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Role of Polyamine Biosynthesis and Transport in *Streptococcus pneumoniae* Pathogenesis, Physiology and Vaccine Design

Pratik Shah

March 31st 2009

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Characterization of a *S. pneumoniae* surface protein as a vaccine antigen

Contribution of pneumococcal polyamine biosynthesis and transport to it's physiology and pathogenesis

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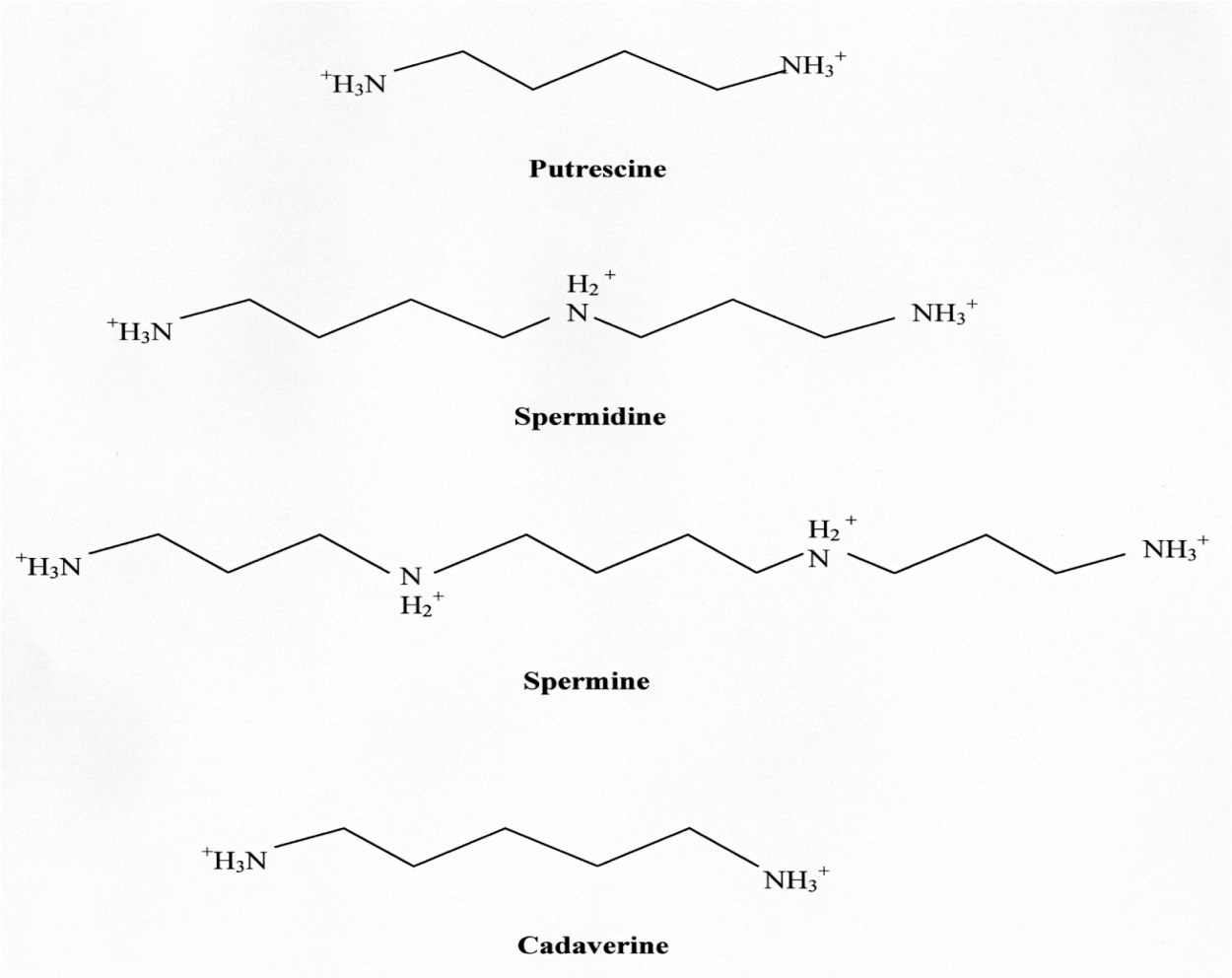
Polyamines



Structures of microbial polyamines

Shah P and E. Swiatlo Mol. Micro.(2008) **68** (1), 4-16

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Sources of bacterial polyamines

