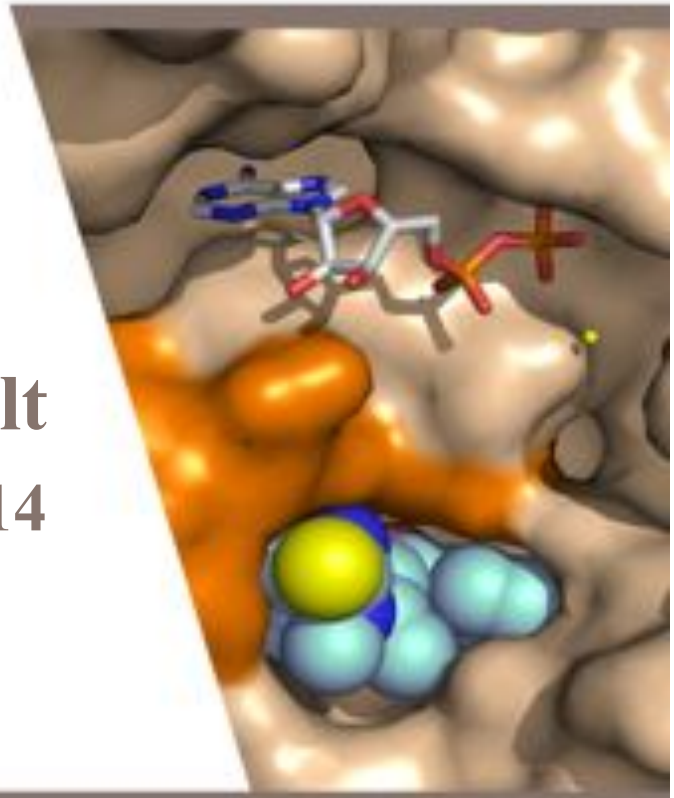
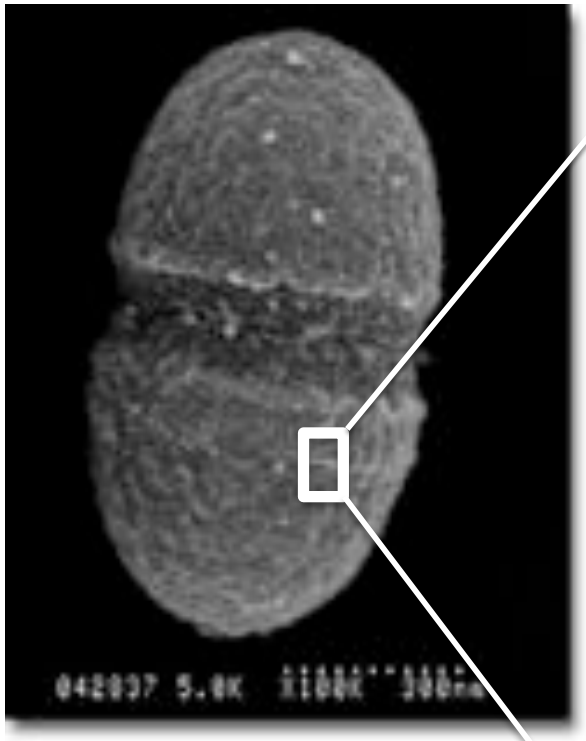
From a static structural description of the bacterial cell wall to a dynamical view of its biosynthesis: what can we learn from NMR?



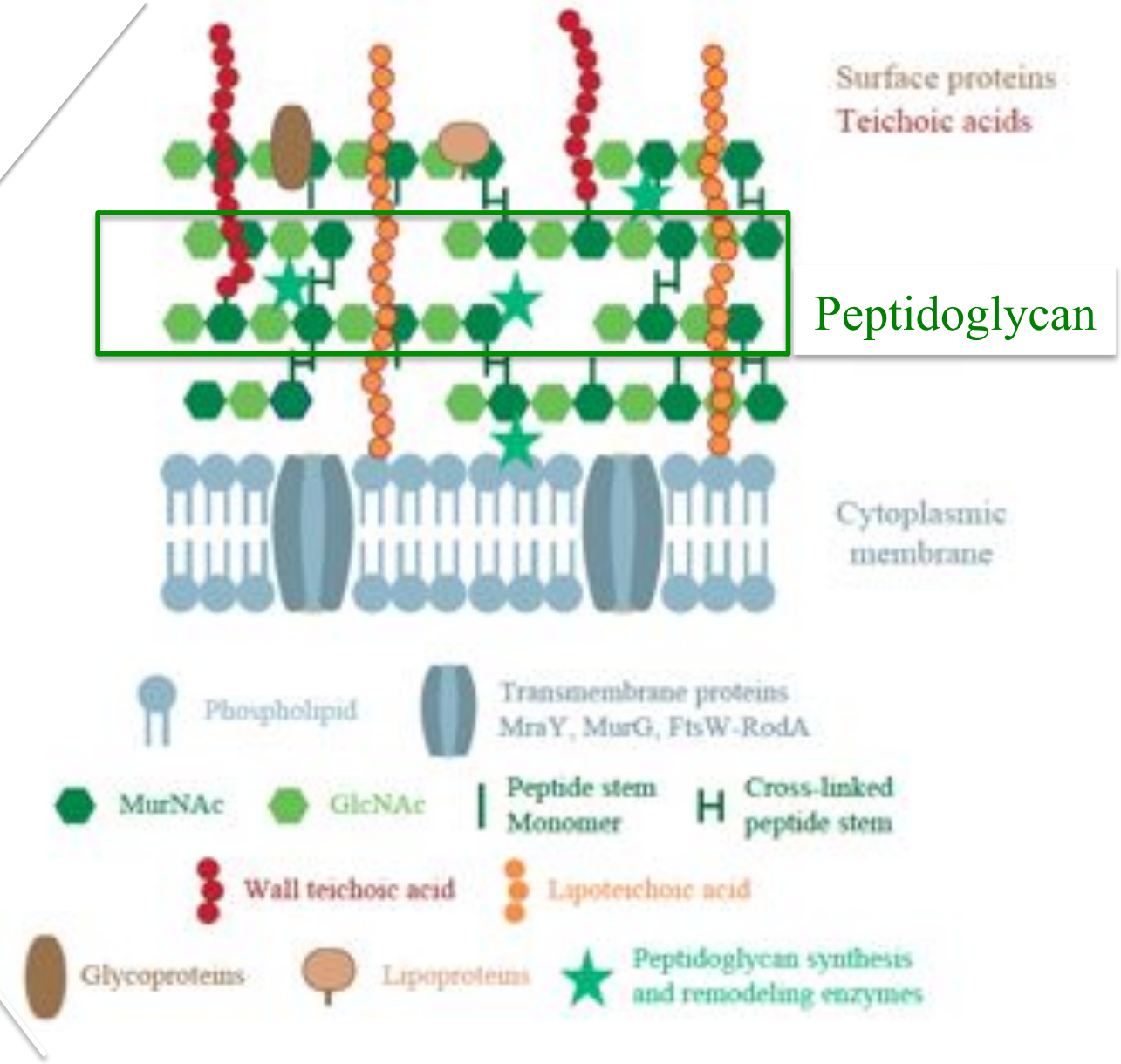
Catherine Bougault

Ecole RéNaFoBiS, Oléron, June 4th 2014

Bacterial cell wall: *Enterococcus faecium*

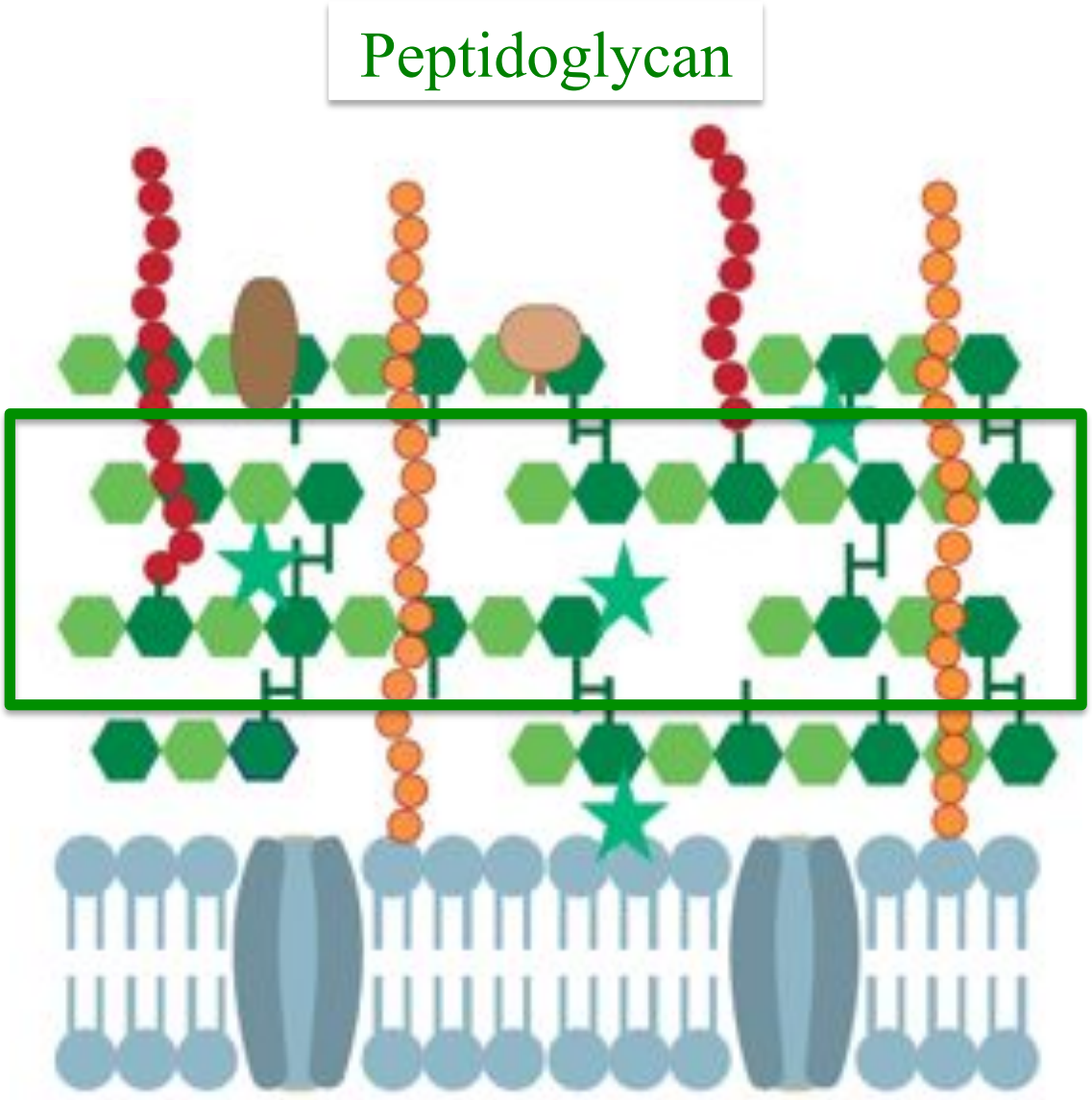


Enterococcus faecium
Gram-positive bacteria
Photo Credit: N. Shankar,
University of Oklahoma Health Science Center



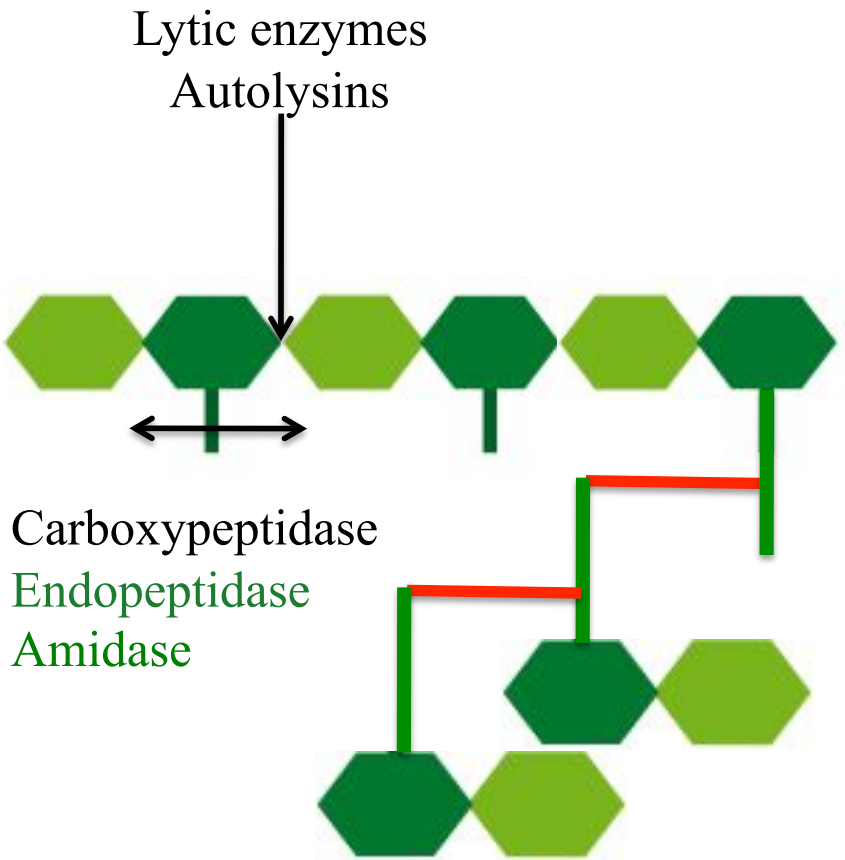
Enterococcus faecium peptidoglycan

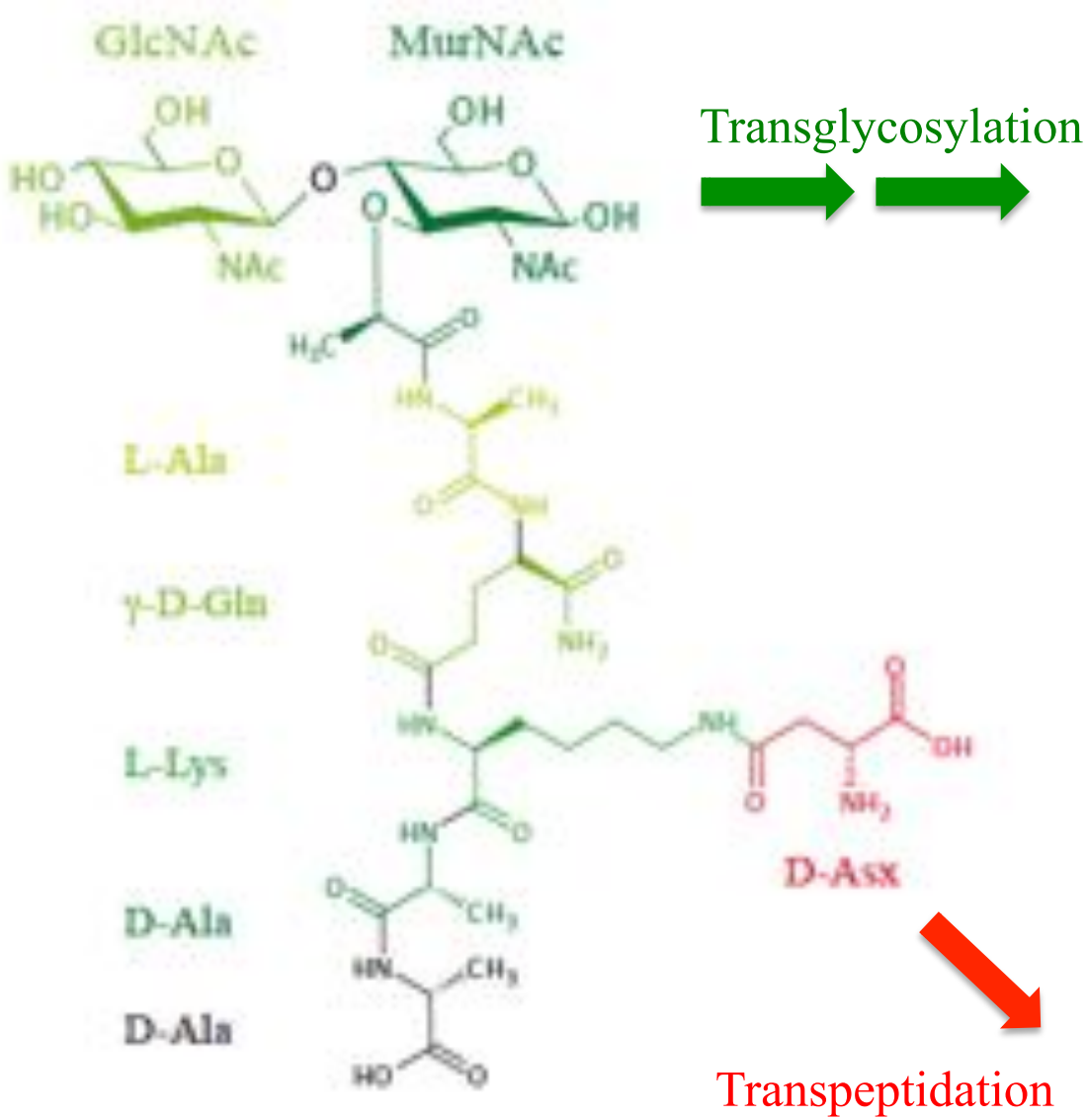
- Scaffold for surface proteins/polymers
- Prevents lysis due to turgor pressure
- Dictates cell morphology and growth
- Unique to bacteria



Key antibiotic target, but increased resistance issues

Enterococcus faecium peptidoglycan

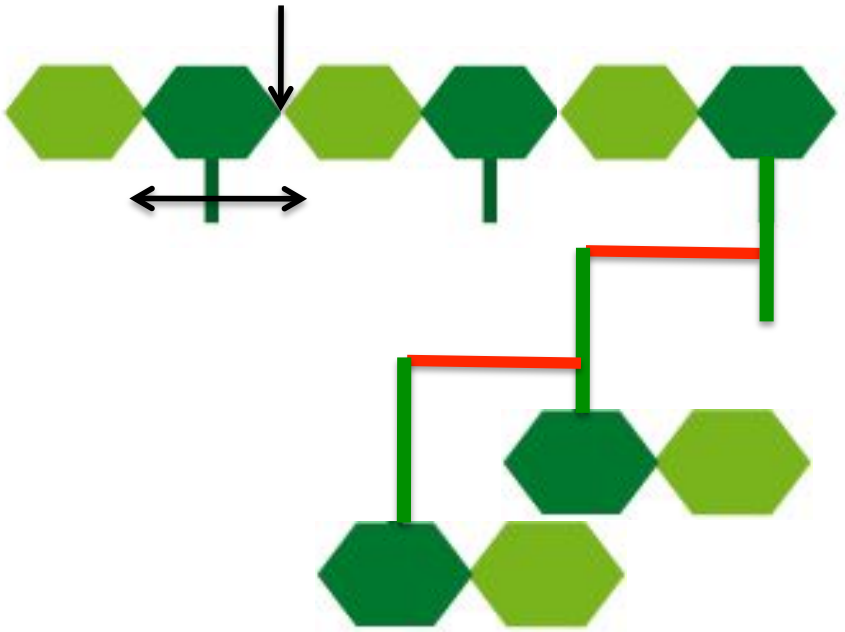




Typas et al, Nature Reviews, 2011, *10*, 123-136

Billot-Klein, et al, Biochem. J., 1996, *313*, 711–715

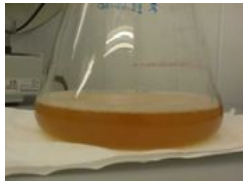
Enterococcus faecium peptidoglycan



Questions to be addressed

- 3D structure
- Oligosaccharide chain length
- Chemical variability in peptide stem
- Degree and nature of cross-linking
- Evolution of macroscopic physical properties along the cell cycle and in relation with antibiotic stress

Peptidoglycan sample preparation for NMR



Cell culture
^{13}C, ^{15}N-labeling

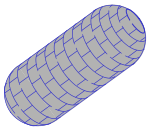
Cell harvesting

Centrifugation

Collection of bacteria

Boiling in SDS

Enzymatic treatments
Washing steps

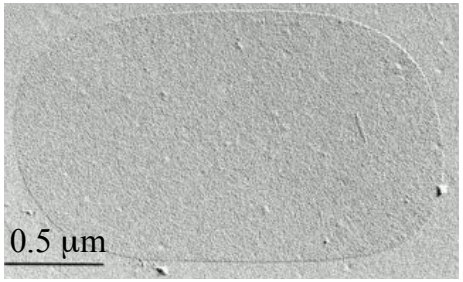


Sacculi

Ultra centrifugation

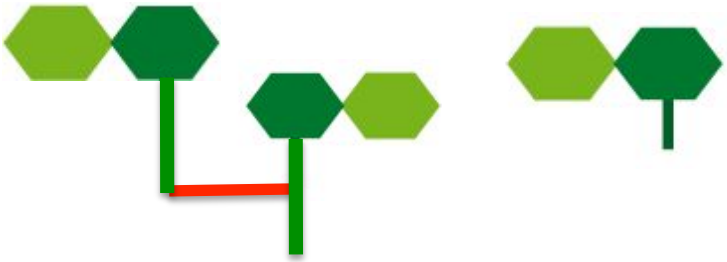
Murein hydrolase digestion

Intact hydrated, fully ^{13}C,^{15}N-labeled sacculi



Höltje, J Microbiol. Mol. Biol. Rev,. 1998, *62*, 181-203

Muropeptide fragments



Intact ^{13}C, ^{15}N-labeled bacteria

Insoluble !
(3×10^6 kDa)

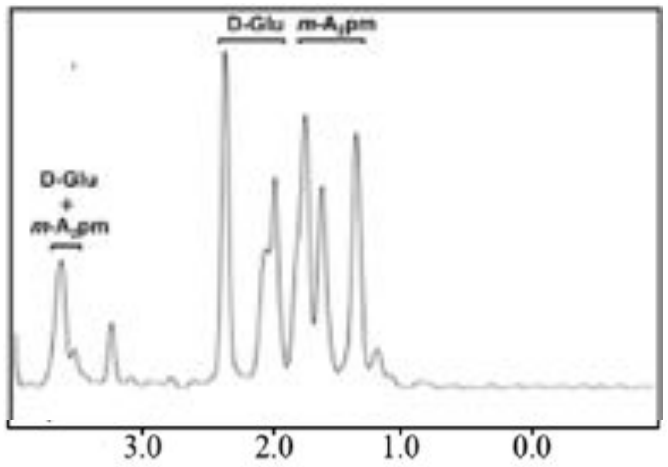
Solid-State NMR

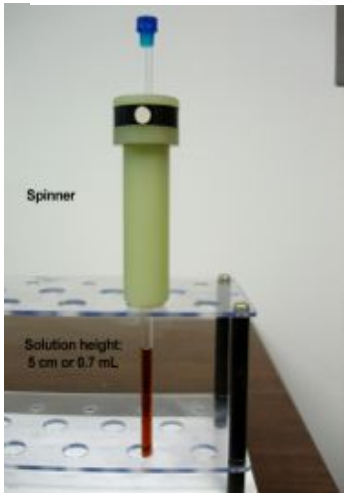
Liquid-state NMR
SEC - Mass spectrometry

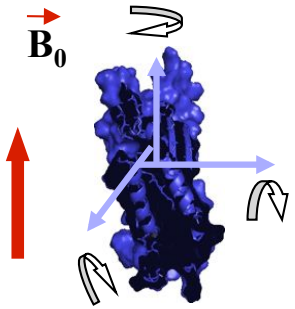
Peptidoglycan sample preparation for NMR

