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Endotoxin-free E. Coli hosts for vaccine discovery and production

David Bramhill
Research Corporation Technologies

Endotoxin-Free *E. coli* Hosts for Vaccine Discovery and Production

VACCINE TECHNOLOGY IV

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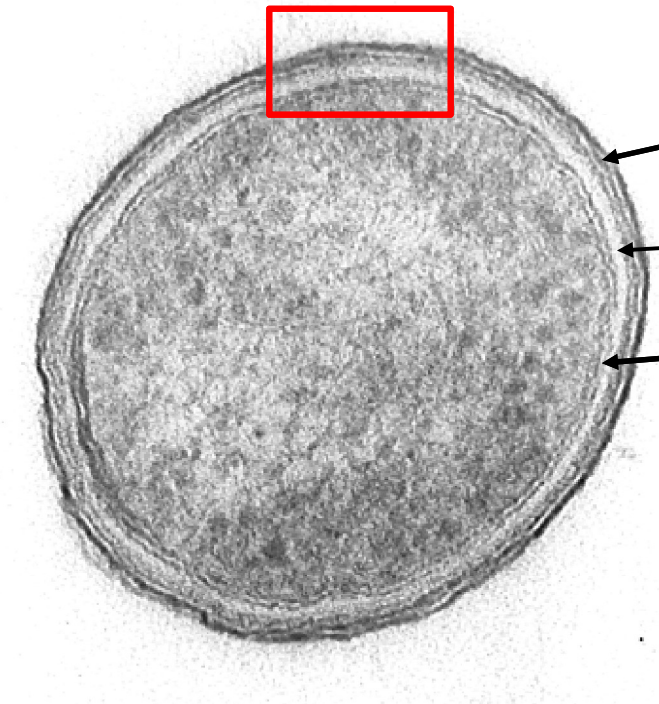
Outline

- Life without LPS
  - Is it possible?
  - How?
- Making it irreversible
- Characterizing LPS-free strains
- Potential applications for vaccines
- Crabs

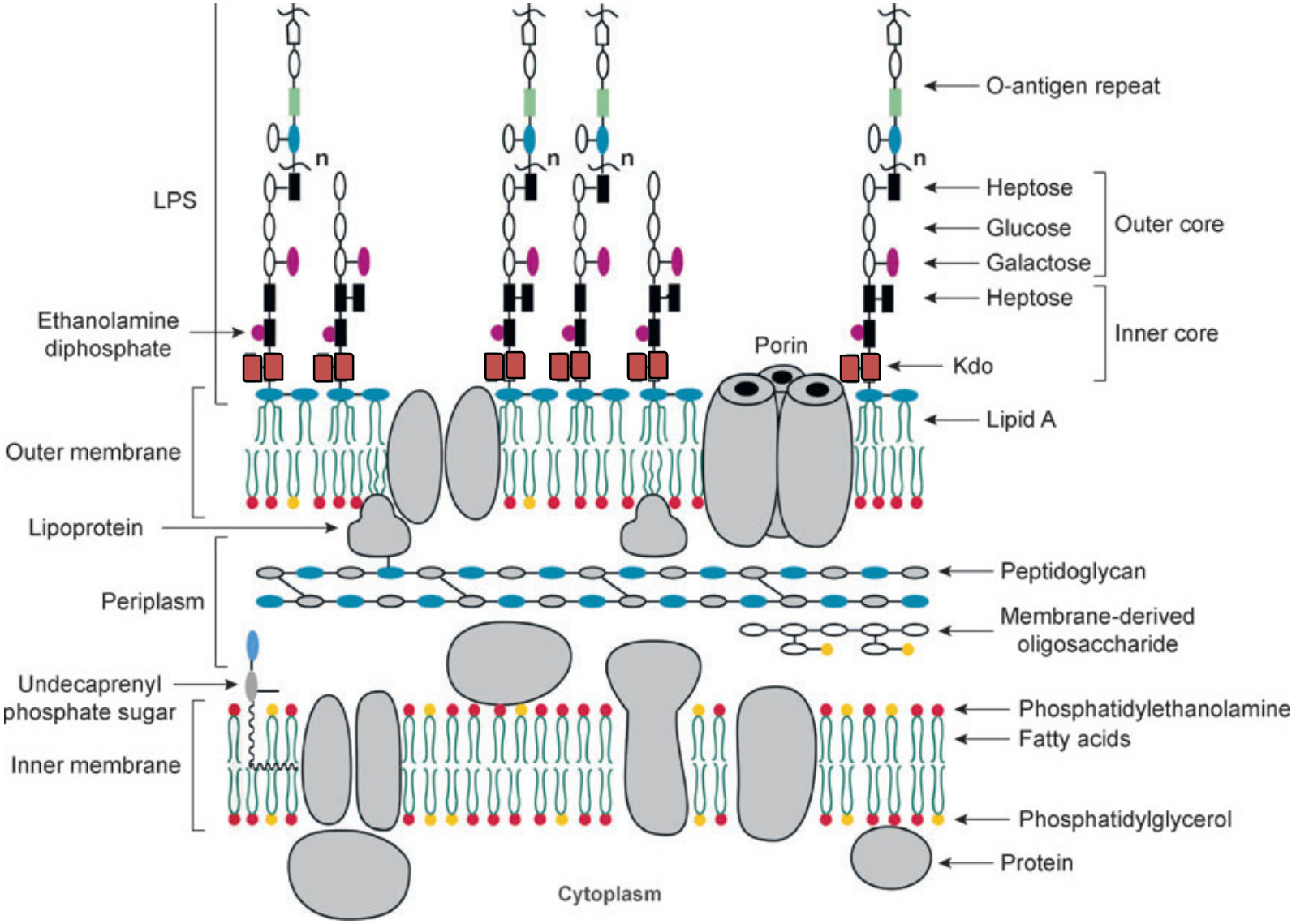
The cell envelope of Gram-negative bacteria



~2 x 10^6 LPS molecules per cell

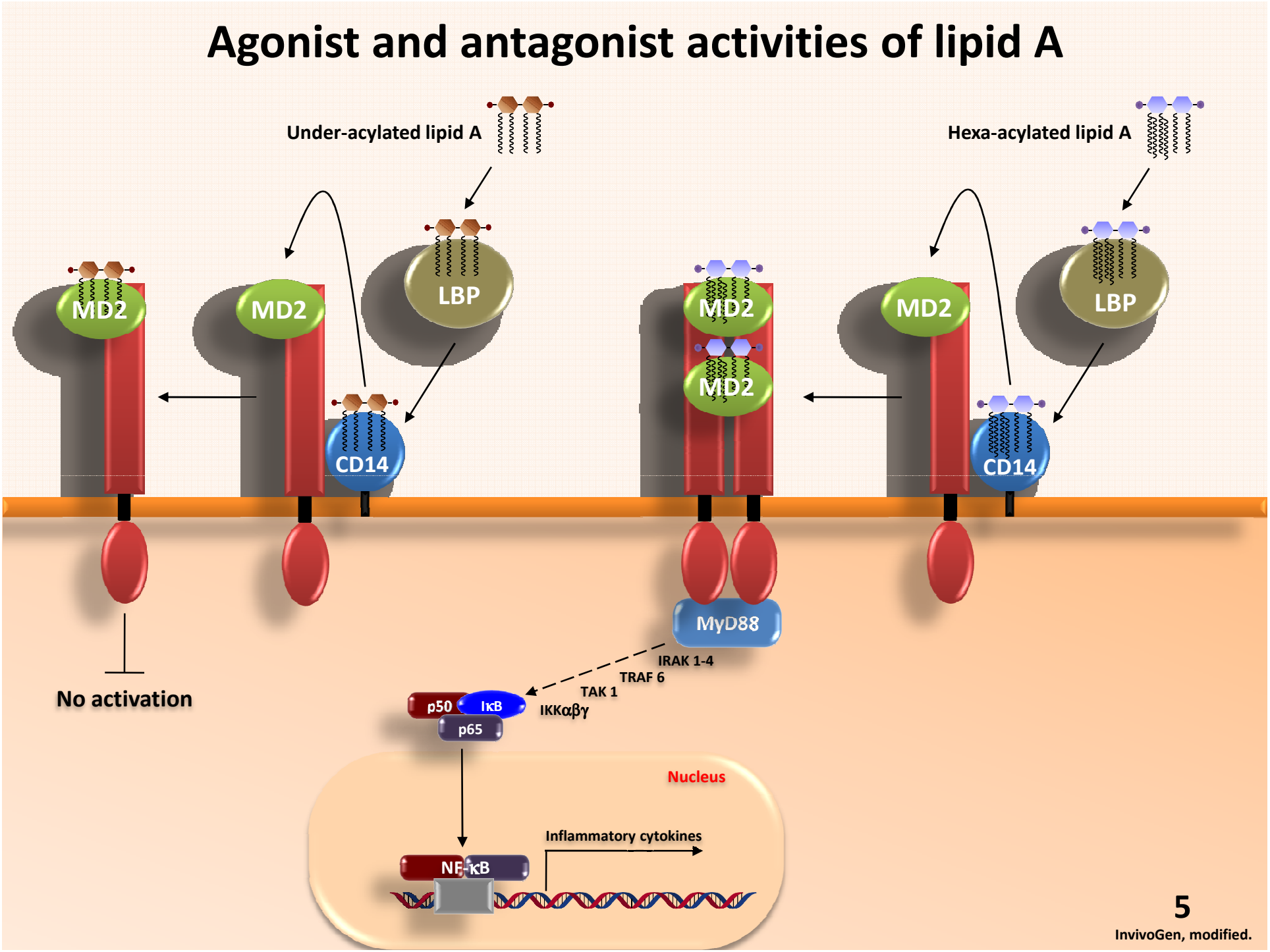
30% of the total outer membrane gross weight.

Inner and Outer Membrane Components



Reatz

Agonist and antagonist activities of lipid A

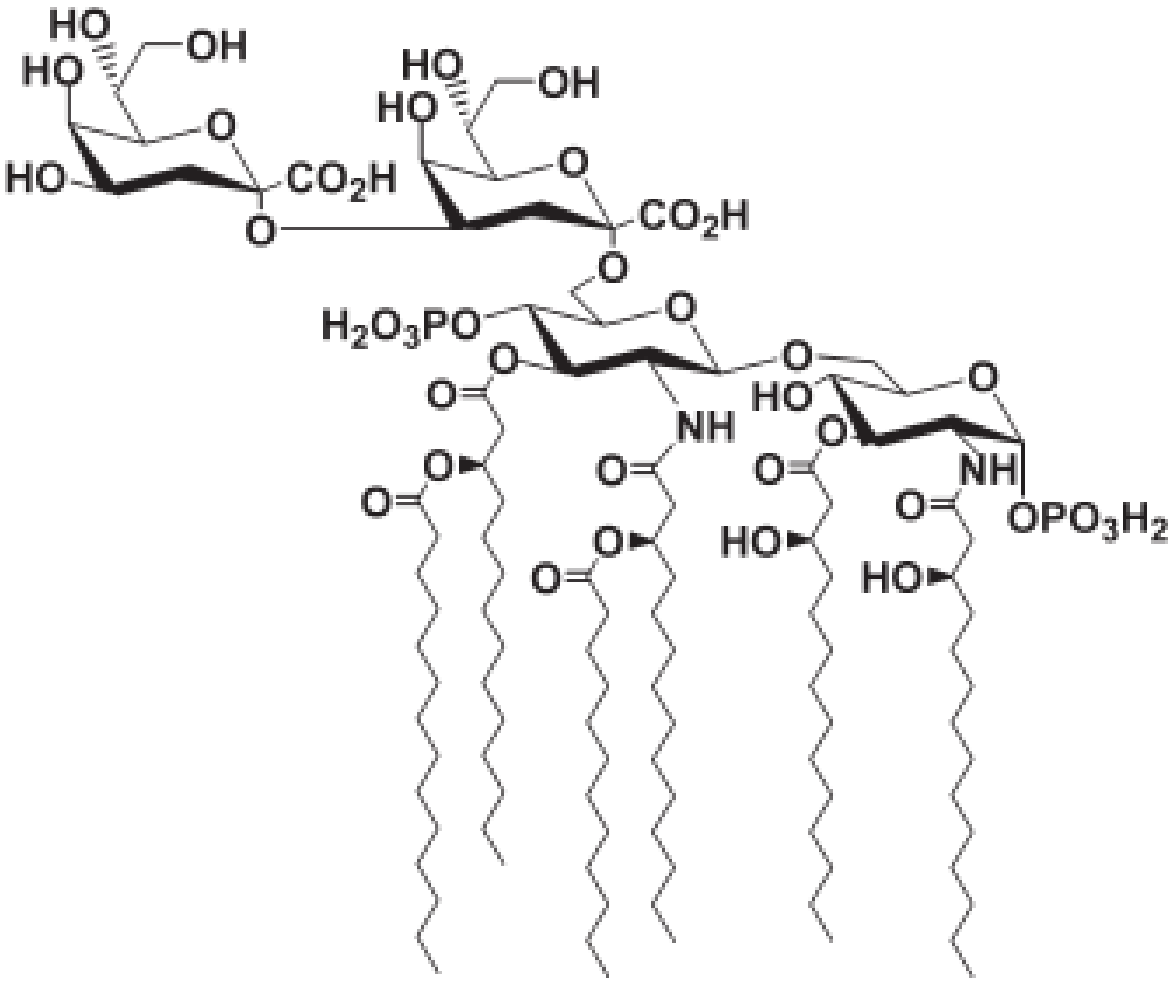


InvivoGen, modified.

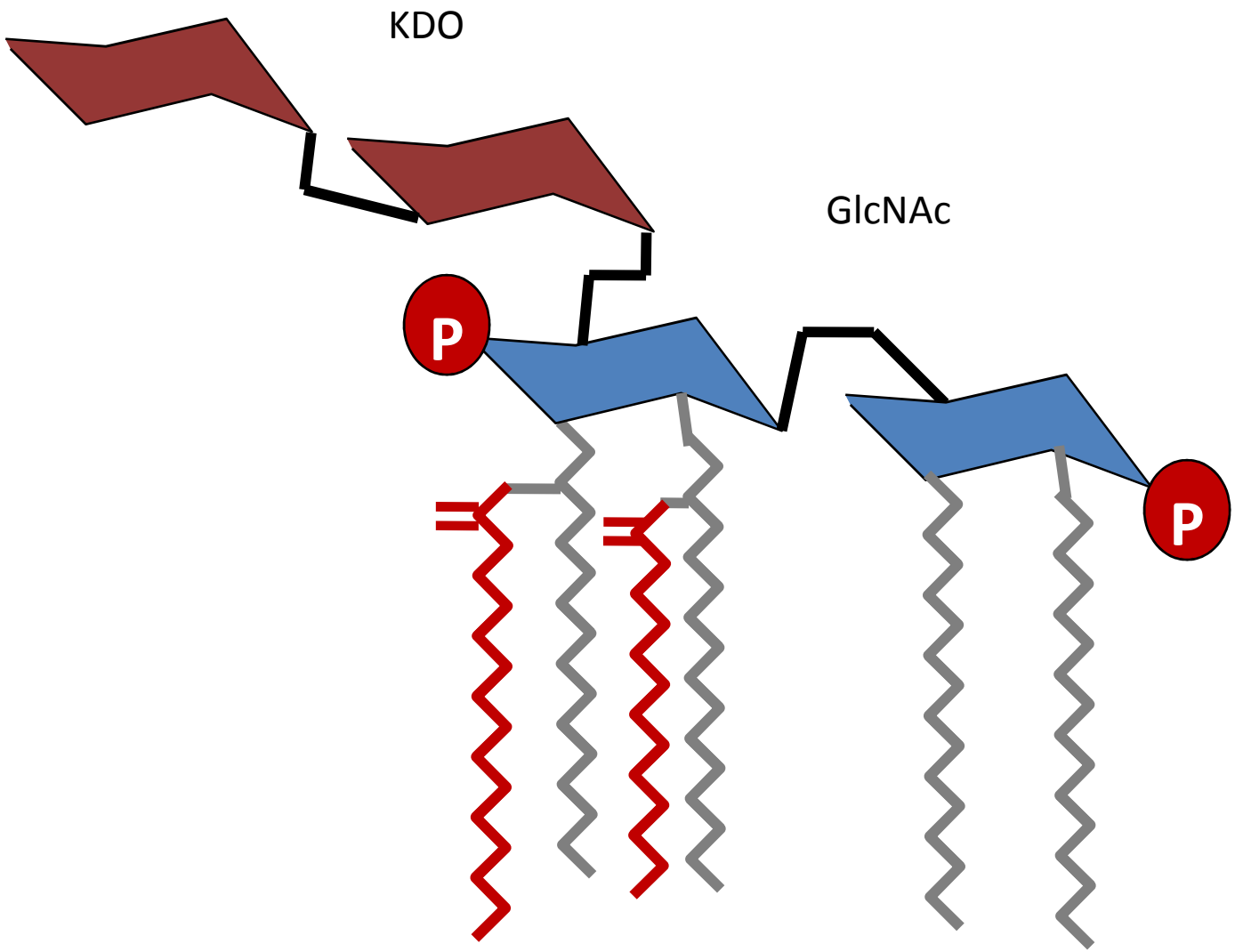
The challenge of endotoxin removal from biological samples

Method	Disadvantages
Ultrafiltration	• Only useful for small proteins. • Would fail if interactions between endotoxin and proteins cause endotoxin monomers to permeate with proteins through the membrane. • Inefficient for proteins which can be damaged by physical forces.
Activated carbon	• Adsorbing activity for both endotoxin and protein.
Surfactants	• Expensive, would add significant costs to a manufacturing process. • May affect the bioactivity of the protein of interest. • Certain amounts of surfactant always remain in the protein solution. Removal may lead to product loss.
Anion-exchange chromatography	• Adsorbing capacity is high for both endotoxin and acidic protein. • No selectivity to adsorb endotoxin.
Histamine- and histidine-immobilized Sepharose	• Removing capacity dependent on the ionic strength. • Biological activity of histamine.
Polymyxin B-immobilized Sepharose	• Protein losses due to the ionic interaction between the cationic region of polymyxin B and net-negatively charged proteins at low ionic strength. • Polymixin B is physiologically active.

