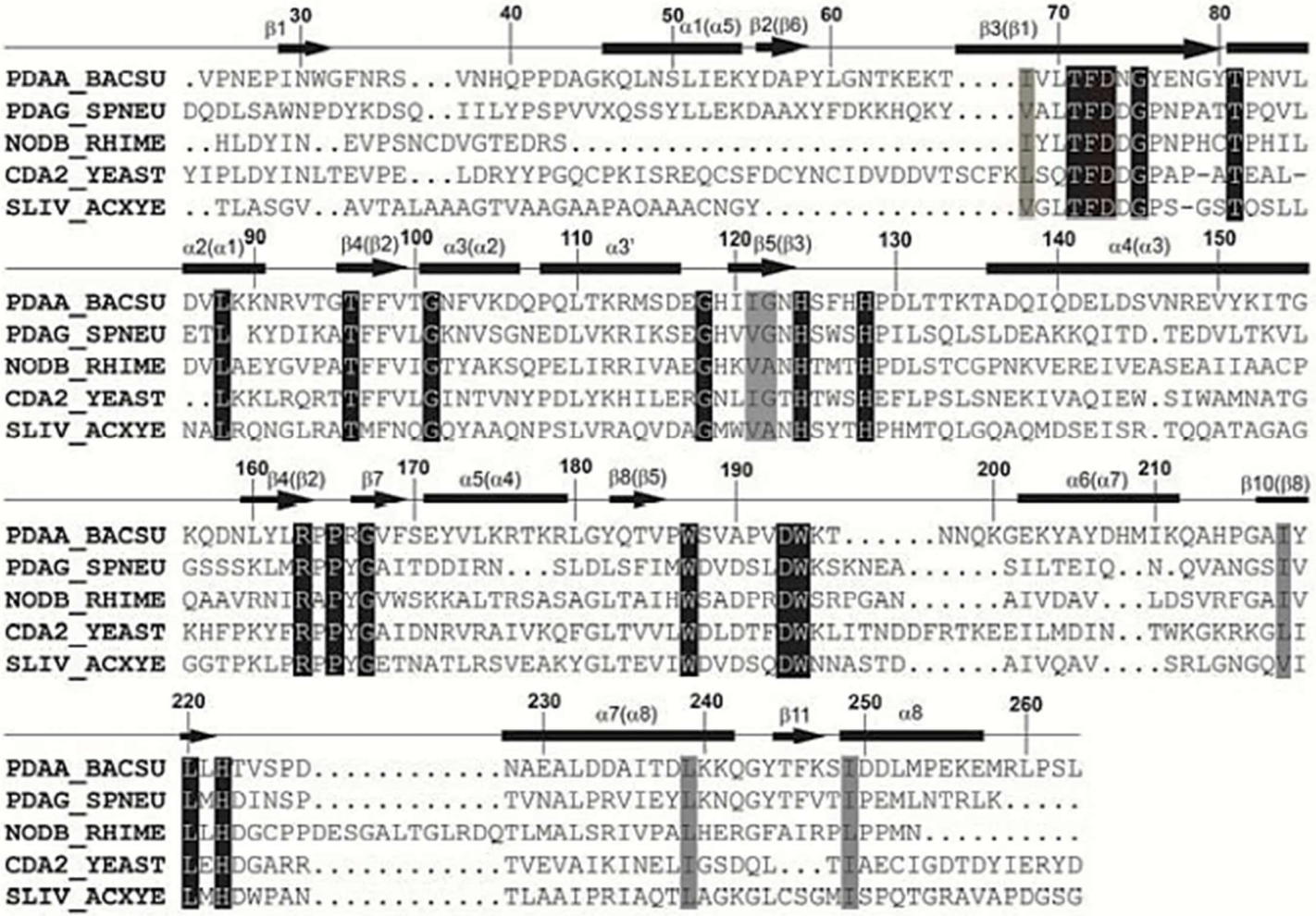
CE4_SlAXE_like
CE4_HpPgdA_like
CE4_MrCDA_like
Superfamilies
CE4_SF superfamily
Multi-domains
CDA1
spore_ylxY
spore_pdaA
pepcterm_polyde

C


β1 30 40 50 α1(α5) β2(β6) 60 β3(β1) 70 80
PDAA_BACSU .VPNEPINWGFNRS...VNHQPPDAGKQLNSLIEKYDAPYLGNTKEKT....IVLTFDDGYENGYTPNVL
PDAG_SPNEU DQDLSAWNPDYKDSQ..IILYPSPVVXQSSYLLEKDAAXYFDKKHQKY....VALTFDDGPNPATTPQVL
NODB_RHIME ..HLDYIN..EVPSNCDVGTEDRS.....IYLTFDDGPNPHCTPHIL
CDA2_YEAST YIPLDYINLTEVPE...LDRYYPGQCPKISREQCSFDCYNCIDVDDVTSCFKLSQTFDDGPAP-ATEAL-
SLIV_ACXYE ..TLASGV..AVTALAAAGTVAAGAAPAQAAACNGY.....VGLTFDDGPS-GSTQSLL
α2(α1) 90 β4(β2) 100 α3(α2) 110 α3' 120 β5(β3) 130 140 α4(α3) 150
PDAA_BACSU DVLKKNRVTGTFFVTGNFVKDQPQLTKRMSDEGHIIGNHSFHHPDLTTKTADQIQDELDSVNREVYKITG
PDAG_SPNEU ETL KYDIKATFFVLGKNVSGNEDLVKRIKSEGHVVGNHSWSHPILSQLSLDEAKKQITD.TEDVLTKVL
NODB_RHIME DVLAEYGVPATFFVIGTYAKSQPELIRRIVAEGHKVANHTMTHPDLSTCGPNKVEREIVEASEAIIAACP
CDA2_YEAST ..LKKLRQRTTFFVLGINTVNYPDLYKHILERGNLIGTHTWSHEFLPSLSNEKIVAQIEW.SIWAMNATG
SLIV_ACXYE NALRQNGLRATMFNQGQYAAQNPSLVRAQVDAGMWVANHSYTHPHMTQLGQAQMDSEISR.TQQATAGAG
160 β4(β2) β7 170 α5(α4) 180 β8(β5) 190 200 α6(α7) 210 β10(β8)
PDAA_BACSU KQDNLYLRPPRGVFSEYVLKRTKRLGYQTVPWSVAPVDWKT......NNQKGEKYAYDHMIKQAHPGAIY
PDAG_SPNEU GSSSKLMRPPYGAITDDIRN...SLDLSFIMWDVDSLDWKSKNEA......SILTEIQ..N.QVANGSIV
NODB_RHIME QAAVRNIRAPYGVWSKKALTRSASAGLTAIHWSADPRDWSRPGAN......AIVDAV...LDSVRFGAIV
CDA2_YEAST KHFPKYFRPPYGAIDNRVRAIVKQFGLTVVLWDLDTFDWKLITNDDFRTKEEILMDIN..TWKGKRKGLI
SLIV_ACXYE GGTPKLPRPPYGETNATLRSVEAKYGLTEVIWDVDSQDWNNASTD......AIVQAV....SRLGNGQVI
220 230 α7(α8) 240 β11 250 α8 260
PDAA_BACSU LLHTVSPD.....NAEALDDAITDLKKQGYTFKSIDDLMPEKEMRLPSL
PDAG_SPNEU LMHDINSP.....TVNALPRVIEYLKNQGYTFVTIPEMLNTRLK.....
NODB_RHIME LLHDGCPPDESGALTGLRDQTLMALSRIVPALHERGFAIRPLPPMN.....
CDA2_YEAST LEHDGARR.....TVEVAIKINELIGSDQL...TIAECIGDTDYIERYD
SLIV_ACXYE LMHDWPAN.....TLAAIPRIAQTLAGKGLCSGMISPQTGRAVAPDGSG