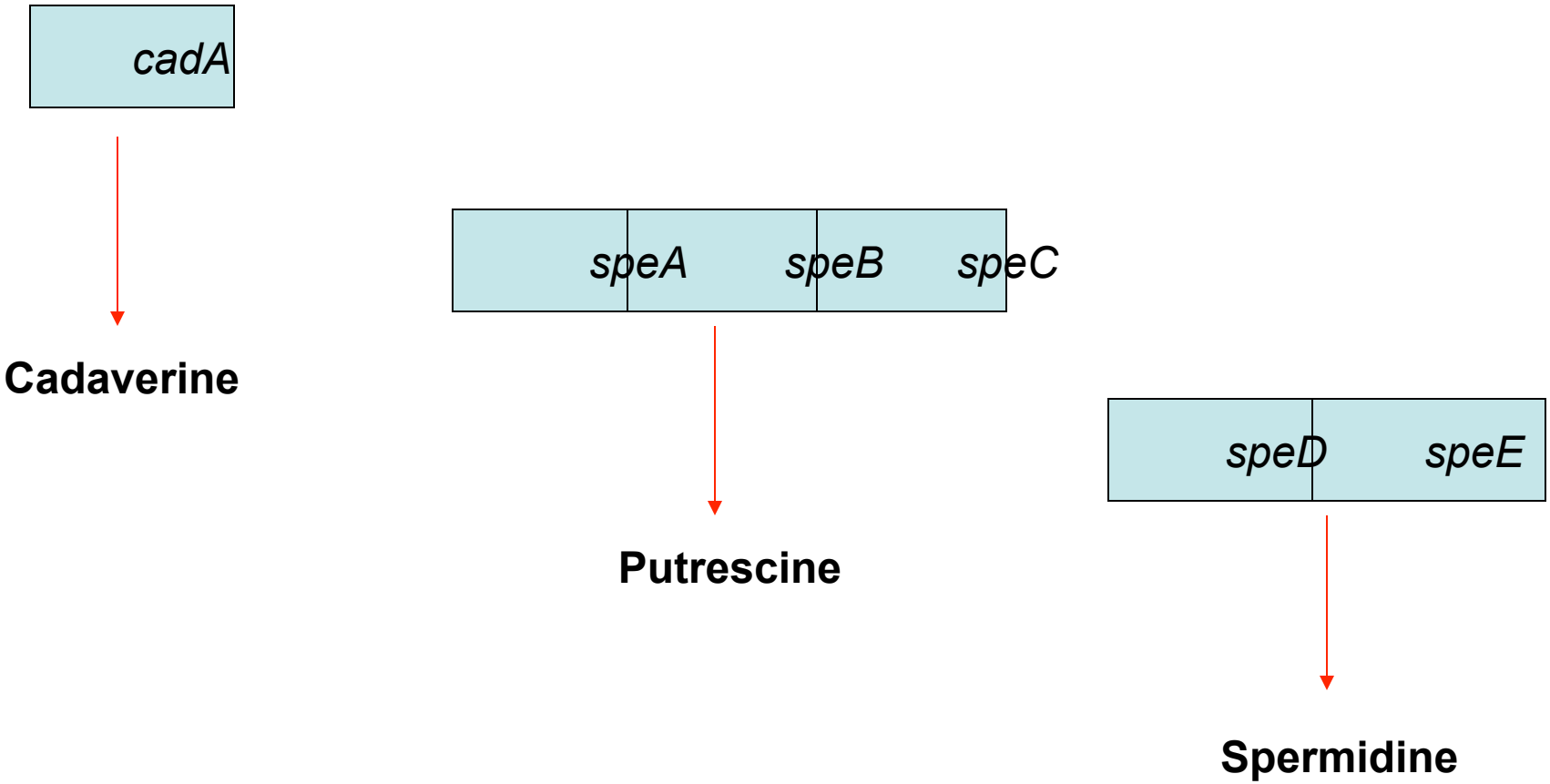
- ***De novo* synthesis**

- Enzymes convert precursor amino acids into polyamines

- **Uptake from the environment**

- Energy dependent polyamine transport systems

E. coli polyamine biosynthesis genes



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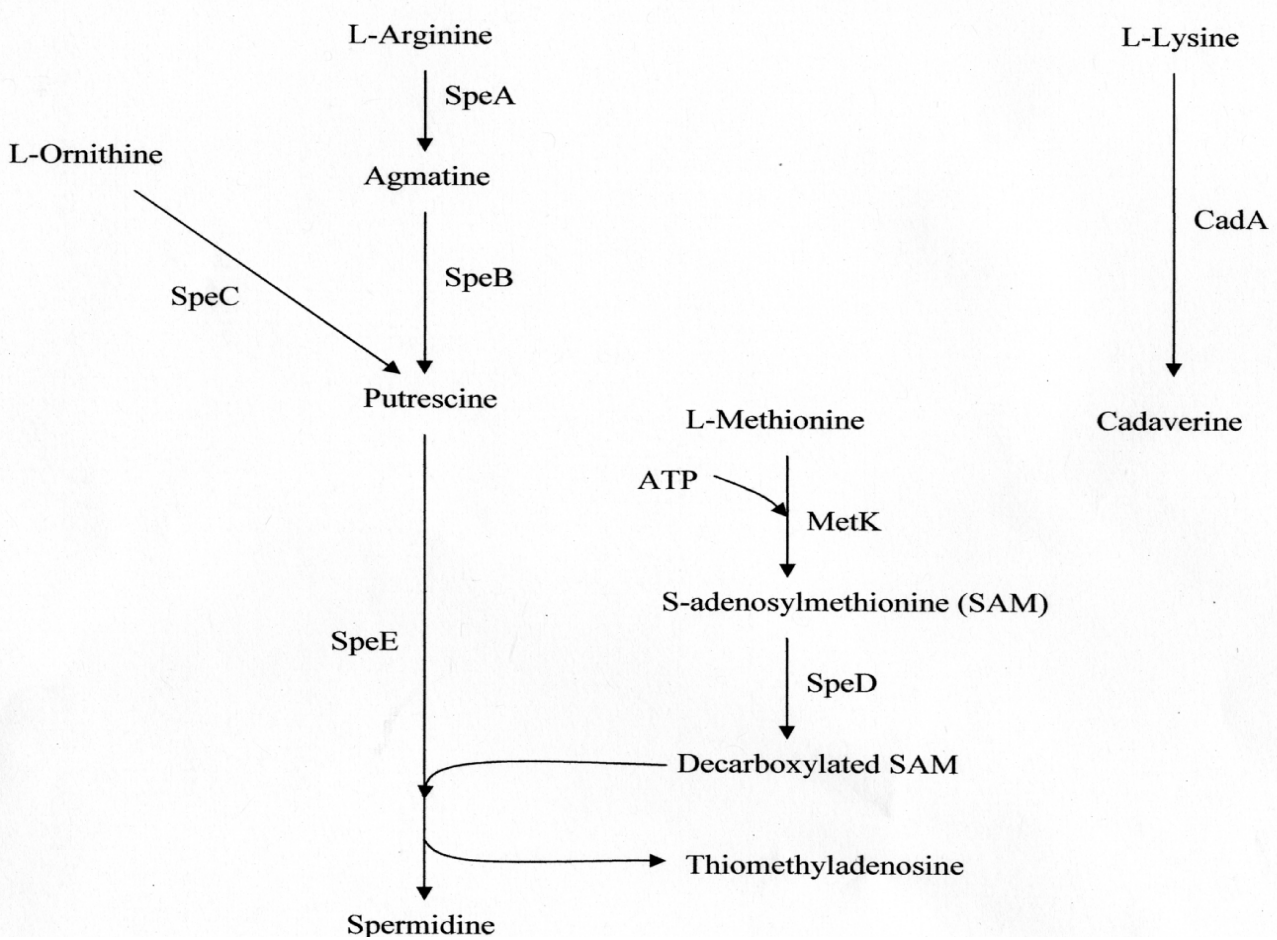
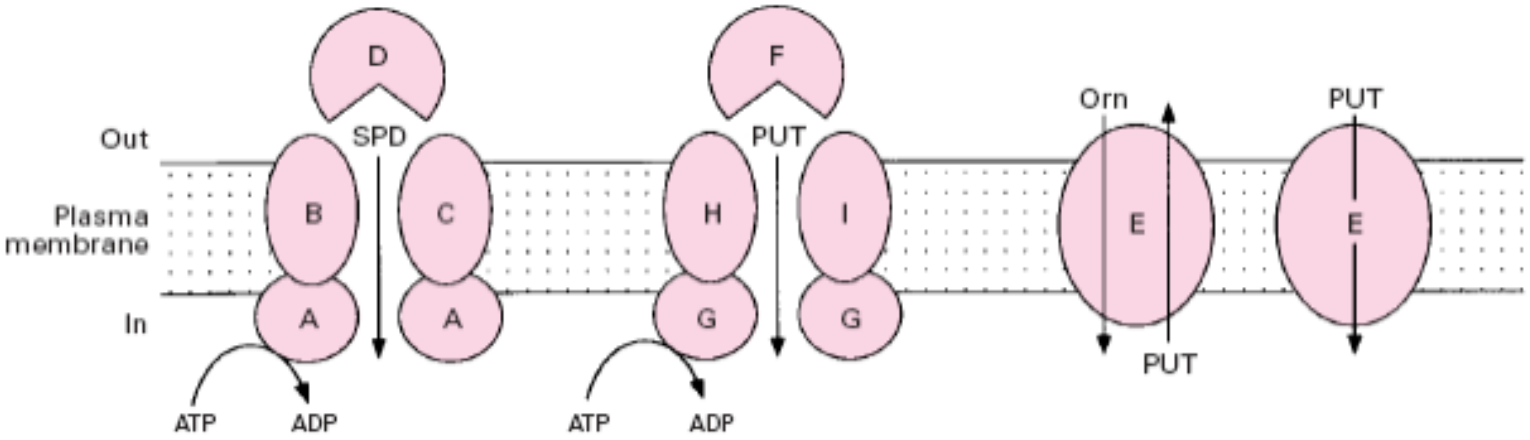
Polyamines biosynthesis in *E. coli*