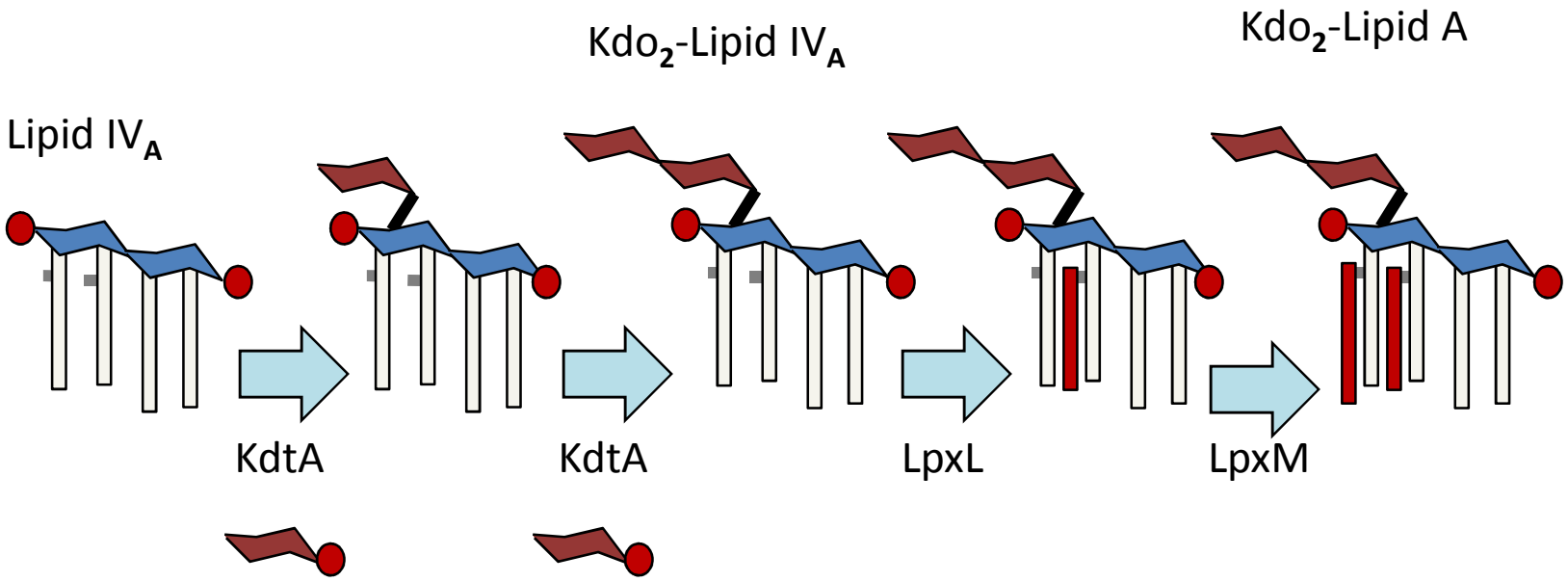
E. coli Kdo_2-LipidA Structure



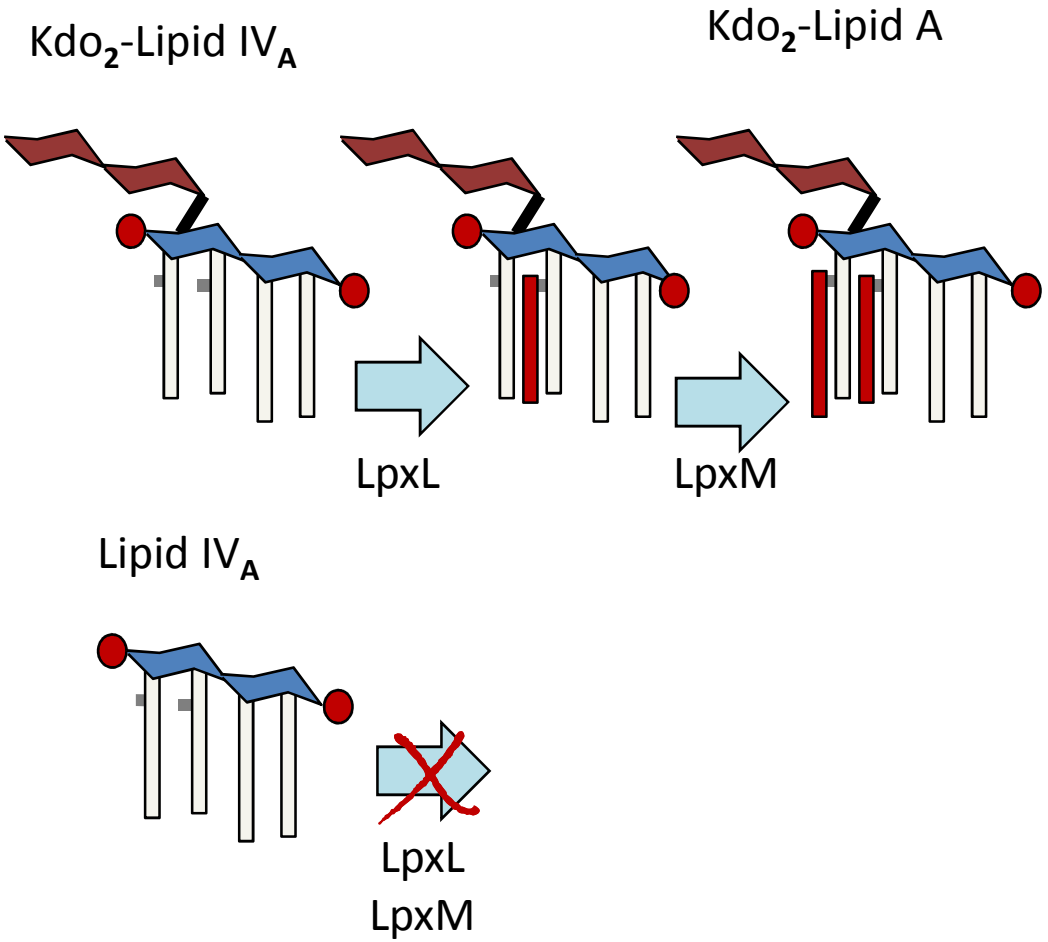
Simplified *E. coli* Kdo_2-LipidA Structure



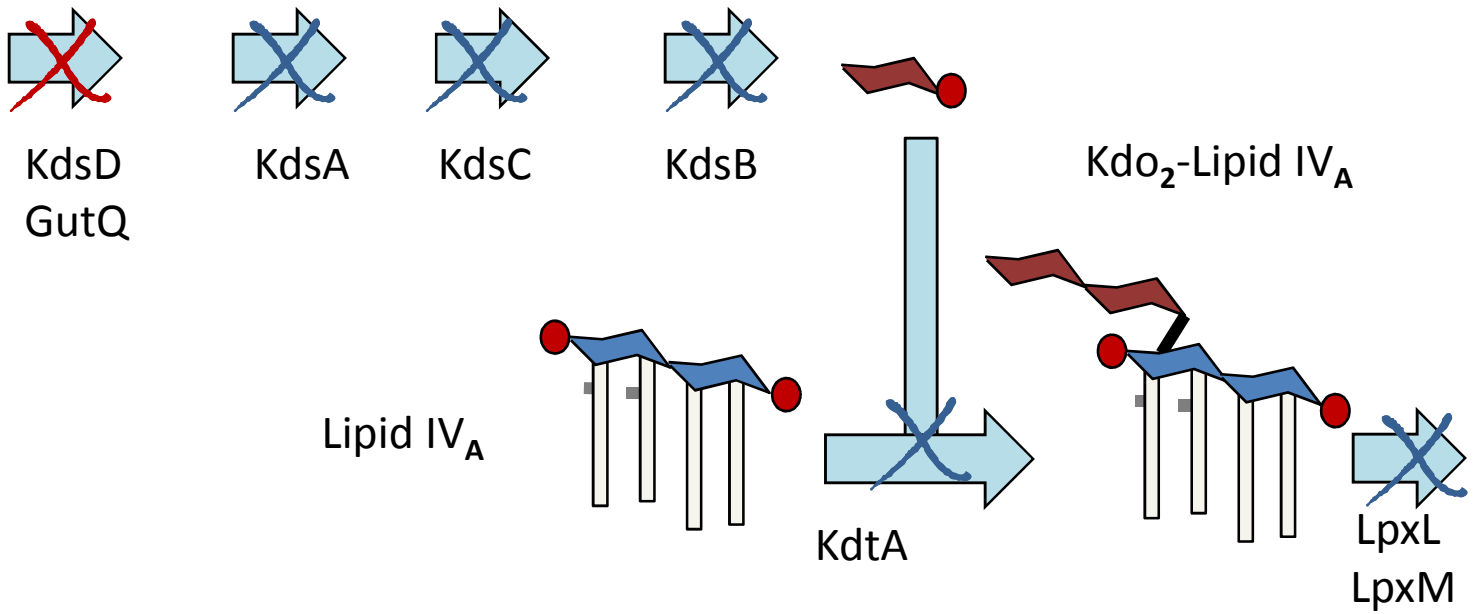
Synthesis of Kdo_2-LipidA



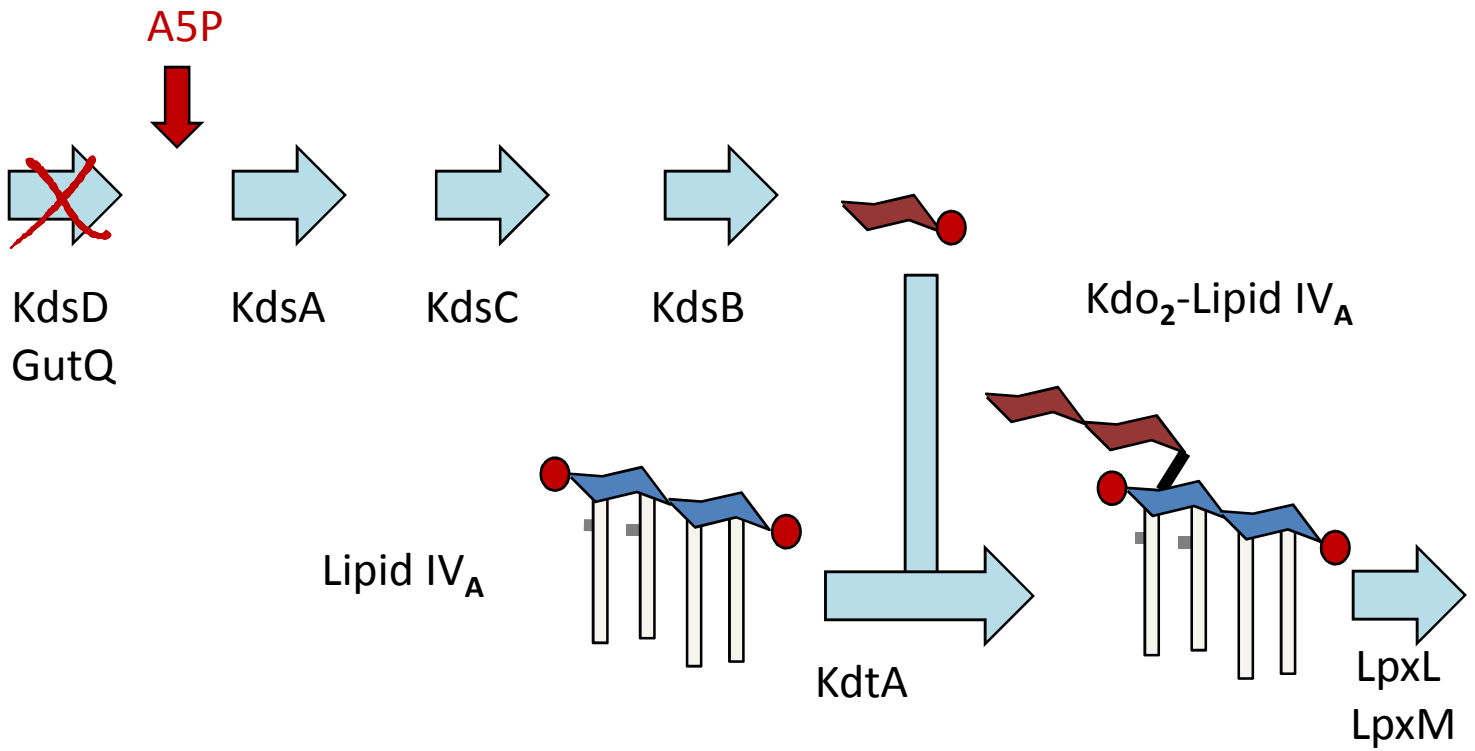
LpxL and LpxM Have an Absolute Requirement for Kdo_2 Substrate



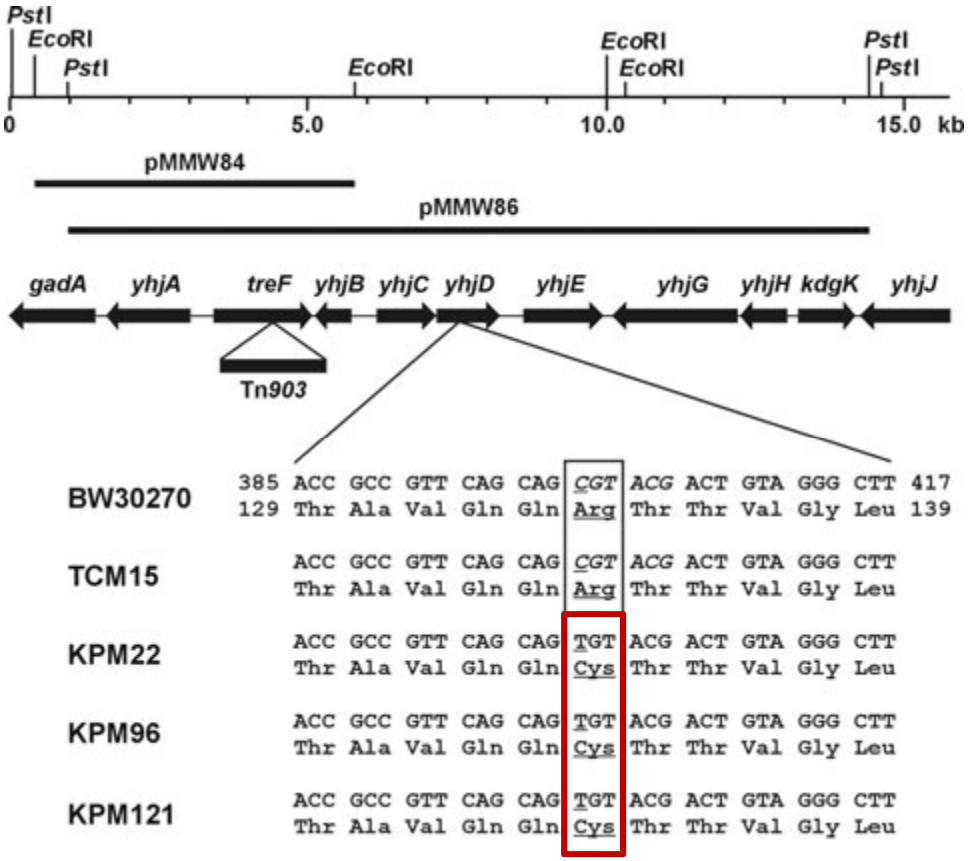
Any Block in Kdo Synthesis or Kdo_2-Lipid IV_A Formation Increases Lipid IV_A Levels