Gene Techniques

Genomics Characterization Principles

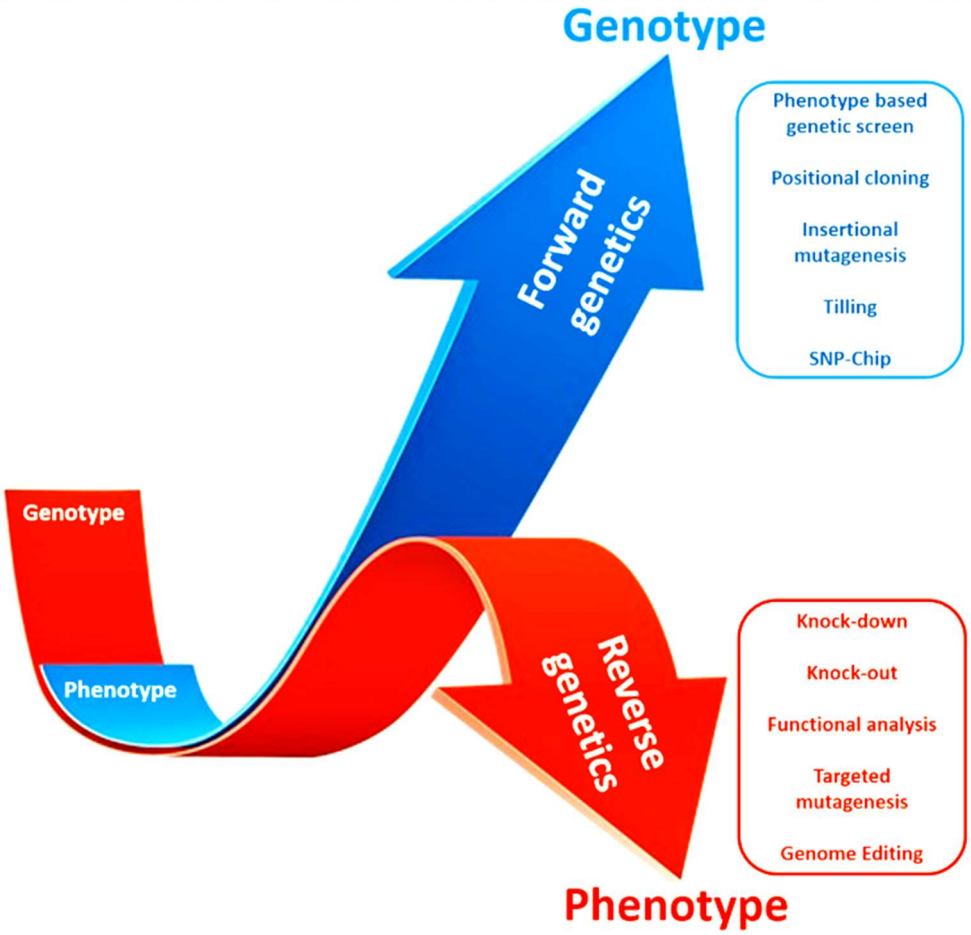
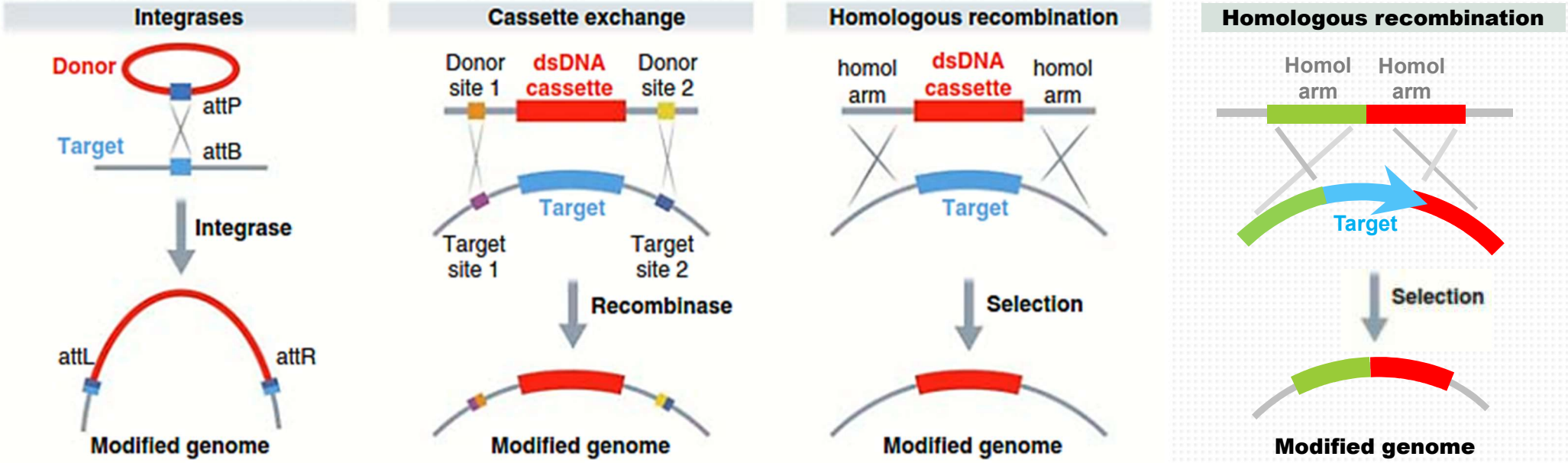


Figure _. **A schematic representation of forward and reverse genetic approaches for identification and characterization of candidate genes.** Forward genetics studies start with the selection of the desired phenotype and culminate with the identification of the responsible gene responsible (depicted in blue arrow). Reverse genetics studies start with the selection of gene of interest and end with the analysis of resulting phenotype (depicted in red arrow)(Ram *et al.*, 2019).

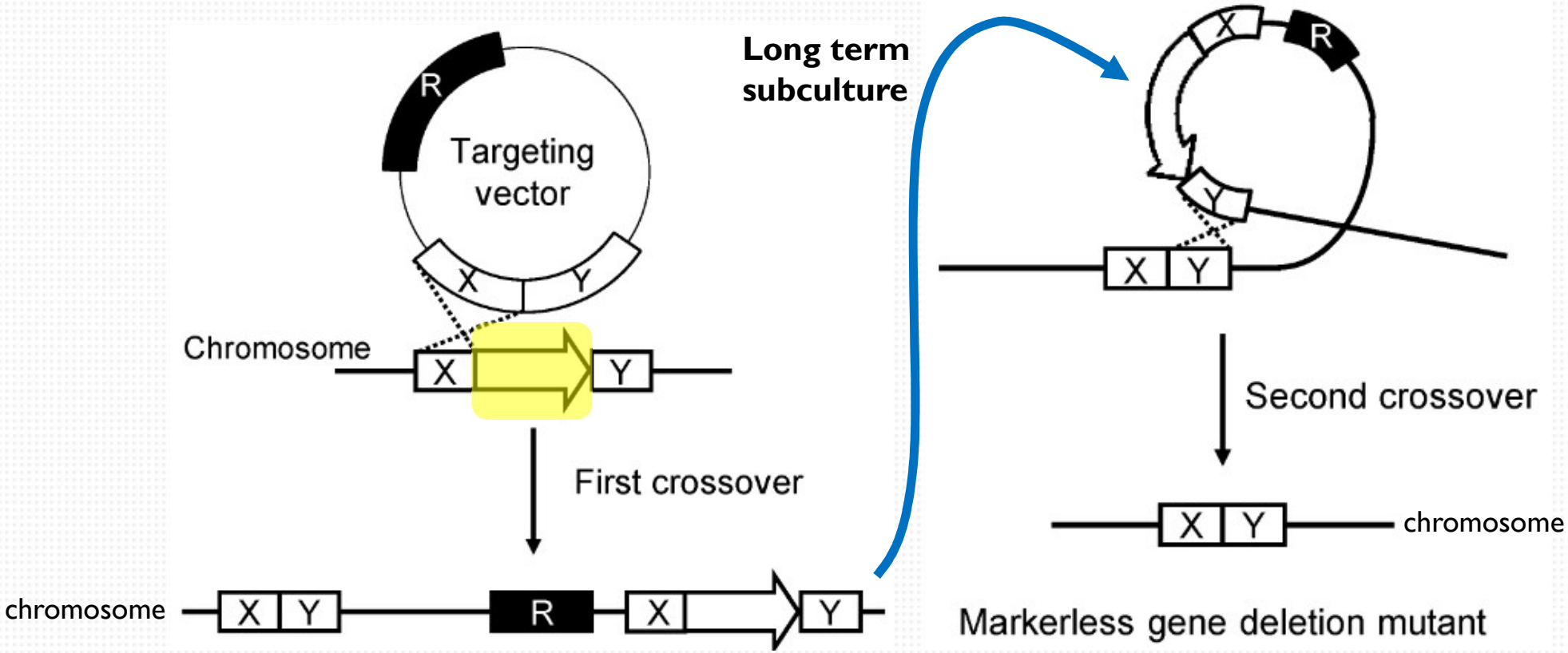
Genomics principle genome editing methods



Gene integration | **Gene replacement** | **Gene replacement** | **Gene knock-out/deletion**