Liquid-state NMR

Fragments





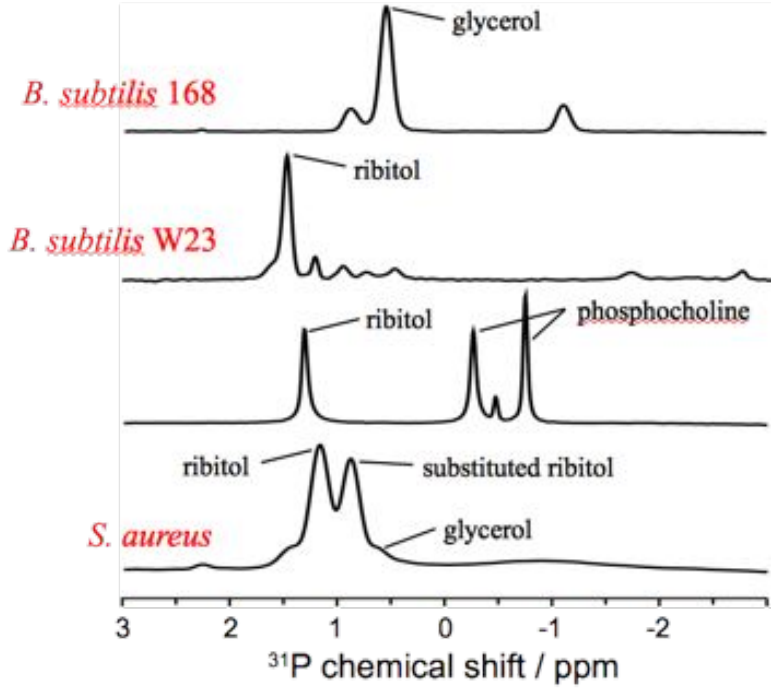


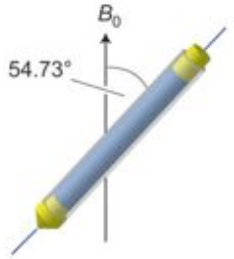
Brownian motion

~ 400 µL of soluble sample

Solid-state NMR

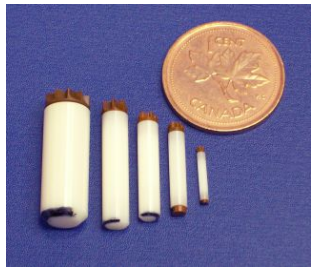
Sacculus





B_0 = 9.4 T
MAS: 9 kHz
SPINAL dec.
at 100 kHz or
CW at 45 kHz

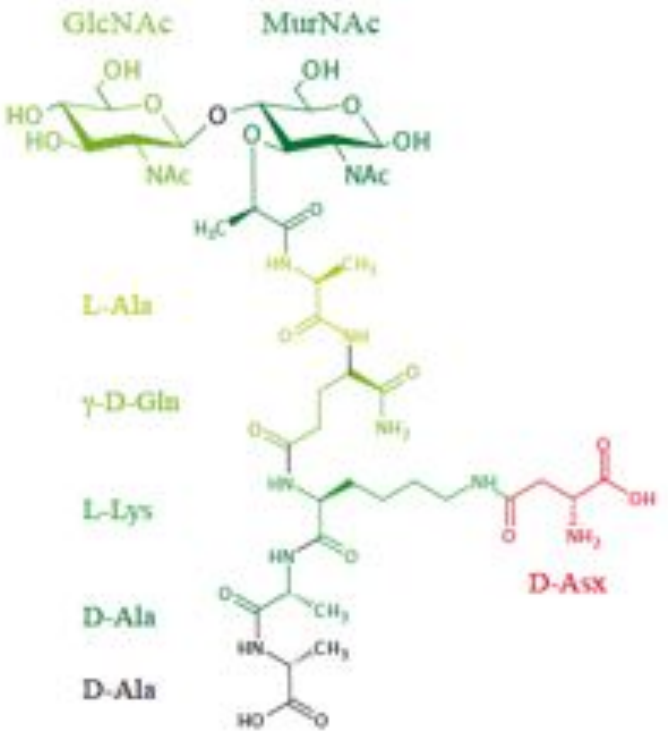
MAS
(Magic Angle Spinning)

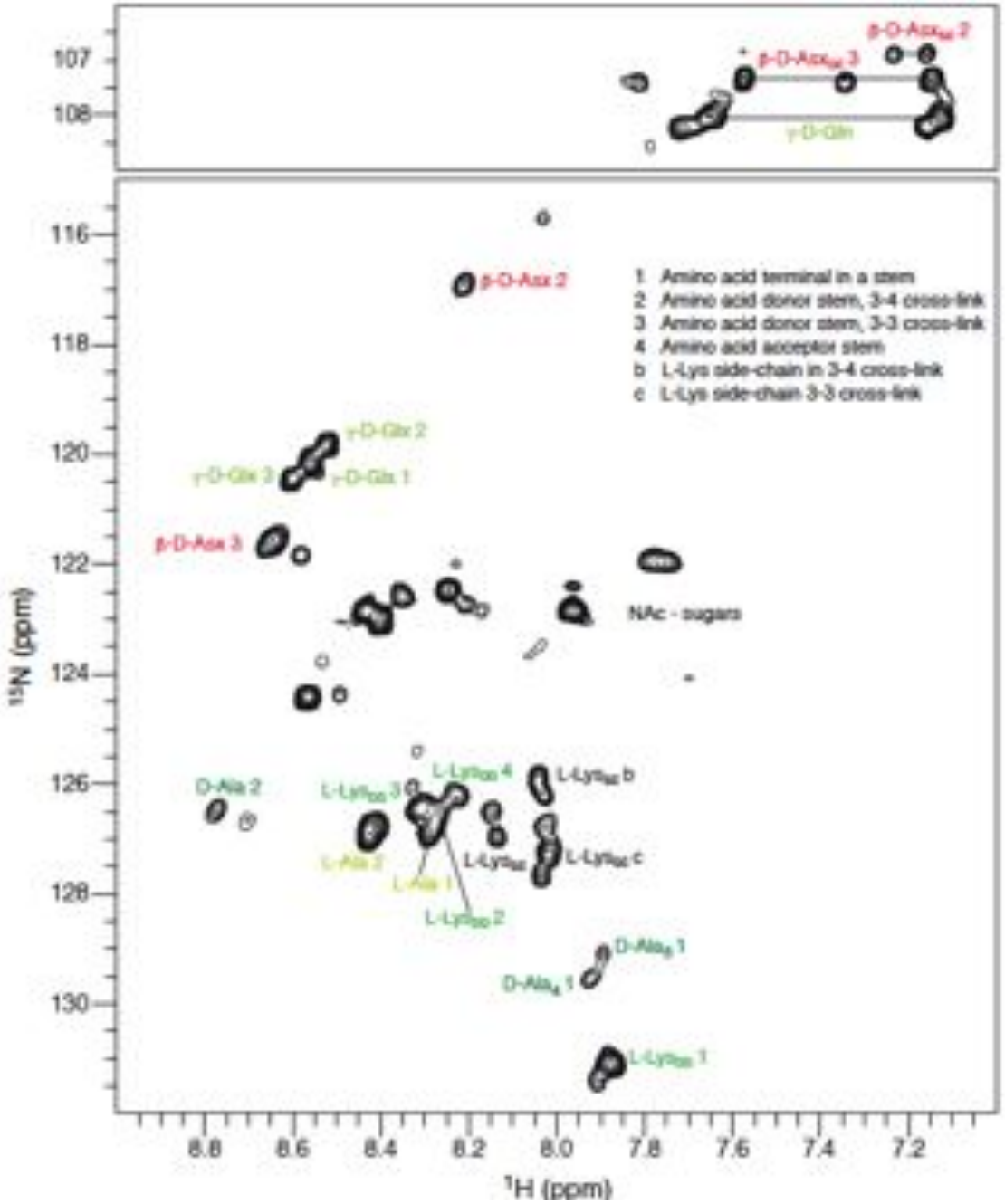


~ 20 µL of hydrated insoluble sample

Correlation experiments on *E. faecium* fragments

^{15}N-HSQC of fragments of *E. faecium* grown under ampicillin stress, pH = 5.3, T = 25°C



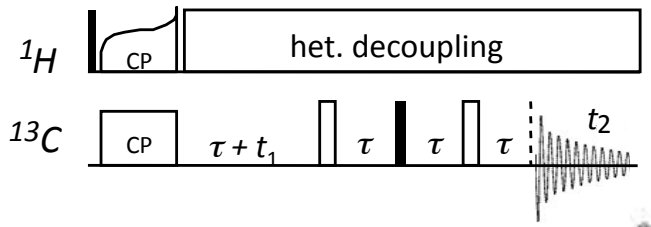


Unambiguous assignments

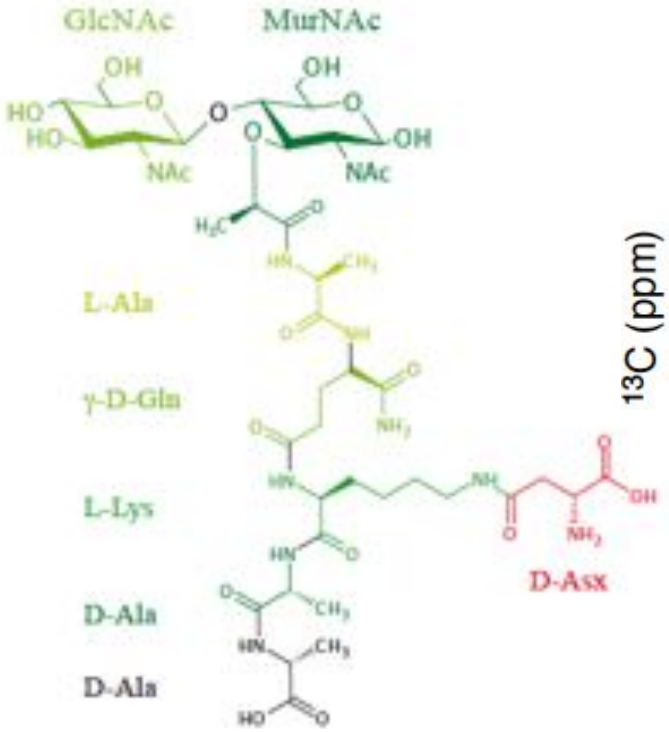
3D heteronuclear experiments: HNCACB, HN(CO)CACB, HNCO

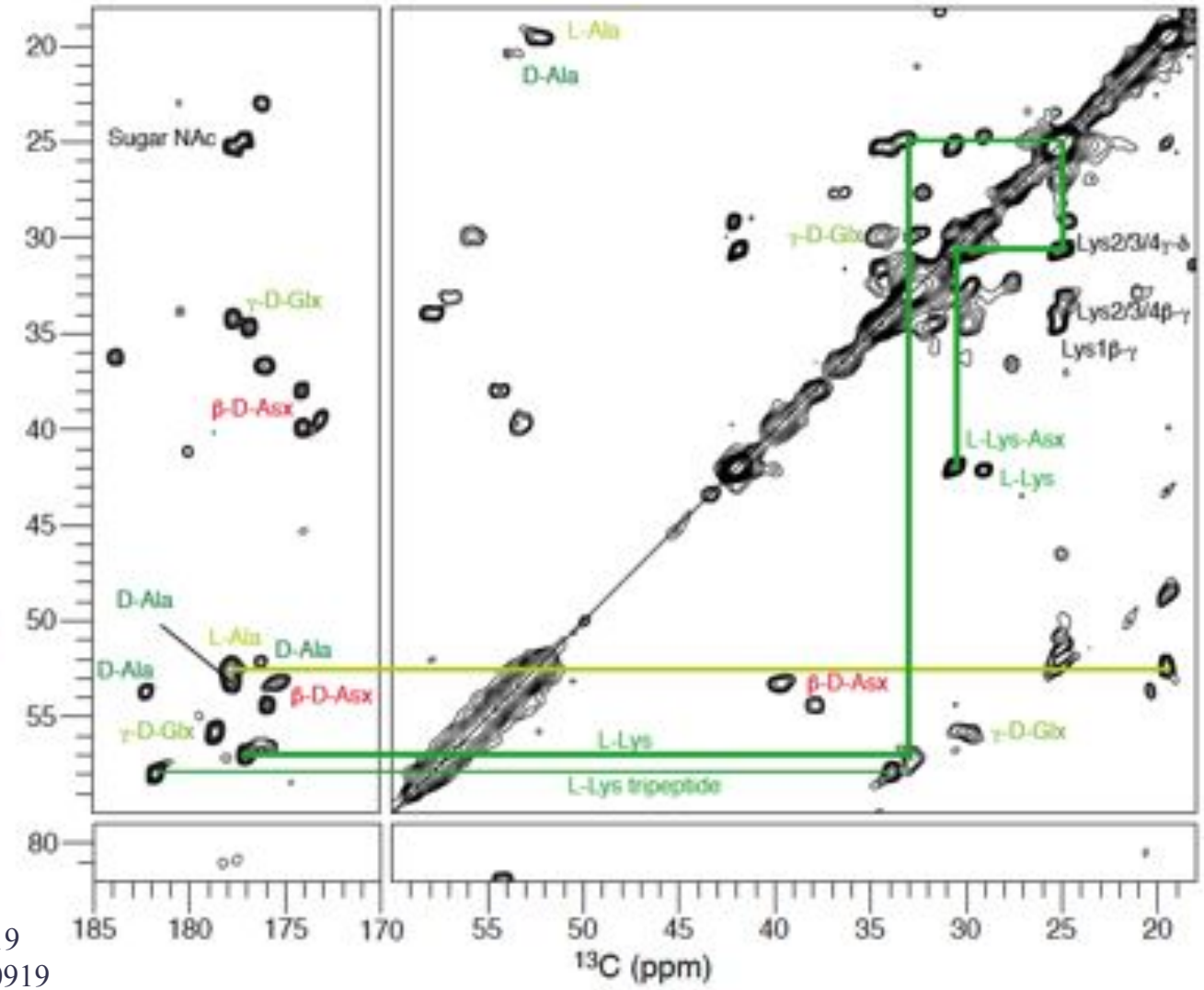
2D heteronuclear experiments: ^{13}C-HSQC, HCCH-TOCSY

J-correlation experiments on *E. faecium* sacculi



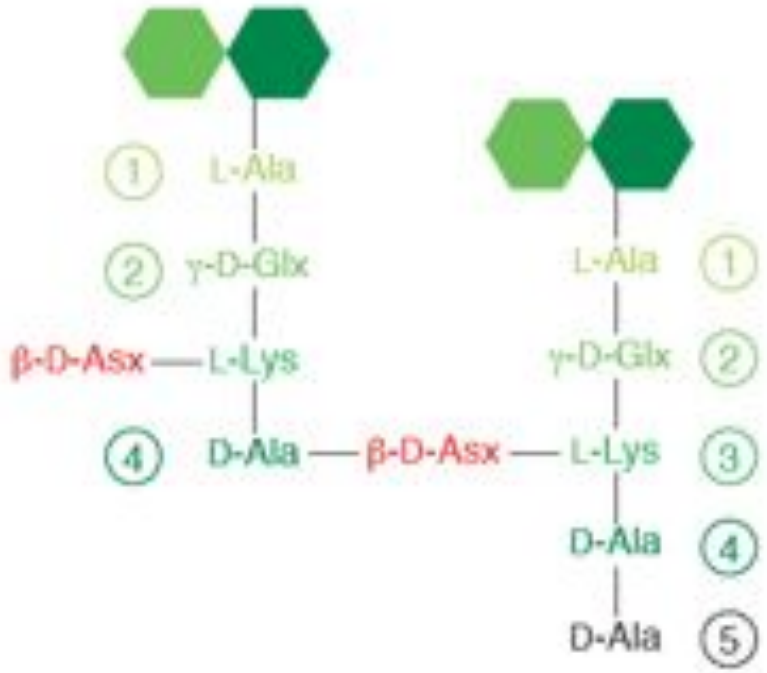
J-correlation experiment on *E. faecium* sacculi grown under ampicillin stress, B_0 = 14 T, MAS 12.5 kHz, SPINAL 9.3 kHz



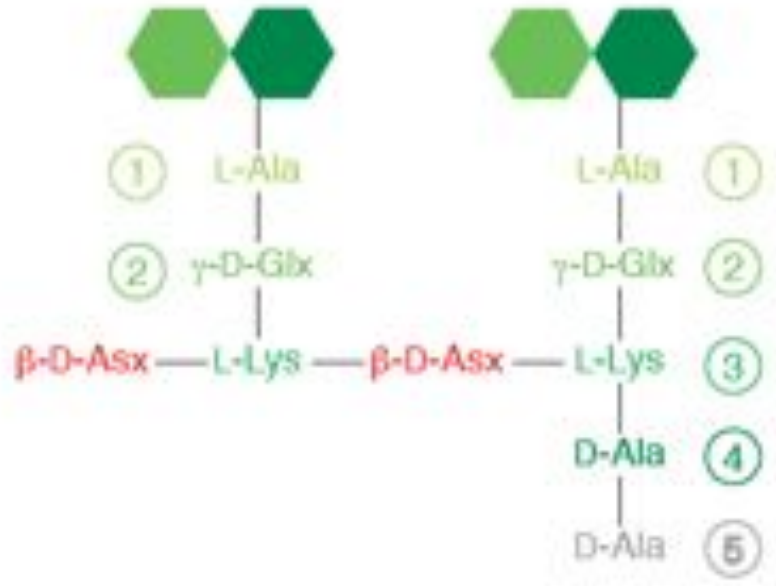


Kern et al, J Am Chem Soc., 2008, *130*, 5618-5619
Kern et al, J Am Chem Soc., 2010, *132*, 10911-10919

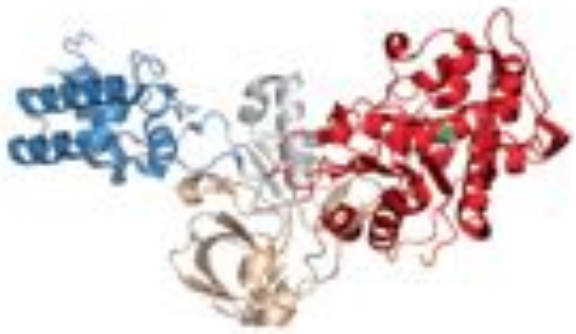
Enterococcus faecium peptidoglycan



Antibiotic stress



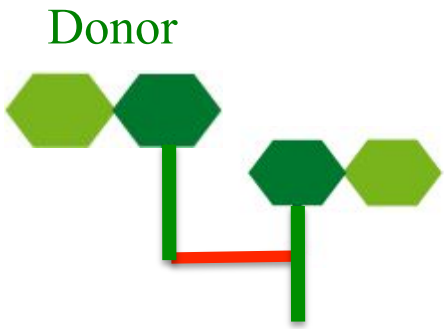
Mainardi et al, J. Biol. Chem., 2000, *275*, 16490-16496
Biarotte-Sorin et al, J. Mol. Biol., 2006, *359*, 533-538
Mainardi et al, J. Biol. Chem., 2007, *282*, 30414-30422



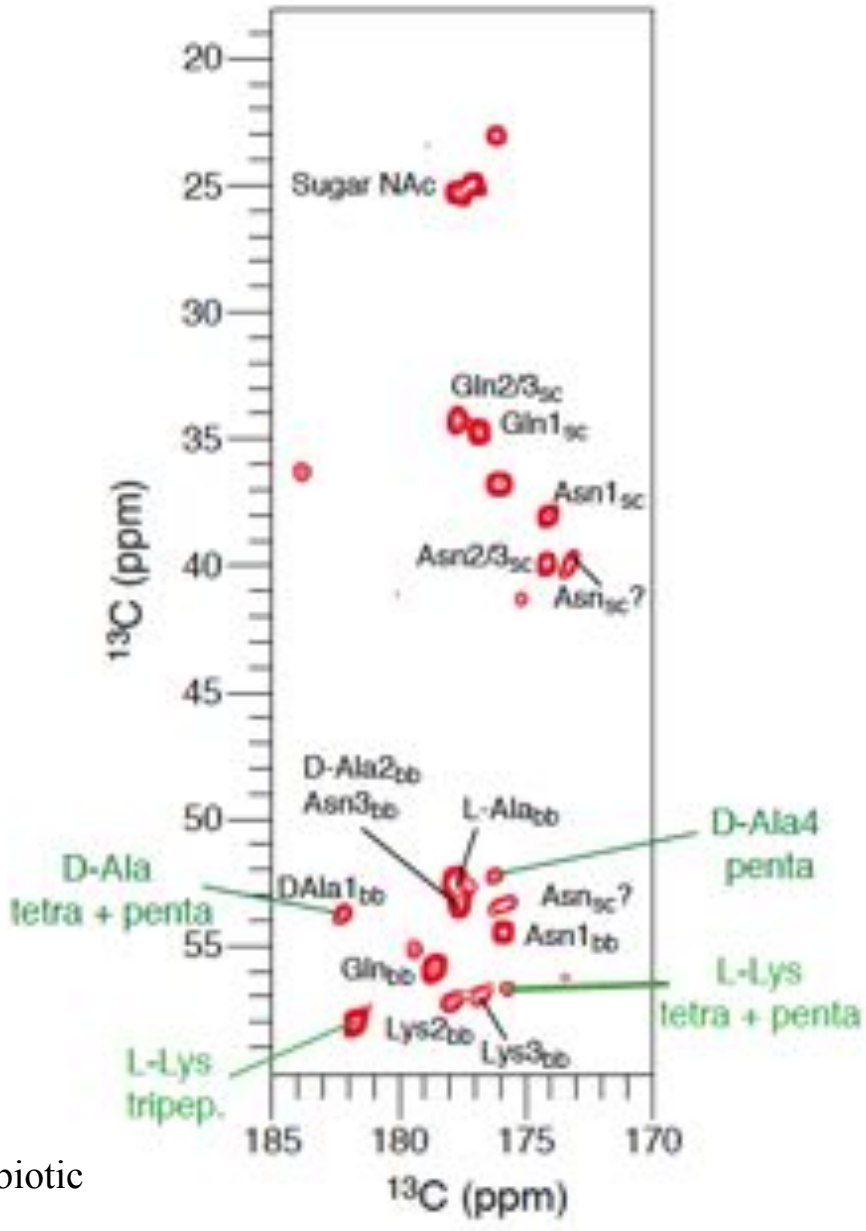
D,D-transpeptidase
E. faecium ampicillin sensitive strain

L,D-transpeptidase
E. faecium ampicillin resistant strain

Qualitative analysis of peptidoglycan composition



Evaluation of average peptide chain length of monomers and acceptor strands



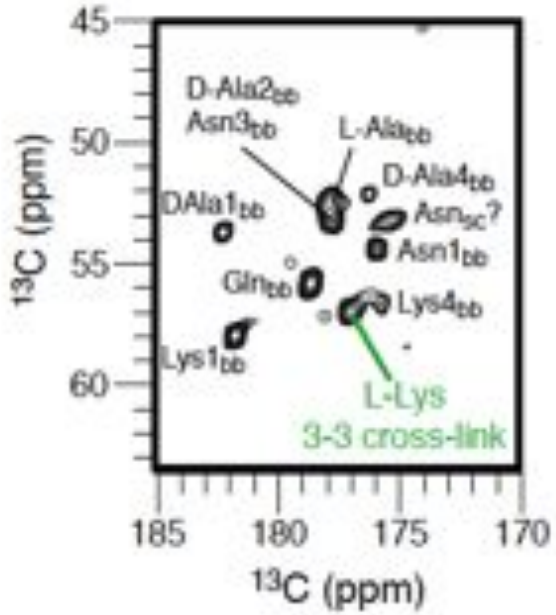
J-correlation experiment on *E. faecium* sacculi grown without antibiotic stress, B_0 = 14 T, MAS 12.5 kHz, SPINAL 9.3 kHz

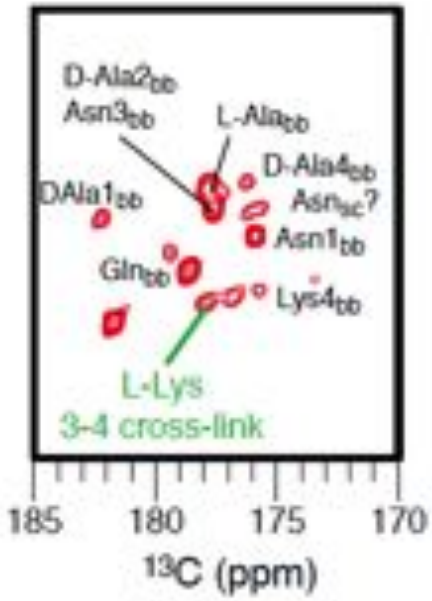
Qualitative analysis of peptidoglycan composition