Addition of A5P Restores Kdo Synthesis in *kdsD gutQ* Mutants

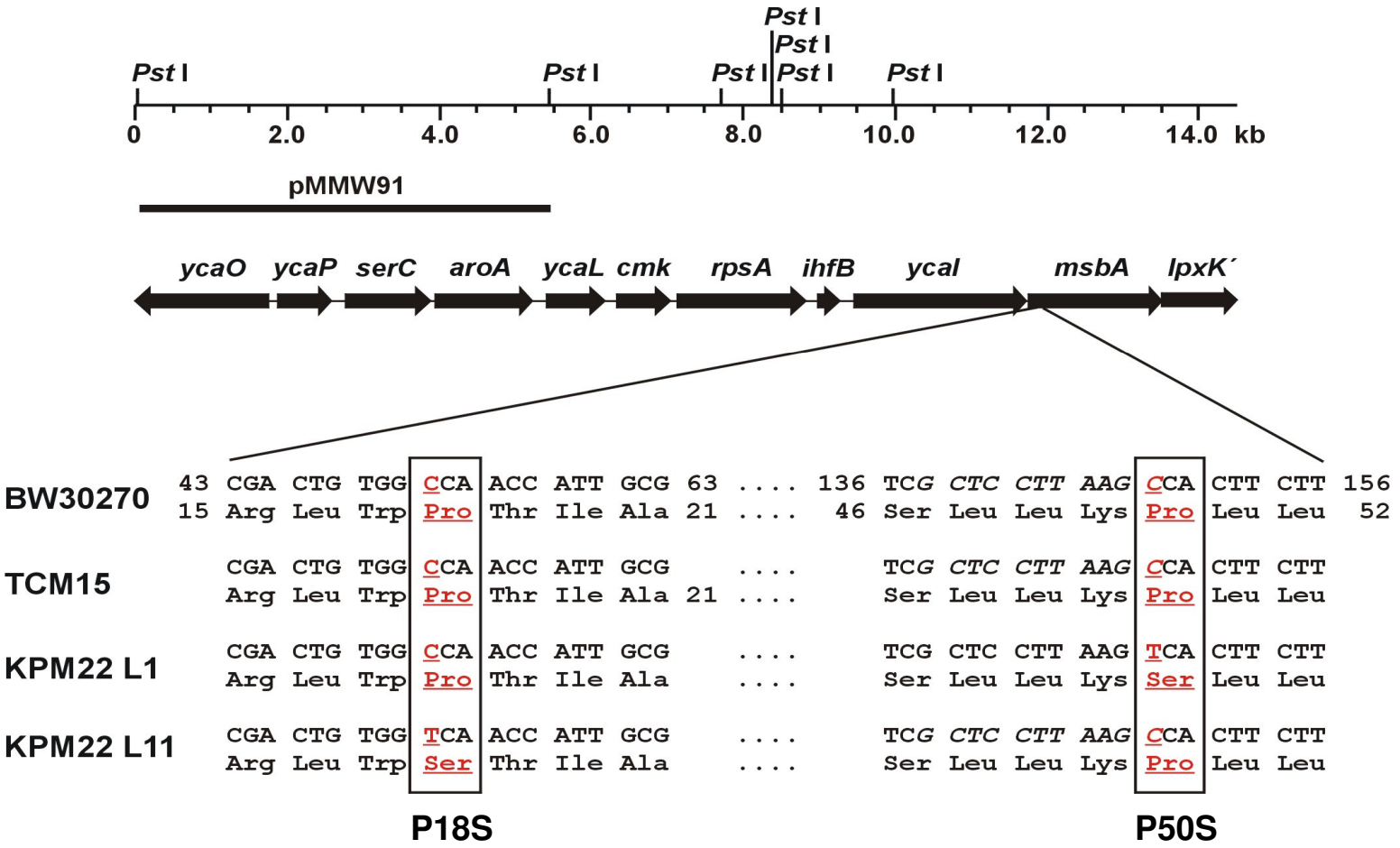


Suppressors in Gene *yhjD* Identified



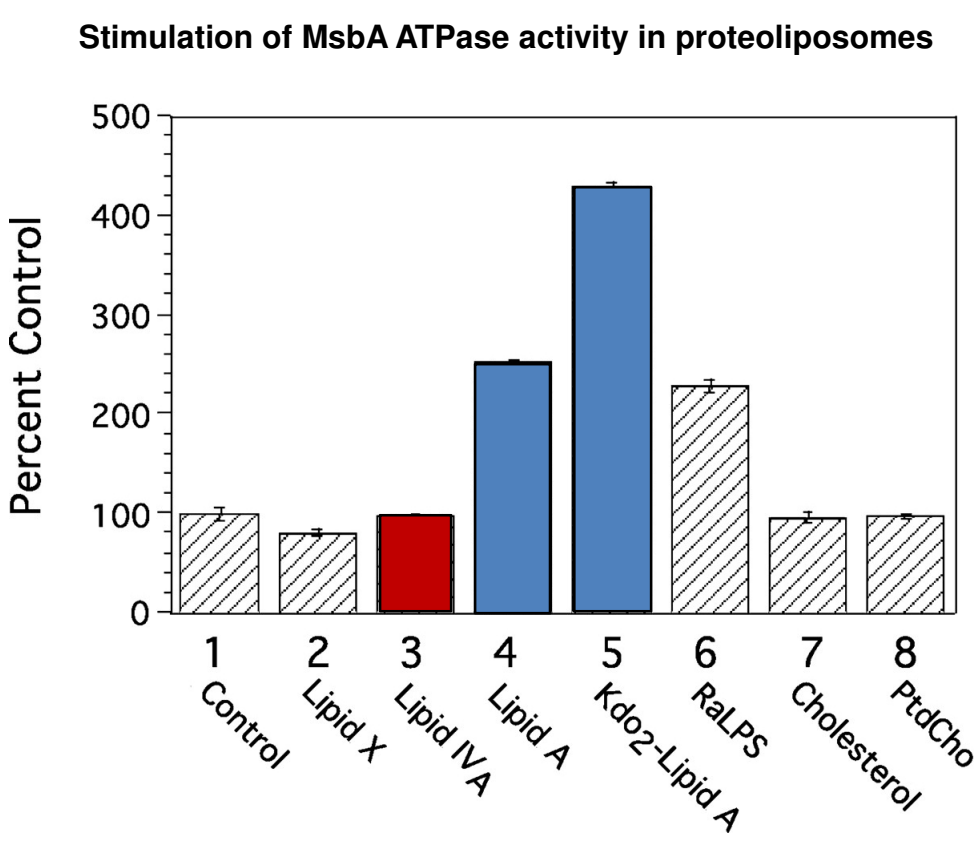
Selected by "weaning" *ΔgutQ ΔkdsD* off A5P
Gain of function: Can't delete *yhjD* as suppressor

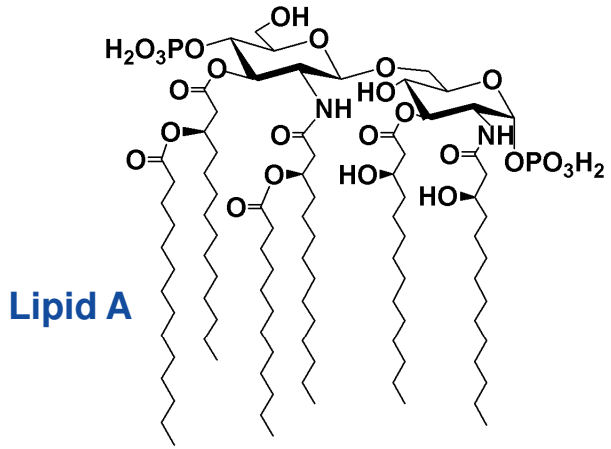
Also *msbA* Suppressors in KPM22-like Mutants

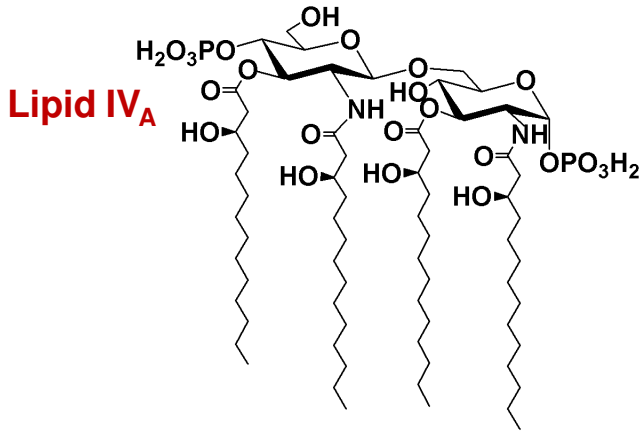


Mamat, U. *et al.* (2008) *Mol Microbiol* 67: 633-648.