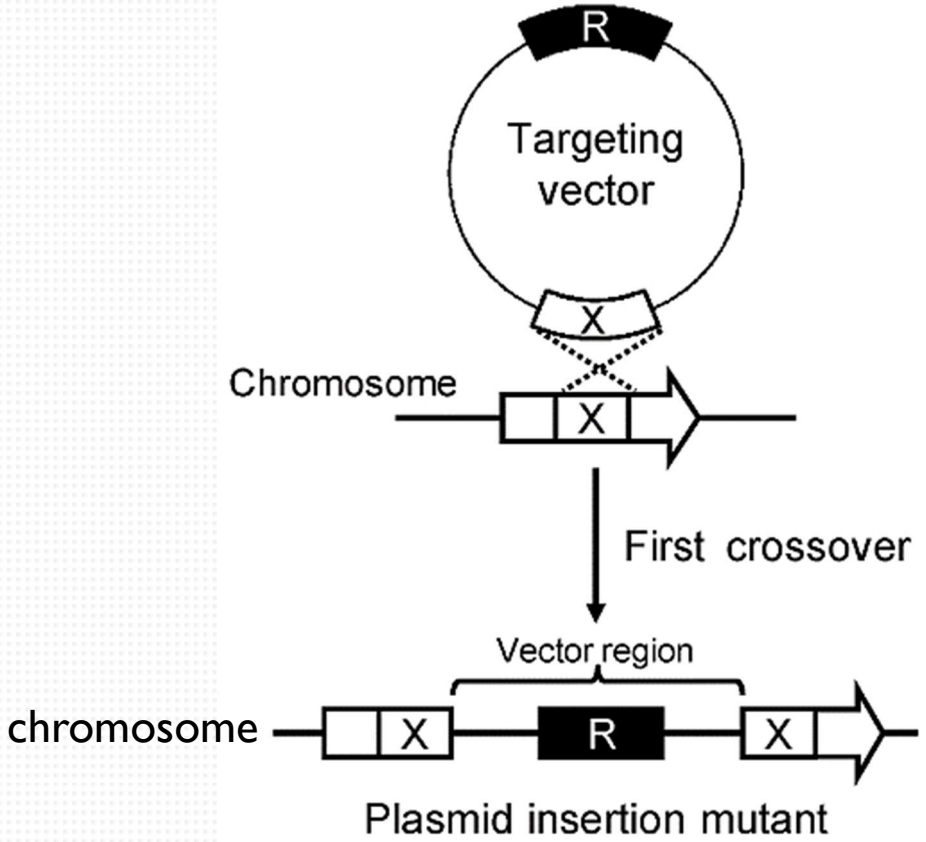
Esvelt & Wang (2013) with an additional gene deletion method

Gene Distruption Methods by Double Recombination



Fukiya *et al.* (2012)

Gene Distruption Methods by Single Recombination



Fukiya *et al.* (2012)

Results & Discussion

Table _. Polysaccharide deacetylase (PDA) encoding genes in *Bacillus licheniformis*

Gene Desig-nation	Protein name	Accession number	Locus tag	Function	Functional status	Strain	References
***yjeA* ***	**YjeA**	YP_090286.1	BLi00655	1. Carbohydrate esterase fam. 4 2. Extracellular DNAse 3. **Endo-1,4-beta xylanase** 4. Similar to **Chitooligo-saccharide deacetylase**	predicted	DSM 13/ ATTC 14580 and F11	Rey, et al. 2004; Veith et al. 2004; Waldeck et al. 2006; Voigt et al. 2006; Voigt et al. 2009
yfjS	**YfjS**	YP_090455.1	BLi00827	1. Delta-lactam biosynthetic de-N-acetylase 2. Similar to Polysaccharide deacetylase 3. Peptidoglycan GlcNAc deacetylase	predicted	DSM 13/ ATTC 14580	Rey, et al. 2004; Veith et al. 2004;
yheN	**YheN**	YP_090640.1	BLi01039	1. Similar to **Endo-1,4-betaxylanase**	predicted	DSM 13/ ATTC 14580	Rey, et al. 2004; Veith et al. 2004; Voigt et al. 2006;Voigt et al. 2009

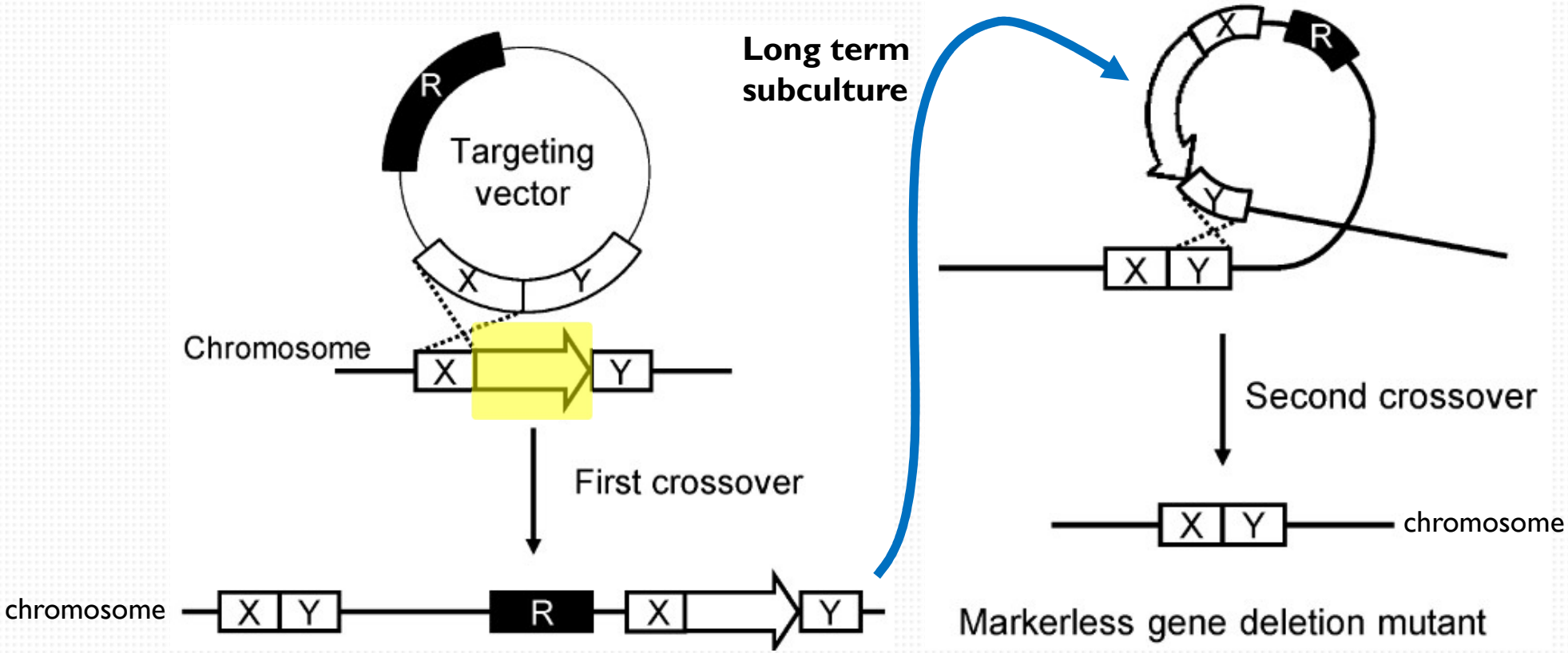
Note: Predicted = putative

Gene designa-tion	Protein name	Accession number	Locus tag	Function	Functional status	Strain	References
ylxY	**YlxY**	YP_091483.1	BLi01895	1. Polysaccharide deacetylase family	predicted	DSM 13/ ATTC 14580	Rey, et al. 2004; Veith et al. 2004
yxkH	**YxkH**	YP_093657.1	BLi04151	1. Polysaccharide deacetylase 2. Carbohydrate esterase family 4	predicted	DSM 13/ ATTC 14580	Rey, et al. 2004; Veith et al. 2004
bli02451 /yheN2	**Bli02451 /YheN2**	YP_092021.1 AAU41828.1	Bli02451	1. **Chitin deacetylase/ xylanase** 2. Peptidoglycan GlcNAc deacetylase	predicted	DSM 13/ ATTC 14580	Rey, et al. 2004; Veith et al. 2004; Wiegand *et al. 2012.*
ybaN	***YbaN* / PdaB**	YP_089842.1	BLi00175	1. Polysaccharide deacetylase	predicted	DSM 13/ ATTC 14580	Rey, et al. 2004; Veith et al. 2004