Ampicillin

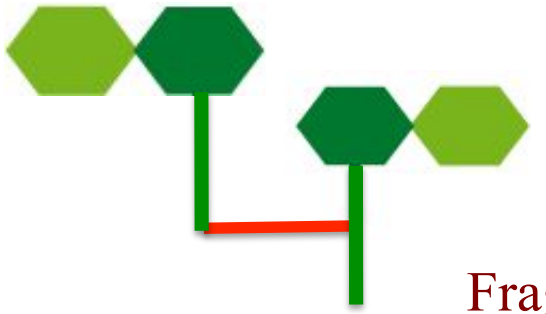
Imipenem

Sacculi



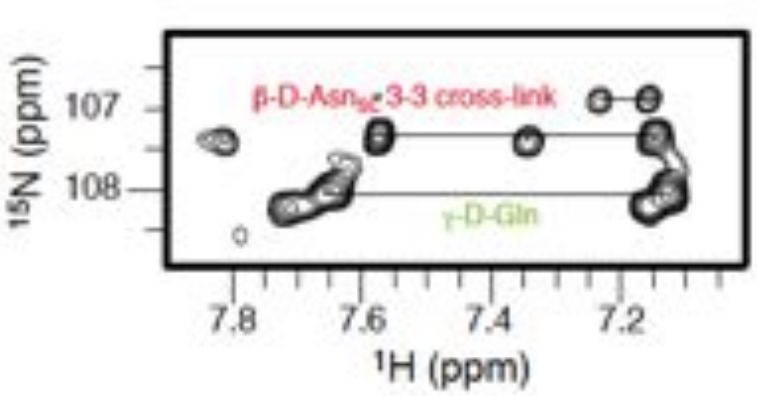


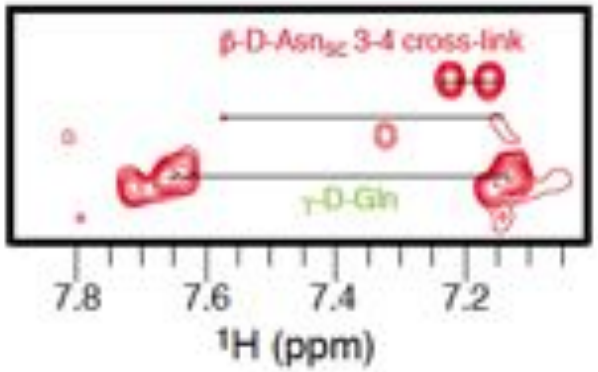
Donor



Acceptor

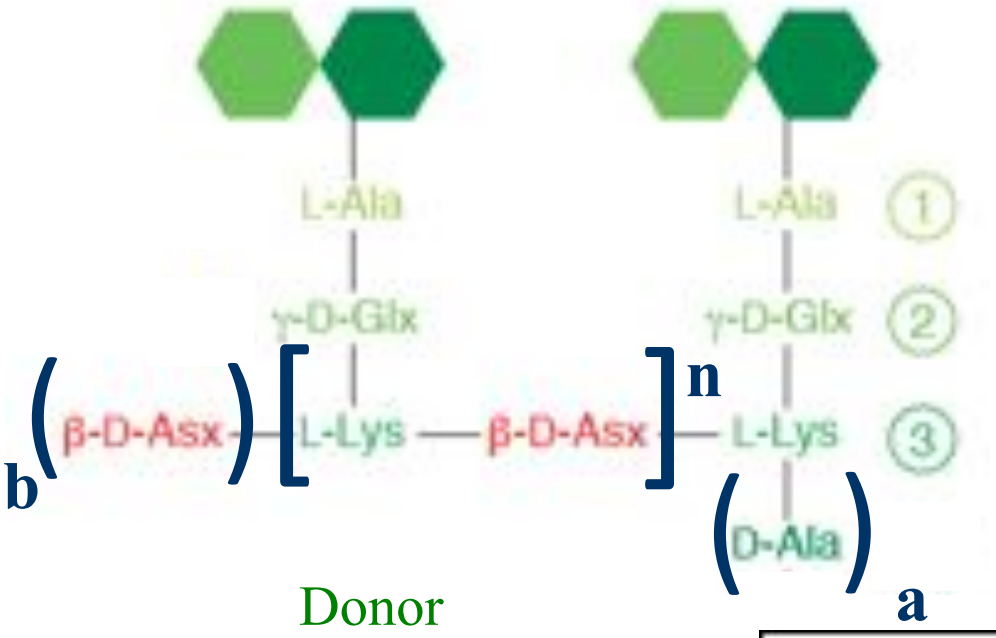
Fragments



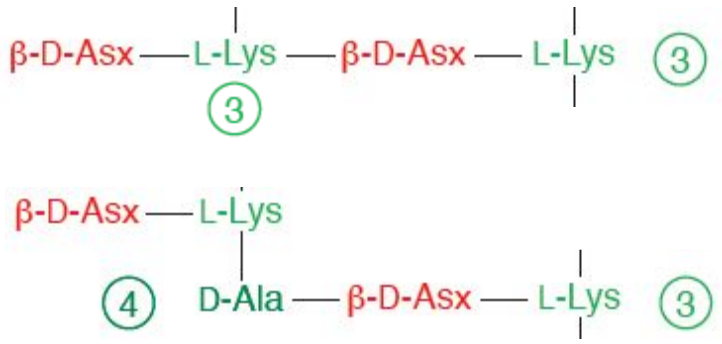


Evaluation of nature and degree of cross-linking

Quantitative analysis of peptidoglycan composition



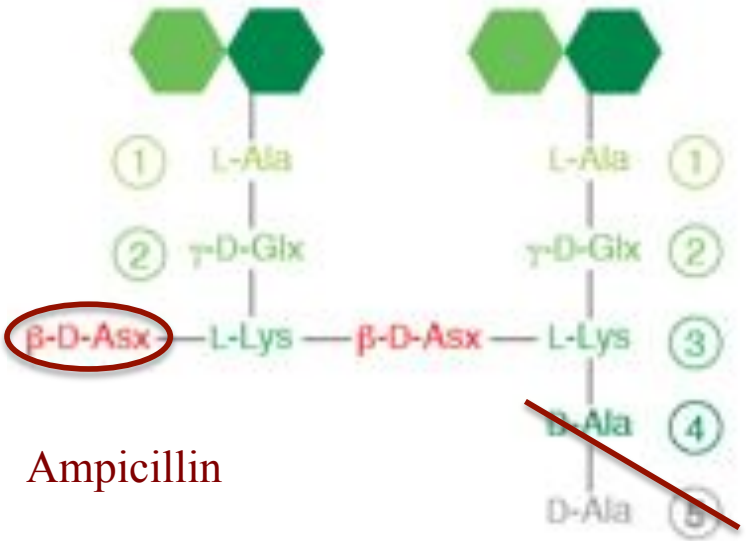
Donor



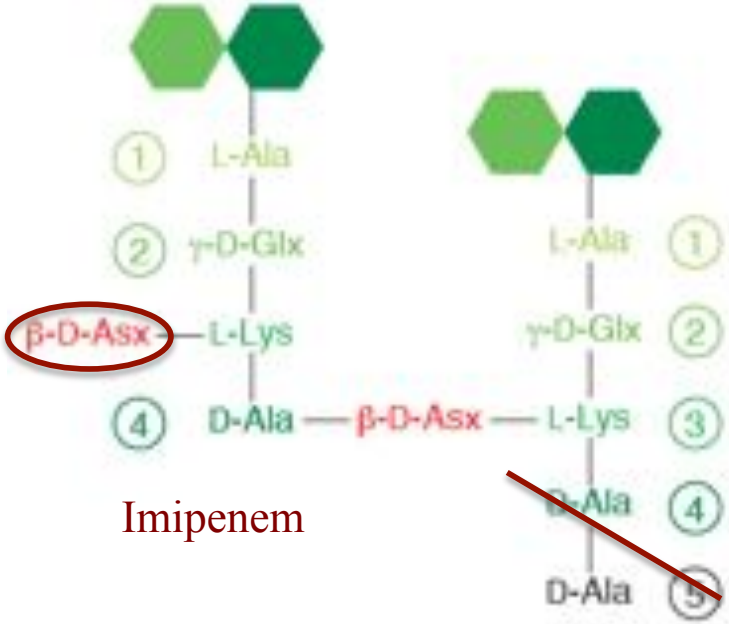
Monomer / Acceptor

			Liquid-state	Solid-state
Monomer/ Acceptor stem	Peptide stem length	a = 0, 1, 2	^{15}N-HSQC NH of termini residues	Cα-COOH connectivities
Presence of bridge on Lys	D-Asx	b = 0, 1	^{13}C-HSQC L-Lys Cε-Hε	L-Lys Cδ-Cε connectivities
Cross-links	Average number per peptide stem	n	^{13}C-HSQC Cα-Hα	L-Lys Cα-CO connectivities
	Nature (3-3 vs 3-4)	r	^{15}N-HSQC D-Asx NH Donor stem	L-Lys Cα-CO Acceptor stem

Evolution of peptidoglycan structure *vs* antibiotic stress



Ampicillin

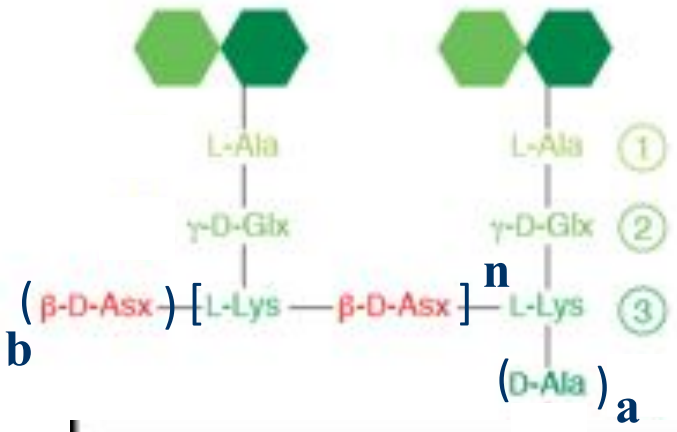


Imipenem

Solid-state quantification on sacculi

		Ampicillin	Imipenem
Monomer/ Acceptor stem	a = 0	78 ± 1 %	82 ± 1 %
	a = 1	22 ± 1 %	18 ± 1 %
	a = 2		
Presence of bridge on Lys	b = 0	24 ± 1 %	19 ± 1 %
	b = 1	76 ± 1 %	81 ± 1 %
Cross-links	n	0.28 ± 0.02	0.25 ± 0.02
	r (3-3 / 3-4)	6.5 ± 0.3	0.75 ± 0.01

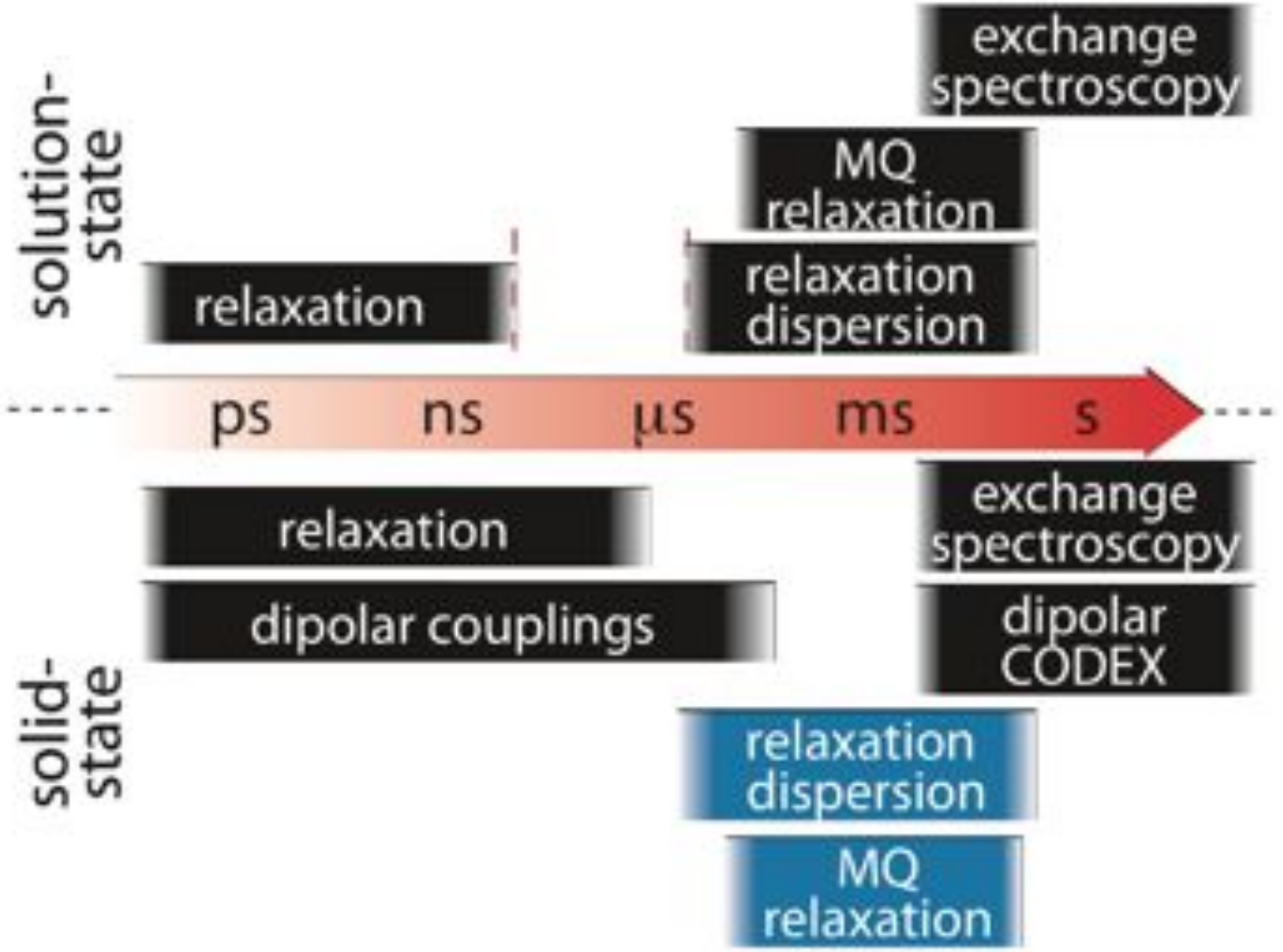
Evolution of peptidoglycan structure *vs* antibiotic stress



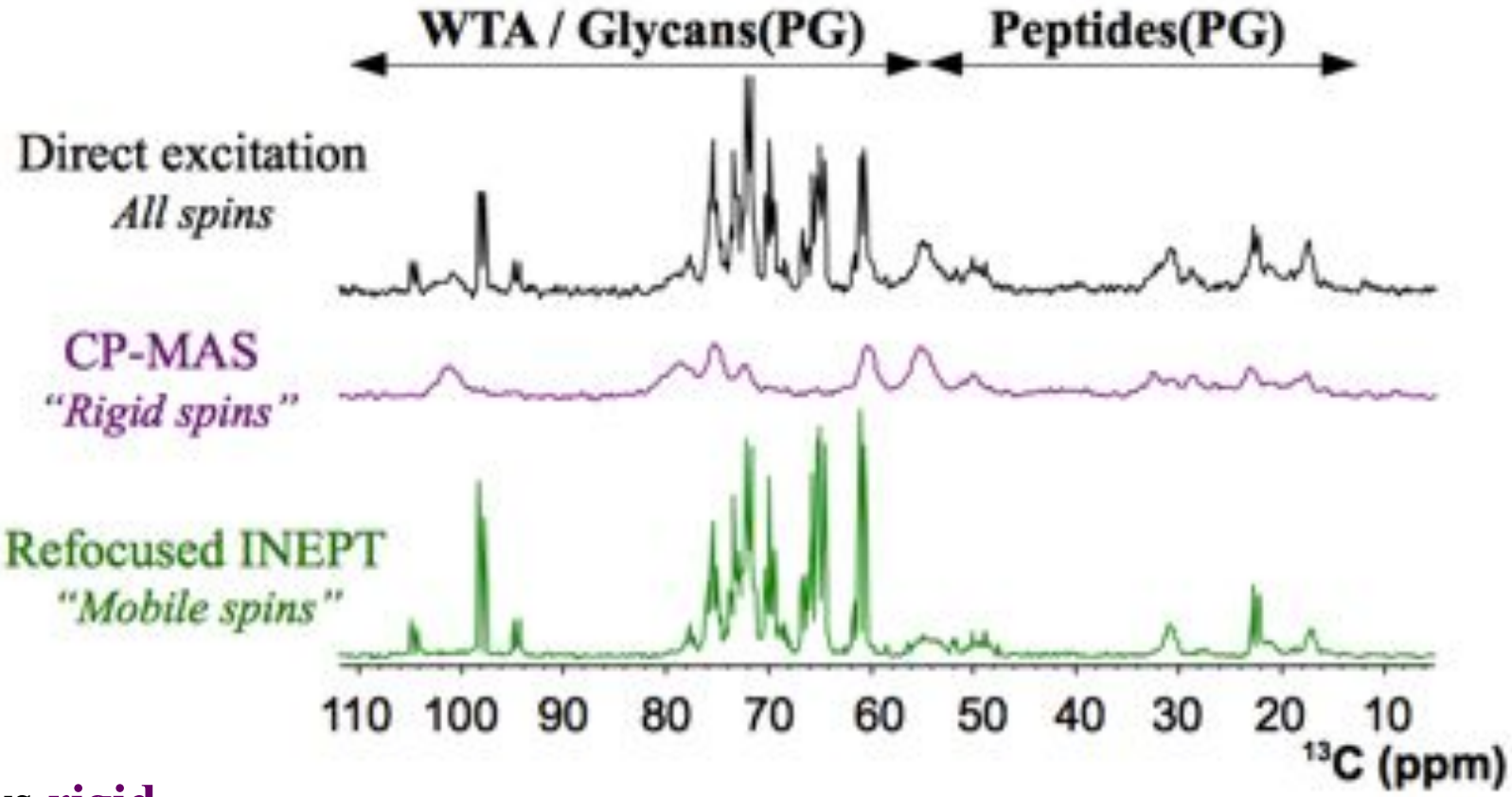
E. faecium M512 grown under ampicillin stress

			Liquid-state	Solid-state
Monomer/ Acceptor stem	Peptide stem length	a = 0	81 ± 1 %	78 ± 1 %
		a = 1	10 ± 1 %	22 ± 1 %
		a = 2	9 ± 1 %	
Presence of bridge on Lys	D-Asx	b = 0	26 ± 1 %	24 ± 1 %
		b = 1	74 ± 1 %	76 ± 1 %
Cross-links	Average number per peptide stem	n	0.28 ± 0.02	0.28 ± 0.02
	Nature (3-3 vs 3-4)	r (3-3 / 3-4)	2.5 ± 0.3	6.5 ± 0.3

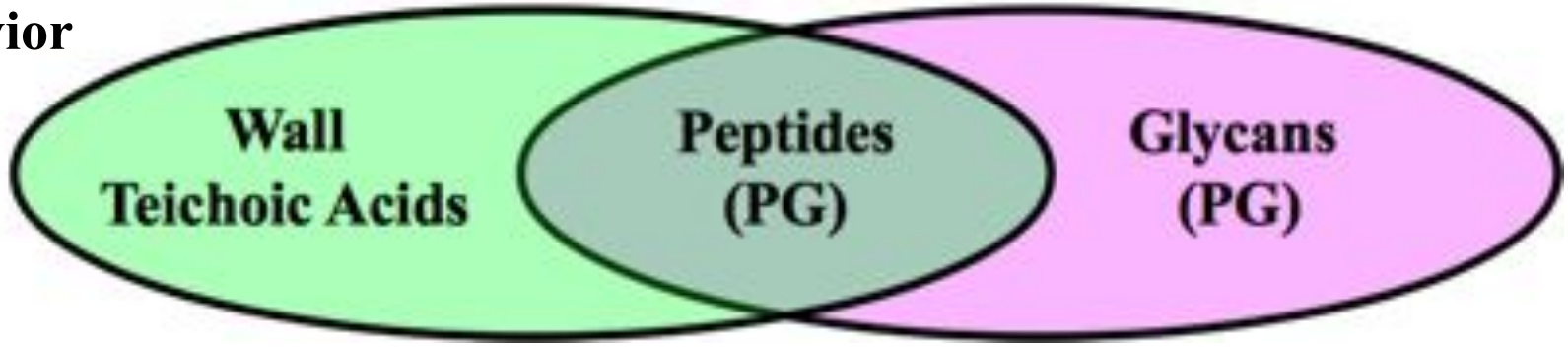
Peptidoglycan dynamics



Different dynamical regimes in peptidoglycan

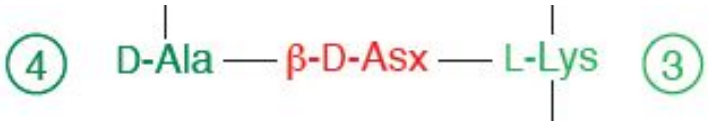


Mobile vs **rigid** behavior

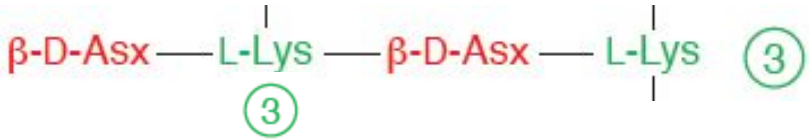


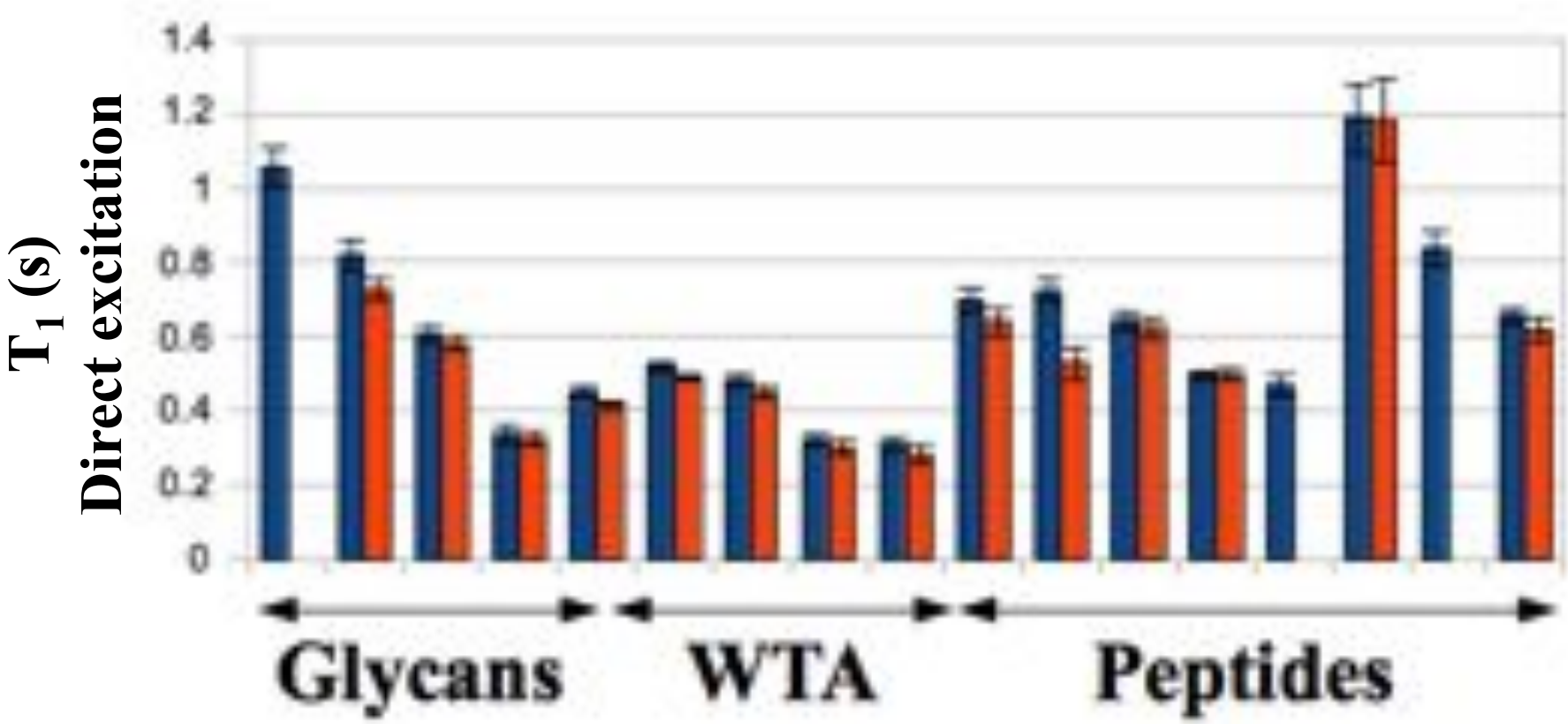
Peptidoglycan dynamics in the ps-ns regime