The Essential Transporter MsbA is Highly Selective for Hexa-Acylated Lipid A/LPS

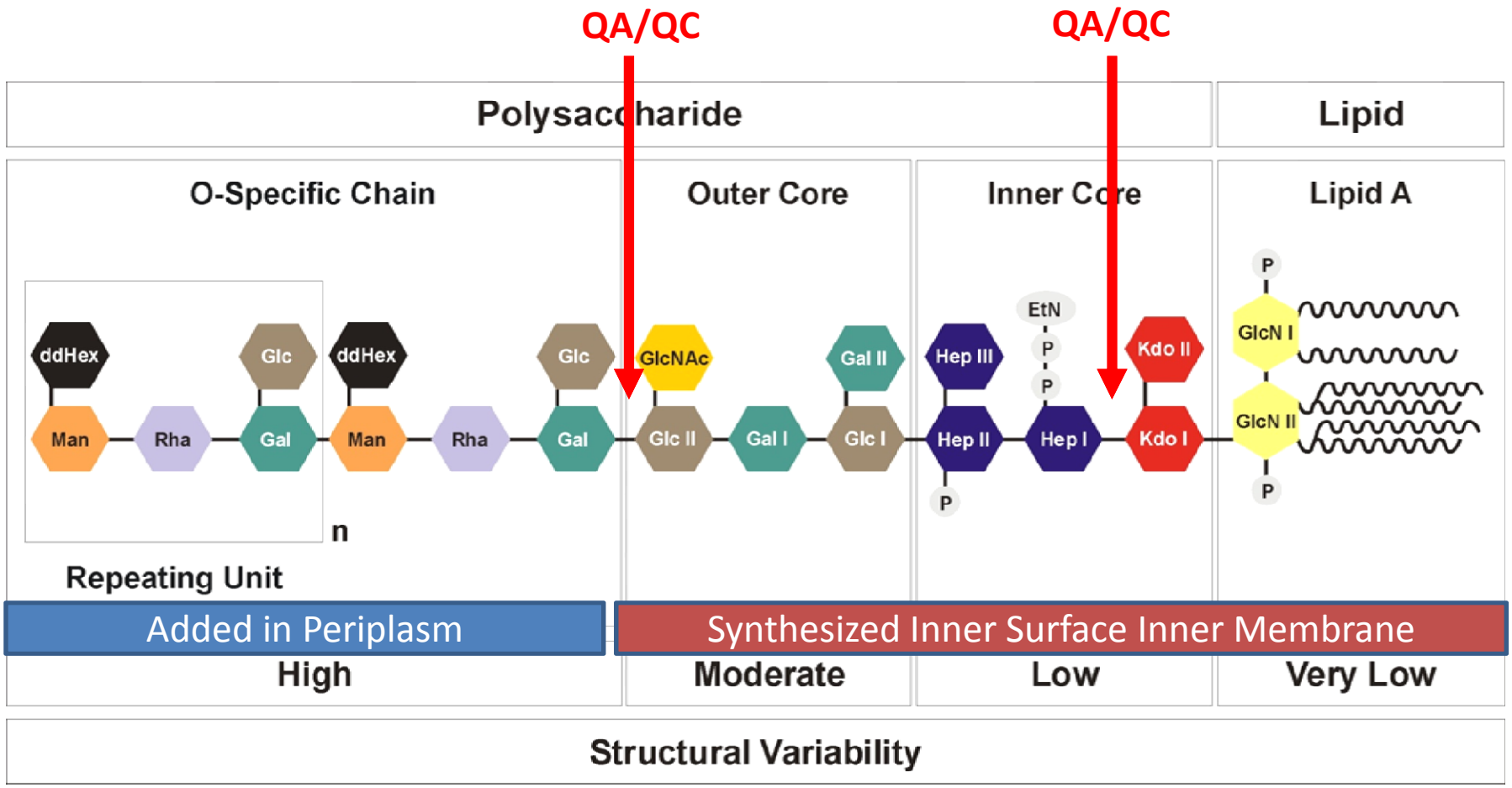




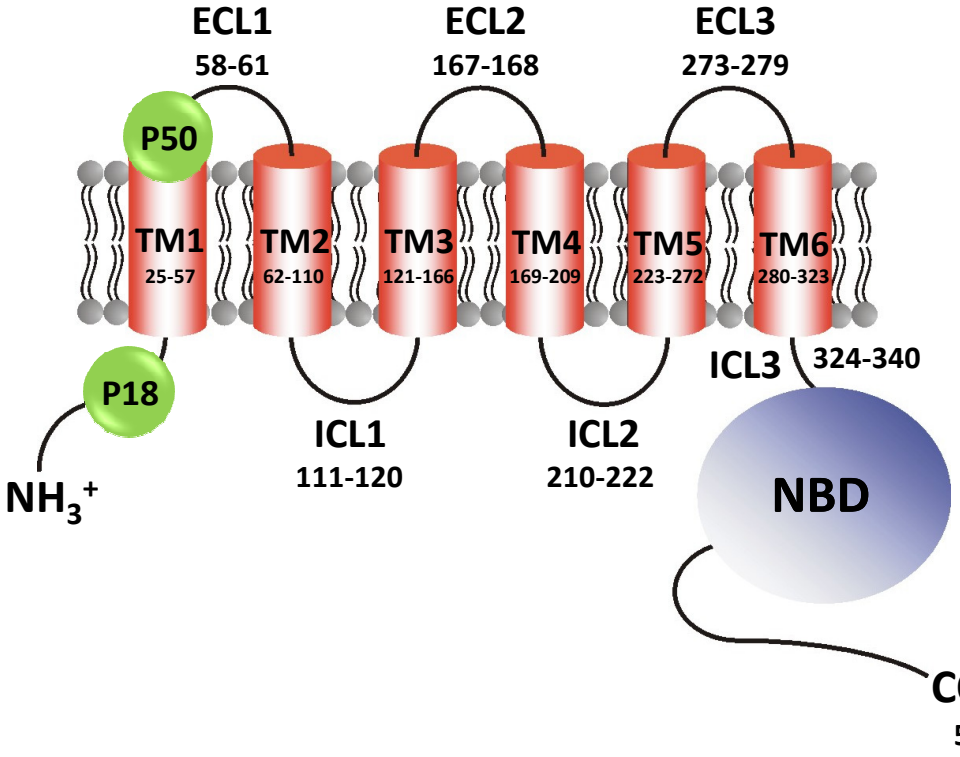


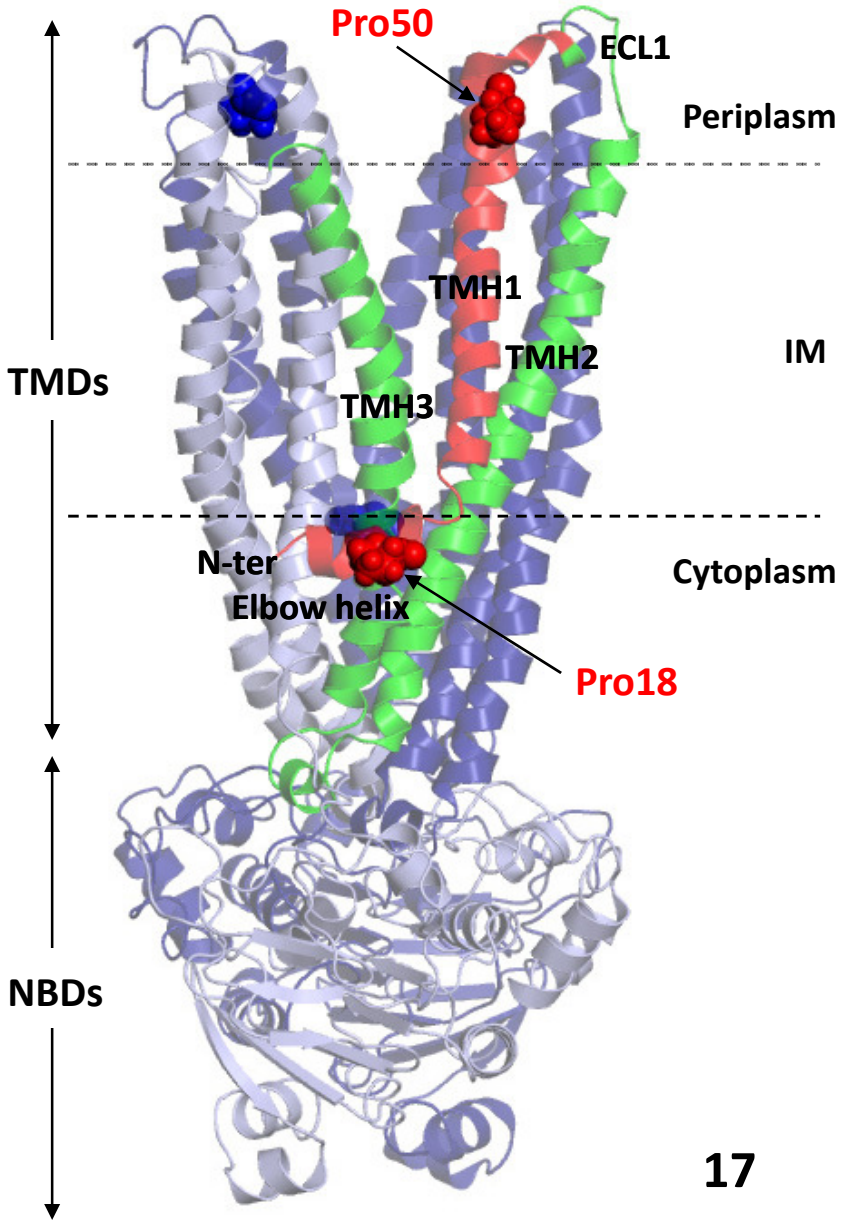
Doerrler, W.T. and Raetz, C.R.H. (2002) *J Biol Chem* 277: 36697-36705.

Schematic architecture of the lipopolysaccharide of Gram-negative bacteria



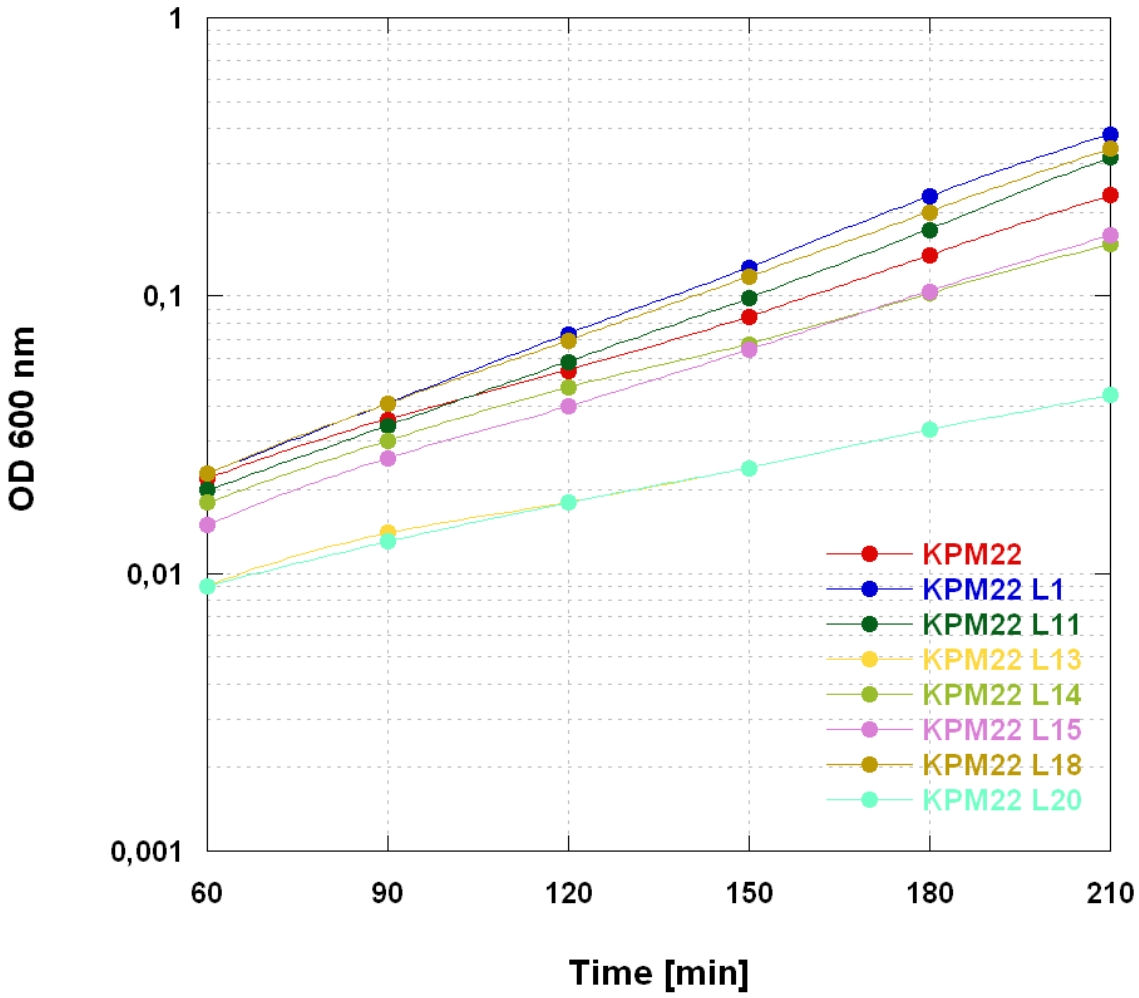
Location of single amino acid substitutions in MsbA suppressor proteins of FreE coli™ strains KPM22 L1 and KPM22 L11





Mamat, U. *et al.* (2008) *Mol Microbiol* 67: 633-648.

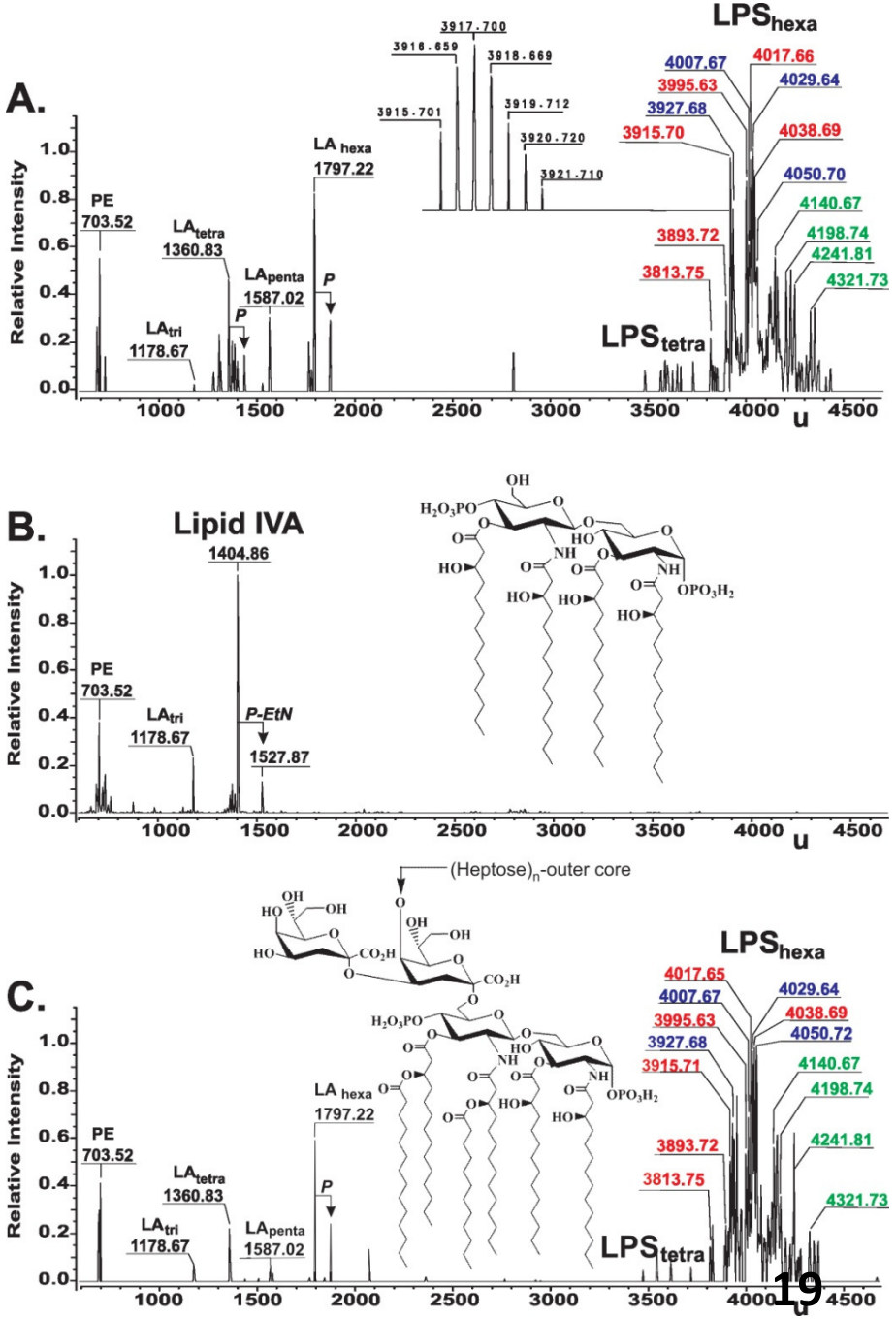
Growth of non-conditional Kdo-pathway mutants at 37°C in LB medium



E. coli strain	Doubling time [min]
BW30270 wt	24
KPM22	40
KPM22 L1	37
KPM22 L11	39
KPM22 L13	59
KPM22 L14	44
KPM22 L15	40
KPM22 L18	36
KPM22 L20	55

Charge deconvoluted electrospray ionization Fourier transform ion cyclotron (ESI FT-ICR) mass spectra in negative ion mode of purified LPS samples from *E. coli* BW30270 (A), and FreE coli™ strains KPM22 (B) and KPM25 (C)

The predominant LPS-related peak in KPM22 is the precursor lipid IV_A.



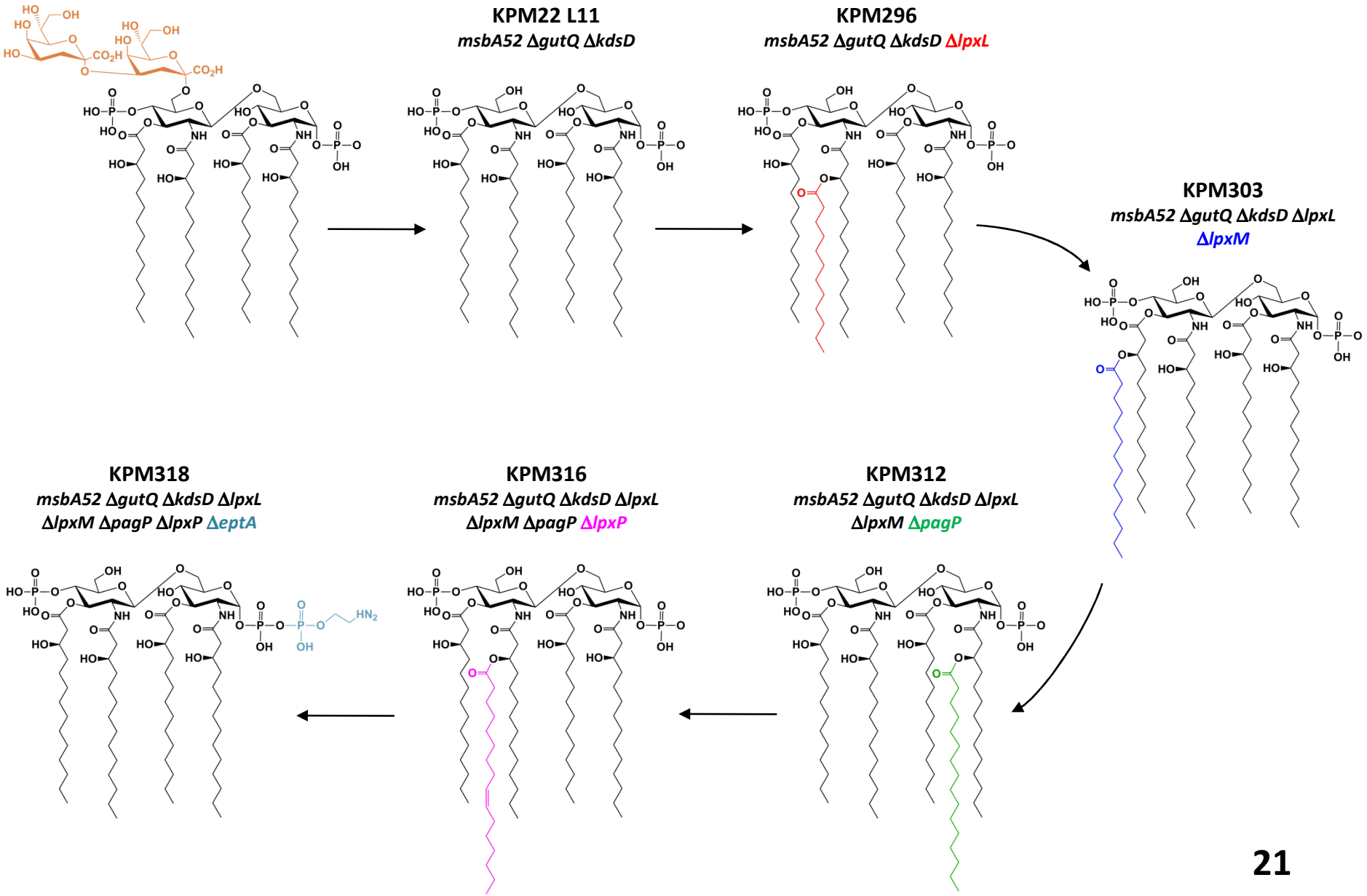
Meredith, T.C. *et al.* (2006) *ACS Chem Biol* 1: 33-42.