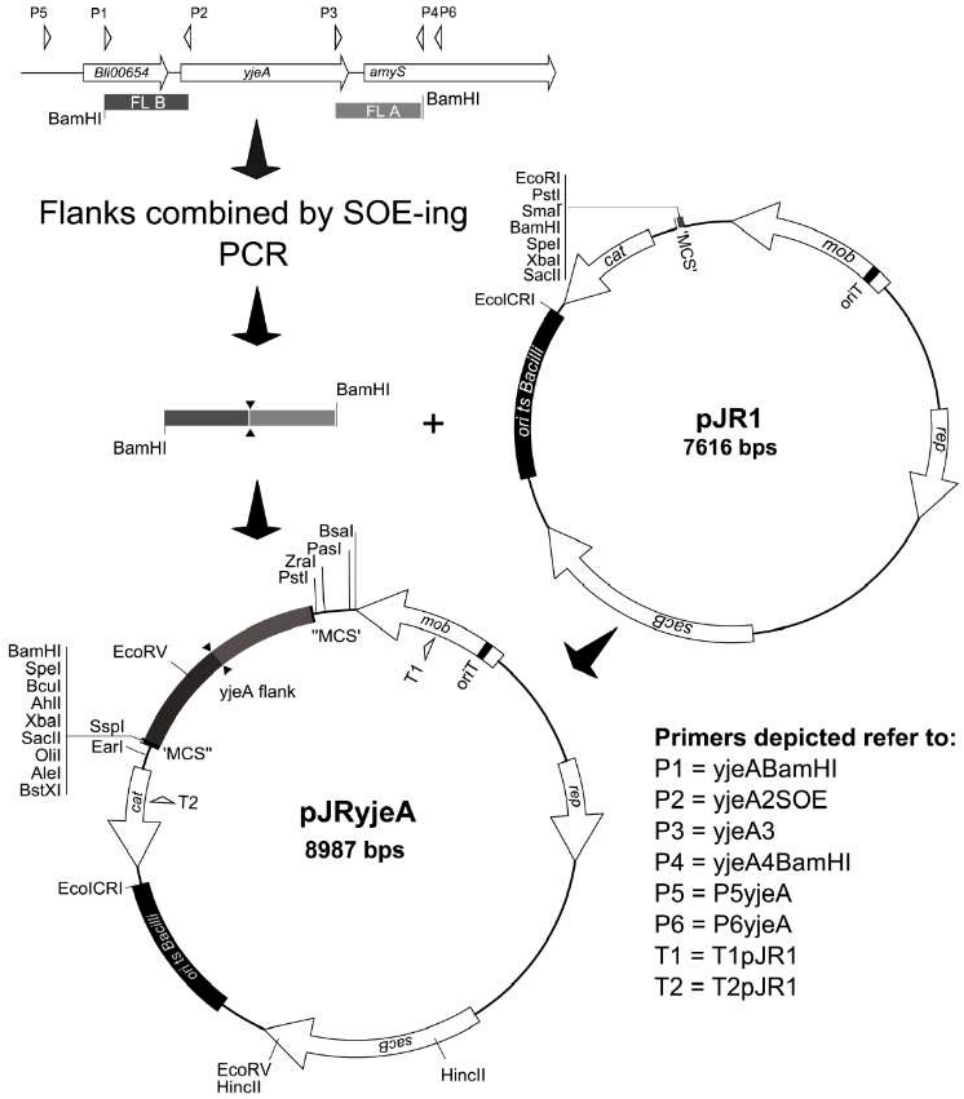
* **All PDAs are putative**; the genes investigated in this study are in bold face. The data in the table were taken from the CAZY database (http://www.cazy.org/CE4_bacteria.html), the Combrex database (http: //combrex.bu.edu/) and the NCBI database (http://www.ncbi.nlm.nih.gov/). Accession numbers refer to unique identifiers given to the protein sequences in GenBank (http://www.ncbi. nlm. nih.gov/genbank/); Locus tags refer to synonyms for the loci in the genomes.

Note: Predicted = putative

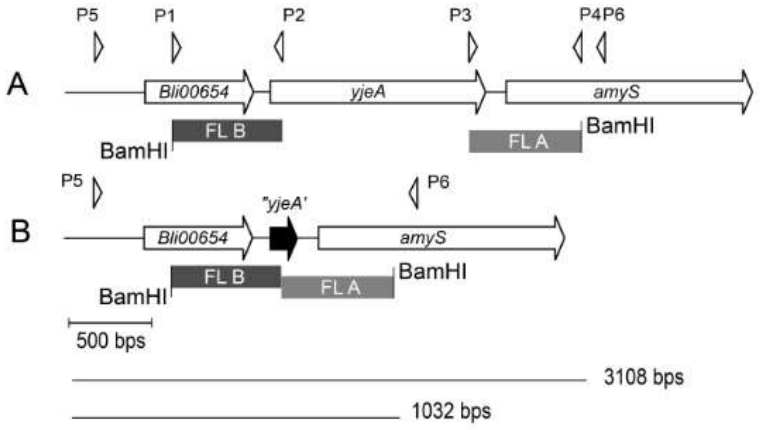
Gene Distruption Methods by Double Recombination

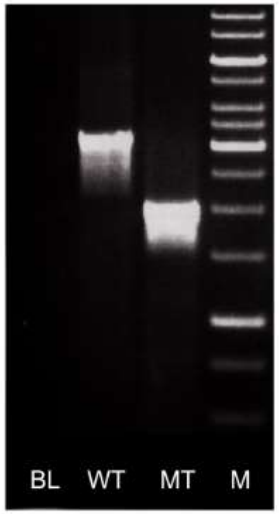


Fukiya *et al.* (2012)



- Amplification of flank A and flank B from the genome of *B. licheniformis*
- Combine of two flanks by SOE-ing PCR
- Insert the hybrid flank into shuttle plasmid **pJR1** (a suicide plasmid that has been constructed for *Bacillus* strains).
- Resulting plasmid **pJRyjeA**
- **1st** Transformation into ***E. coli* S17-1**
- **2nd** Tranformation by "**Conjugation system**" that available in conjugative ***E. coli* S17-1** strain into ***Bacillus*** (cell) strains
- Conjugants selected by Pasteurization.
- Mutant selection by colony PCR