E. faecium **ampicillin sensitive strain**

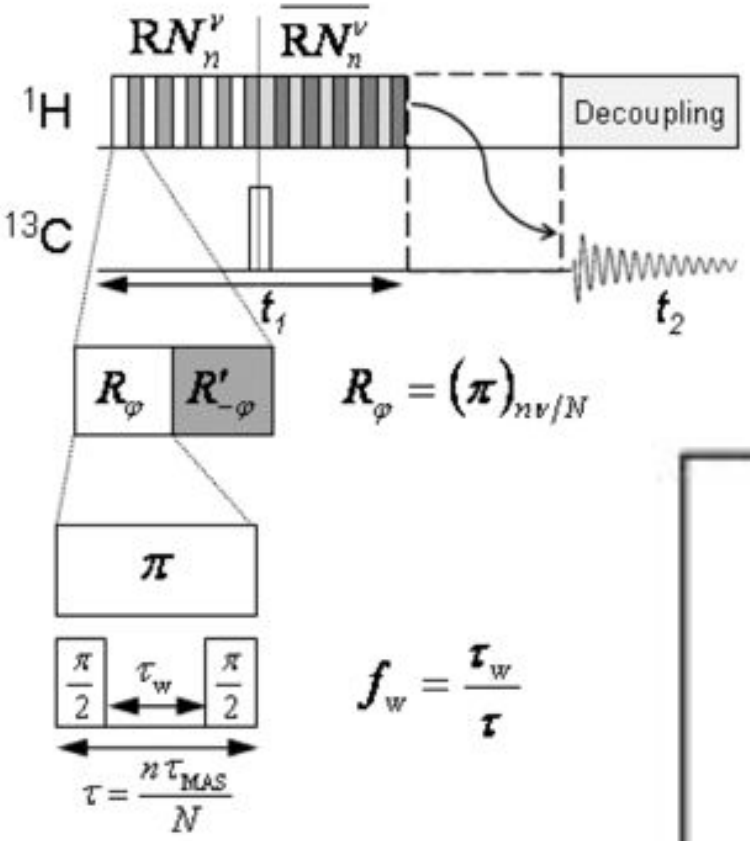


E. faecium **ampicillin resistant strain**

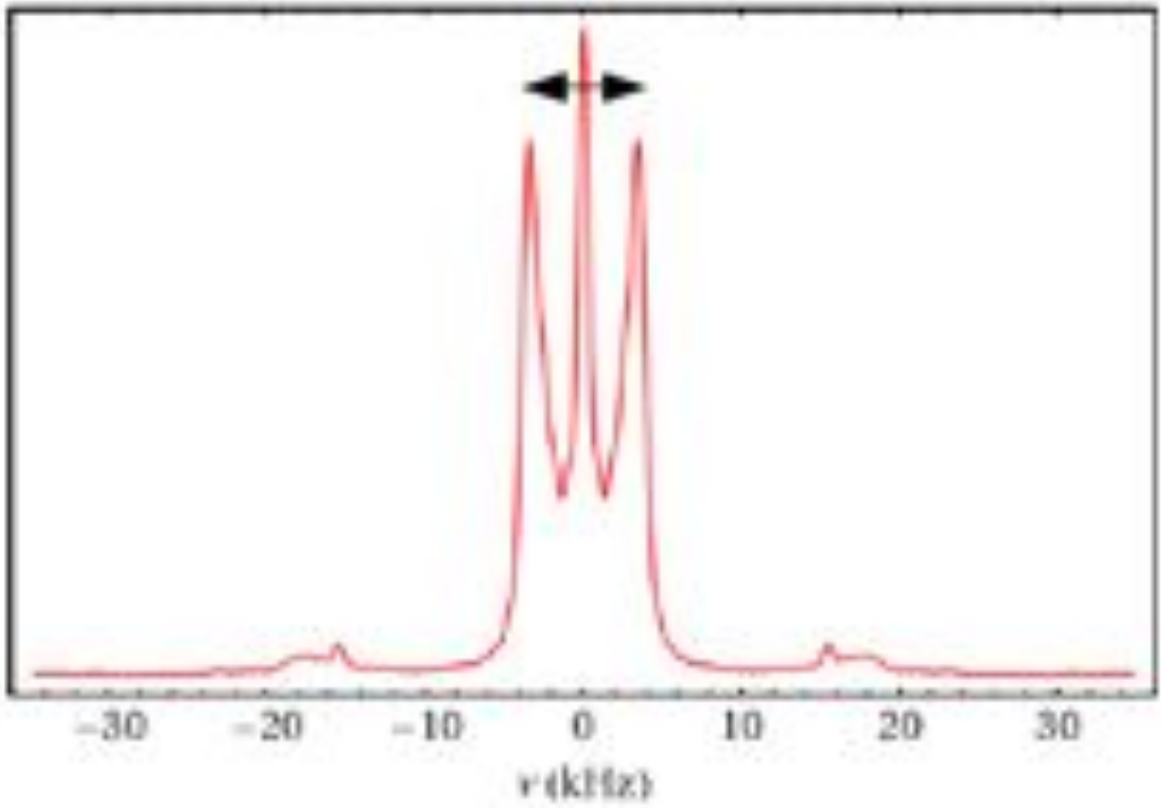




Peptidoglycan dynamics in the ps-µs regime

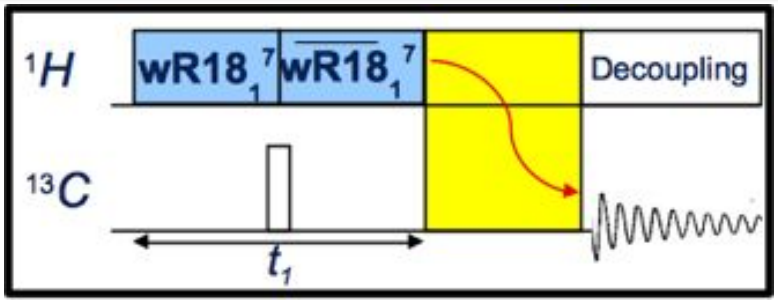


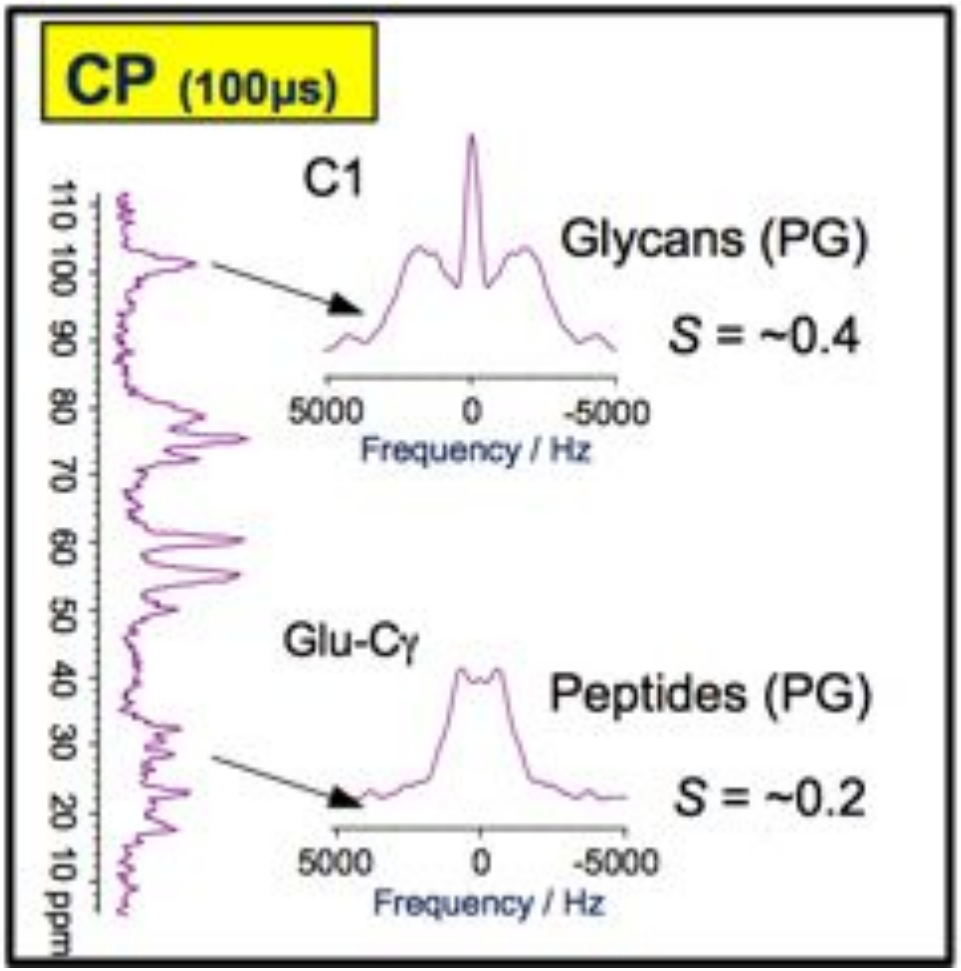
wR-PDLF sequence optimized with f_w = 33% to minimize sensitivity of proton RF errors
MAS = 12.5 kHz

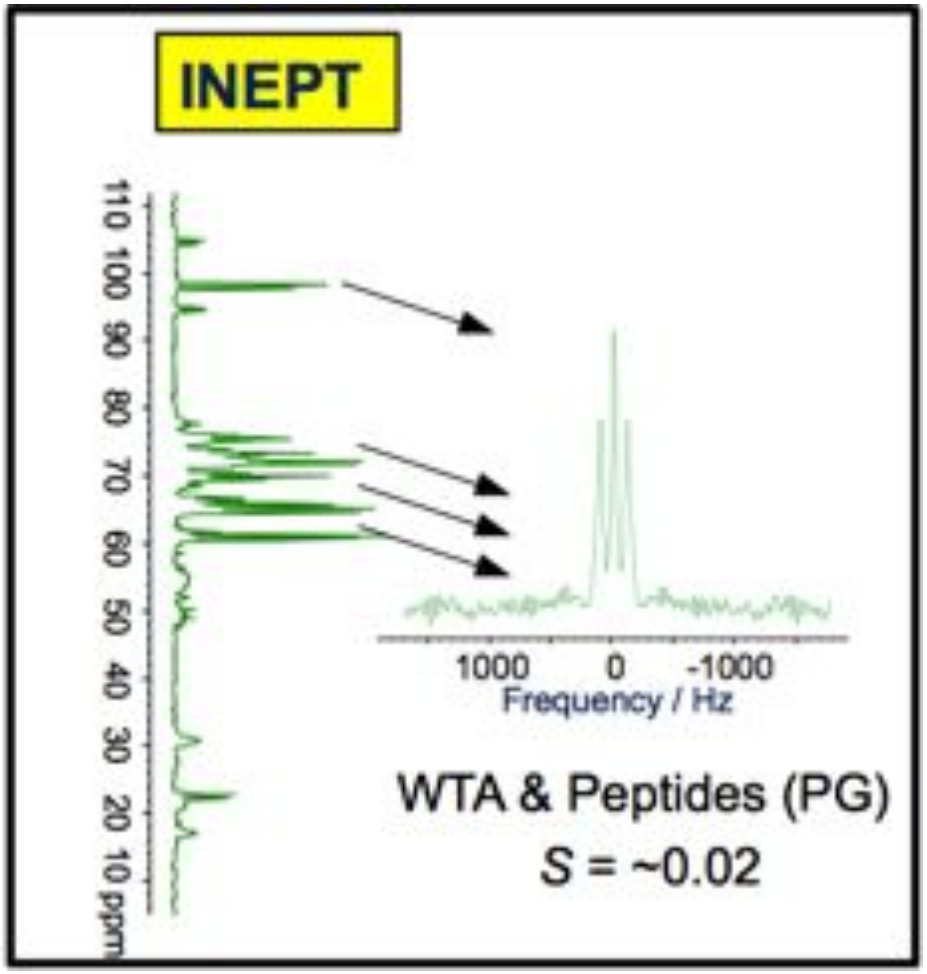


$$S = \frac{\text{residual } {}^1D_{CH}}{\text{static } {}^1D_{CH}}$$

Peptidoglycan dynamics in the ps-µs regime



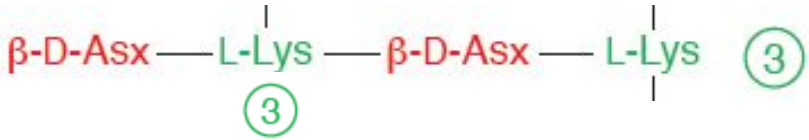


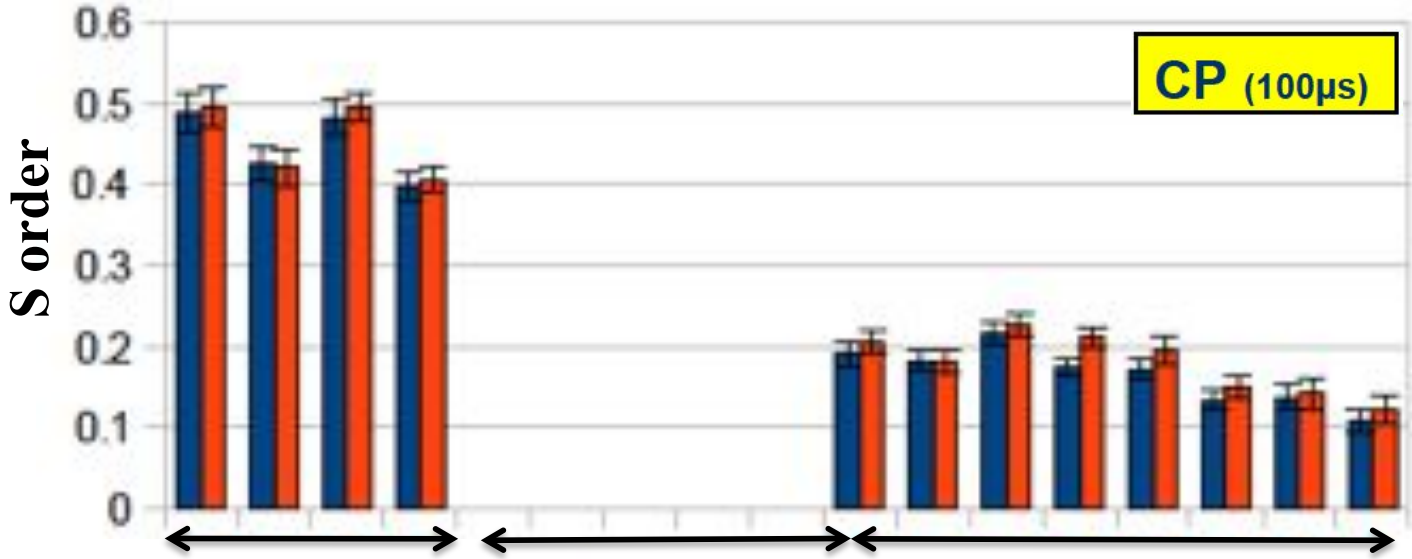


Peptidoglycan dynamics in the ps-ns regime

E. faecium **ampicillin sensitive strain**

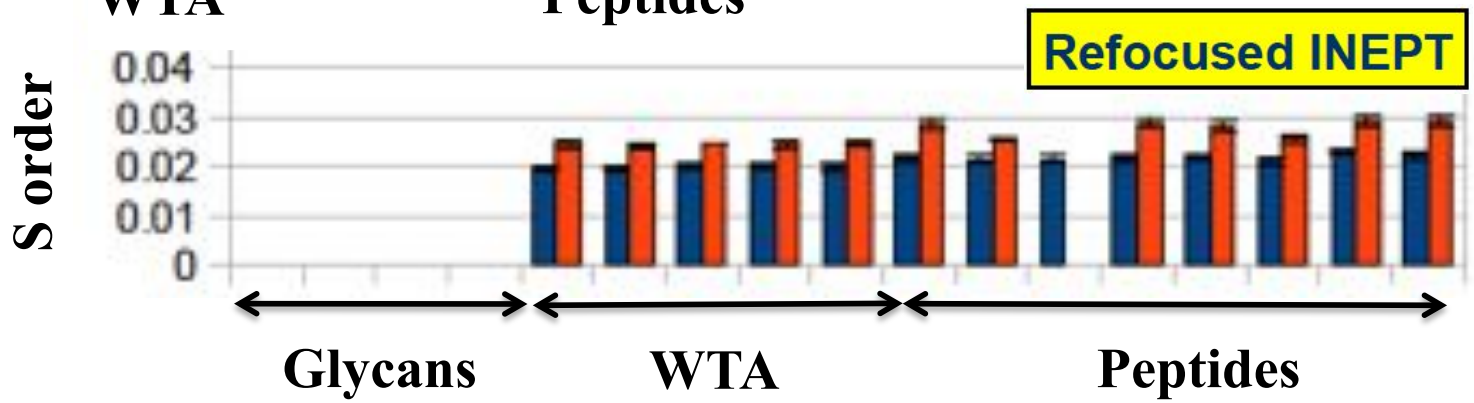
E. faecium **ampicillin resistant strain**