Prevent Regaining of LPS

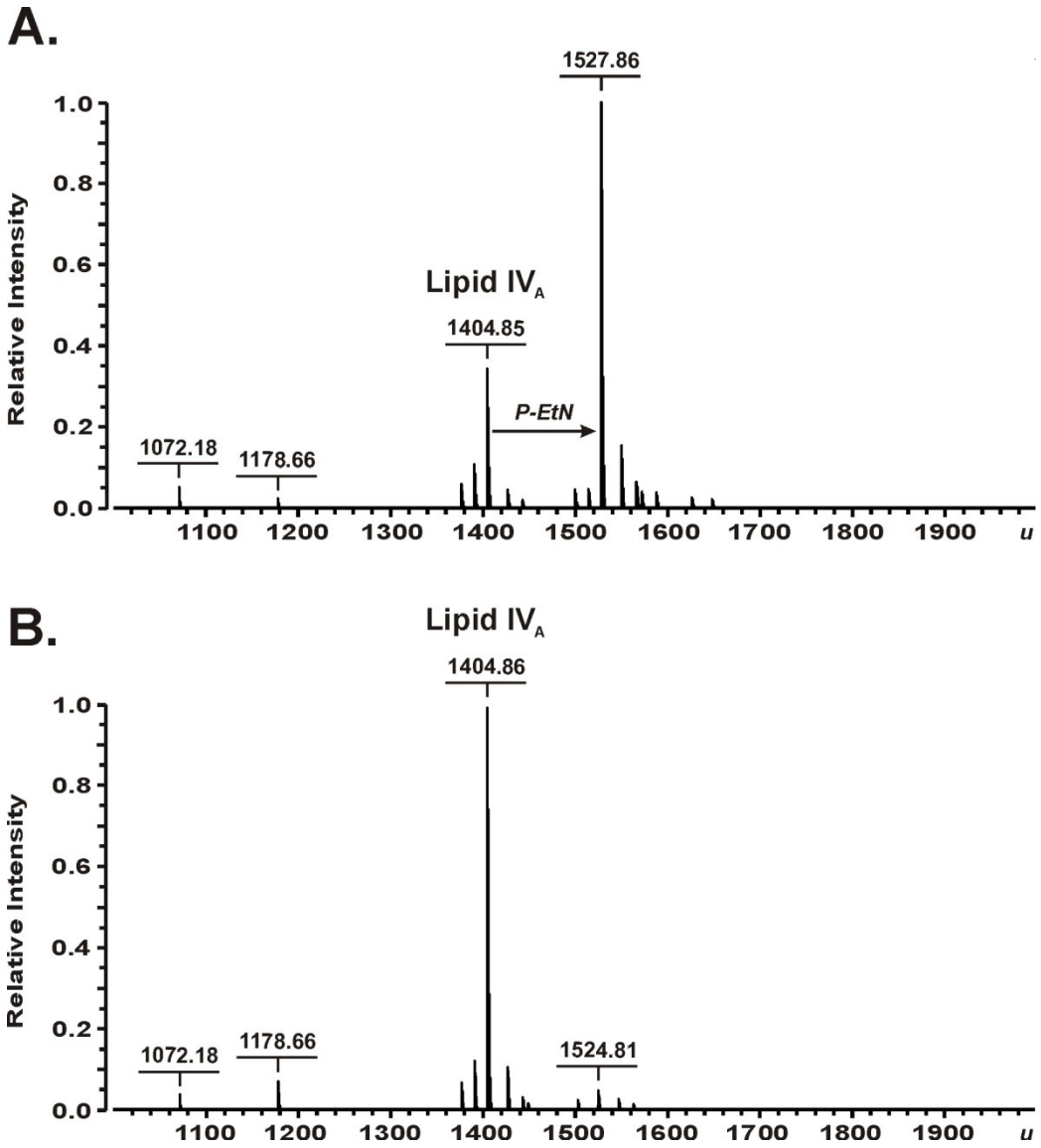
Construction of *E. coli* derivatives that

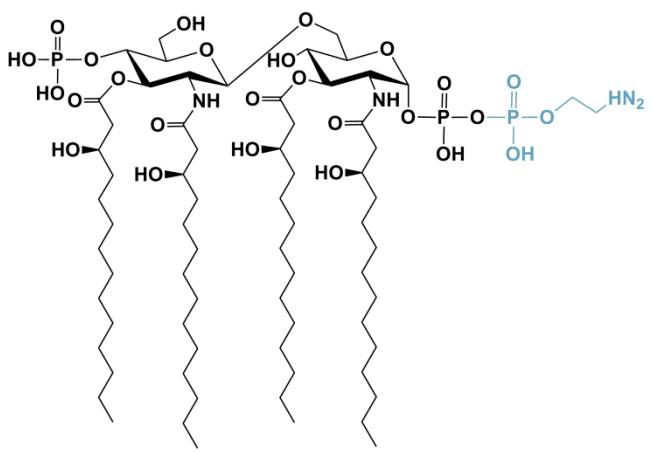
- **Entirely lack LPS**
- **Cannot regain the ability to synthesize active LPS**
- **Viable, able to grow exponetially**

Construction of FreE coli™ KPM318

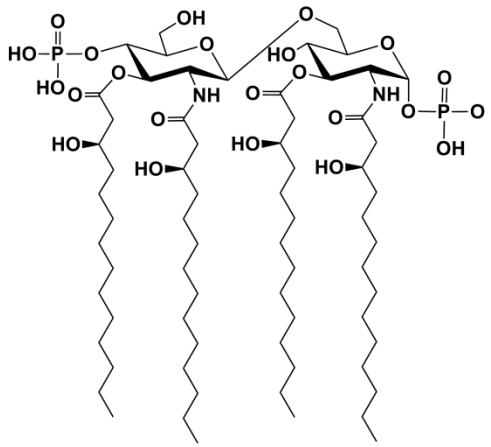


ESI FT-ICR mass spectra in negative ion mode of LPS samples isolated from FreE coli™ strains KPM316 (A) and KPM318 (B)





m = 1527.86 u



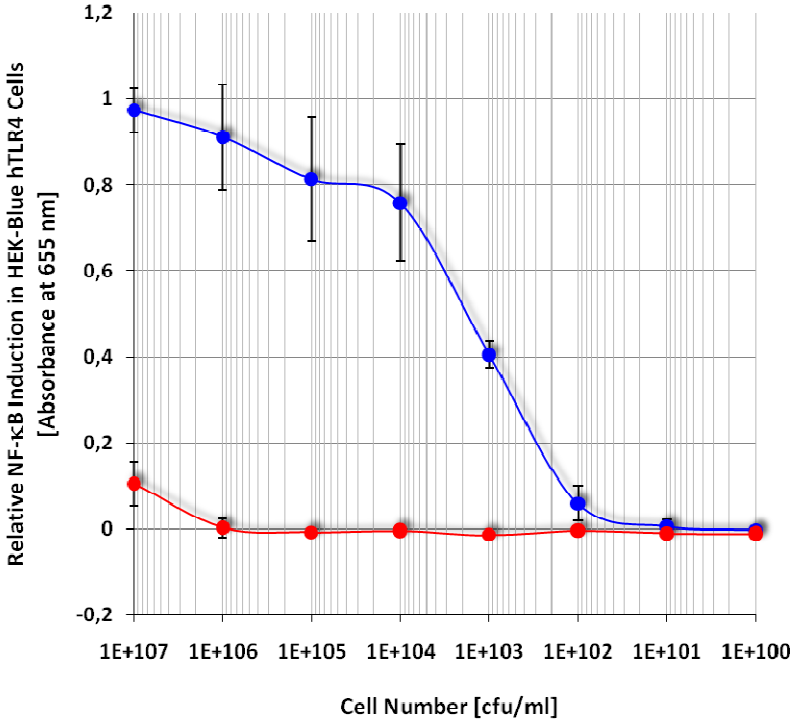
m = 1404.86 u

Human TLR4/MD2 Assays

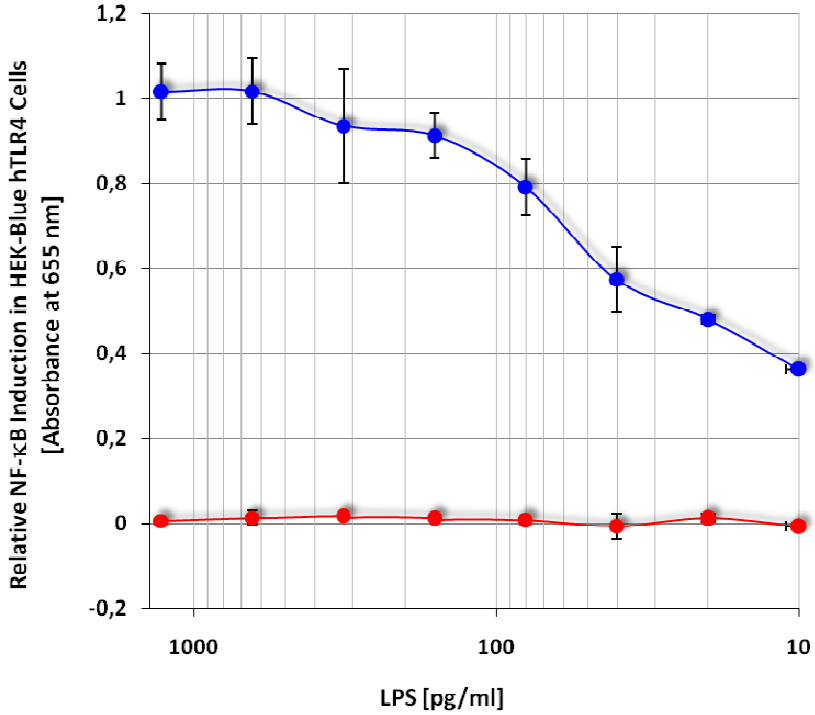
- HEK-Blue cell line based
  - Overexpressing TLR4/MD2
  - Phosphatase reporter gene linked to NFkappaB
- HEK line independently expressing TLR4/MD2
  - IL-8 secretion monitored
- Human macrophages – primary isolates from subjects
  - TNF-alpha production measured

The bacterial cells (A) and the LPS/lipid IV_A (B) of FreE coli™ KPM318 are virtually free of hTLR4/MD2-stimulating activity

A. Whole Bacterial Cells in Assay

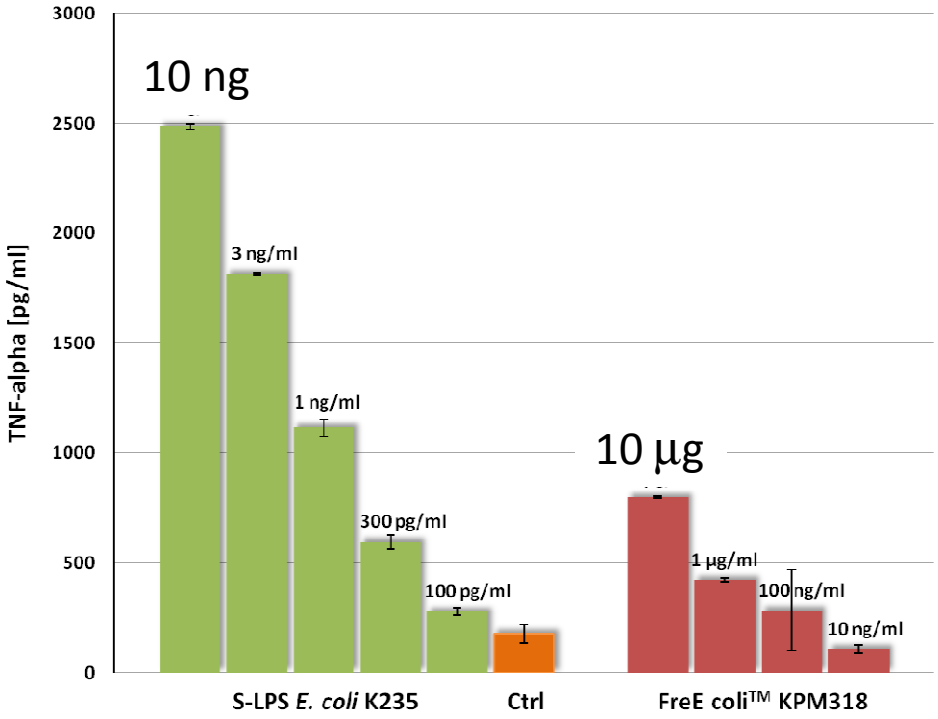


B. Outer Membrane Extracts in Assay

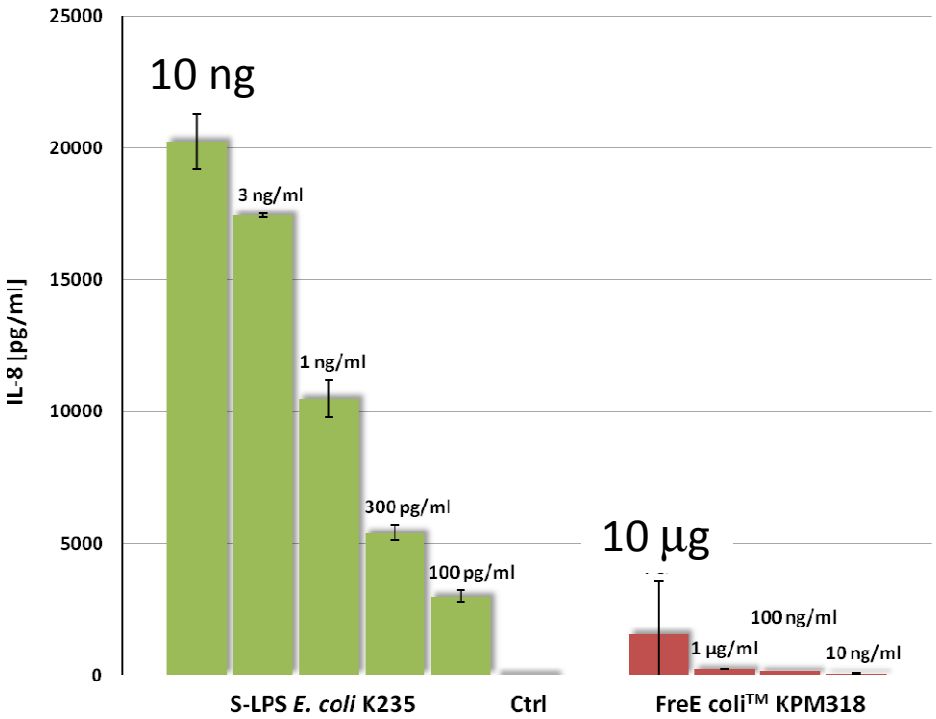


Stimulation of human macrophages and HEK293 hTLR4/MD2 #33 Cells by LPS/lipid IV_A of FreE coli™ KPM318