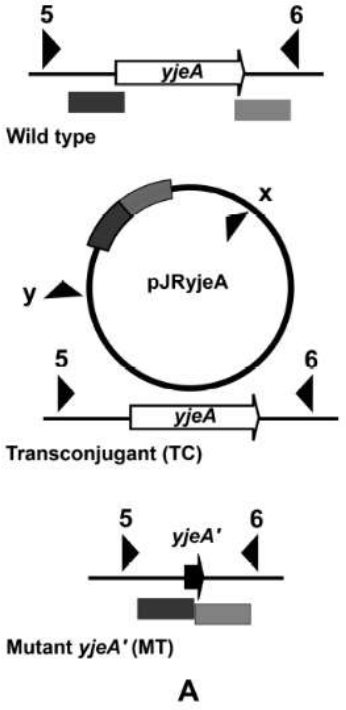


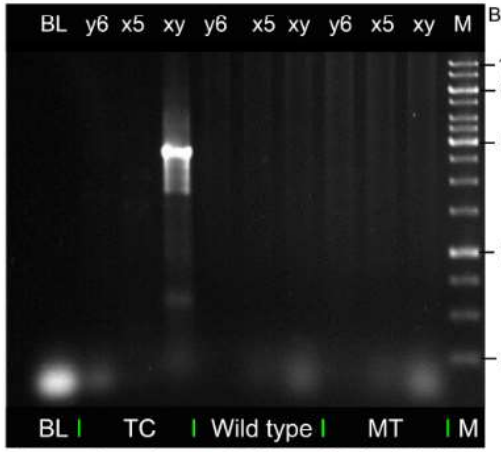


C

Mutant screening of "Clean deletion" of *yjeA* gene

Confirmation of "Clean deletion" by Analytic PCR



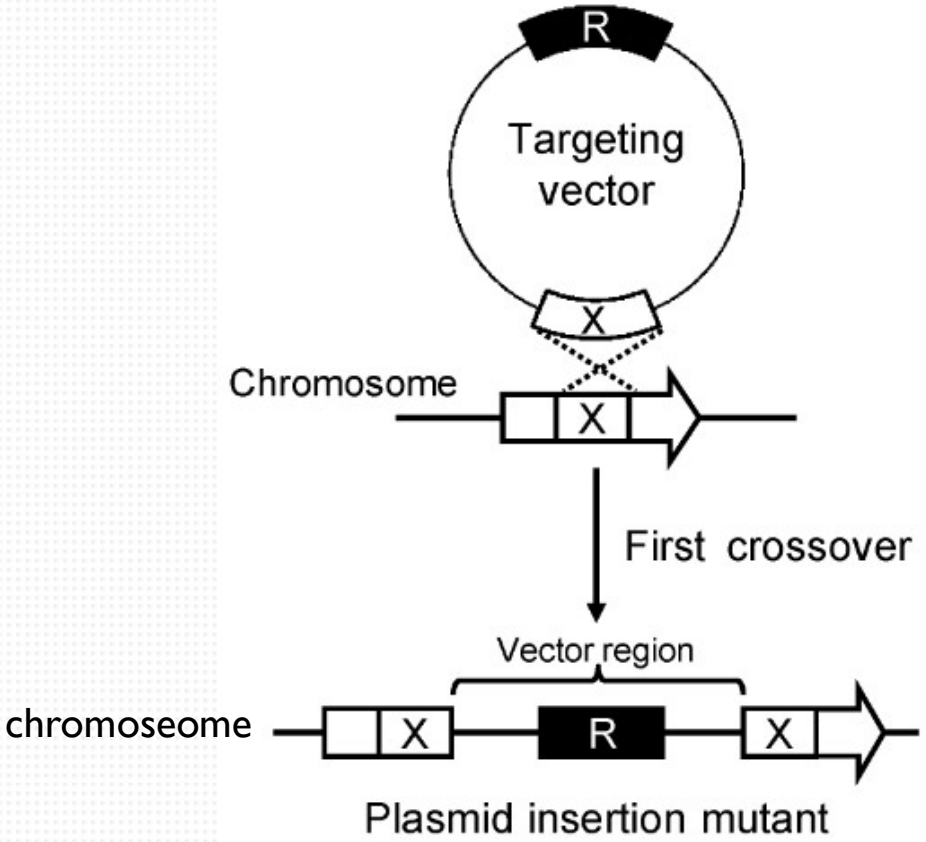


Primer pairs and genomic DNA used in Analytical PCR:

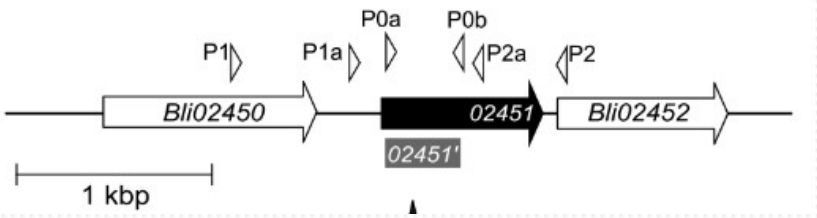
xy	= primer pair of x with y
x5	= primer pair of x with 5
y6	= primer pair of y with 6
Wild type	= genomic DNA of B. licheniformis MW3
TC	= genomic DNA of B. licheniformis MW3 containing mobilizable plasmid pJRyjeA after transconjugation
MT	= genomic DNA of B. licheniformis MW3 Δ*yjcA*
BL	= no Genomic DNA nor plasmid DNA added into PCR reaction

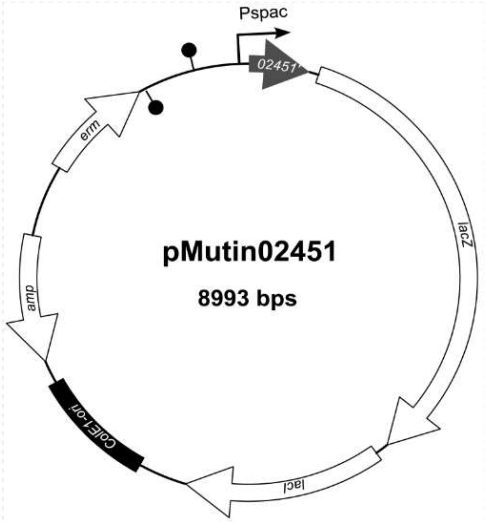
B

Gene Distruption (plasmid Insertion) Methods

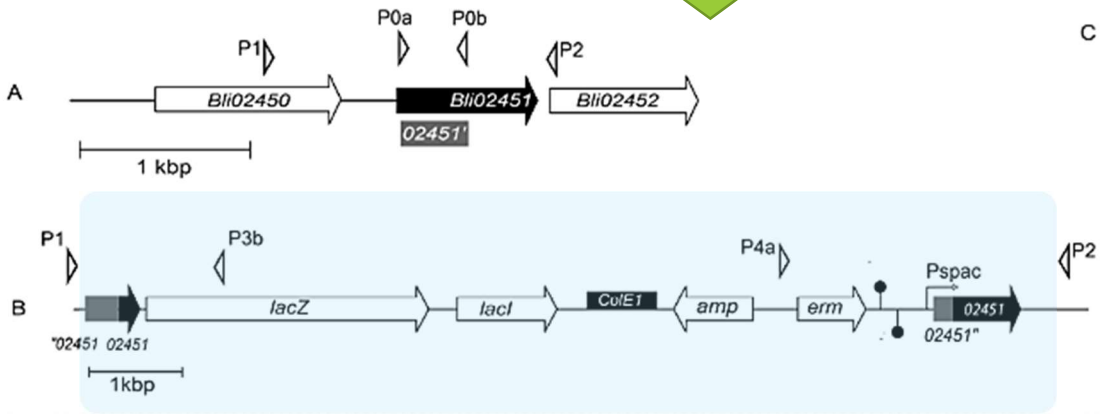


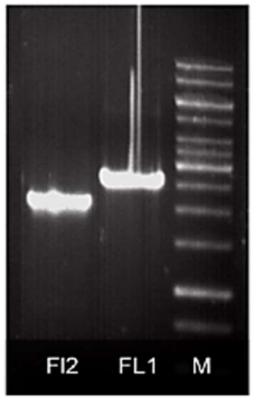
Fukiya *et al.* (2012)

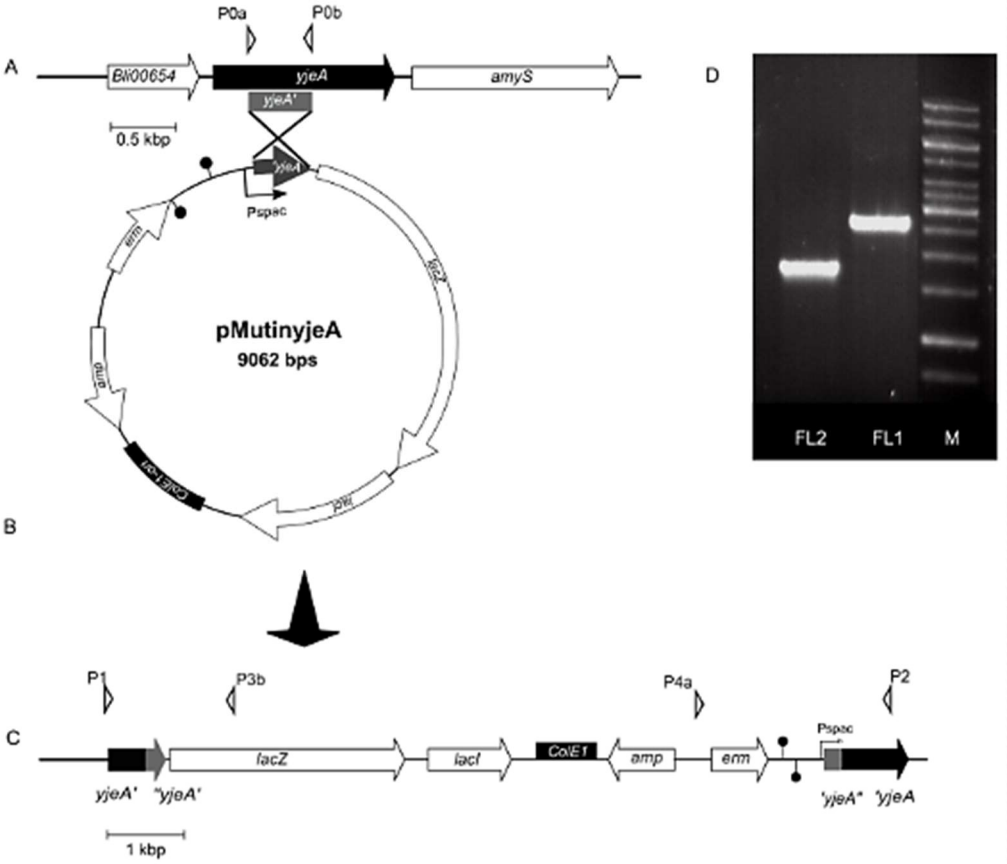




- Amplifying a single flank (of ***bli02451***).
- Insert into an **integration vector pMutin2**.
- **Protoplast Transformation** into *Bacillus* cell.
- Single recombination of the ***bli02451*** flank into the *Bacillus* genome was selected by by colony PCR






A
P0a
P0b
Bli00654
yjeA
amyS
yjeA'
0.5 kbp
yjeA
Pspac
erm
lacZ
pMutinyjeA
9062 bps
amp
ColE1 ori
lacI
B
C
P1
P3b
P4a
P2
lacZ
lacI
ColE1
amp
erm
Pspac
yjeA'
'yjeA'
'yjeA"
'yjeA
1 kbp