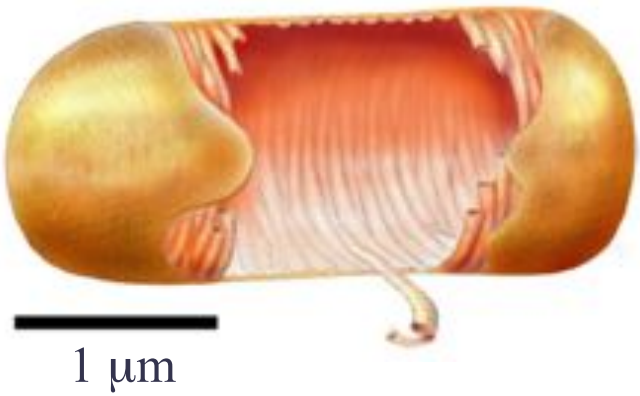


Rigid fraction

Mobile fraction



Enterococcus faecium peptidoglycan



Questions to be addressed

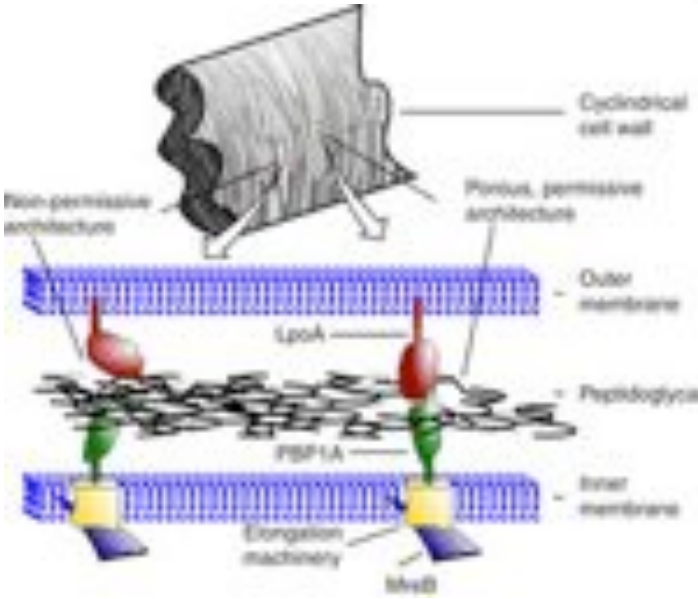
- 3D structure

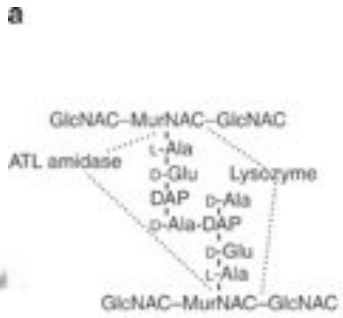
Bacillus subtilis

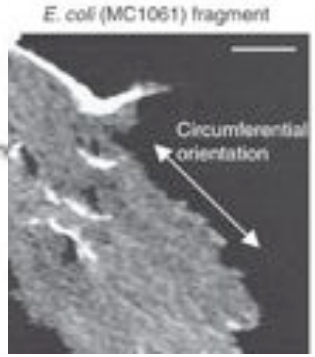
AFM peptidoglycan structural model

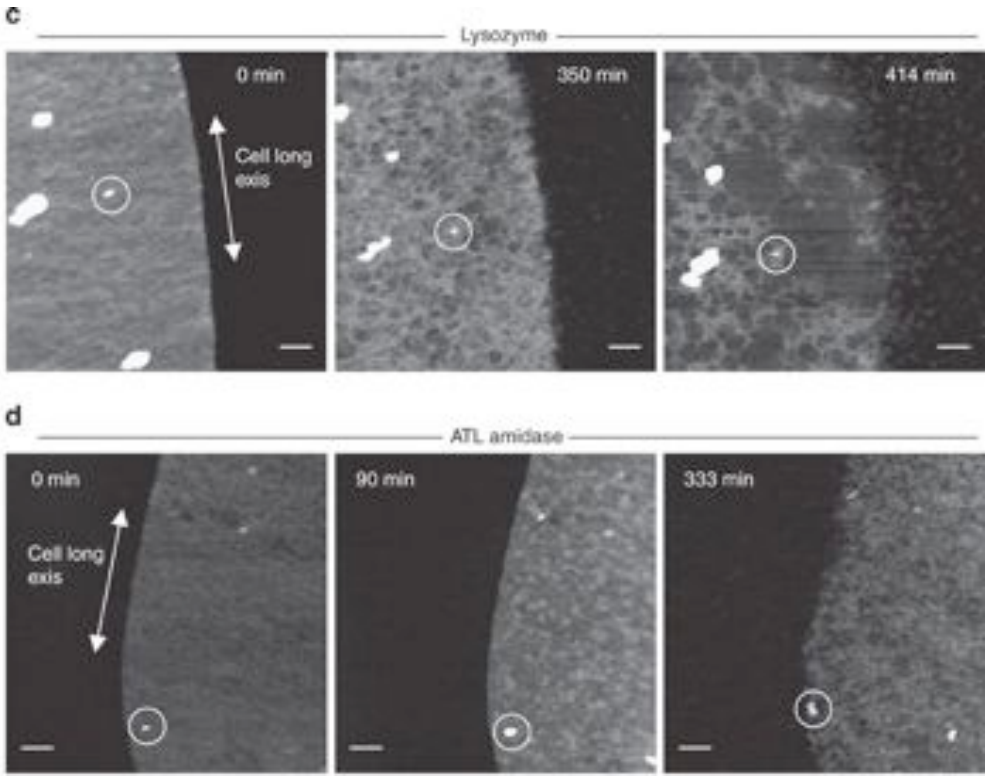
Hayhurst et al PNAS 2008, *105*, 14603

R. D. Turner, A. F. Hurd, A. Cadby, J. K. Hobbs, S. J. Foster, Nature Comm., 2013, 4, 1493

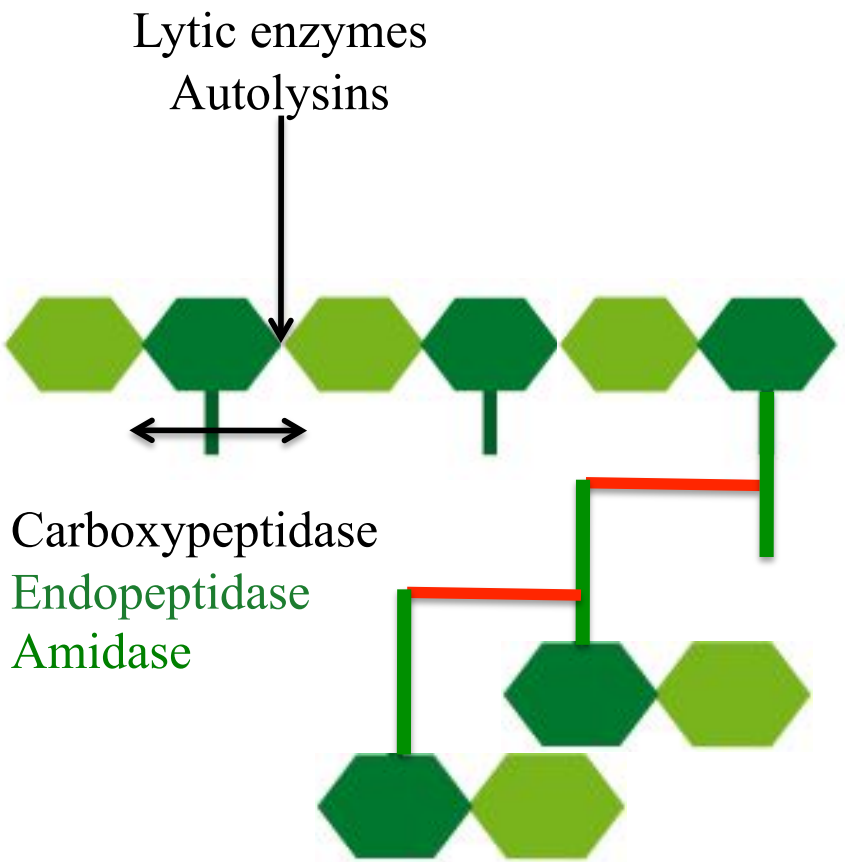


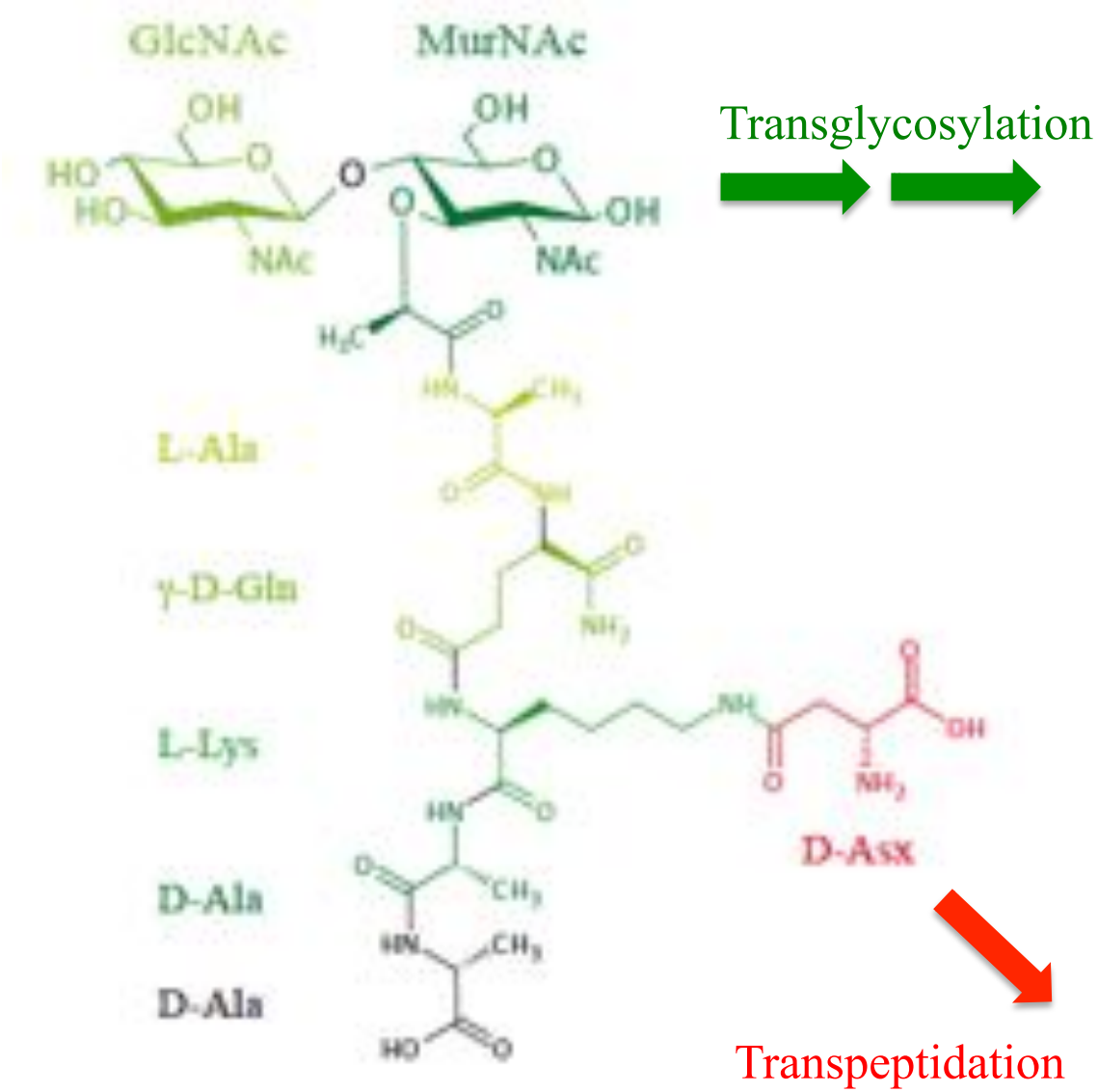






Insight into the peptidoglycan biosynthesis

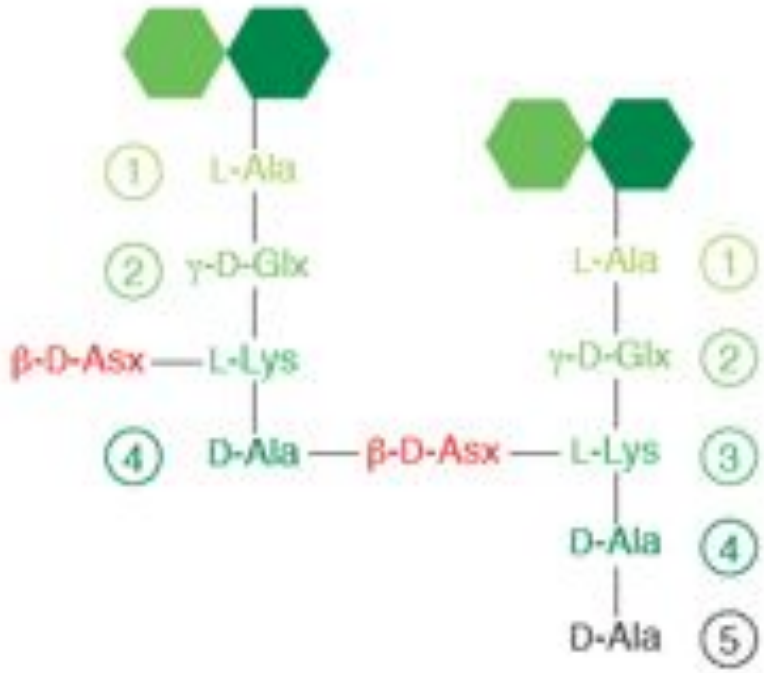




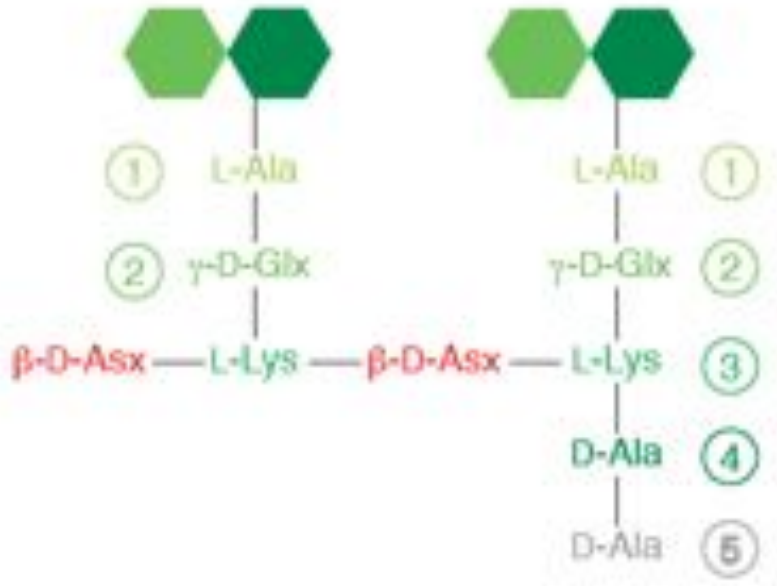
Typas et al, Nature Reviews, 2011, *10*, 123-136

Billot-Klein, et al, Biochem. J., 1996, *313*, 711–715

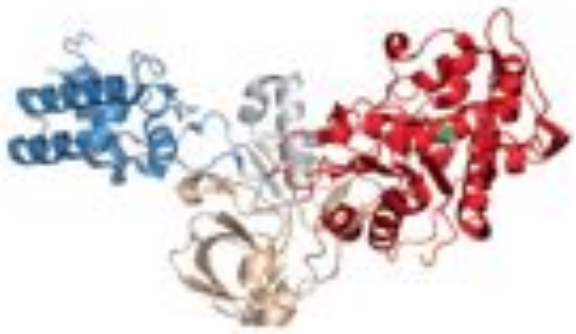
Insight into the peptidoglycan biosynthesis



Antibiotic stress



Mainardi et al, J. Biol. Chem., 2000, *275*, 16490-16496
Biarotte-Sorin et al, J. Mol. Biol., 2006, *359*, 533-538
Mainardi et al, J. Biol. Chem., 2007, *282*, 30414-30422



D,D-transpeptidase
E. faecium ampicillin sensitive strain

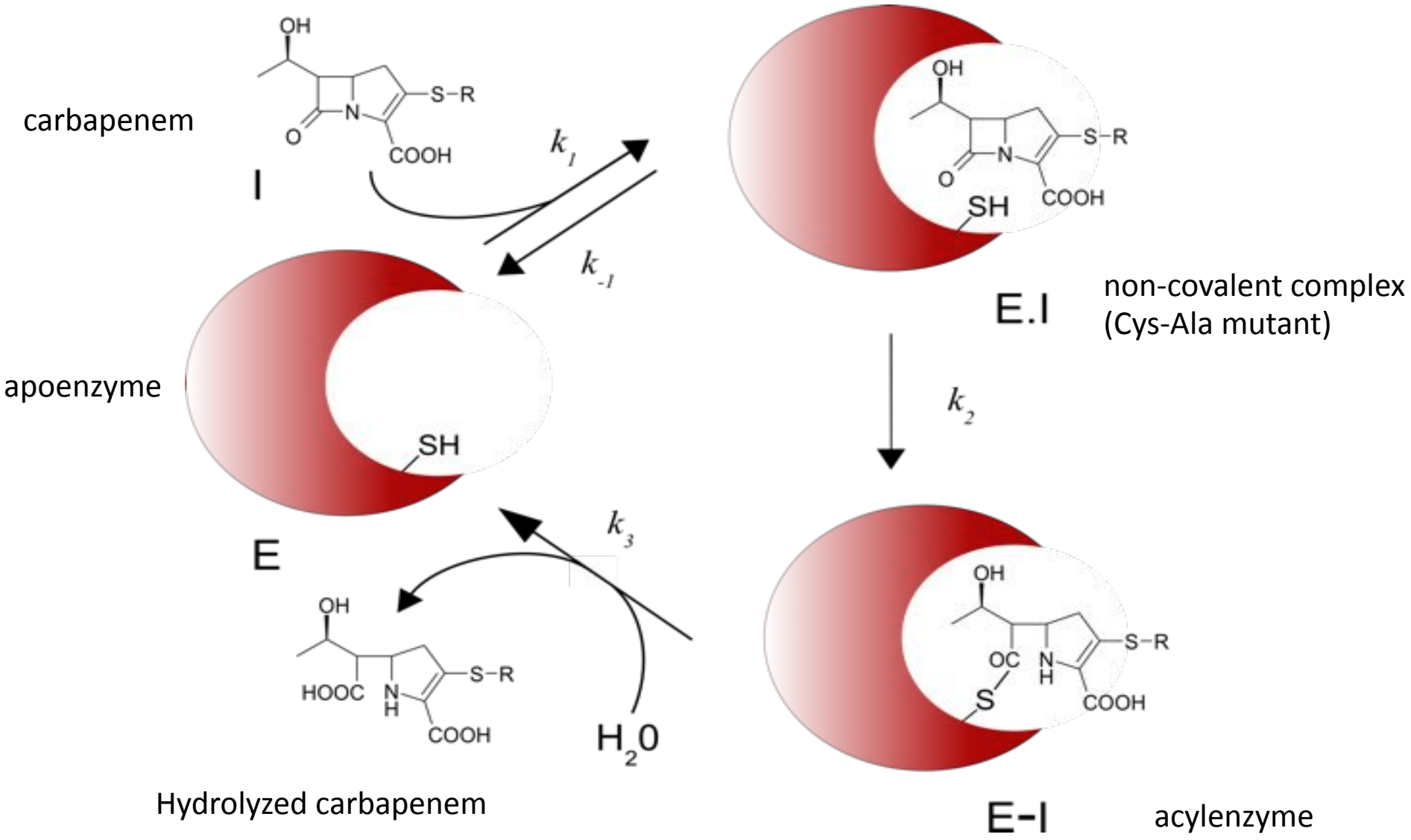
L,D-transpeptidase
E. faecium ampicillin resistant strain

Insight into the peptidoglycan biosynthesis

E. coli	*E. faecium*	*M. tuberculosis*
Exponential phase	**Wild-type strain**	**Dormant forms**
PBPs 96% LDts 4%	PBPs 97% LDts 3%	PBPs 20% LDts 80%
Stationary phase	**Ampicillin resistant**	
PBPs 90% LDts 10%	PBPs 0% LDts 100%	

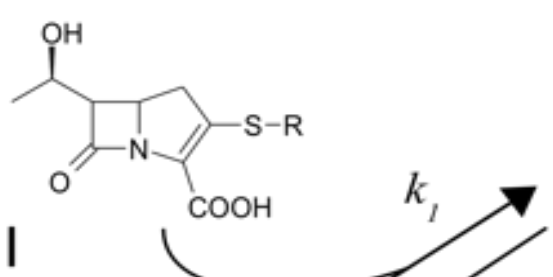
Mainardi et al., *J. Biol. Chem.*, 2005, 38146-38152
Lavollay et al., *J. Bact.*, 2008, 4360-4368

Mechanism of Ldt inhibition by carbapenems



Mechanism of Ldt inhibition by carbapenems

β-lactam

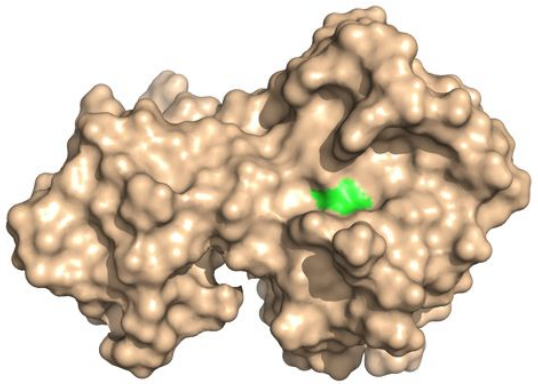


I

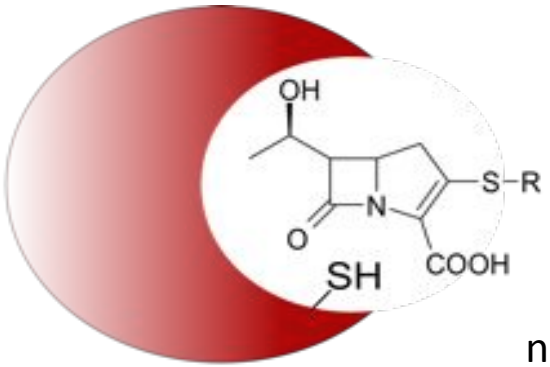
k_1

k_{-1}

apoenzyme



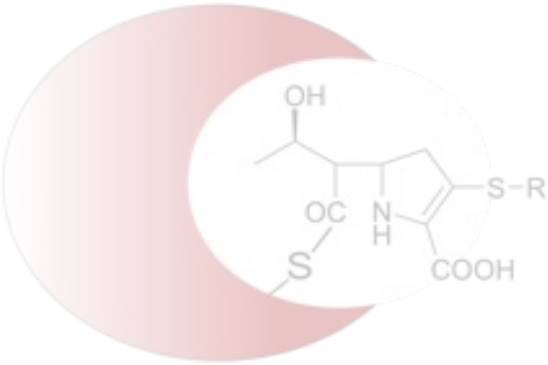
E



E.I

non-covalent complex
(Cys-Ala mutant)

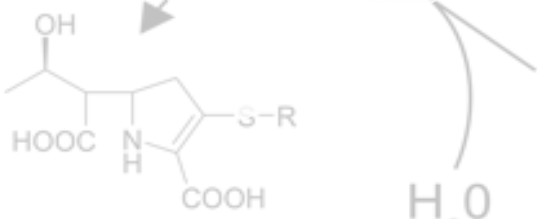
k_2



E-I

acylenzyme

k_3

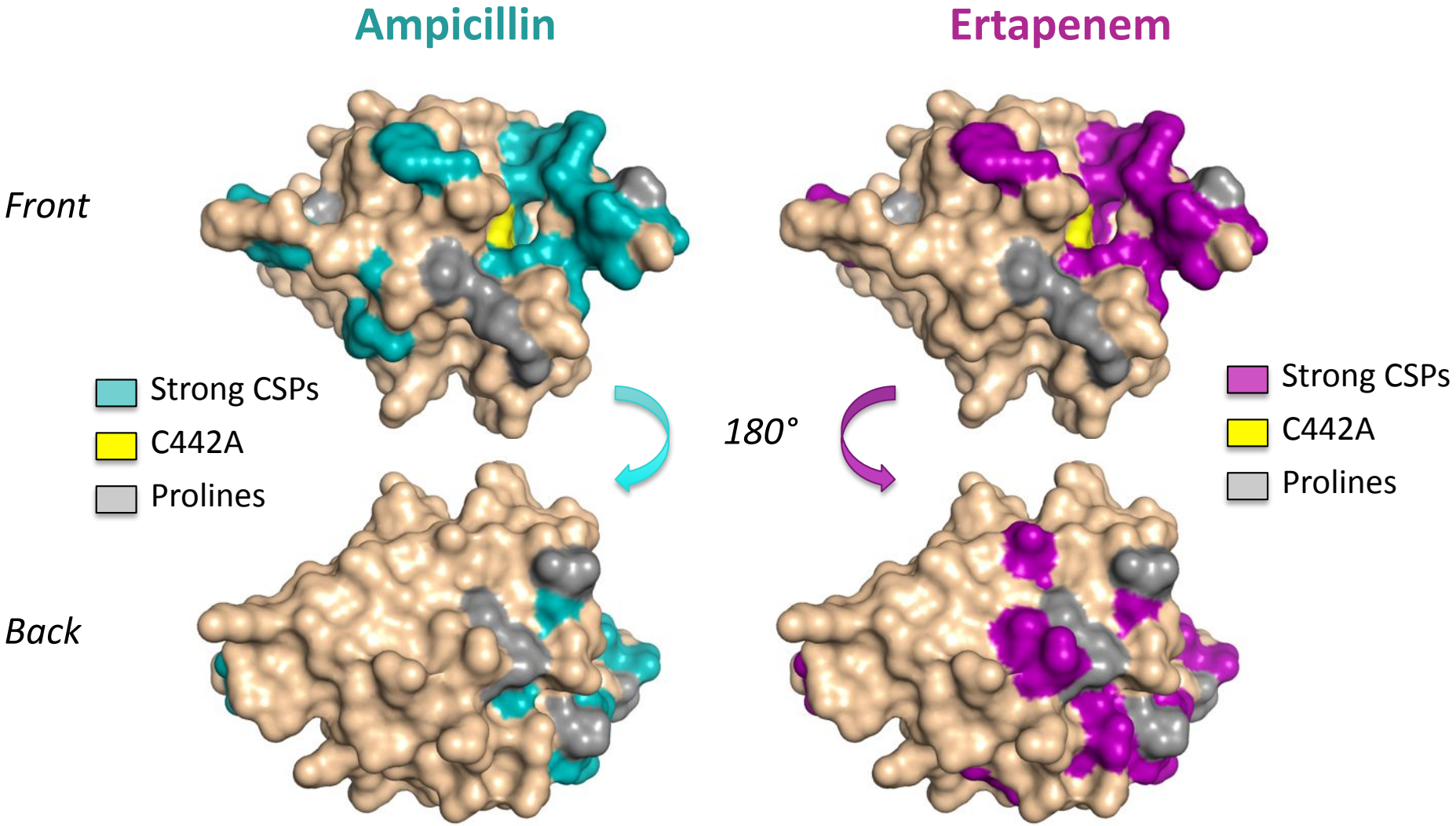


H_2O

Hydrolyzed carbapenem

Mechanism of Ldt inhibition by carbapenems

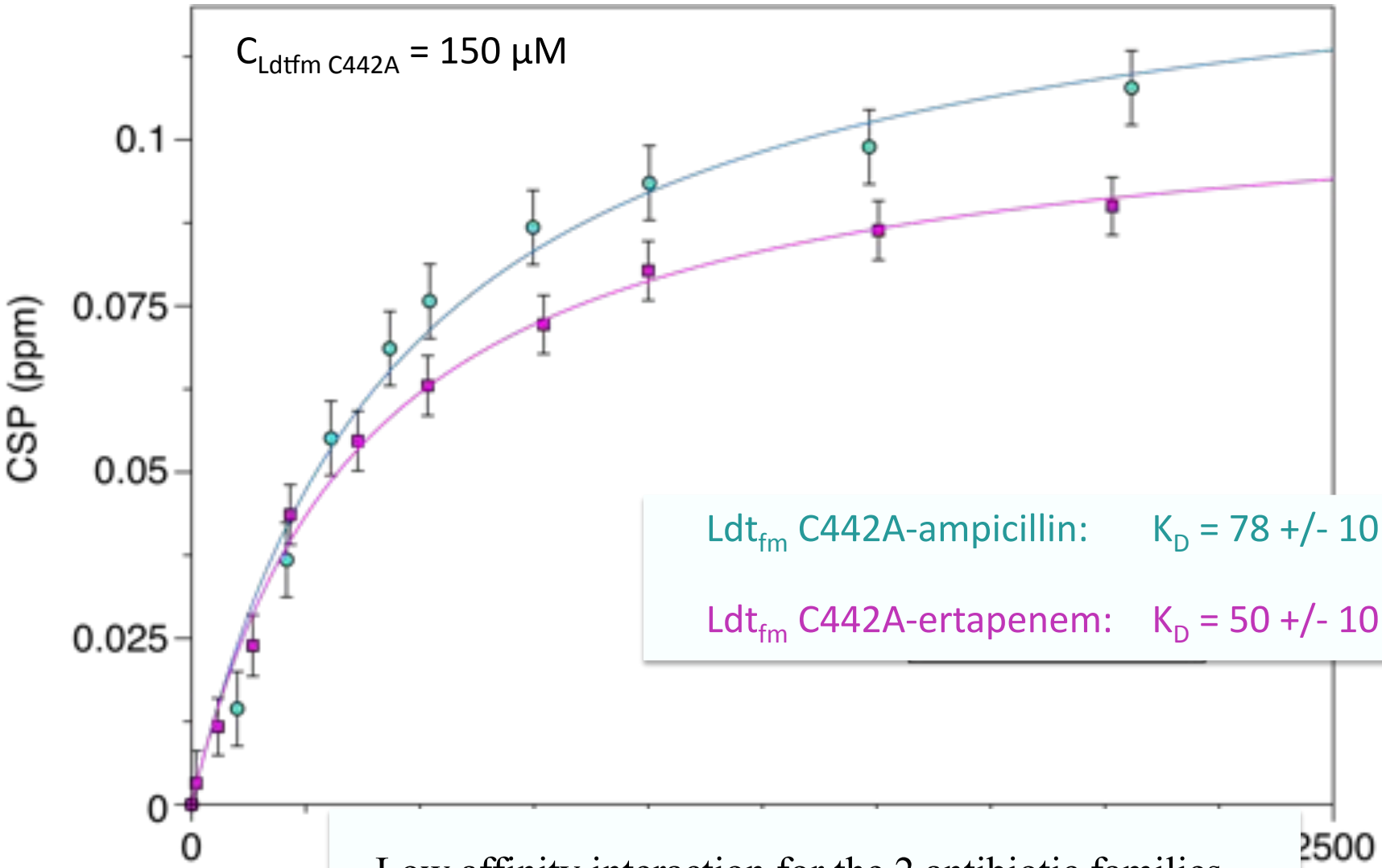
Chemical Shift Perturbations for antibiotics from penam and carbapenem families:



Ertapenem and ampicillin bind into the same cavity of the protein

Mechanism of Ldt inhibition by carbapenems

K_D determination: fit all residues with CSP > 0.03 ppm with a single K_D value



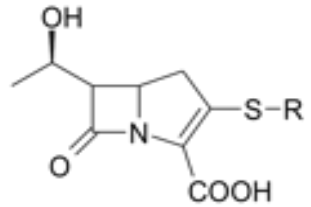
Ldt_{fm} C442A-ampicillin: K_D = 78 +/- 10 mM

Ldt_{fm} C442A-ertapenem: K_D = 50 +/- 10 mM

Low affinity interaction for the 2 antibiotic families

Mechanism of Ldt inhibition by carbapenems

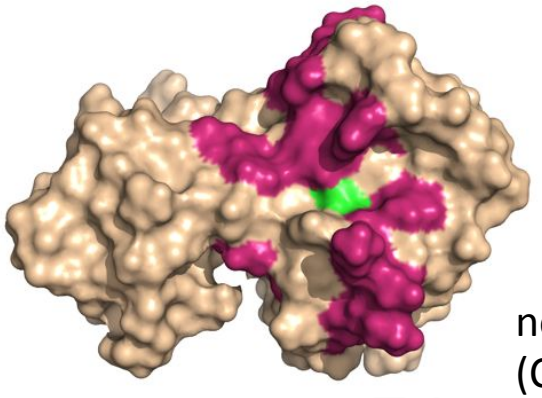
β-lactam



I

k_1

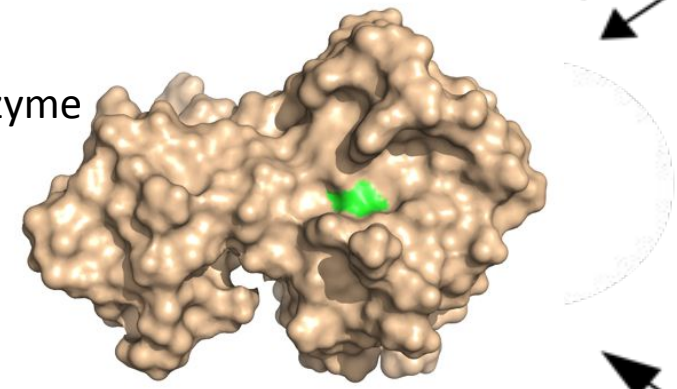
k_{-1}



non-covalent complex
(Cys-Ala mutant)

E.I

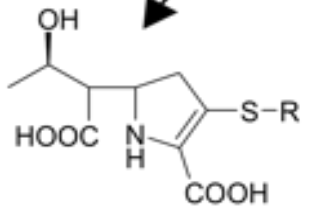
apoenzyme



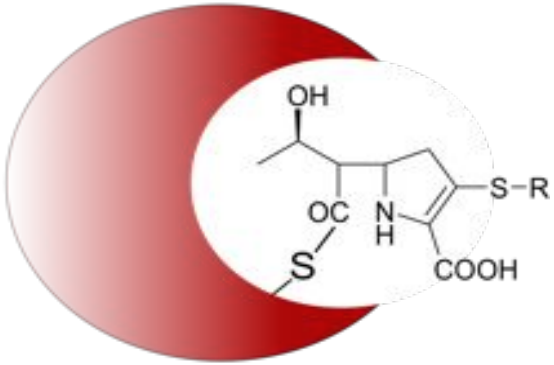
E

k_2

k_3



H_2O



Hydrolyzed carbapenem

E-I

acylenzyme

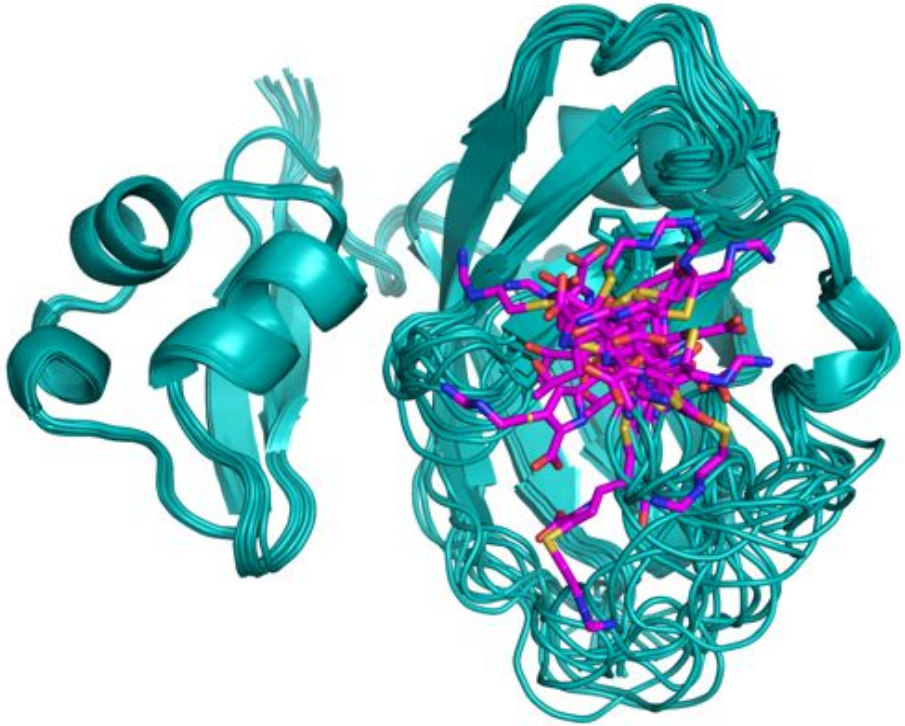
Mechanism of Ldt inhibition by carbapenems

Acylenzyme
Ldt_{fm}-ertapenem

Both apo- and acyl-enzymes reveal
slight conformational exchange contributions

Lecoq et al., *ACS Chem. Biol. 2013*

Acylenzyme
Ldt_{Bs}-imipenem

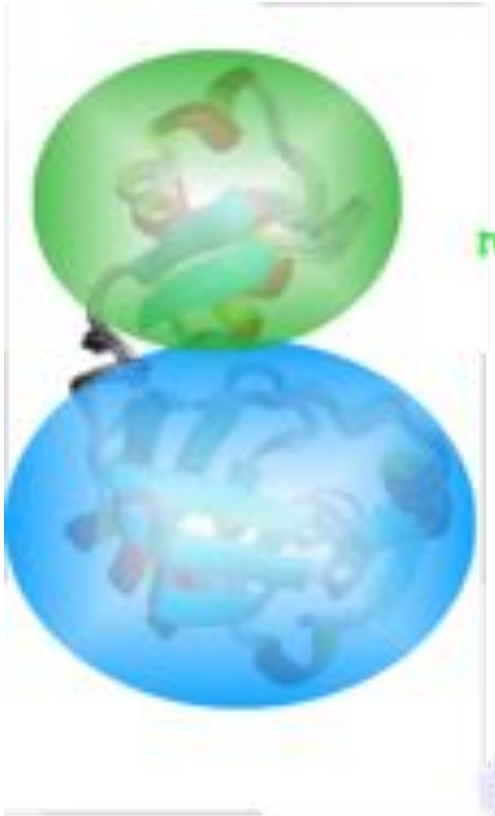


Only acylenzyme reveals
conformational exchange contributions

Lecoq et al., *Structure* 2012

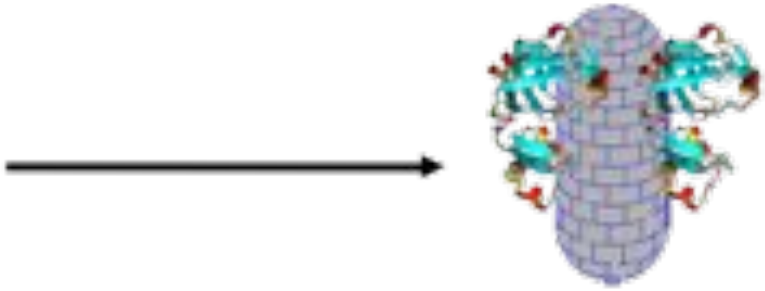
➢ Dynamics has more impact in the NMR of Ldt_{Bs} in comparison to Ldt_{fm}

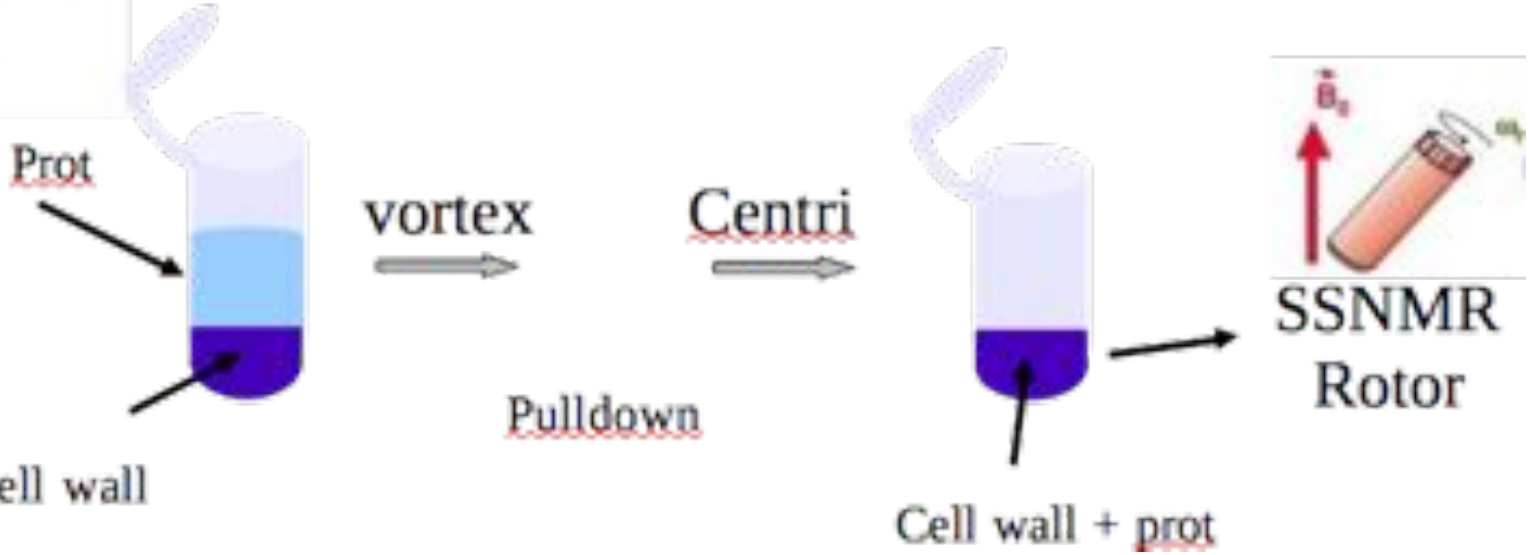
Insight into the peptidoglycan biosynthesis



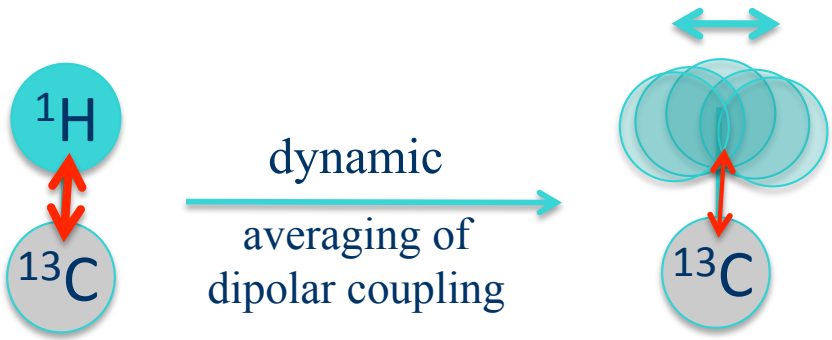
LysM
recognition domain

Catalytic domain

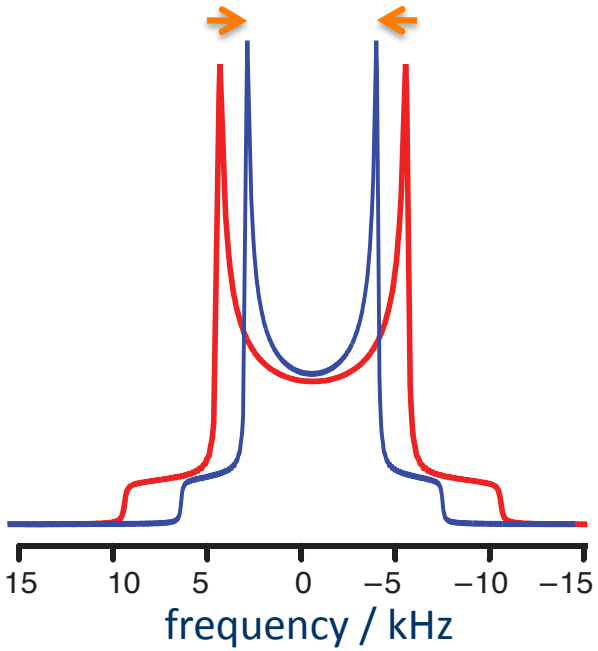




Insight into the peptidoglycan biosynthesis

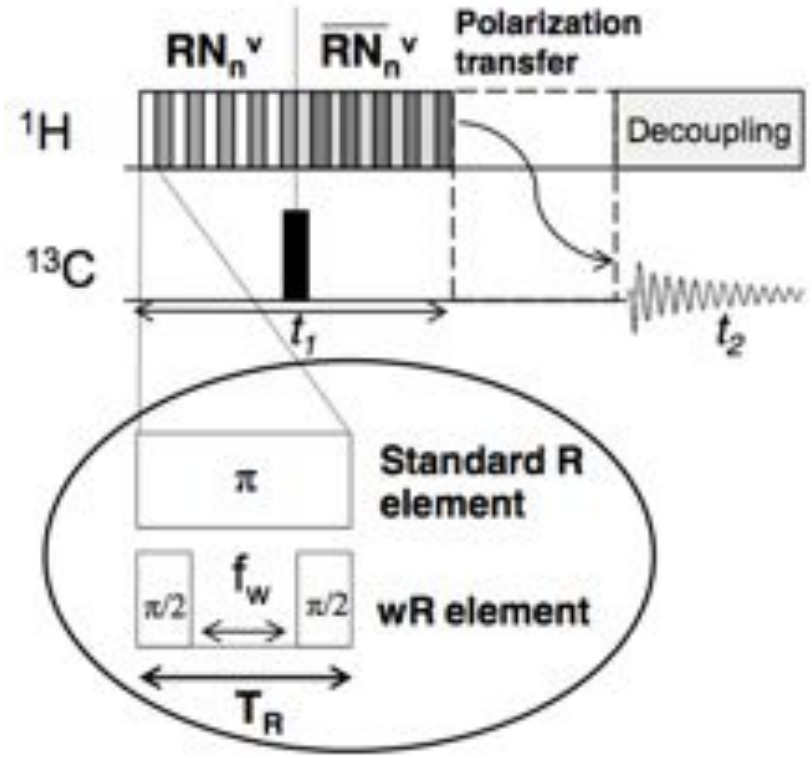


$S = D_{measured} / D_{rigid}$



Measurement of CH dipolar couplings

Windowed R18 sequence
Robust with respect to rf inhomogeneity



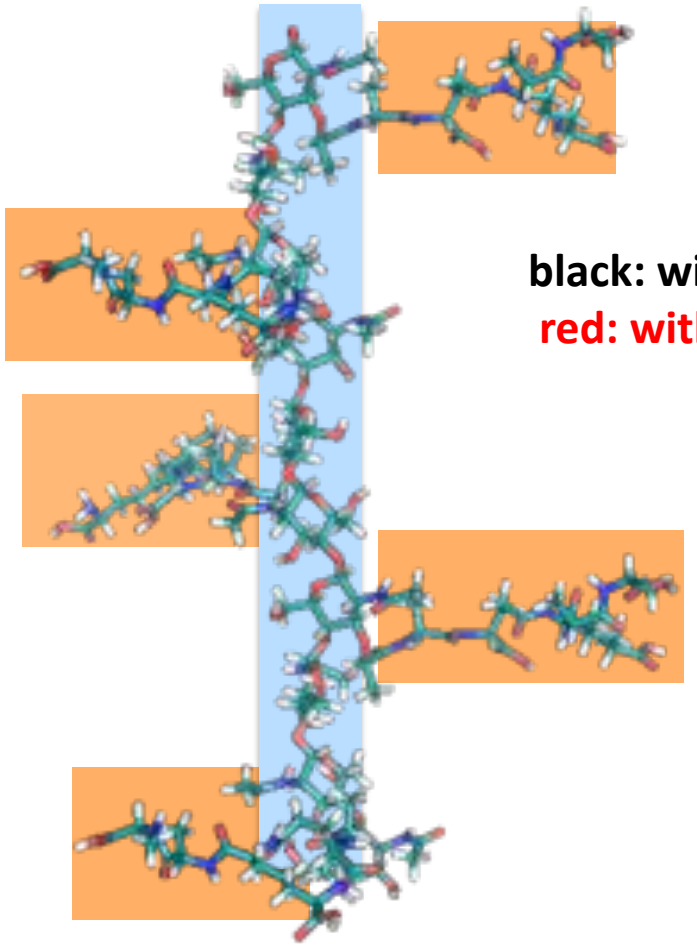
Gansmüller et al., *J. Magn. Res.*, 2013

Insight into the peptidoglycan biosynthesis

Protein binding:

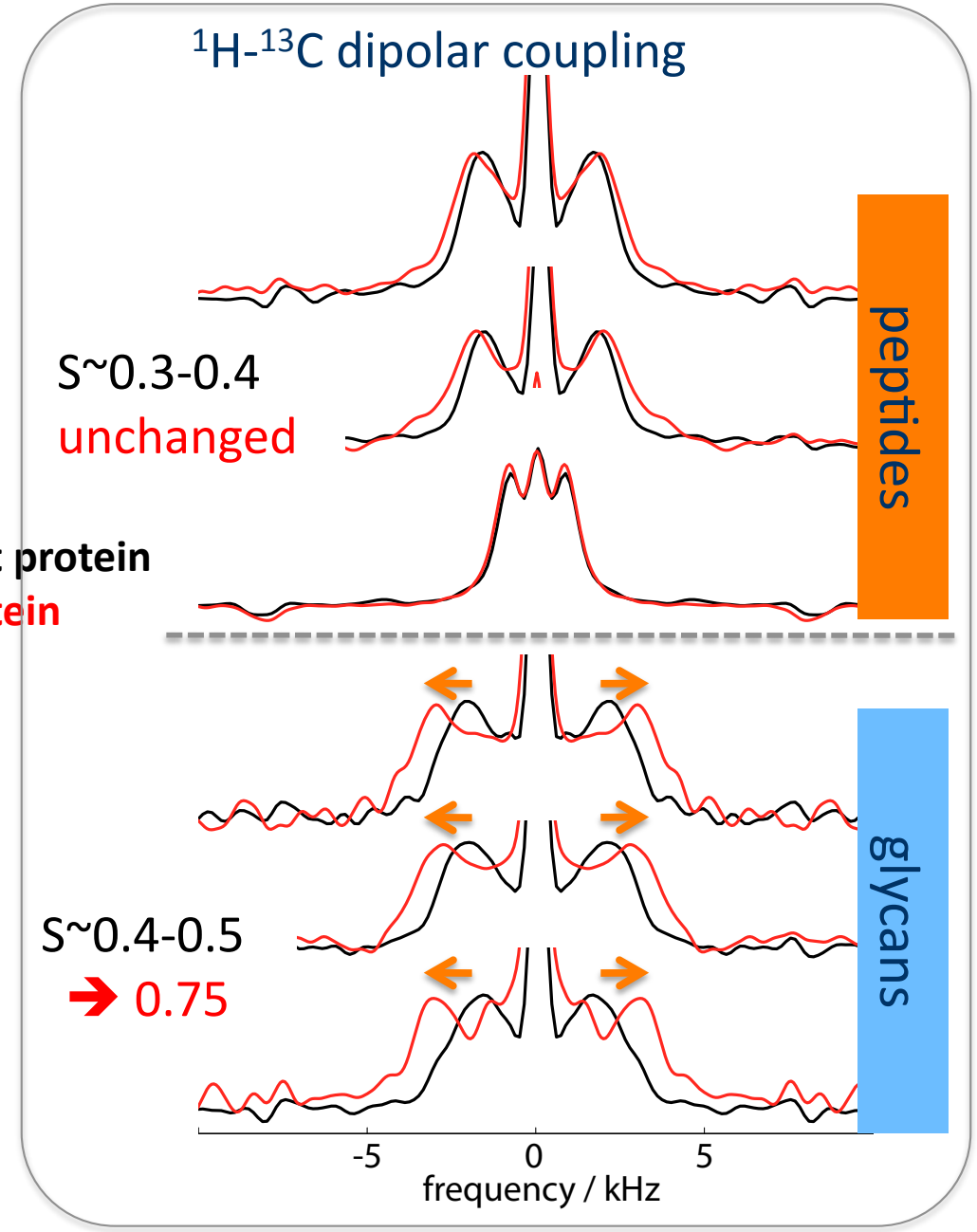
reduction in glycan motional amplitude

peptide parts remain rather flexible



black: without protein

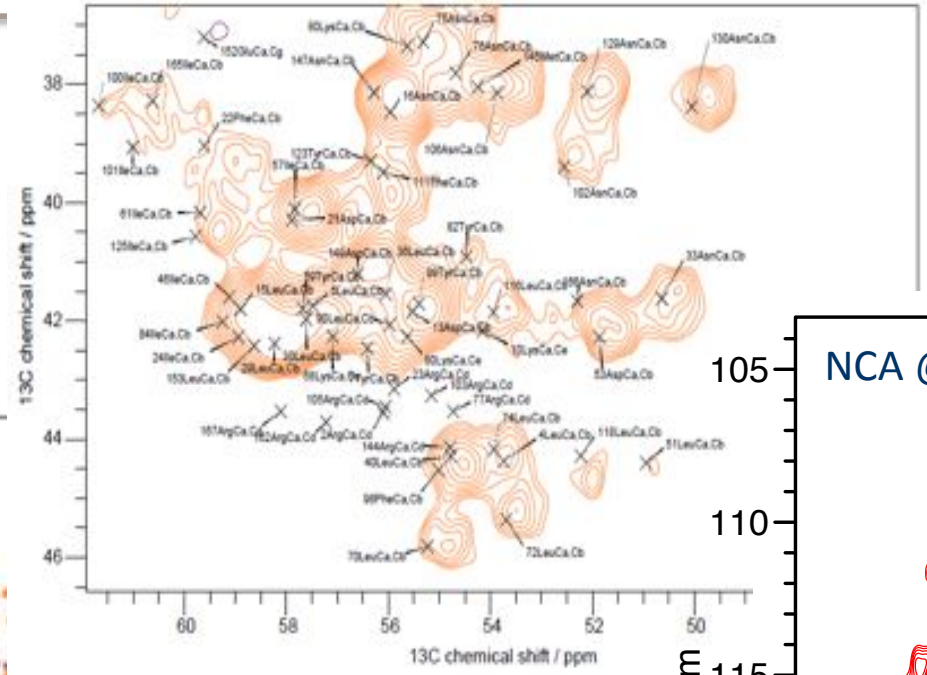
red: with protein

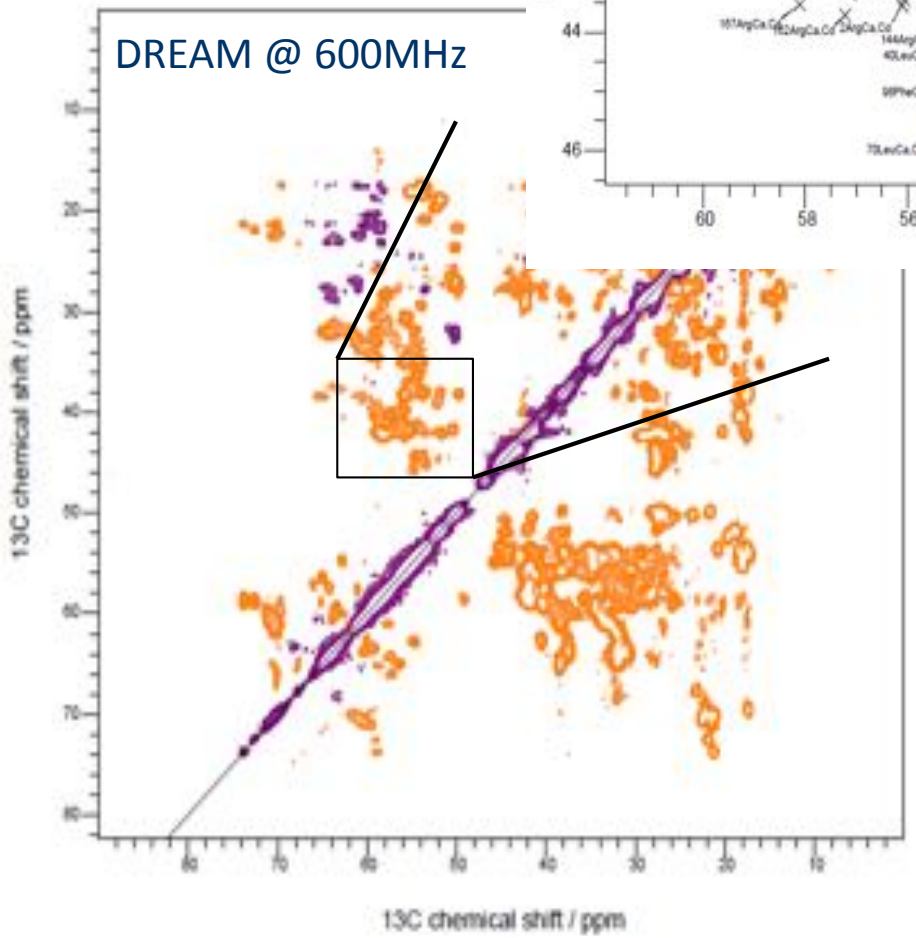


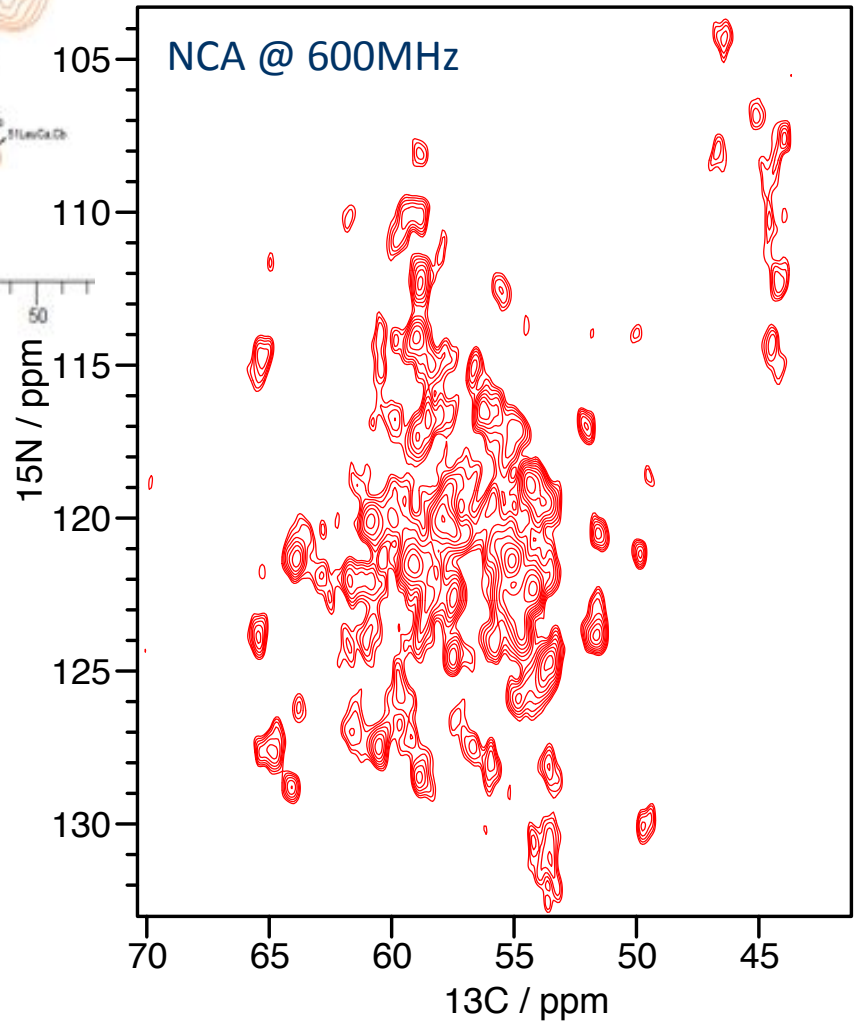
Insight into the peptidoglycan biosynthesis

Challenges:

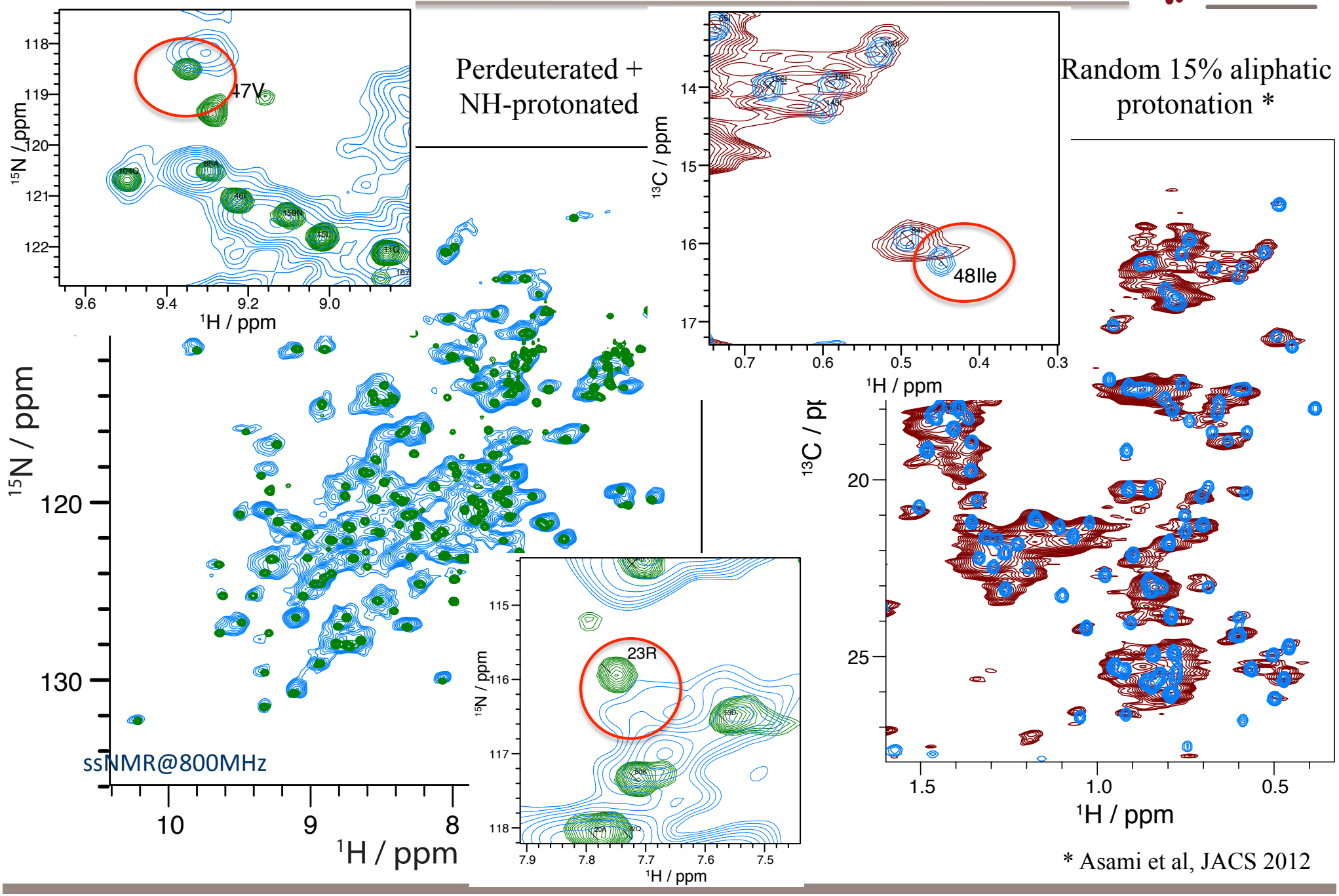
- only ~2mg protein in 3.2mm rotor (≈25uL)
- > 170 residues





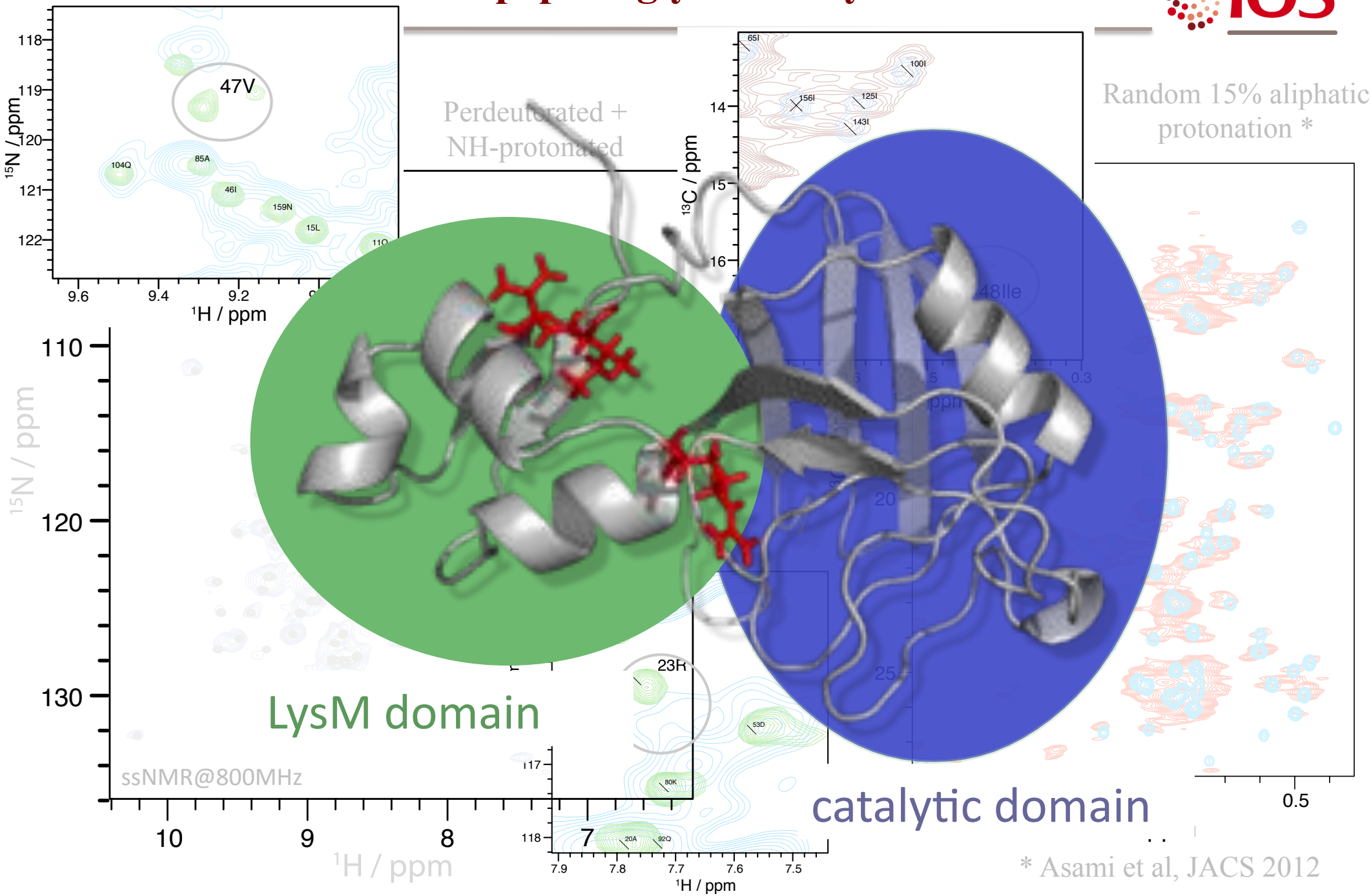


Insight into the peptidoglycan biosynthesis



* Asami et al, JACS 2012

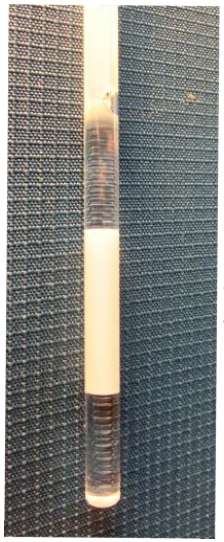
Insight into the peptidoglycan biosynthesis



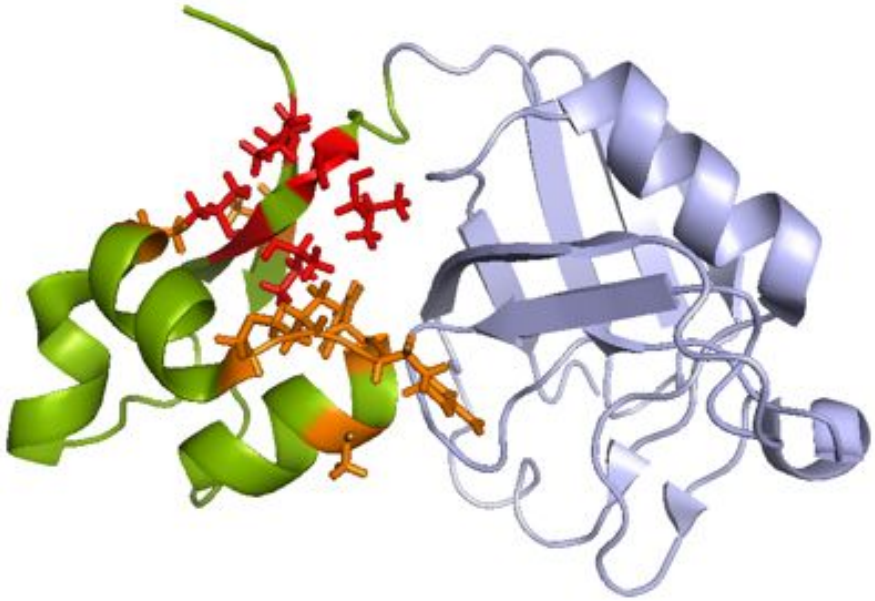
Insight into the peptidoglycan biosynthesis

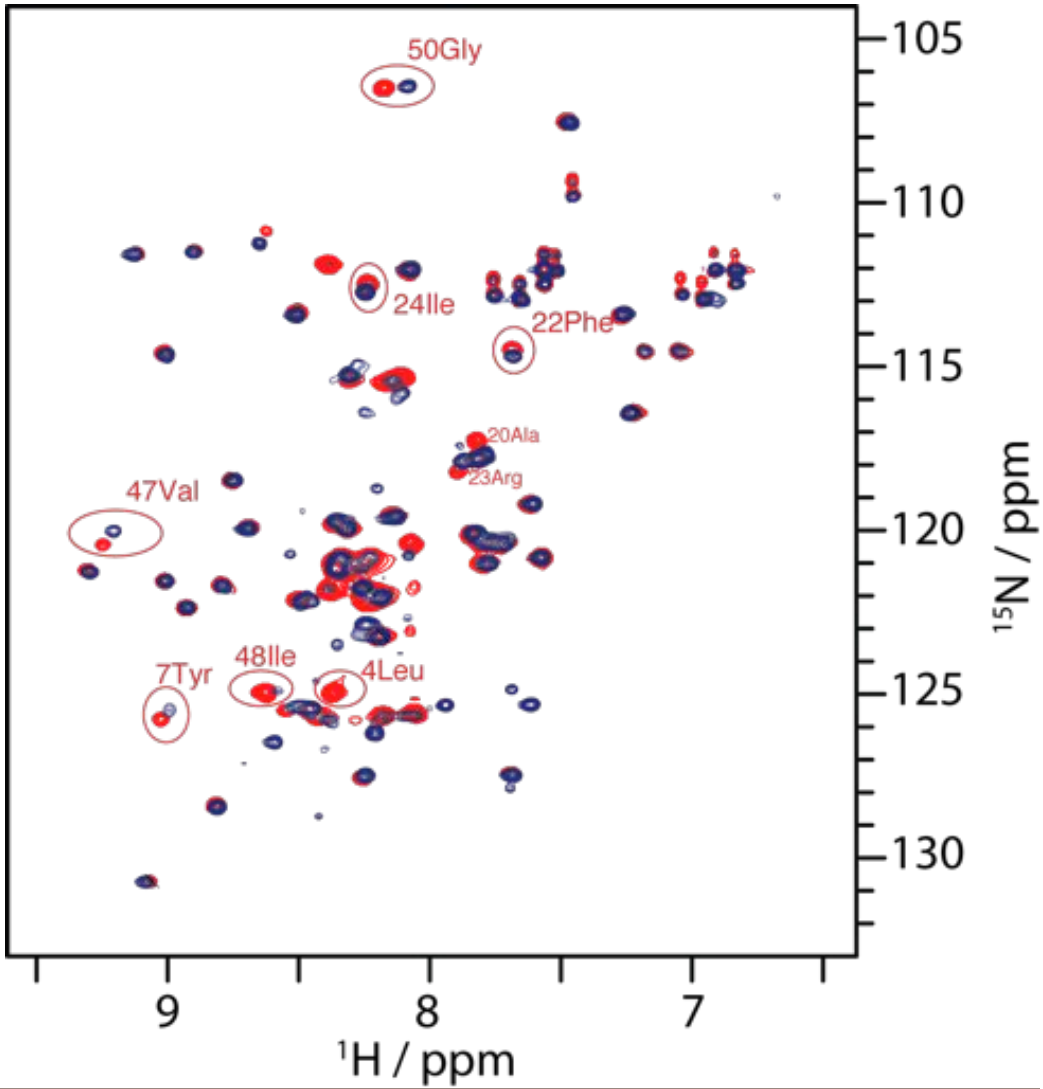
No signal in the solid-state

… but visible in solution !