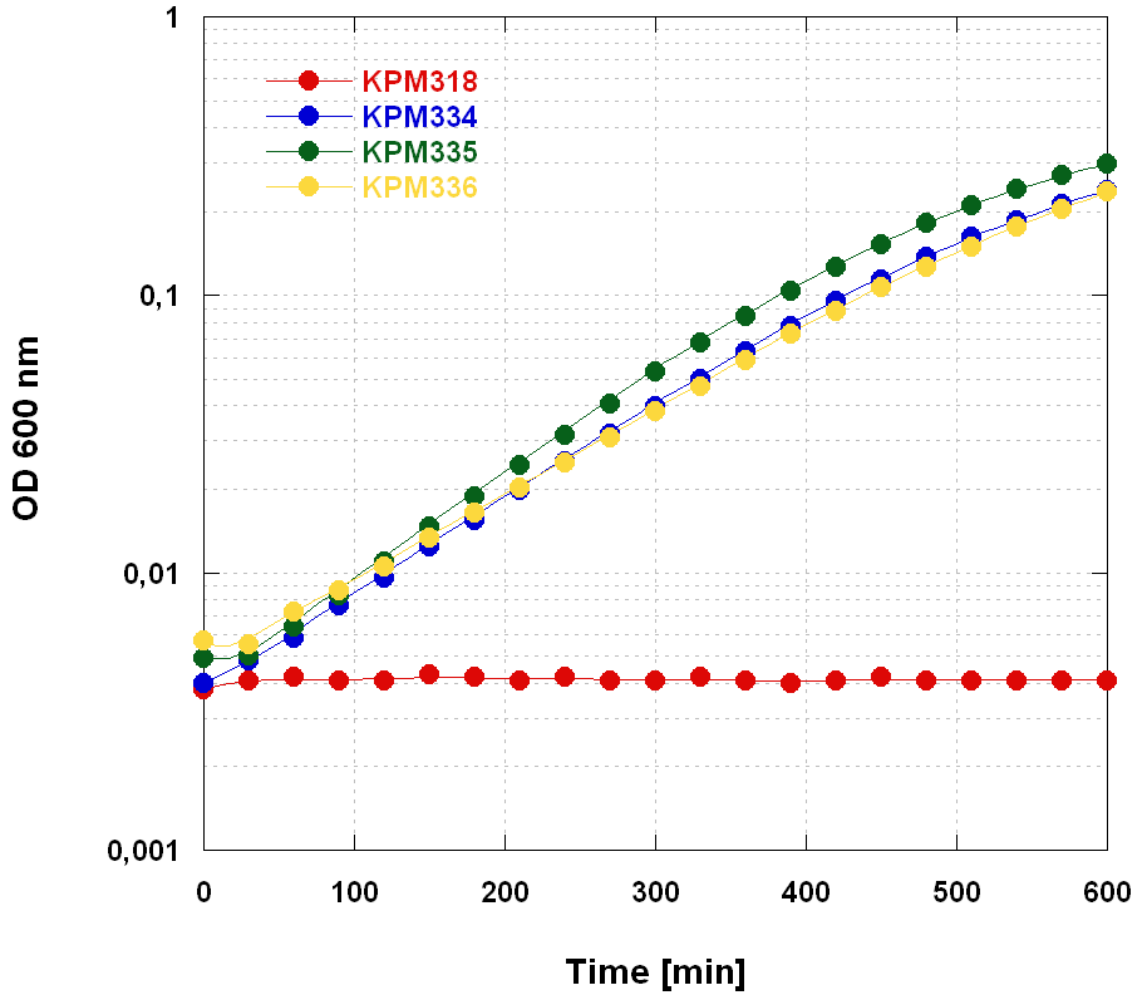
A. Macrophages



B. HEK293-hTLR4/MD-2

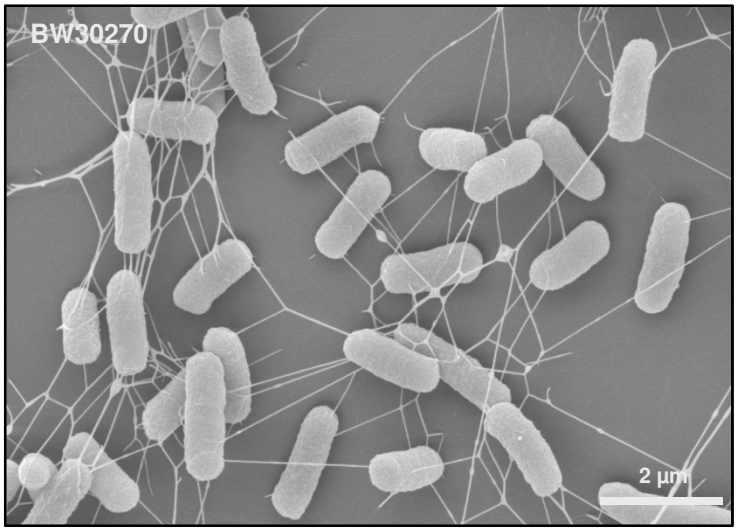


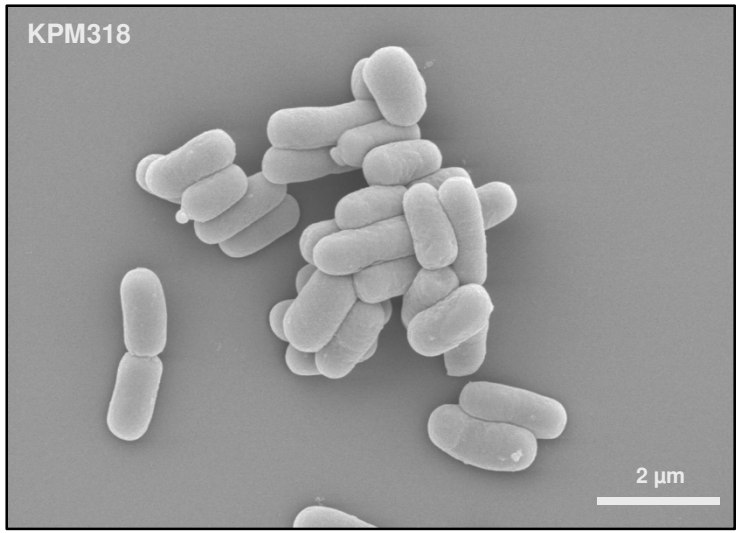
Growth of FreE coli™ KPM318 and temperature-resistant FreE coli™ KPM318 derivatives at 42°C in SB medium

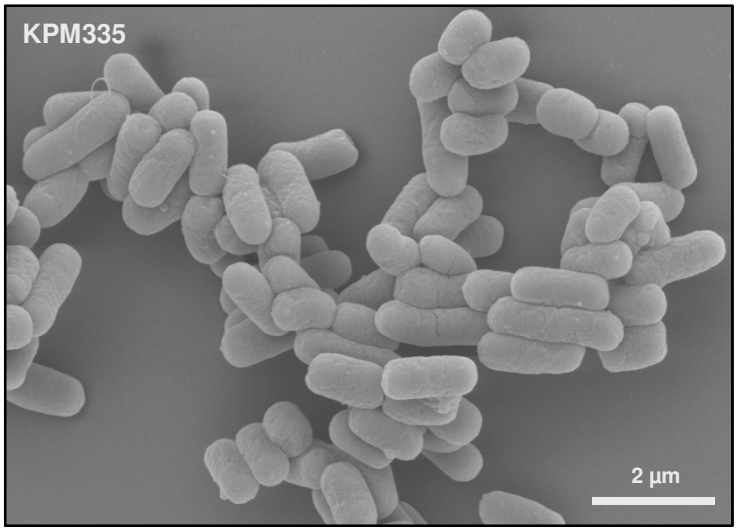


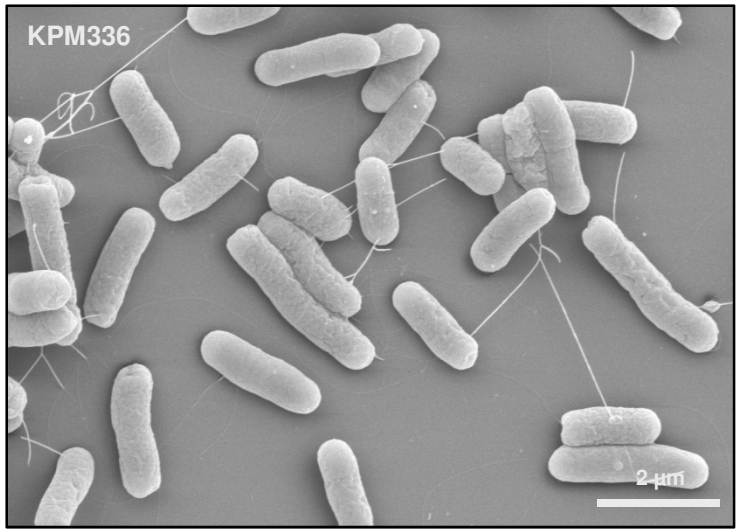
Strain	Doubling time [min]
KPM318	-
KPM334	93
KPM335	86
KPM336	100

Scanning electron micrographs of FreE coli™ KPM318 and temperature-resistant FreE coli™ KPM318 derivatives









Electron micrographs by Manfred Rohde, Helmholtz Center for Infection Research, Braunschweig, Germany.

Potential applications of the LPS-free strains:

Hosts for protein expression

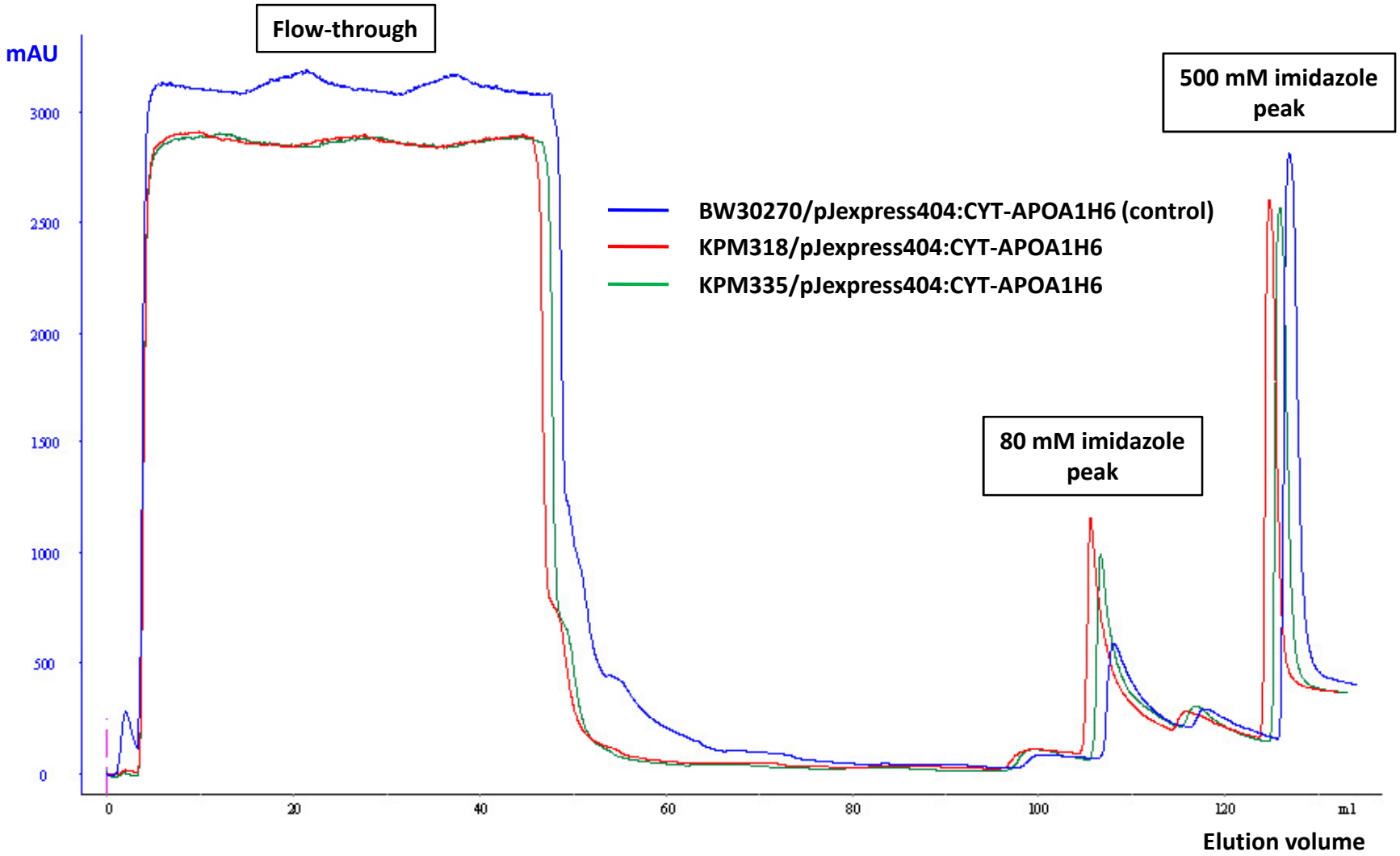
- **research e.g. variant screening, phage display output,**
- **manufacturing, reducing DSP costs**

Hosts for plasmid DNA preparation

- **research e.g. for transient transfection of HEK293 cells**
- **production of DNA vaccine**

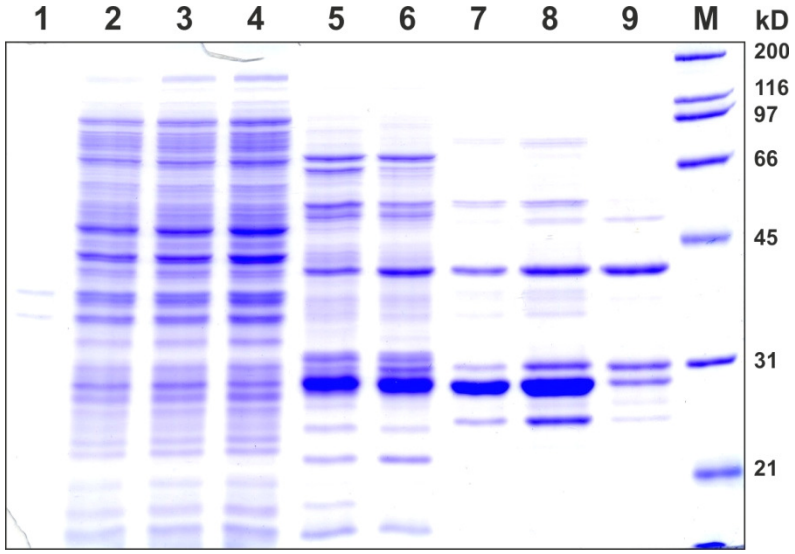
Modify Gram negative pathogens to be LPS-free

FreE coli™ strains KPM318 and KPM335 as hosts for endotoxin-free protein production: Expression and purification ApoA1

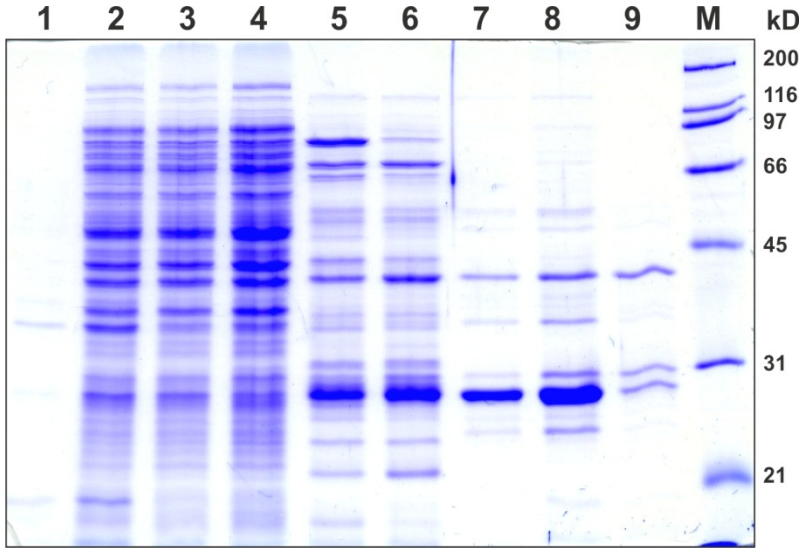


IMAC on HisTrap HP (1 ml) columns

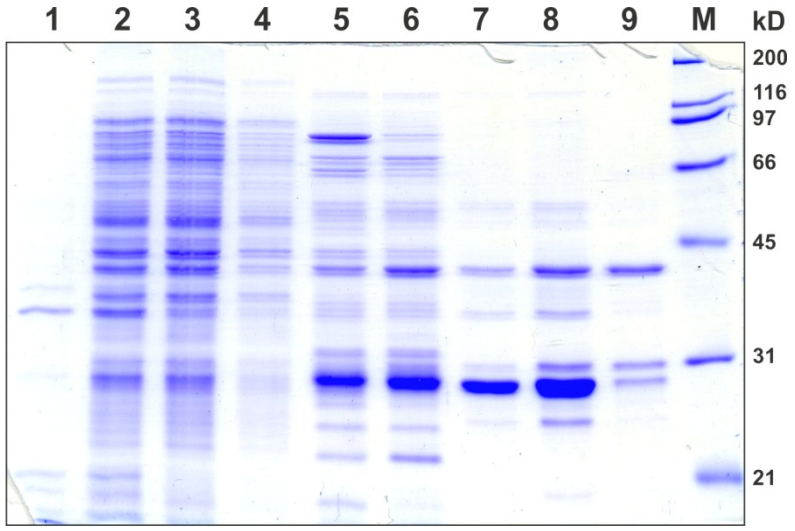
FreE coli™ strains KPM318 and KPM335 as hosts for endotoxin-free protein production: Expression and purification ApoA1



BW30270/pJexpress404:CYT-APOA1H6 (control)



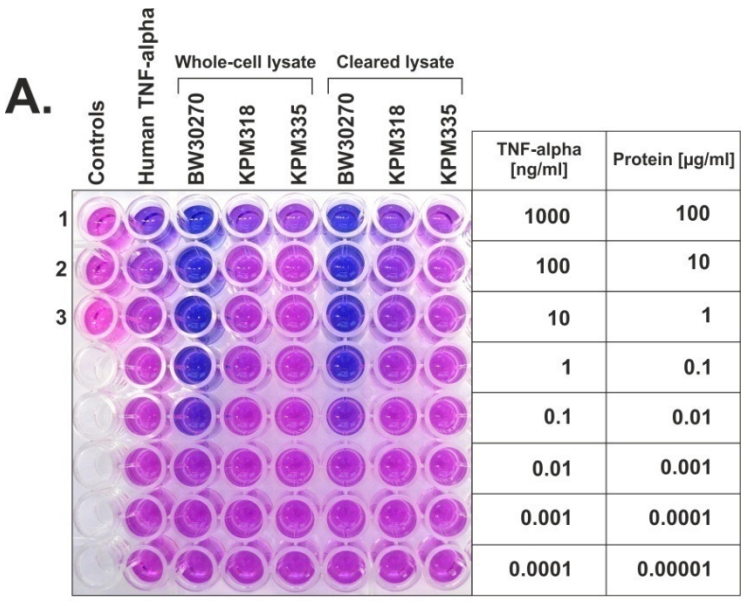
KPM318/pJexpress404:CYT-APOA1H6



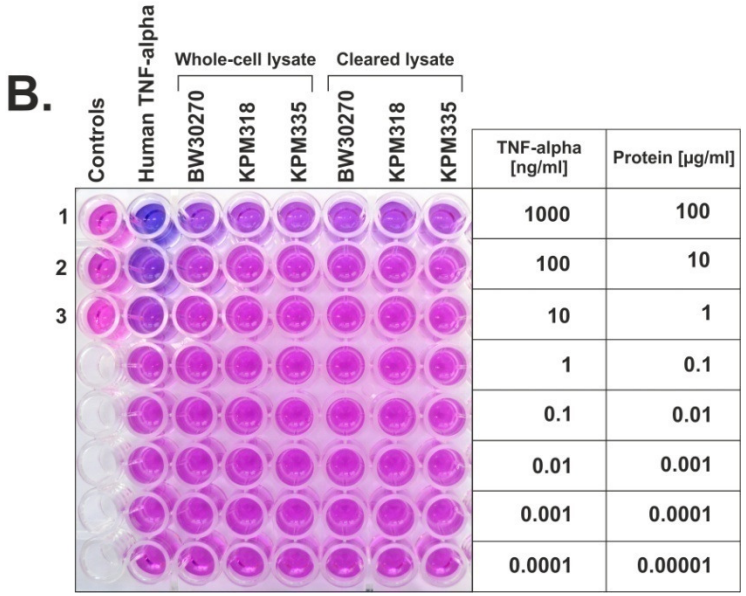
KPM335/pJexpress404:CYT-APOA1H6

1. Insoluble cell fraction
2. Whole-cell lysate
3. Cleared cell lysate
4. IMAC flow-through
5. IMAC 80 mM imidazole peak
6. IMAC 80 mM imidazole peak
7. IMAC 500mM imidazole peak
8. IMAC 500mM imidazole peak
9. IMAC 500mM imidazole peak