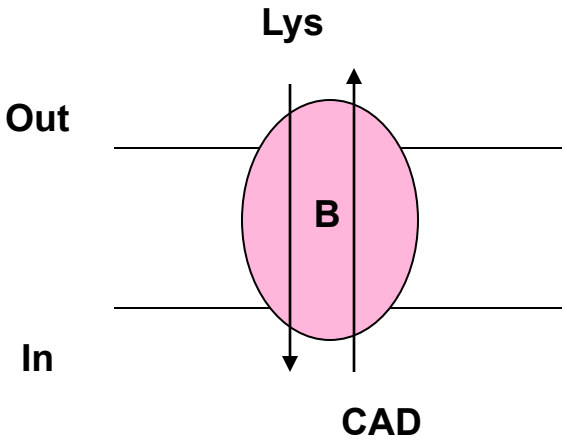


SpeA – arginine decarboxylase, SpeB – agmatine ureohydrolase, SpeC – ornithine decarboxylase, SpeD – SAM decarboxylase, SpeE – spermidine synthase, MetK – methionine adenosyltransferase, CadA – lysine decarboxylase.

Shah P and E. Swiatlo Mol. Micro.(2008) **68** (1), 4-16

Polyamine transport in bacteria





Transport Systems	Polyamine
PotABCD	**Spermidine**
PotE	Putrescine
PotFGHI	Putrescine
CadB	Cadaverine

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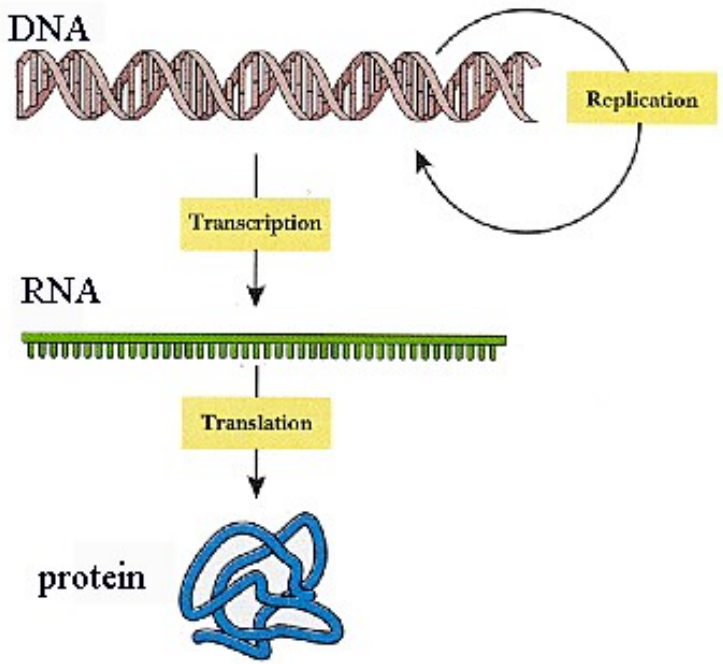
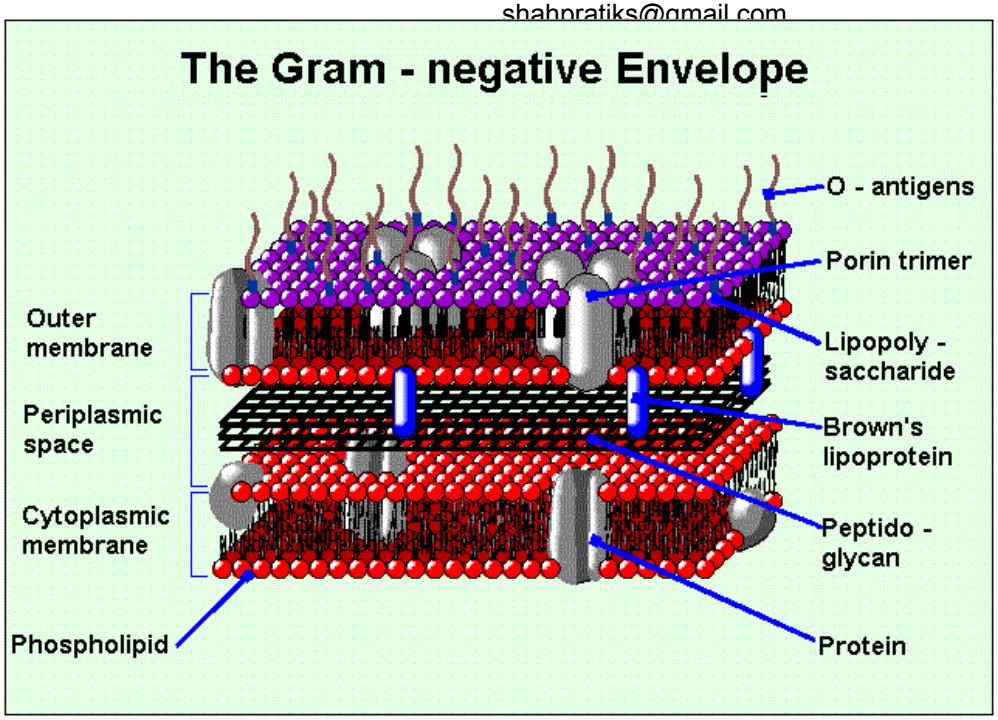
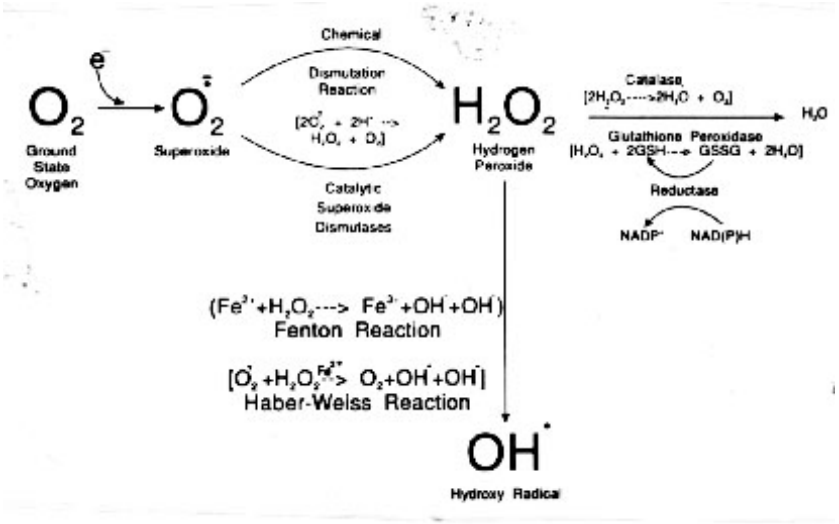
Physiological functions of polyamines in bacteria