D
FL2
FL1
M

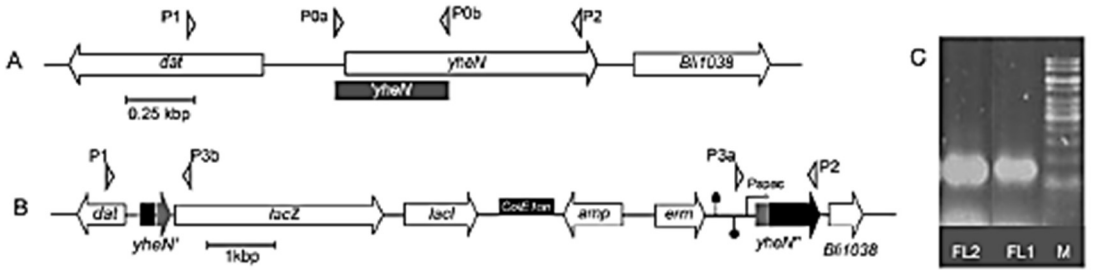

A
P1
P0a
P0b
P2
dat
yheN
Bli1039
yheN
0.25 kbp
B
P1
P3b
P3a
Pspac
P2
dat
yheN'
lacZ
lacI
ColE1ori
amp
erm
yheN"
Bli1038
1kbp
C
FL2
FL1
M

Figure __ **Several mutants strain of the *Bacillus licheniformis*** which generated in this work by gene techniques.

No	Strain	Genotype	References
1	*B. licheniformis* DSM 13	Wild type	DSMZ, Veith, *et al.* (*2004*)
2	*B. licheniformis* MW3	Δ*hsdR1*, Δ*hsdR2*	Waschkau, *et al.* (2008)
3	Bli MW3 Δ*yfjS*	Δ*hsdR1*, Δ*hsdR2*, Δ*yfjS*	Borgmeier (this lab.)
4	Bli MW3 Δ*yjeA*	Δ*hsdR1*, Δ*hsdR2*, Δ*yjeA*	This study
5	Bli MW3.01*	Δ*hsdR1*, Δ*hsdR2* :: *Bli02451pMutin2*	This study
6	Bli MW3.02*	Δ*hsdR1*, Δ*hsdR2*, Δ*yfjS*:: *yjeApMutin2*	This study
7	Bli MW3.03*	Δ*hsdR1*, Δ*hsdR2*, Δ*yfjS*:: *Bli02451pMutin2*	This study
8	Bli MW3.04*	Δ*hsdR1*, Δ*hsdR2*, Δ*yjeA*::*Bli02451pMutin2*	This study
9	Bli MW3.05*	Δ*hsdR1*, Δ*hsdR2*, Δ*yfjS*:: *ylxYpMutin2*	This study
10	Bli MW3.06*	Δ*hsdR1*, Δ*hsdR2*, Δ*yfjS*:: *yheNpMutin2*	This study
11	Bli MW3.07*	Δ*hsdR1*, Δ*hsdR2*, Δ*yfjS*::*yxkHpMutin2*	This study

Phenotype Analysis

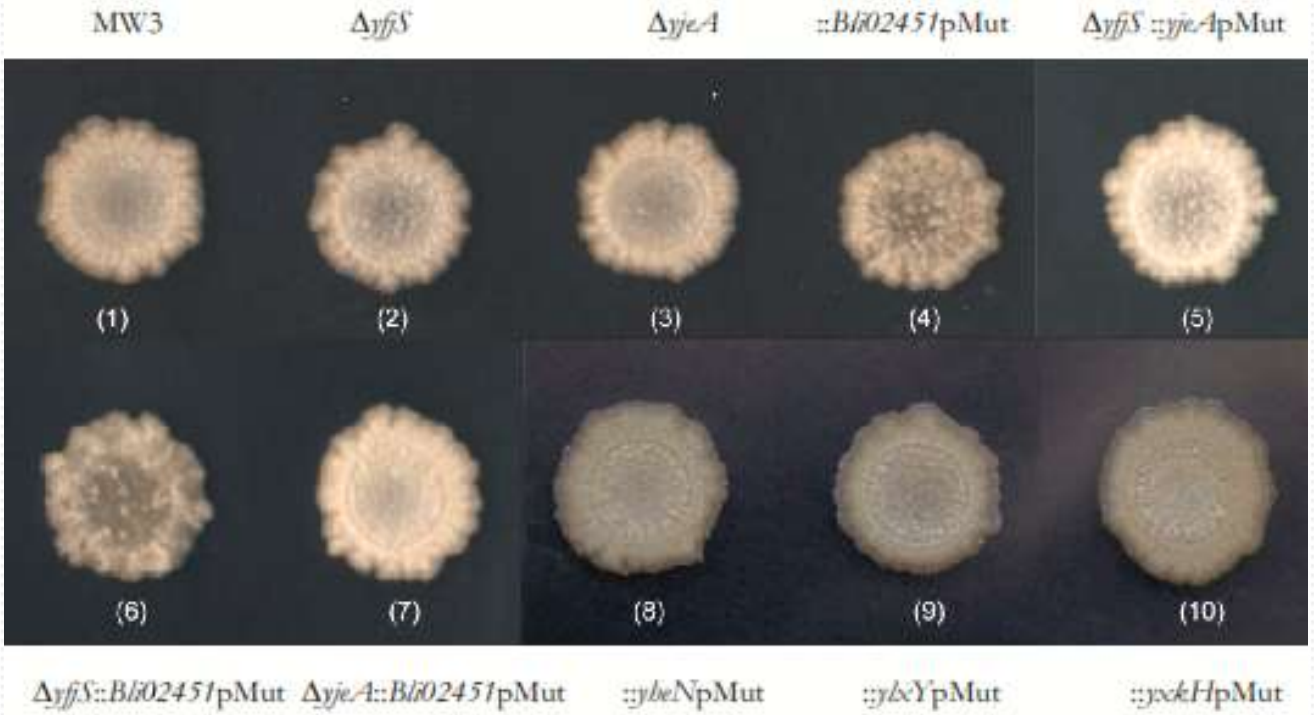


Figure _ *B. licheniformis*; Colony phenotype of the wild type MW3 and the mutants after 72 hours at 30°C grown on LB agar. The relevant genotype of each mutant strain is given in close proximity to the respective colony. The colonies formed by strains formed by strains *::Bli02451pMut and ΔyfjS::Bli02415pMut differ from all other colonies by their* dotted appearance. The colony number of (5) and (7) are whitish.

Growth curves of the *B. licheniformis* MW3 knock–out mutants

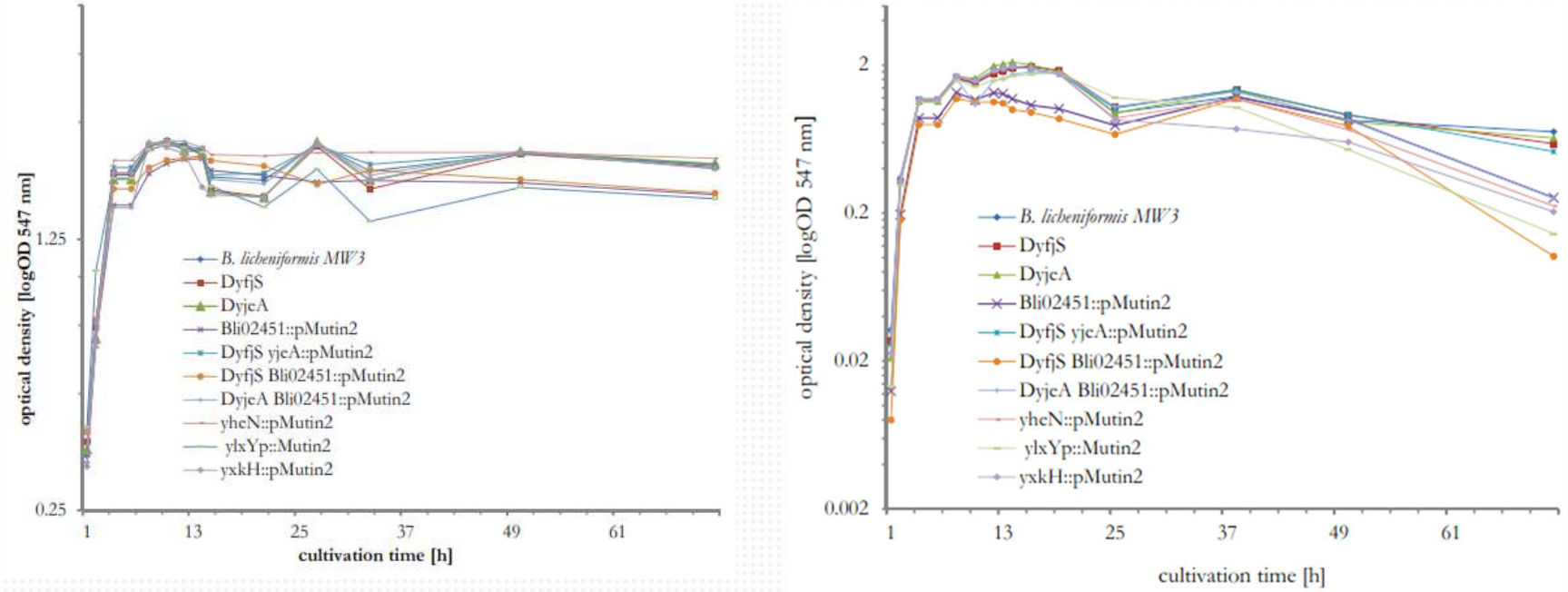


Figure_ .A). Growth curves of the *B. licheniformis* MW3 knock–out mutants in LB medium at the initial pH 7.5; B). The strain mutants growth in minimal media; in comparison to the wild type MW3. Cultivation was done in M9 medium at the initial pH 7.4. Strain designations along with the corresponding graphs are mentioned as such as in Figure 3.10. The data resulted from two different experiments (mean values)