Expression and purification of ApoA1: Stimulation of HEK-Blue™ hTLR4 (A) and Null2 (B) cells by protein samples of BW30270/pJexpress404:CYT-APOA1H6 and FreE coli™/pJexpress404:CYT-APOA1H6 strains



TNF-alpha [ng/ml]	Protein [µg/ml]
1000	100
100	10
10	1
1	0.1
0.1	0.01
0.01	0.001
0.001	0.0001
0.0001	0.00001



TNF-alpha [ng/ml]	Protein [µg/ml]
1000	100
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0.1	0.01
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0.001	0.0001
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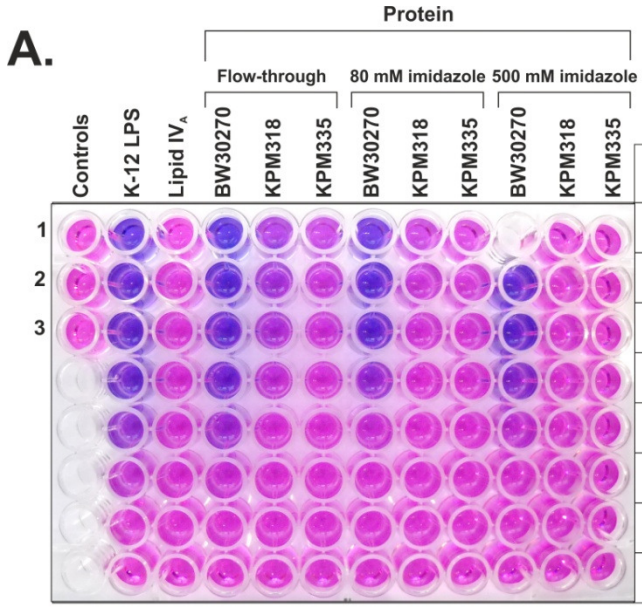
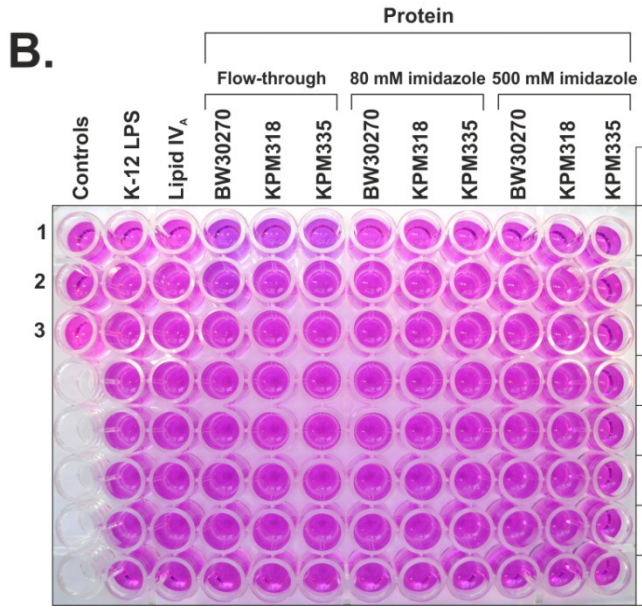
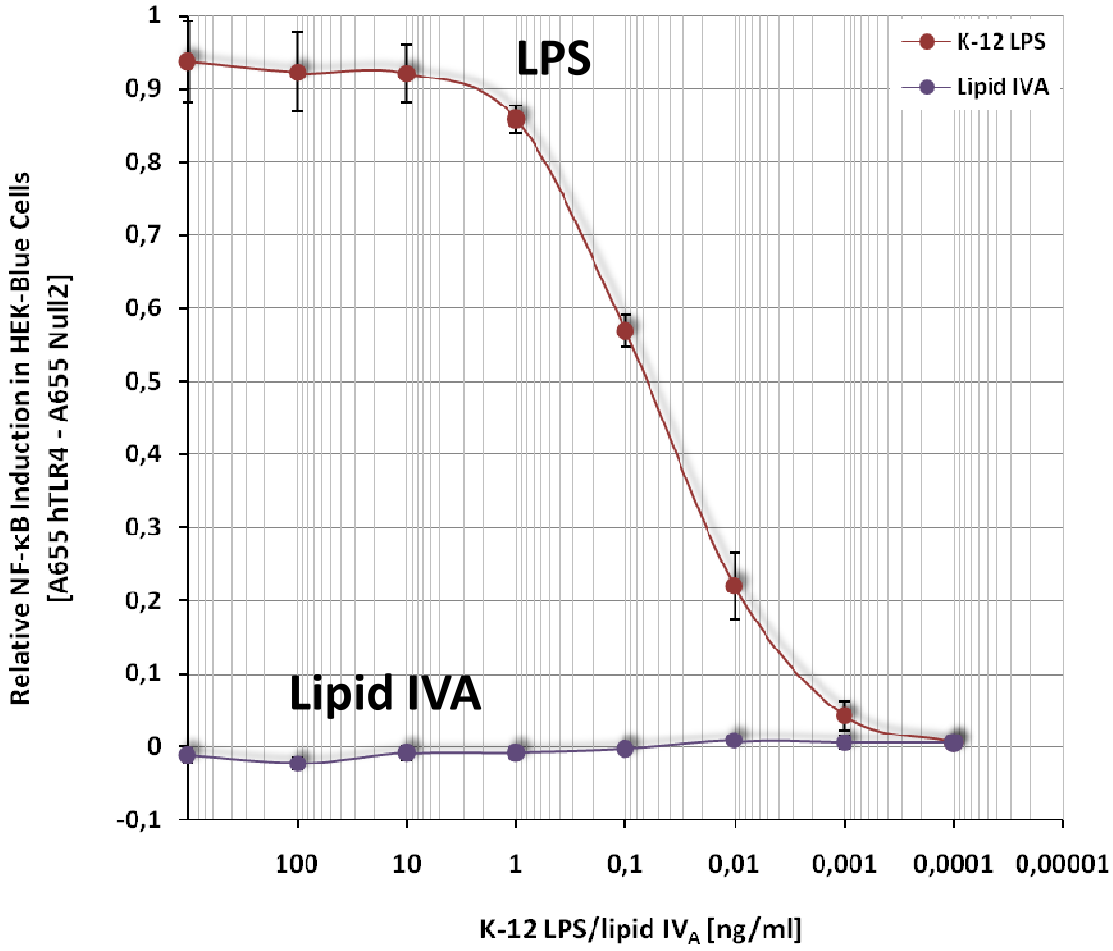


LPS/lipid IV_A [ng/ml]	Protein [µg/ml]
1000	100
100	10
10	1
1	0.1
0.1	0.01
0.01	0.001
0.001	0.0001
0.0001	0.00001

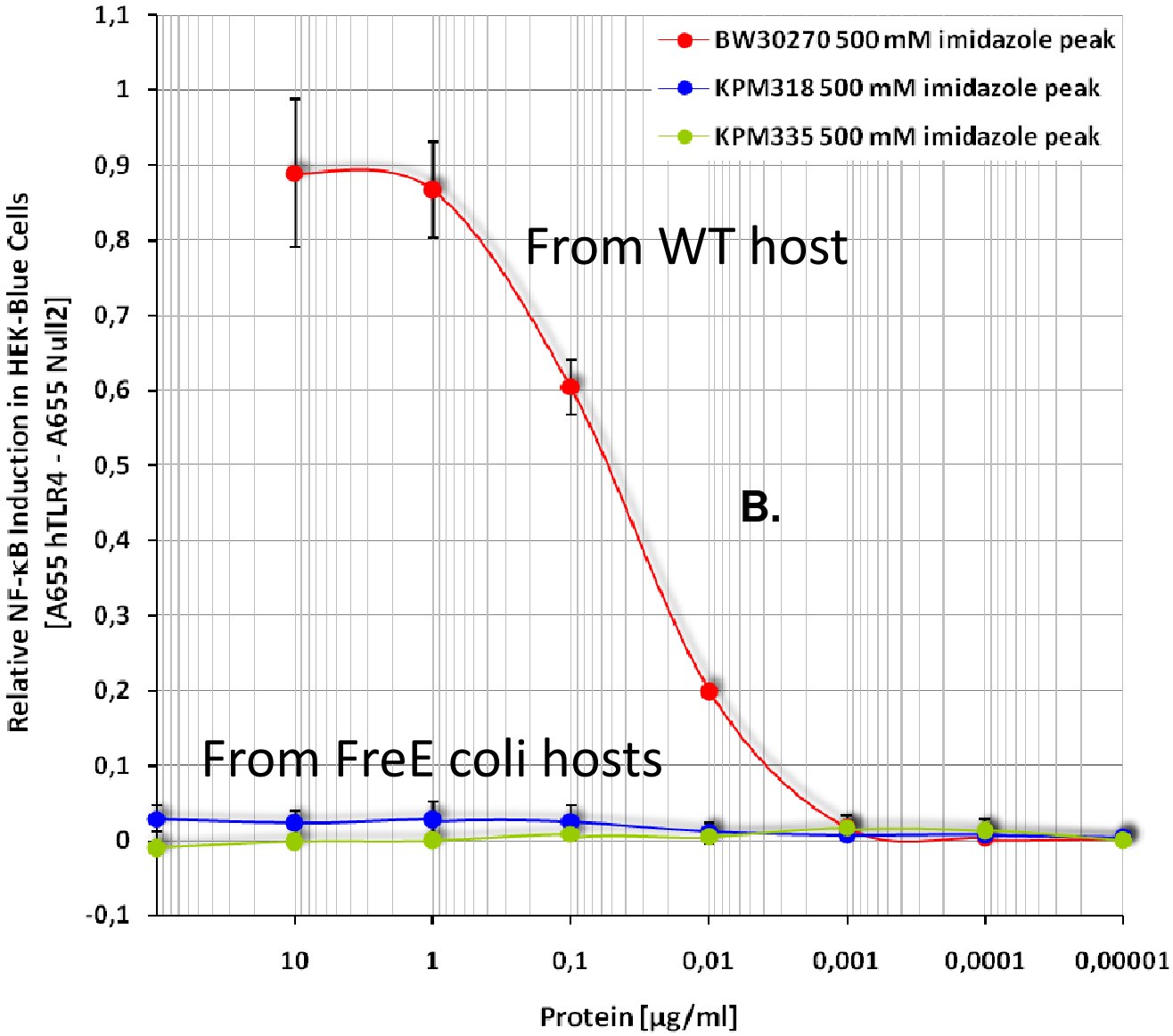


LPS/lipid IV_A [ng/ml]	Protein [µg/ml]
1000	100
100	10
10	1
1	0.1
0.1	0.01
0.01	0.001
0.001	0.0001
0.0001	0.00001

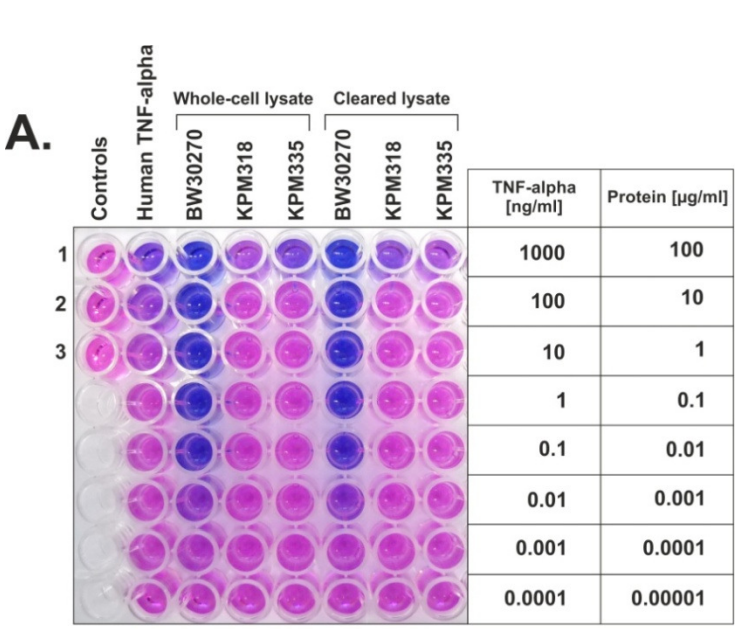
LPS and Lipid IVA in HEK-Blue TLR4/MD-2 Assay



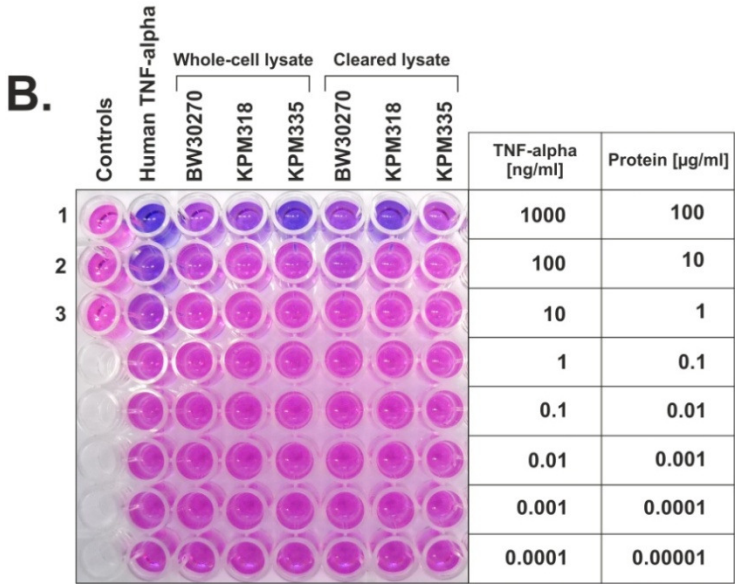
NF-κB Induction in HEK-Blue™ hTLR4/MD2 Assay by Purified ApoA1



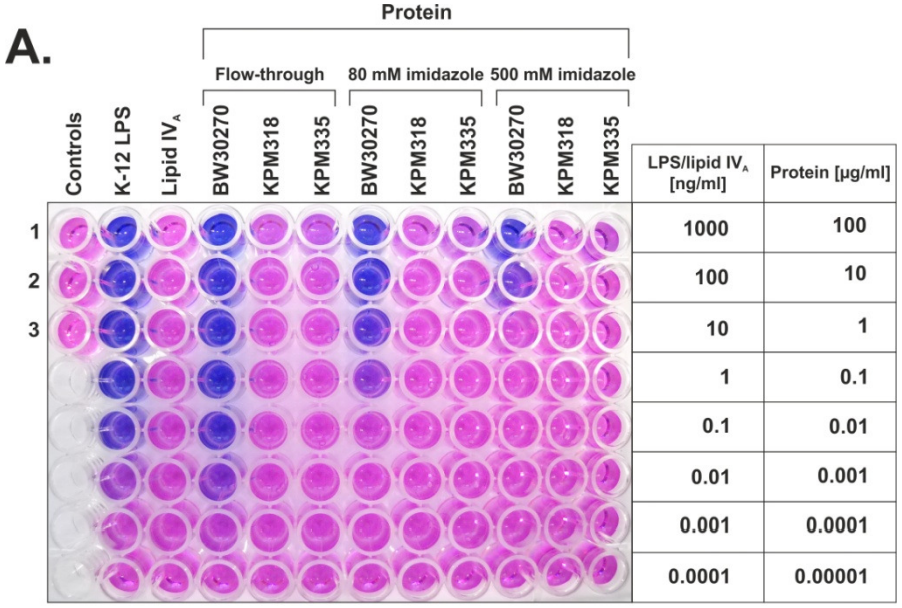
Expression and purification of VHm36: Stimulation of HEK-Blue™ hTLR4 (A) and Null2 (B) cells by protein samples of BW30270/pJexpress404:CYT-VHM36H6 and FreE coli™/pJexpress404:CYT-VHM36H6 strains