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Microbial pathogenesis and polyamines

Pathogens and polyamines

A

Ornithine —***speABC***→ Putrescine

↓

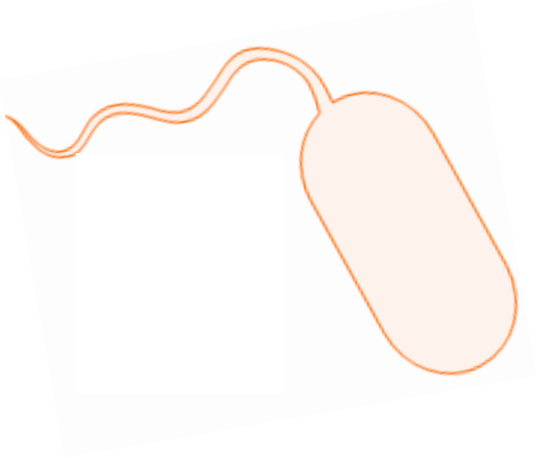
Δ *speABC*

Reduces *Yersinia pestis* biofilm formation

Proteus mirabilis

Inactivation of *speAB* genes in *P. mirabilis* leads to loss of swarming phenotype that can be reversed by exogenous putrescine

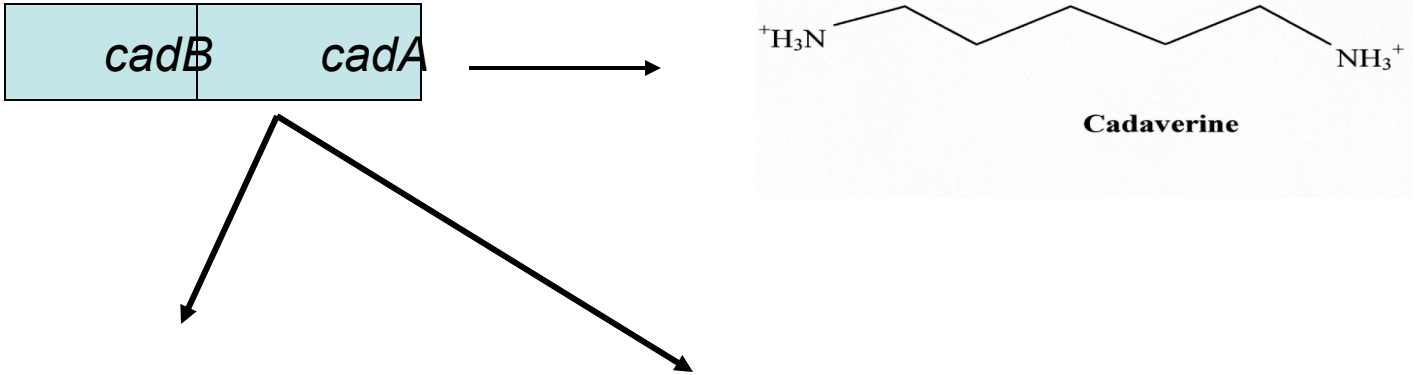
B

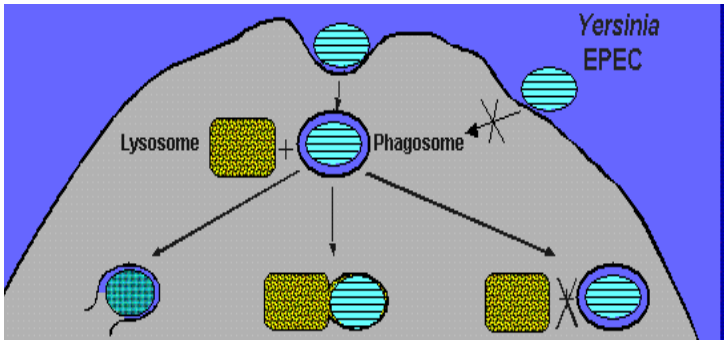


Regulate colicin production in *E. coli*

Polyamines as "antivirulence" molecules

+ + Cadaverine biosynthesis and transport genes in *Shigella*





Reduced enterotoxin activity

Prevents escape from phagolysosomes

Shigella

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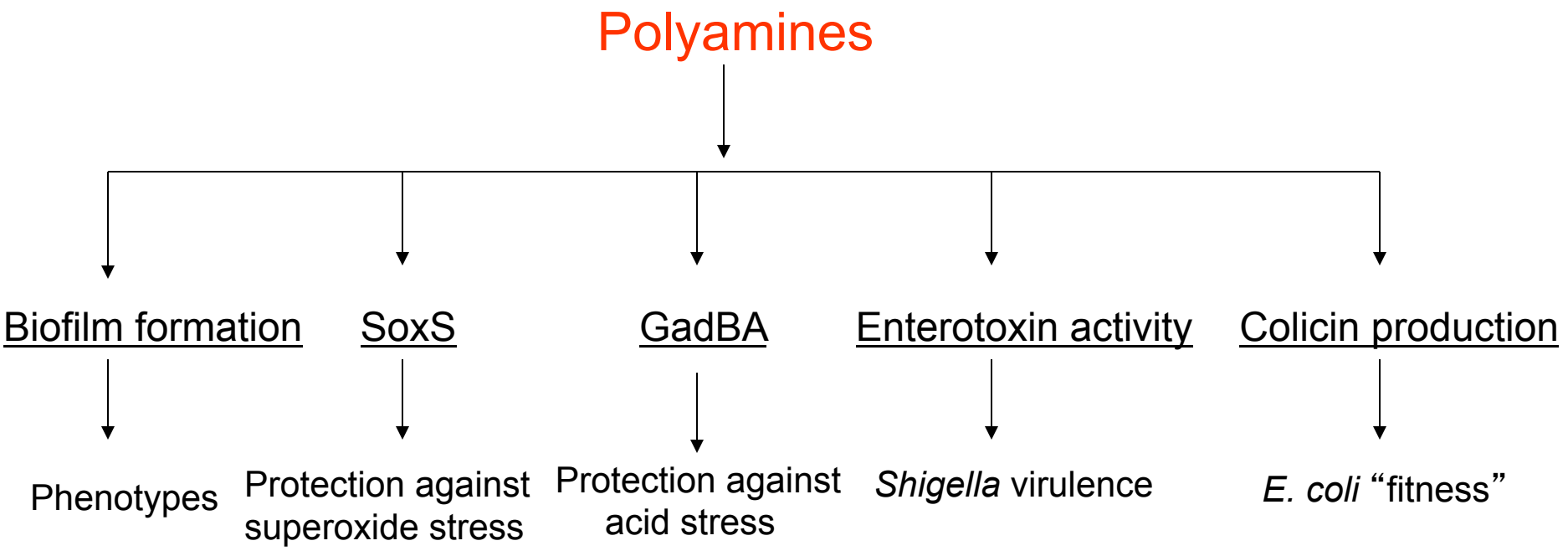
Polyamines as "regulators" of multiple gene expression cascades