Sporulation of the *B. licheniformis* PDA knock-out Mutants

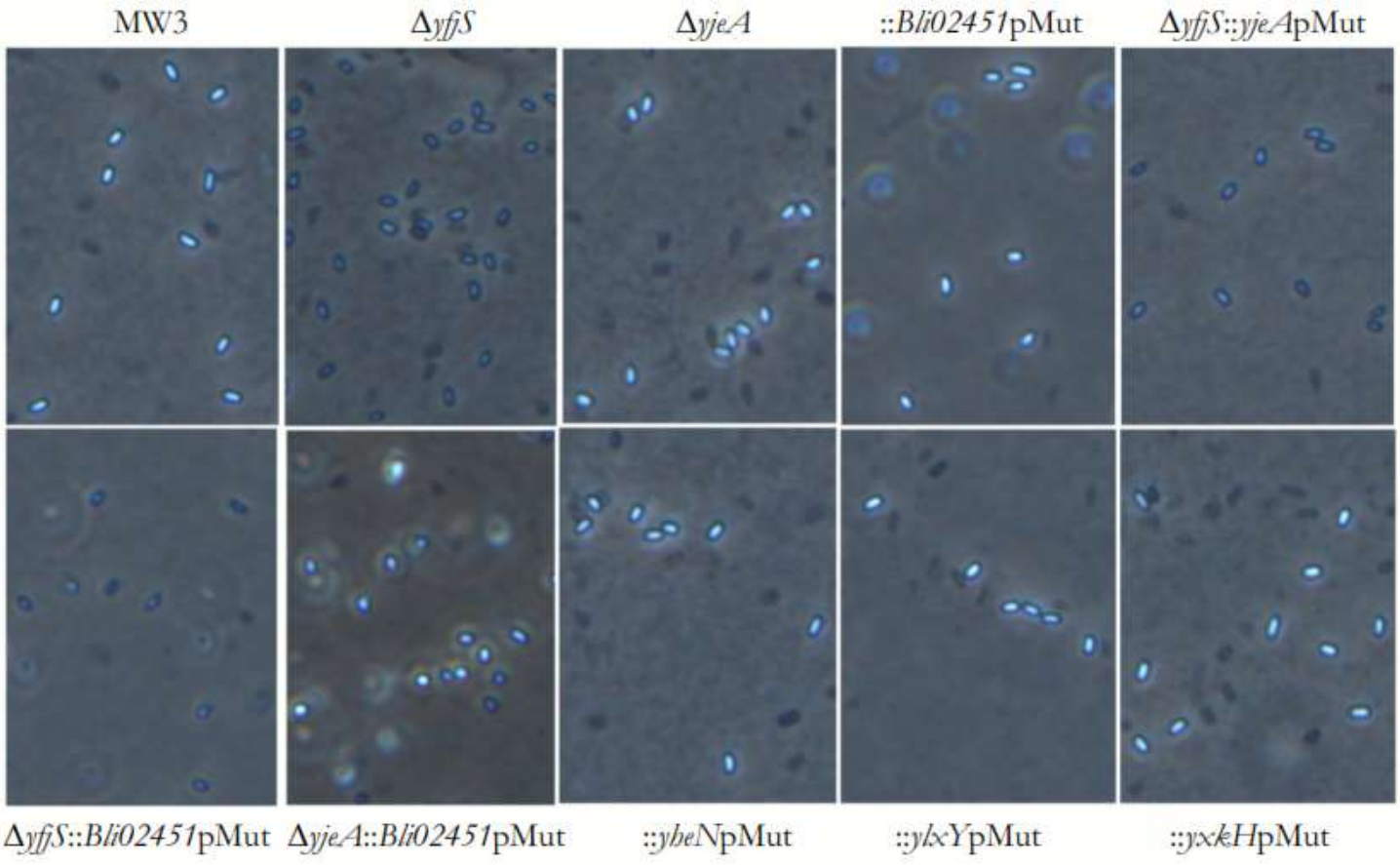


Figure _ Spore formation of the *B. licheniformis* PDA knock-out mutants. The spores were obtained from cultures on Schaeffer's sporulation medium (2x SG medium)

Spore Germination of Spores of the *B. licheniformis pda* genes knockout Mutants

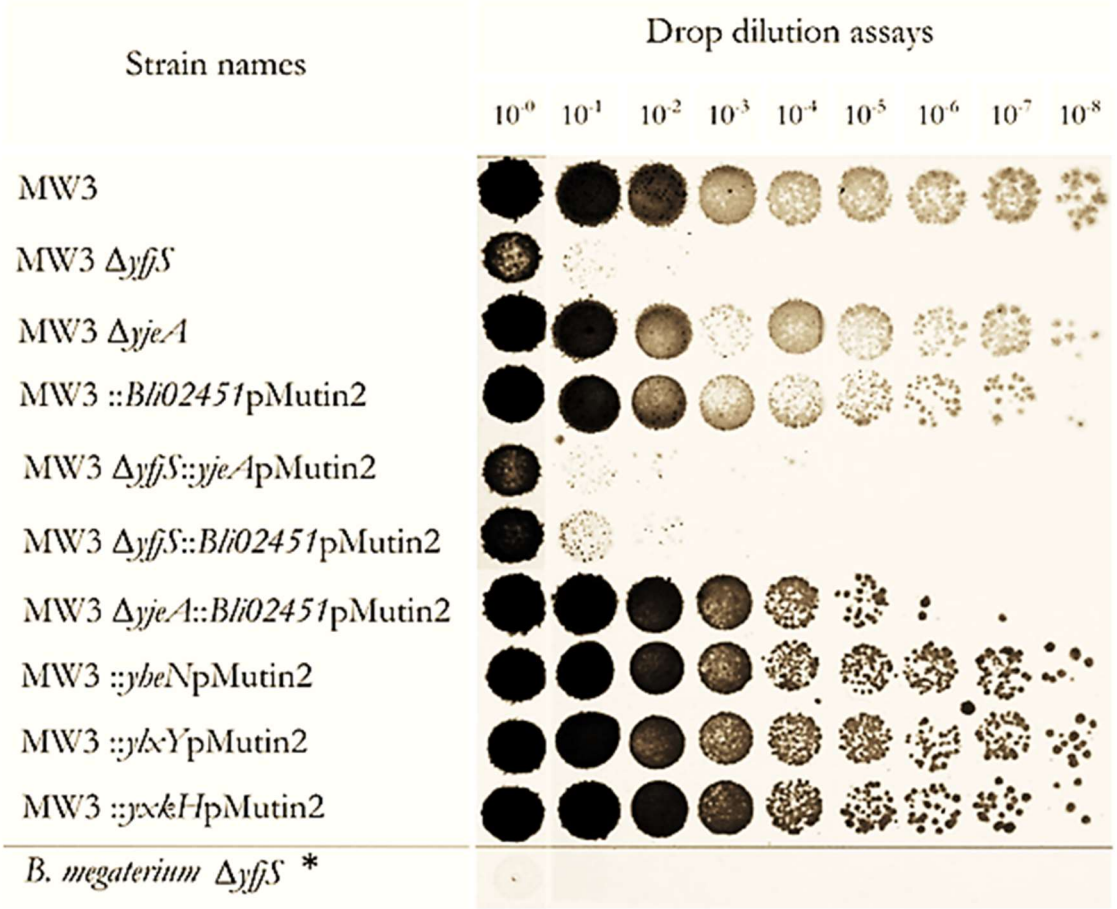


Figure _ *B. licheniformis,* drop dilution assays for recording the impacts on spore germination of the knock out mutations. The *yfjS deletion mutant of B. megaterium* (Borgmeier and Meinhardt, *unpublished*) served as an internal control. The cultures were normalized to 10^8 spores per ml and subsequently heated for 20 minutes at 80 °C to kill remaining vegetative cells.