Shigemi tube
with cell wall

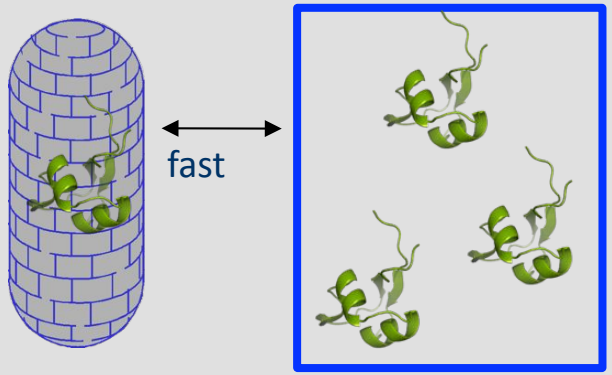




Insight into the peptidoglycan biosynthesis

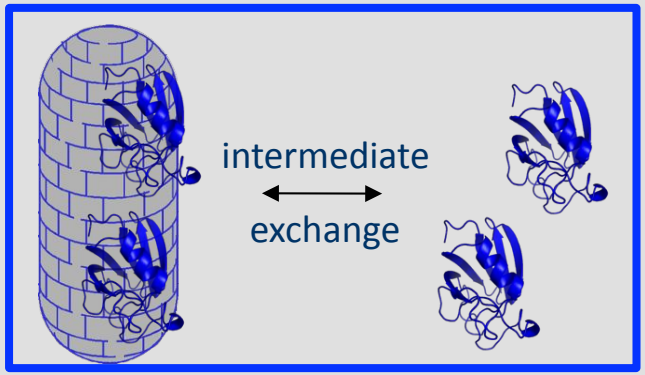
LysM domain alone

weak interaction



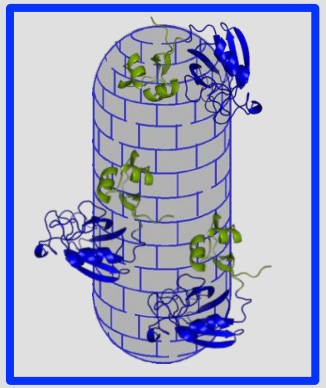
catalytic domain alone

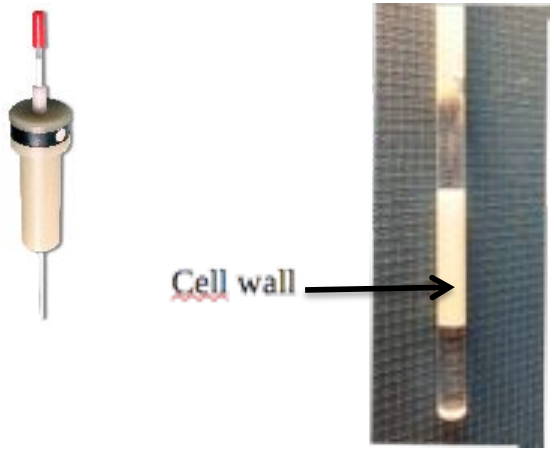
intermediate interaction



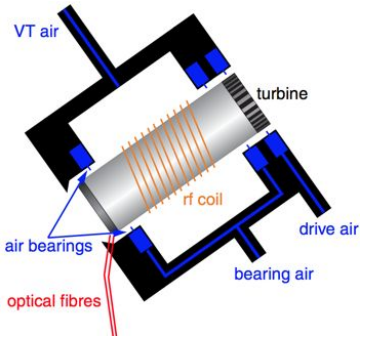
full-length protein

rigidly attached

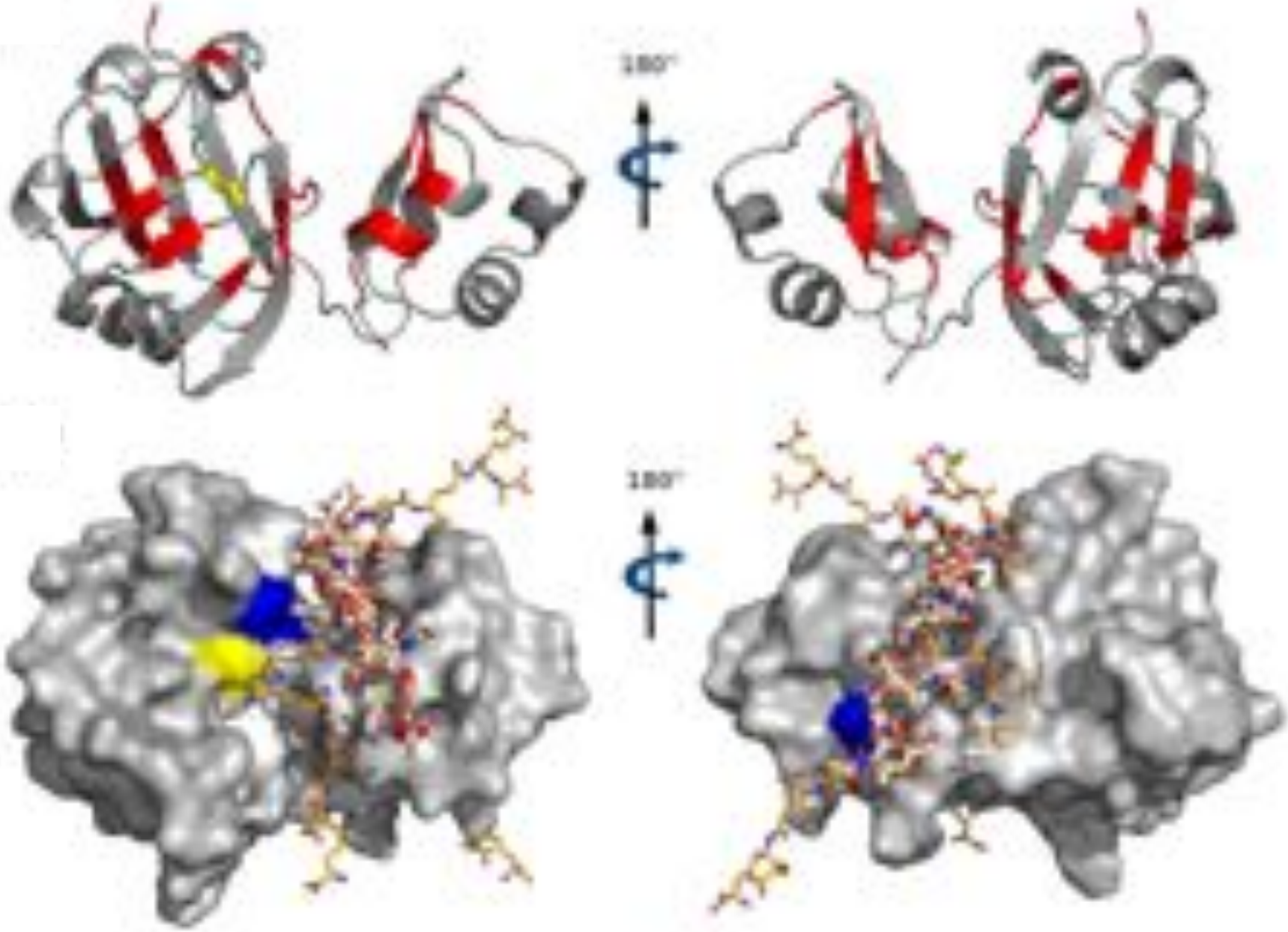




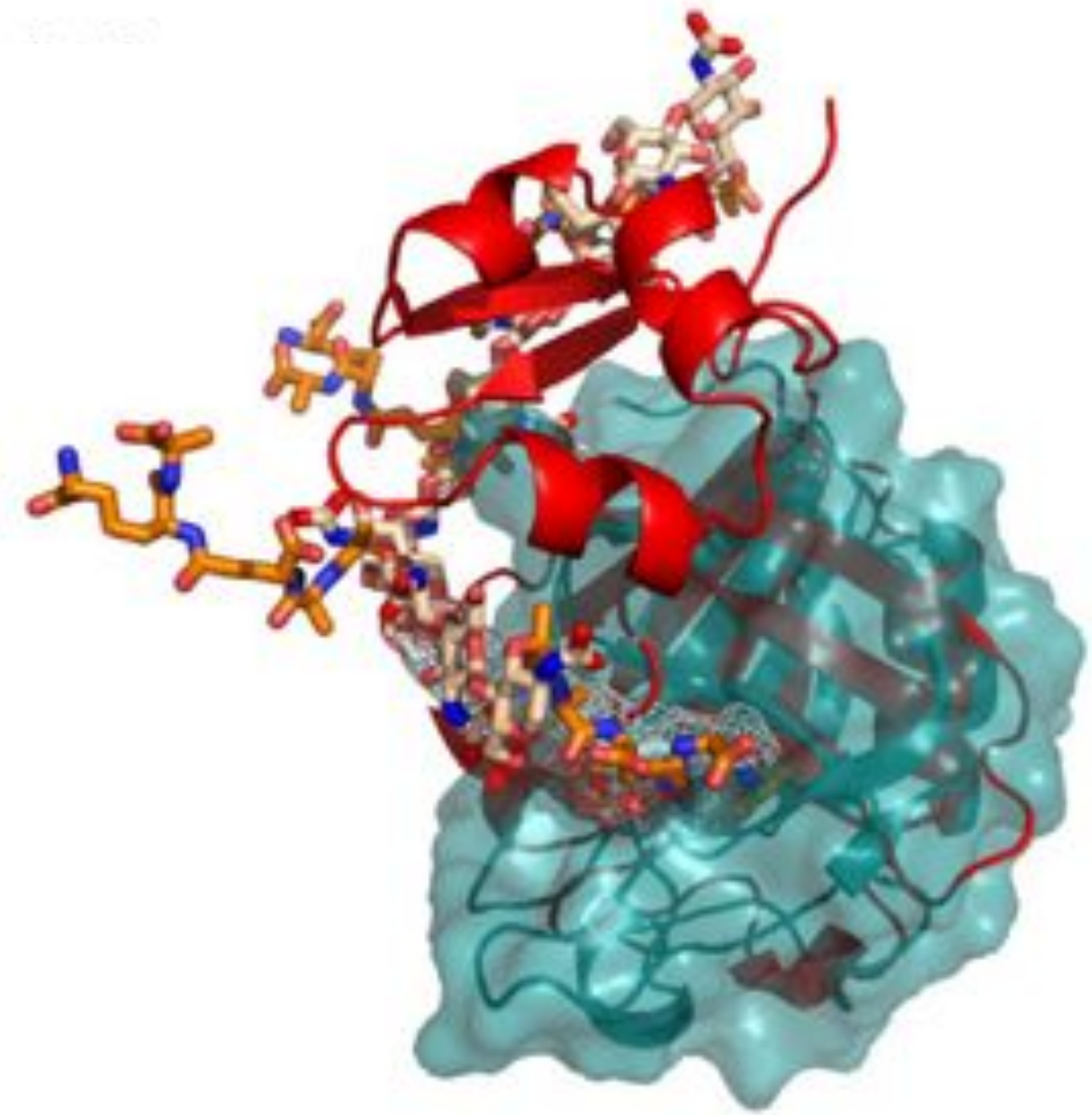
Affinity depends on the presence of both domains



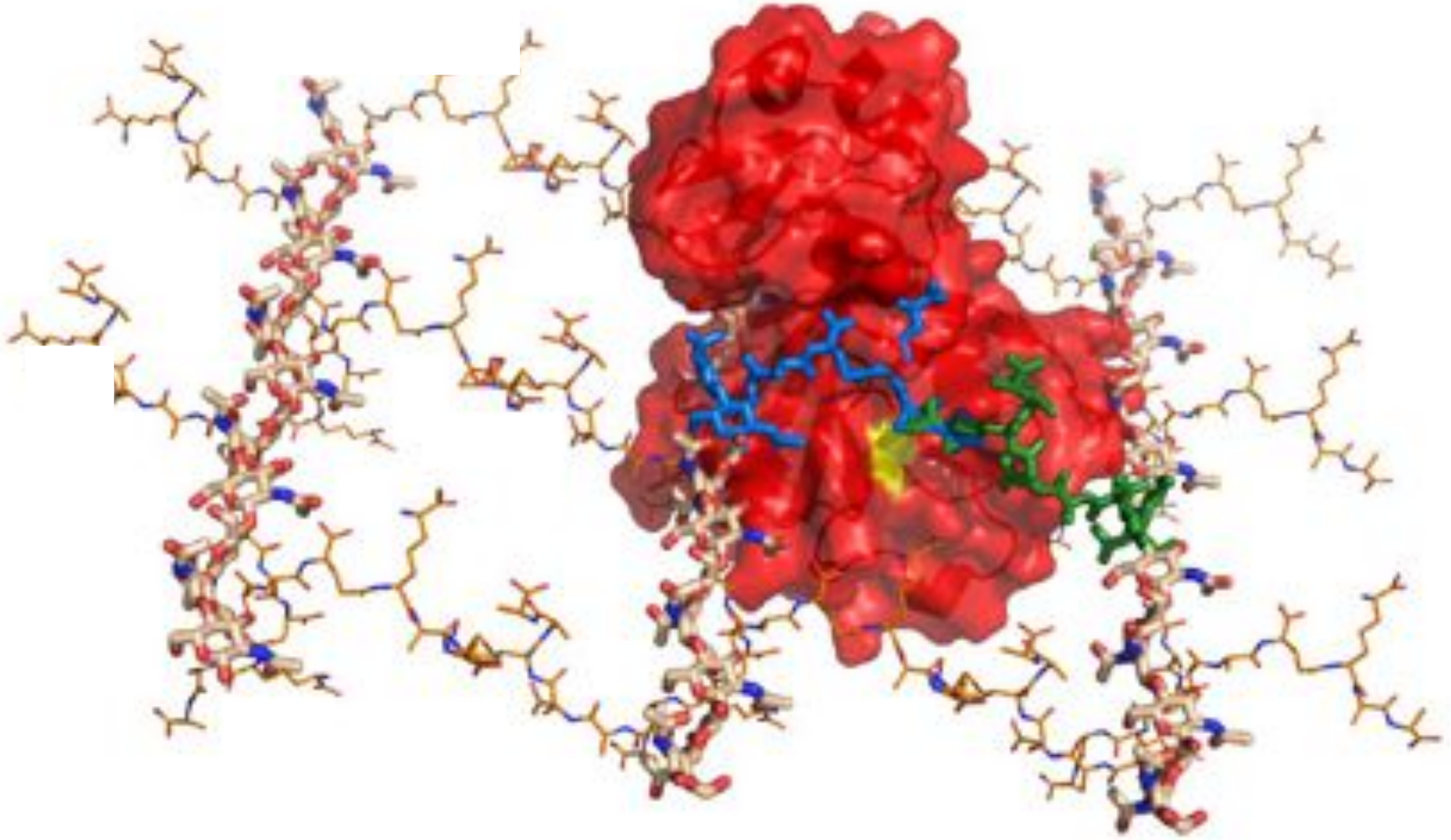
Insight into the peptidoglycan biosynthesis



Insight into the peptidoglycan biosynthesis



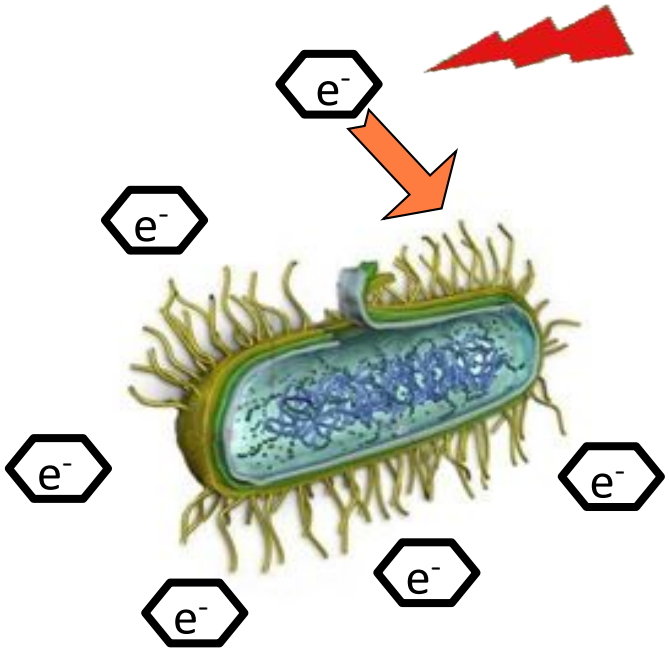
Insight into the peptidoglycan biosynthesis



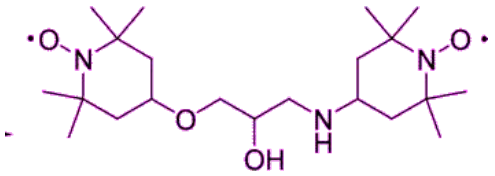
DNP-enhanced NMR

T ~ 100 K : compatible with cell survival

Gyrotron



DNP @ CEA Grenoble

e- = 

TOTAPOL

NMR signal of the cell surface nuclei ?

Gyrotron on
Gyrotron off

^{13}C chem. shift / ppm

^{13}C, ^{15}N-labeled *B. subtilis*

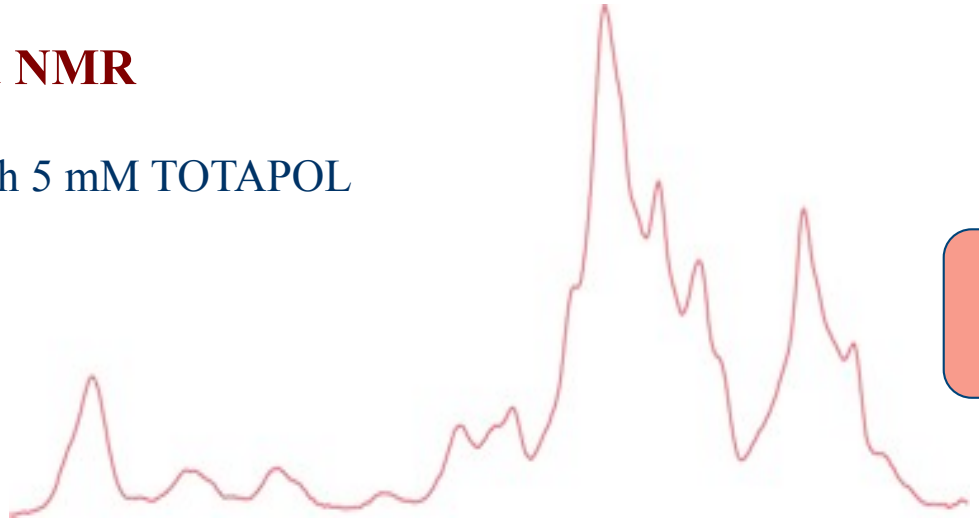
Takahashi et al, J. Am. Chem. Soc.,2013, *135*, 5105-5110

Linebroadening vs. Absolute Sensitivity Ratio

DNP-enhanced NMR

washed 1x with 5 mM TOTAPOL
T ~ 100 K

3.2 mm rotor
16 scans



→ Compare S/N per unit time :

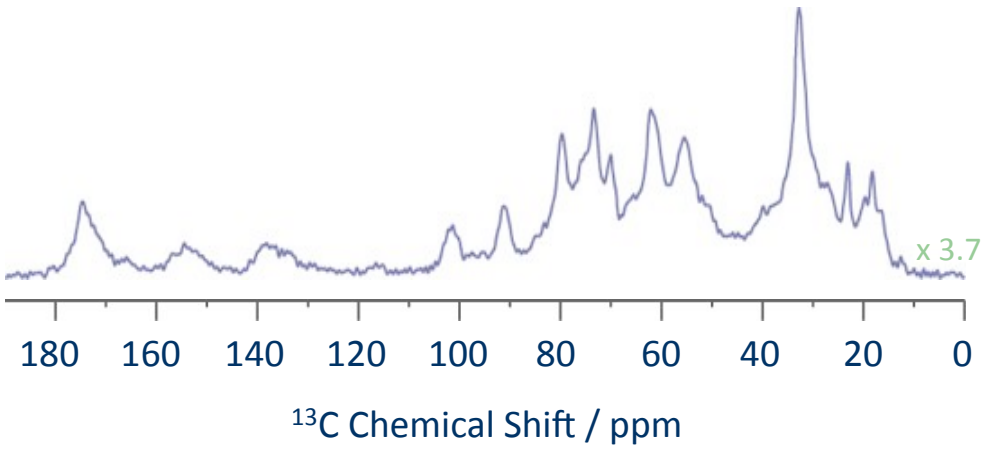
Absolute Sensitivity Ratio ASR

Takahashi et al, Angew. Chem.., 2012, *51*, 11766-11769

Conventional NMR

no radicals
T ~ 0 ºC

4 mm rotor
256 scans



lipids

sugars

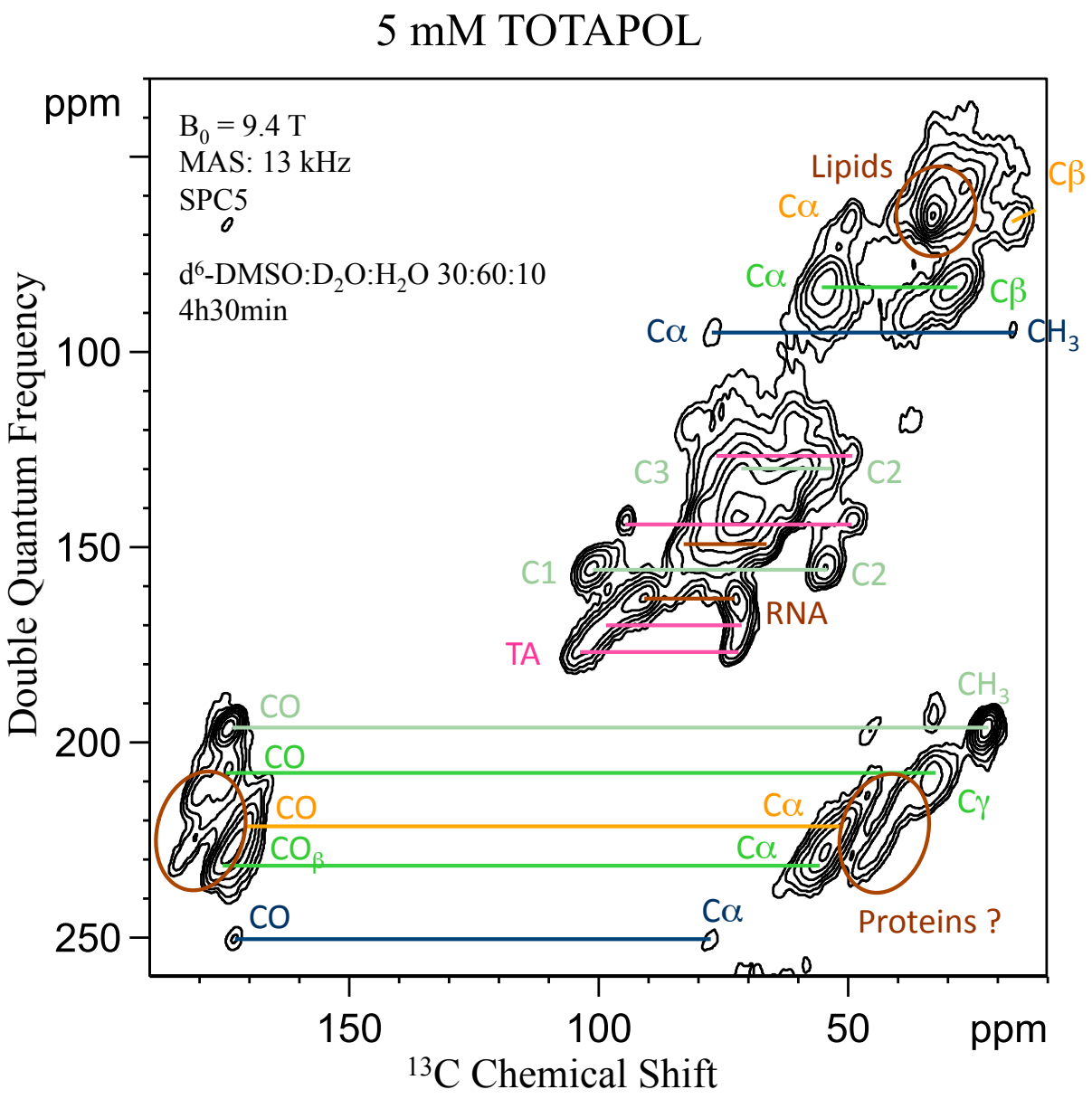
ASR = 9 - 24

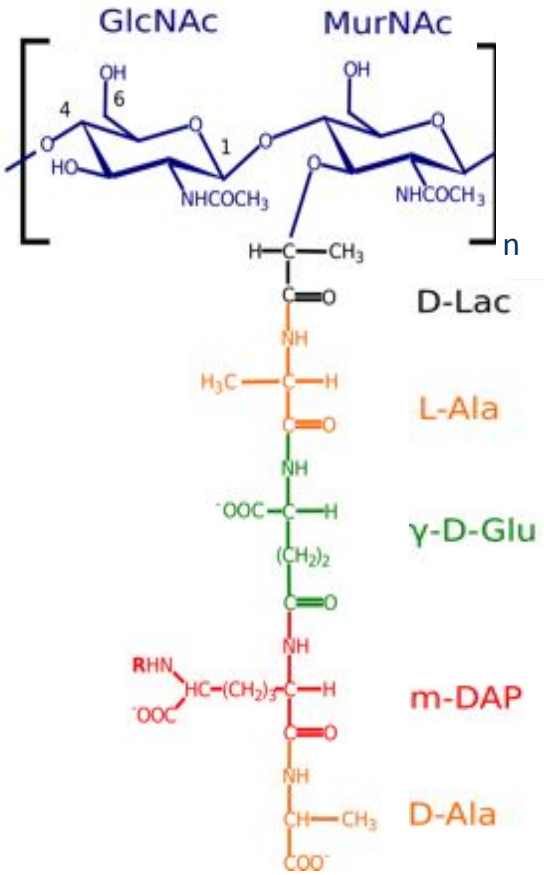
→ Time saving of up to 600 !

^{13}C, ^{15}N-labeled *B. subtilis*

Takahashi et al, J. Am. Chem. Soc., 2013, *135*, 5105-5110

Cell-Wall-Signals Enhancement in Entire Cells

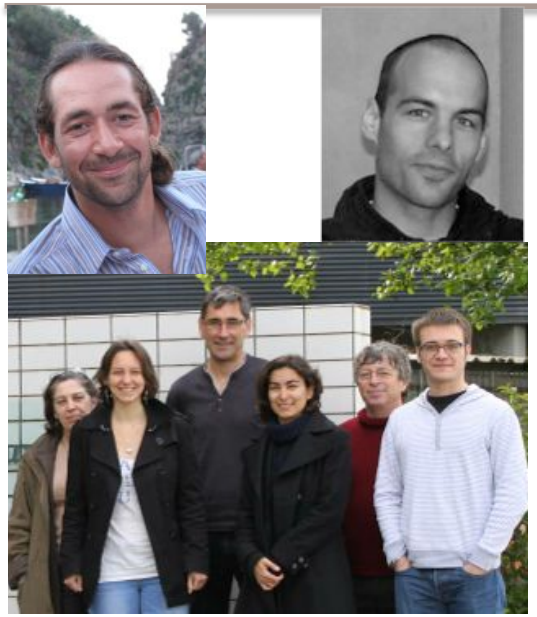




Cell wall polymers dominate the spectrum at low TOTAPOL concentration

Takahashi et al, J. Am. Chem. Soc., 2013, *135*, 5105-5110

Acknowledgments



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