Polyamines may regulate expression of pneumococcal virulence

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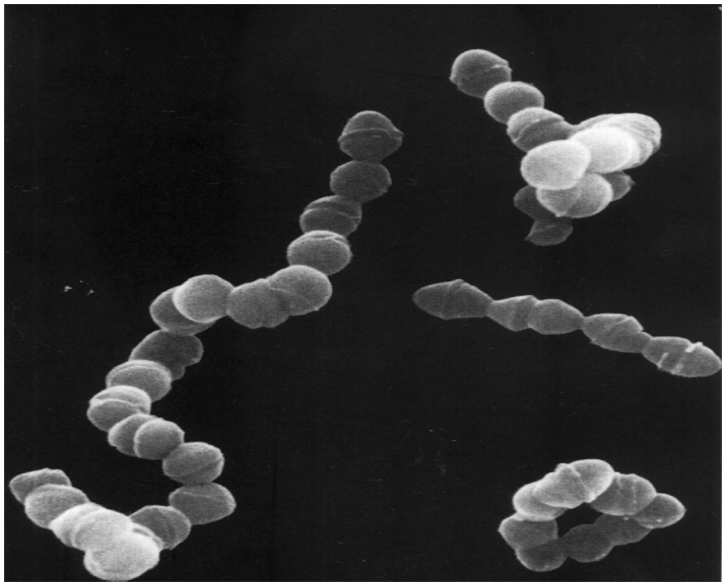
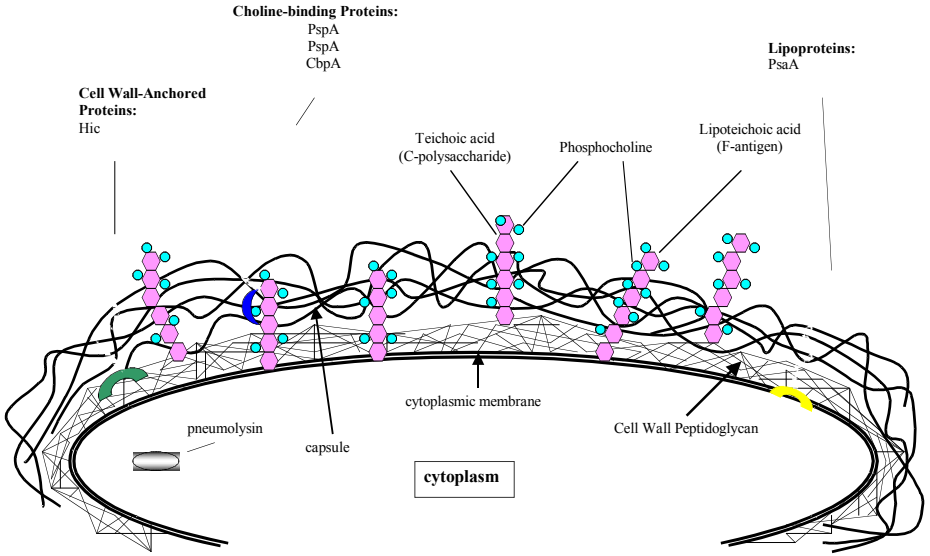
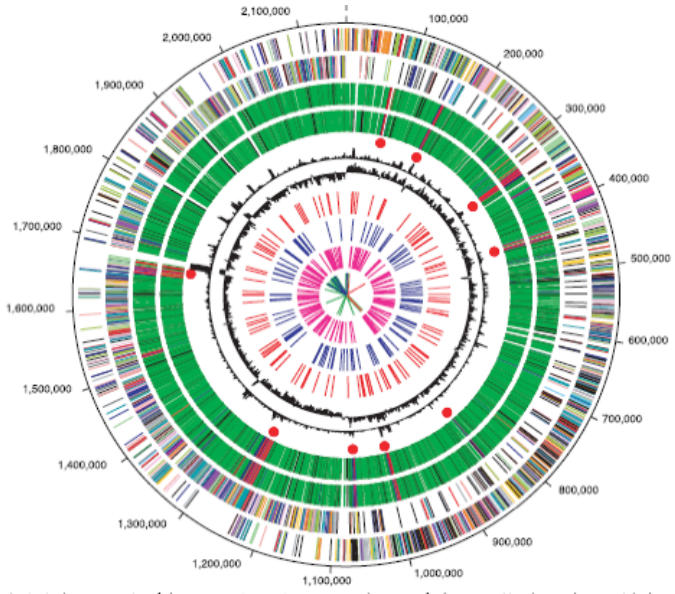
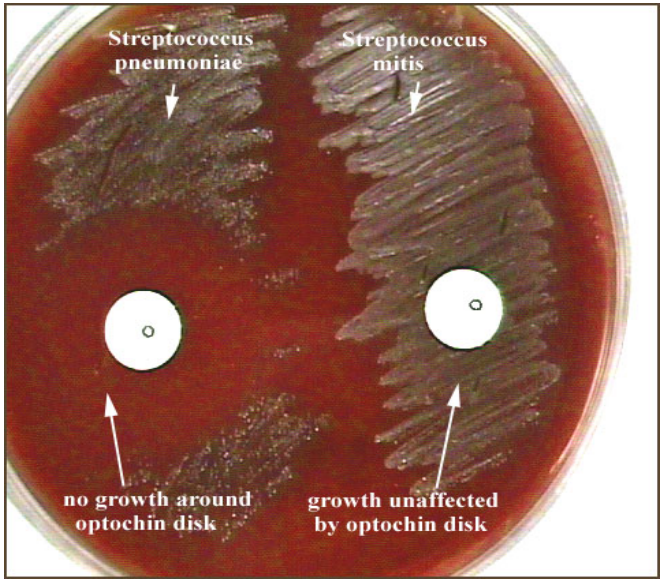
Streptococcus pneumoniae