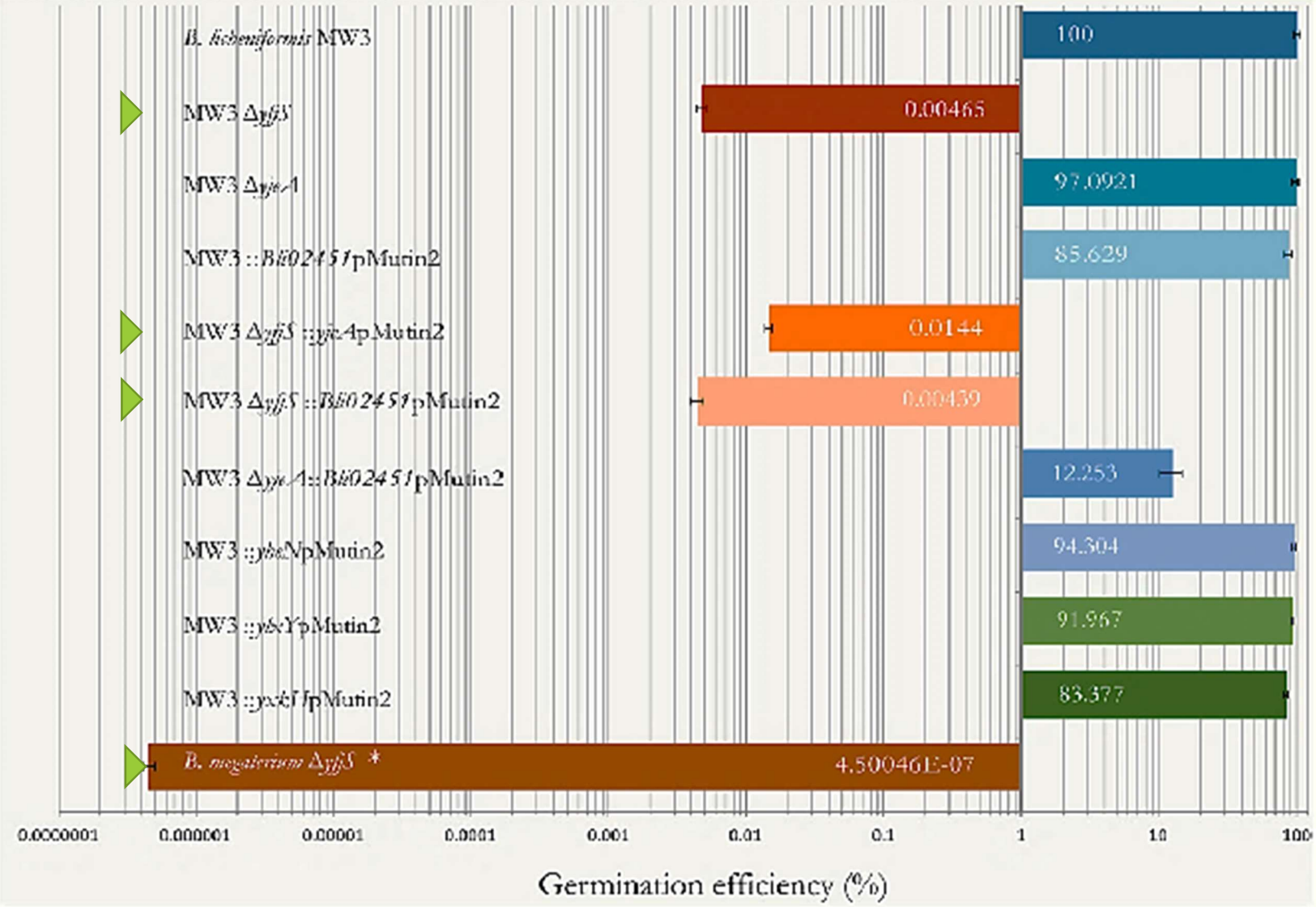
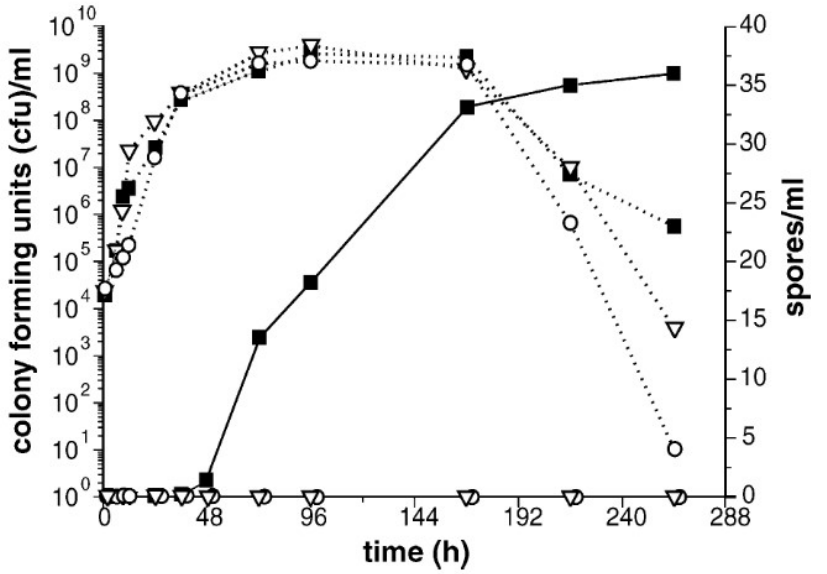
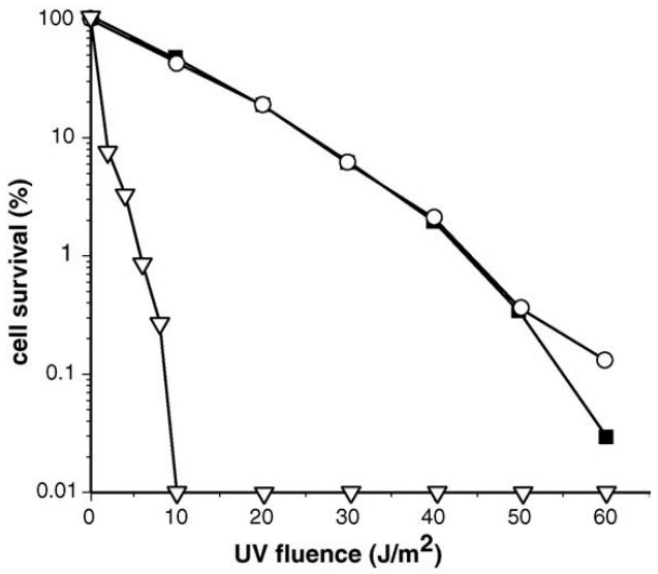


Figure _ Germination efficiency by calculation the number of viabel cells of the *B. licheniformis* PDA knockout mutants, after Pasteurization.

Previous study

Sporeless Mutants by *spoIV* and *recA* knock-out





(■) ***B. licheniformis* MD1** (wild type); (○) ***B. licheniformis* MD1.1** ***(Δ spoIV);*** (▽) ***B.licheniformis*** MD1.2 ***(Δ spoIV, ΔrecA).***

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Conclusion

- We had successfully in generating several mutants related to sporulation and germination i.e. *Bacillus licheniformis* MD1.1 (Δ*spoIV*); *B.licheniformis* MD1.2 (Δ *spoIV*, Δ*recA*) (**Sporeless mutants**); *B. licheniformis* Δ*yfjS*, *B. megaterium* Δ*yfjS*, *and other* two double mutants (**Germinative defected spore**)
- Mutant *B. licheniformis* MW3 Δ*yfjS* produce less than100% germination spore defect.
- **Those mutants ready to use in production of industrial products**.
- Need to know why the *B. licheniformis* MW3 Δ*yfjS* mutant is not 100% unviable.
- We had isolated several potential strains from **North & East Kalimantan** i.e. *B. licheniformis* K1.2.4, *Bacillus* sp P.A2.5, *Paenibacillus polymyxa* A.B2.8, *Bacillus* sp A.B2.10, *B. subtilis* A.C1.2, *Brevibacillus brevis* P.B3.4, *Bacillus flexus*, and *Bacillus paramycoides* PYA211. These strains have ability in secretion several industrial enzymes such as proteases, chitin deacetylases, lipases, etc.

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- **Yeni Fitriani**
- **Hajar Nailufar**
- **Priyanka Hastri**

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