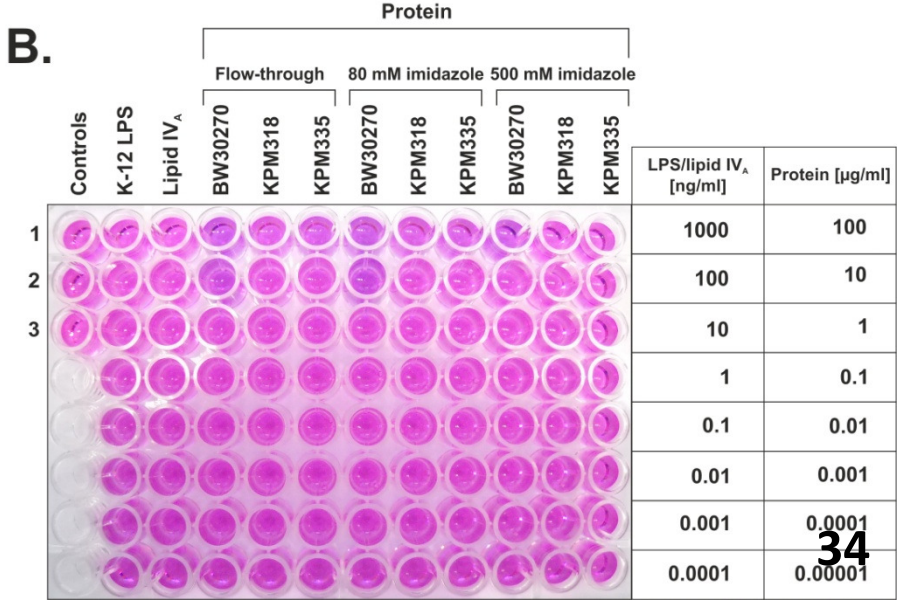
TNF-alpha [ng/ml]	Protein [µg/ml]
1000	100
100	10
10	1
1	0.1
0.1	0.01
0.01	0.001
0.001	0.0001
0.0001	0.00001



TNF-alpha [ng/ml]	Protein [µg/ml]
1000	100
100	10
10	1
1	0.1
0.1	0.01
0.01	0.001
0.001	0.0001
0.0001	0.00001

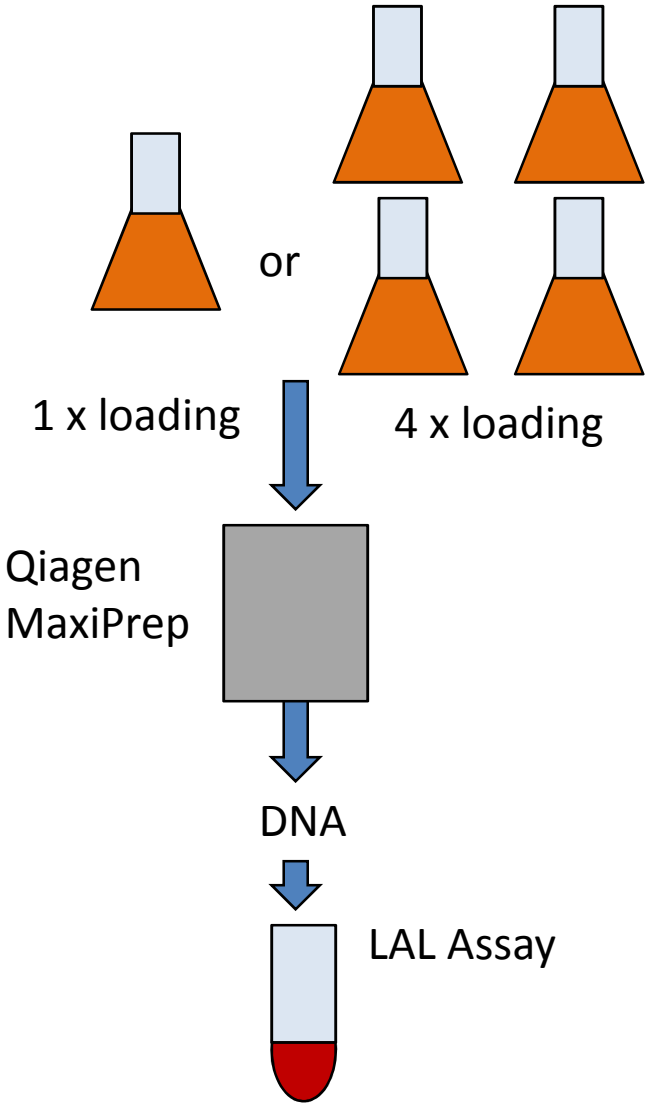


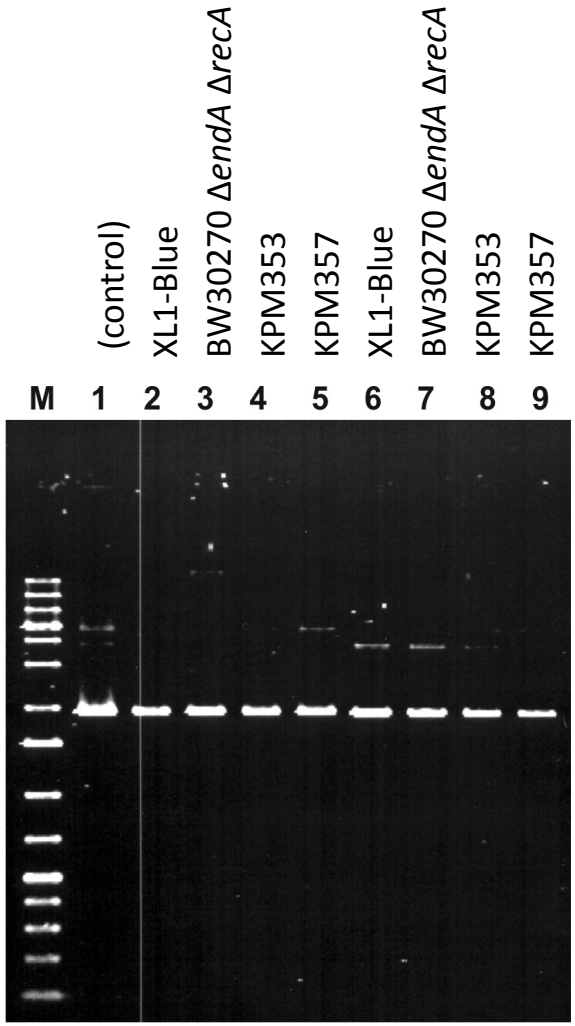
LPS/lipid IV_A [ng/ml]	Protein [µg/ml]
1000	100
100	10
10	1
1	0.1
0.1	0.01
0.01	0.001
0.001	0.0001
0.0001	0.00001



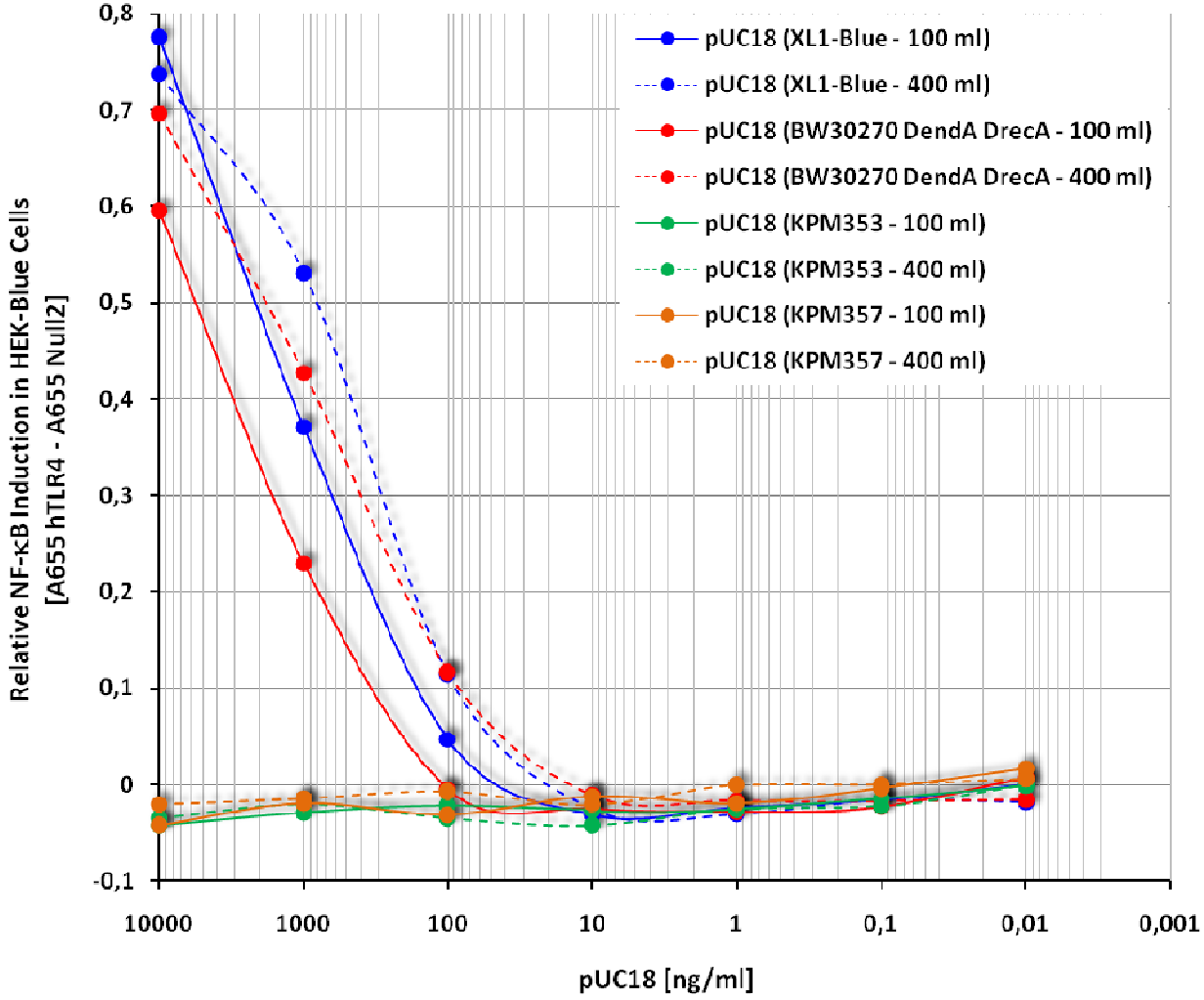
LPS/lipid IV_A [ng/ml]	Protein [µg/ml]
1000	100
100	10
10	1
1	0.1
0.1	0.01
0.01	0.001
0.001	0.0001
0.0001	0.00001

pUC18 Plasmid Isolated from Δ*endA* Δ*recA* FreE coli™ Strains



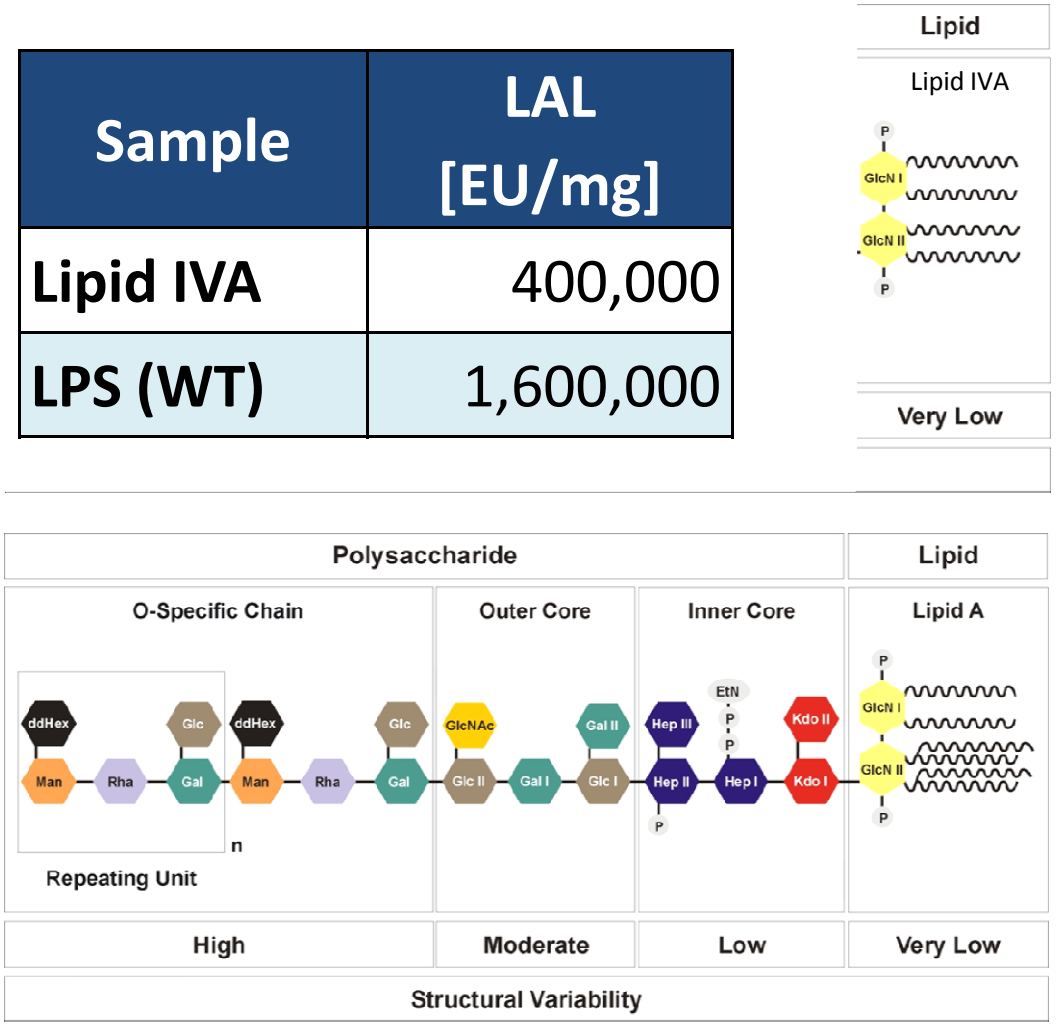


Stimulation of HEK-Blue™ hTLR4 cells by pUC18 plasmid DNA samples isolated from Δ*endA* Δ*recA* derivatives of FreE coli™ strains



What about Horseshoe Crabs?
LAL Assay Activities of LPS and Lipid IVA

Sample	LAL [EU/mg]
Lipid IVA	400,000
LPS (WT)	1,600,000



LAL Assay Activities of Protein Samples

Host Strain	Protein	Protein conc [mg/ml]	Protein yield [mg]	Total EU Sample	EU/mg protein
BW30270	VHm36	5.2	10.3	150.0	15.00
KPM335	VHm36	1.8	3.6	1.6	0.43
BW30270	ApoA1	1.2	2.4	550.0	225.00
KPM335	ApoA1	2.0	4.1	230.0	57.00

LAL Activities of DNA Samples

Strain	Cult vol [ml]	DNA [mg/ml]	DNA yield [µg]	Endotox [EU/ml]	Total EU in prep	EU/mg DNA
XL1-Blue	100	0.86	430	290	140	340
XL1-Blue	400	1.4	700	2,800	1,400	2,000
BW30270 Δ(*endArecA*)	100	0.47	240	150	75	320
BW30270 Δ(*endArecA*)	400	0.81	410	870	430	1,100
KPM353	100	0.37	190	0.11	0.055	0.29
KPM353	400	0.59	290	48	24	83

Summary

- The FreE coli™ strains are viable
- Outer membrane is predominantly lipid IV_A
- Inactivation of seven lipid A-related genes in FreE coli™ strains precludes synthesis of
  - Normal LPS
  - Other TLR4-active lipid IV_A derivatives
- Lipid IV_A activity in LAL is significant
- BUT lipid IV_A is readily removed by single column
- The FreE coli™ strains hold promise for manufacturing of endotoxin-free proteins and plasmid DNA.

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