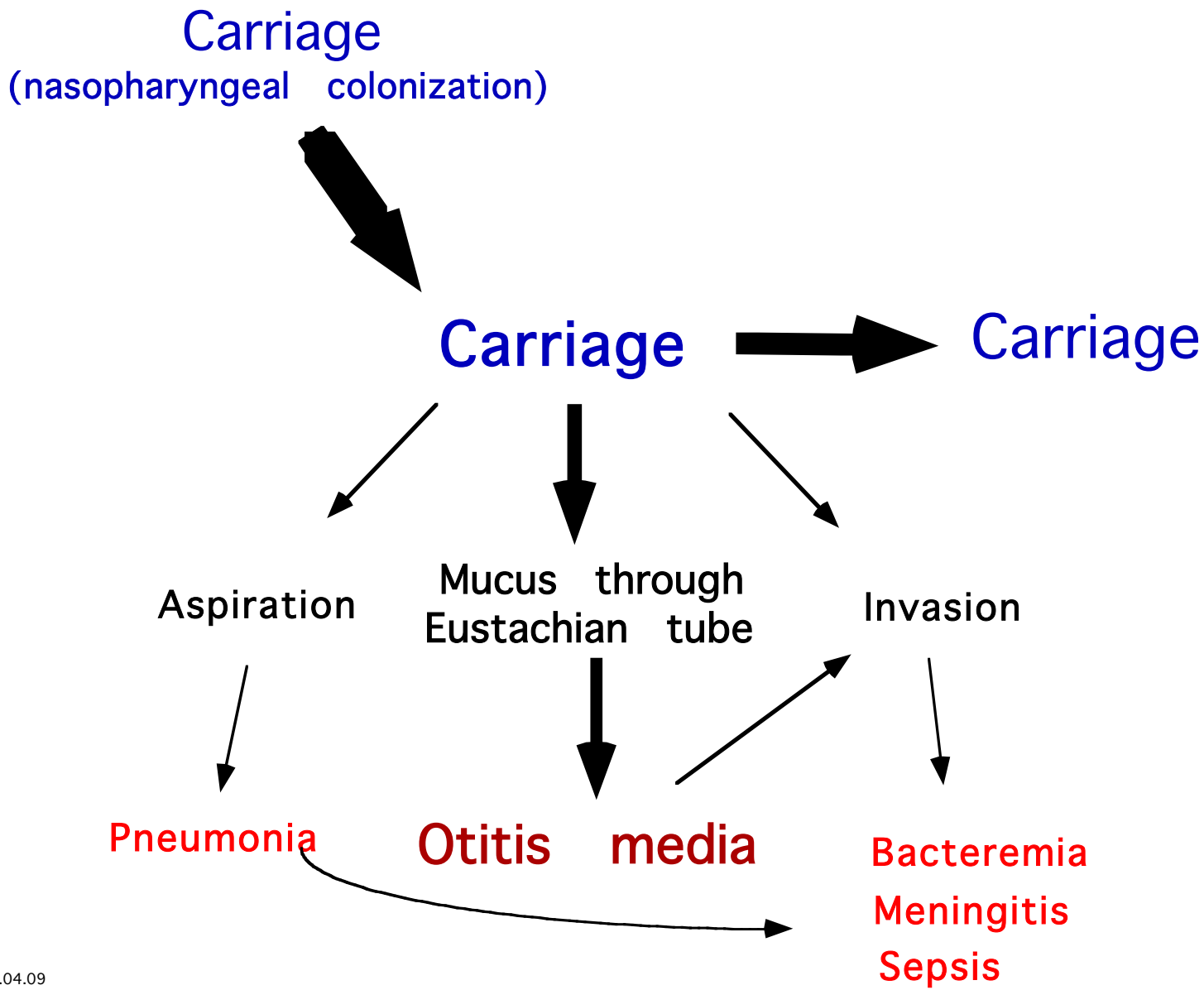








Pneumococcal Disease



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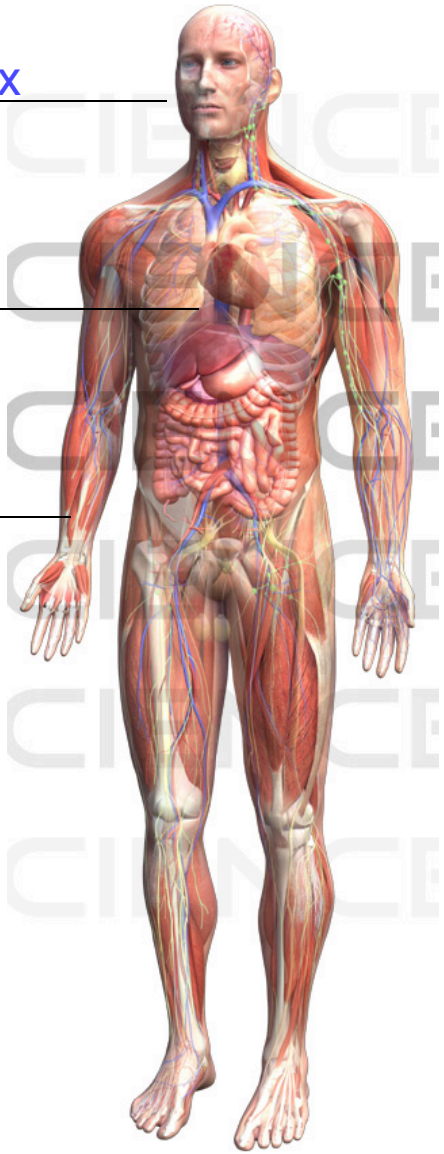
Streptococcus pneumoniae disease

Colonization — Nasopharynx

Pneumonia — Lungs

Invasive Infection — Blood



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Streptococcus pneumoniae disease

- Most common cause of community-acquired bacterial pneumonia
  - 440,000 -- 1,100,000 cases annually in USA (CDC)
  - 36 -- 62% of pneumonia in USA
  - ~ 6-10,000 deaths in the United States annually
  - 1.2 million infant deaths per year, other countries
- Important cause of otitis media, meningitis, bronchitis and sepsis

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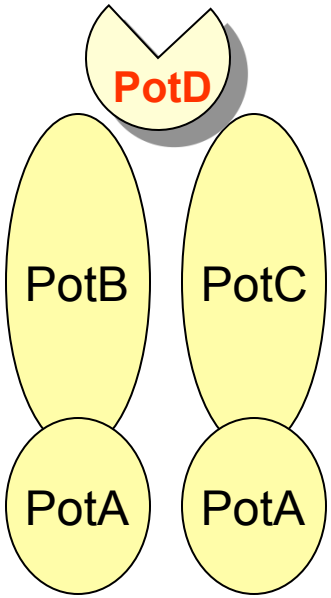
Vaccine protection is limited

- Two vaccines are currently available
  - 23-valent purified polysaccharide
  - 7-valent polysaccharide-protein conjugate

- Multi-drug resistant strains
- ~ 44,000 deaths (US) and 1 million (worldwide)

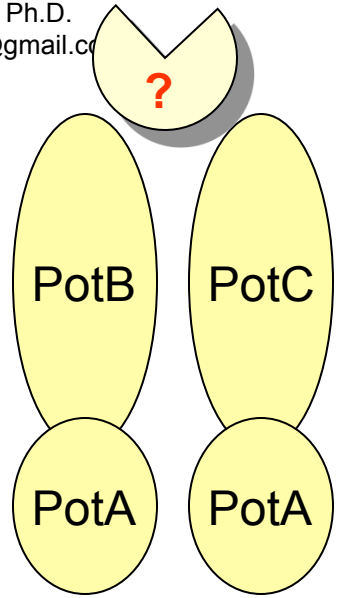
Streptococcus pneumoniae polyamine transport operon



potABCD = *pot* operon = Spermidine / putrescine uptake

A signature tagged mutagenesis study identified pneumococci with deletions in *potB-C* to be attenuated in murine pneumococcal pneumonia

S. pneumoniae PotD



- Homology to *E.coli* PotD protein is significant.
- 1071 base pairs/357 amino acids/41kD
- N terminus leader sequence - Potential extracellular protein
- Does not have a consensus anchor domain

Questions

- **Cellular location**

  Is PotD a cytoplasmic or a secreted protein?

  Hypothesis: PotD is a secreted protein that localizes to the pneumococcal cell surface

- **Suitability as an vaccine antigen**

  Hypothesis: If PotD is secreted, it may be immunogenic

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Section 1

CELLULAR LOCATION AND IMMUNOGENICITY OF *S. PNEUMONIAE* POTD

Hypothesis: PotD is a secreted protein that localizes to the pneumococcal cell surface

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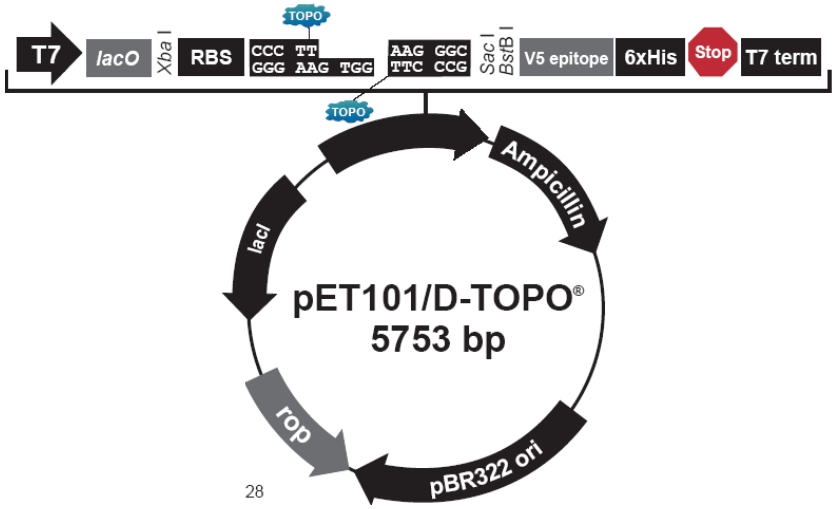
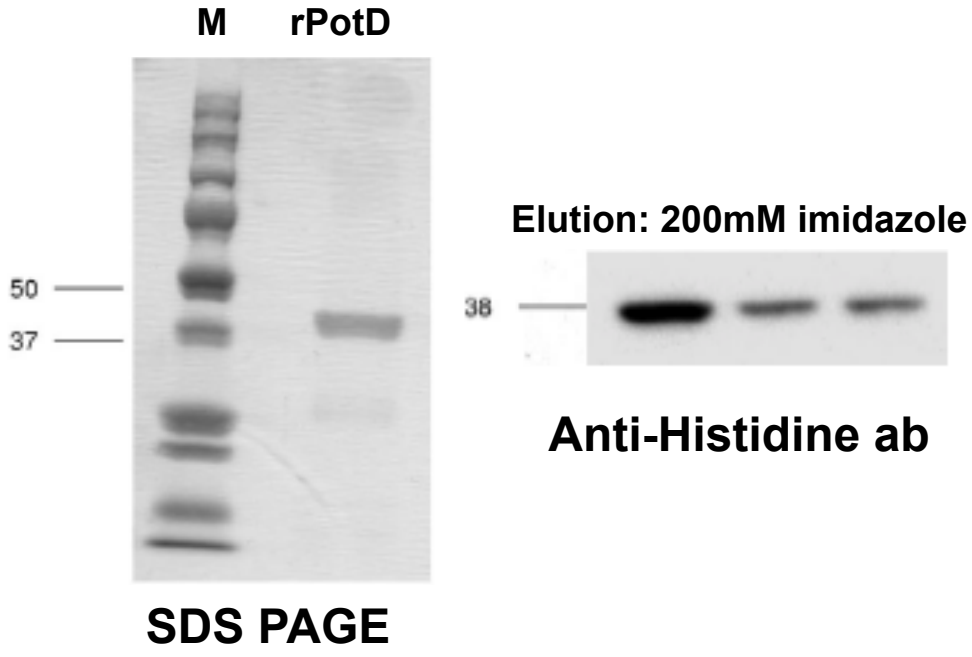
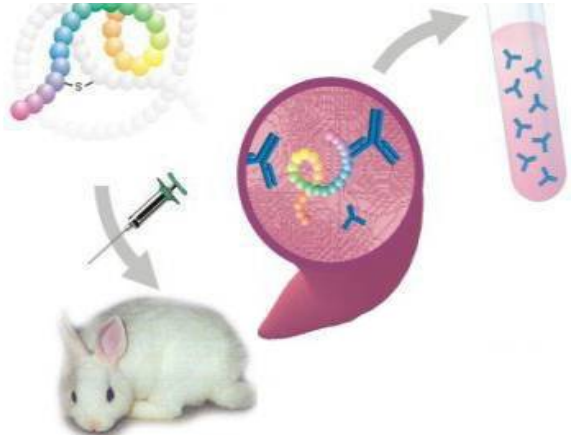
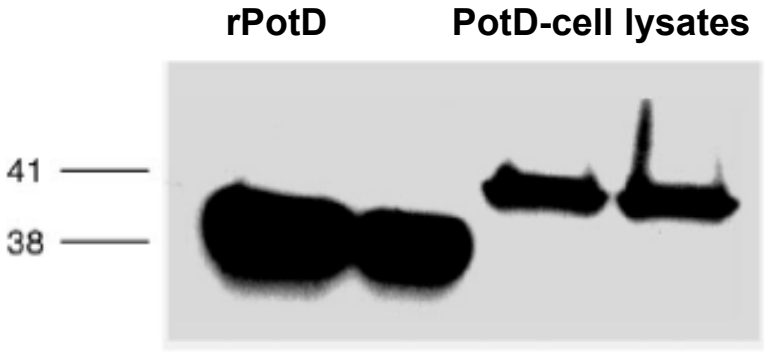
Cloning, expression, purification and generation of polyclonal antiserum









Shah P. and Swiatlo E. Infec.Immu.(2008) **74** (10), 5888-5892

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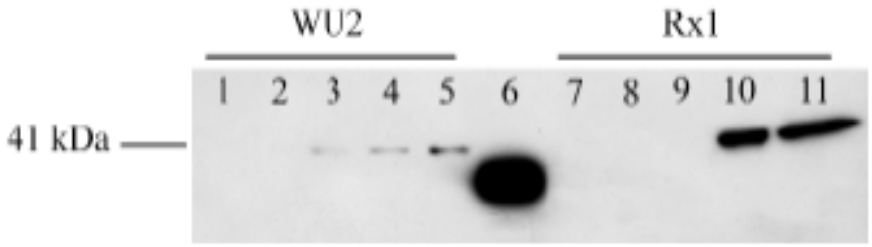
Cellular fractionization and sub-cellular location of PotD



Immunoblot analysis with subcellular fractions of pneumococcal strains WU2 and Rx1. Lanes: 1, 7, secreted proteins; 2, 8, noncovalently attached surface proteins; 3, 9, cell wall-associated proteins; 4, 10, soluble cytoplasmic contents; 5, 11, membranes, insoluble cytoplasmic contents; 6, recombinant PotD (control).

Flow cytometry with PotD antiserum

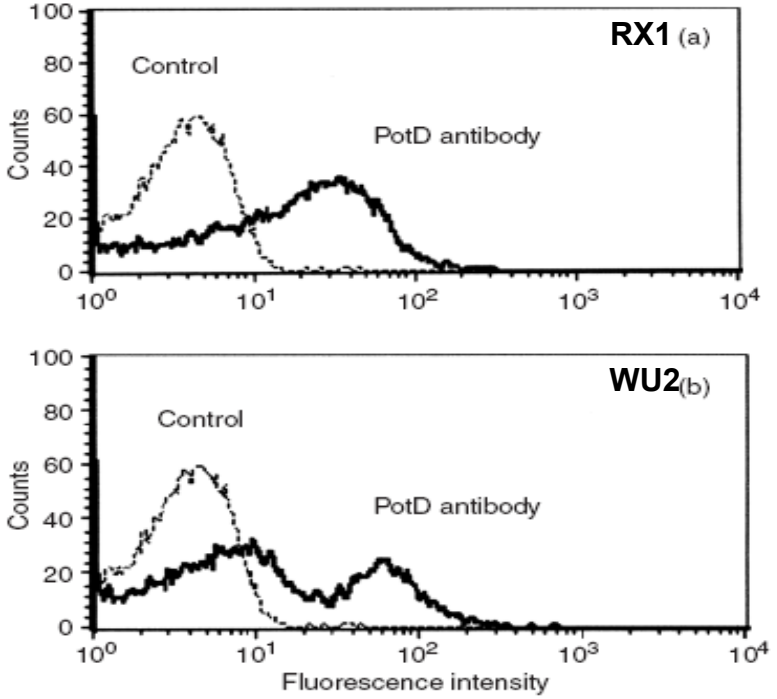


Fig. 1. Flow cytometric measurement of binding of PotD antibodies to the pneumococcal surface. (a) unencapsulated strain Rx1; (b) capsule type 3 strain WU2. Each graph is a representative result of three independent experiments.

PotD is a membrane anchored surface-exposed protein

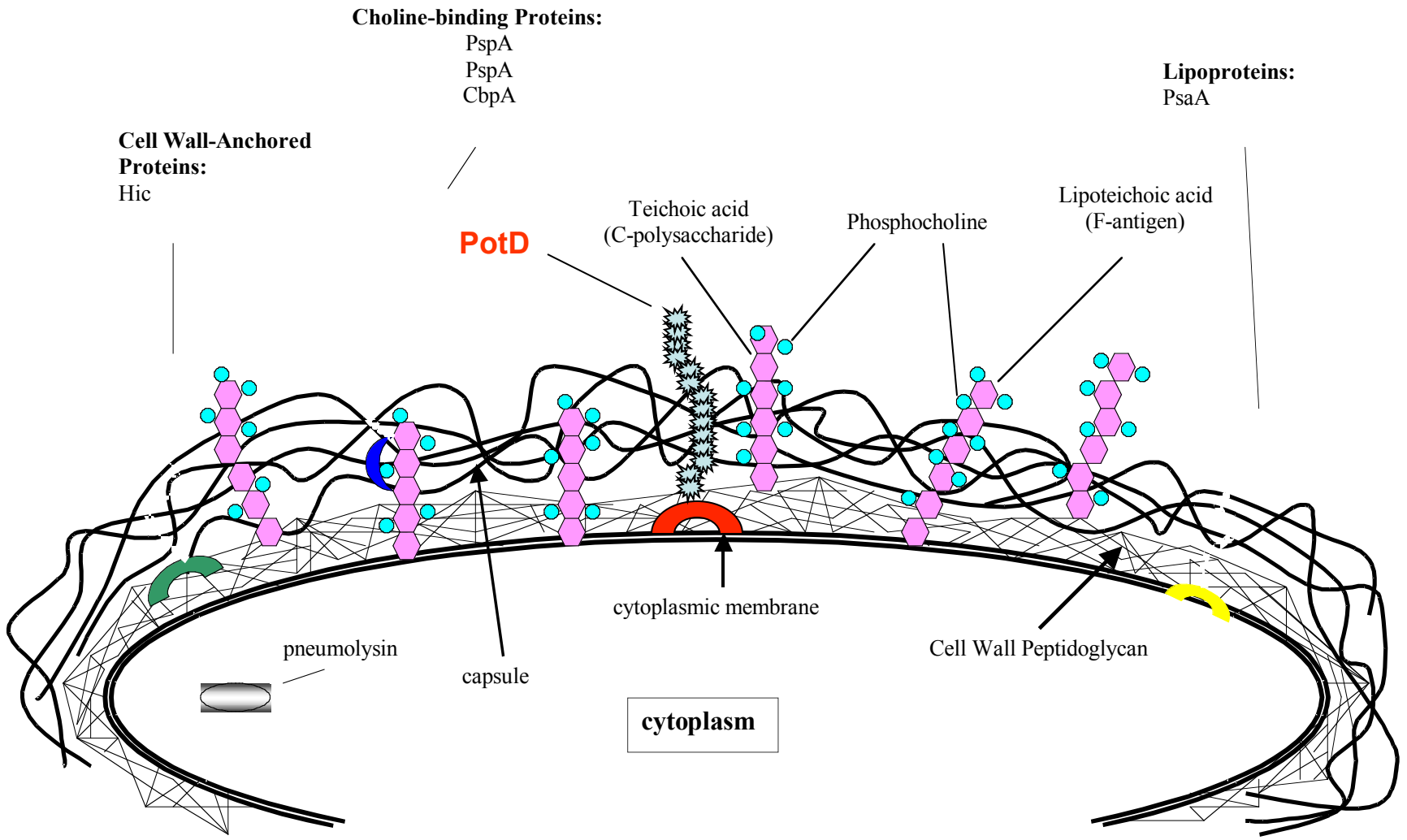
Shah P. *et al.* FEMS Microbiol Lett (2006) **261**, 235-237

PotD is expressed on cell surfaces of different pneumococcal capsular serotypes

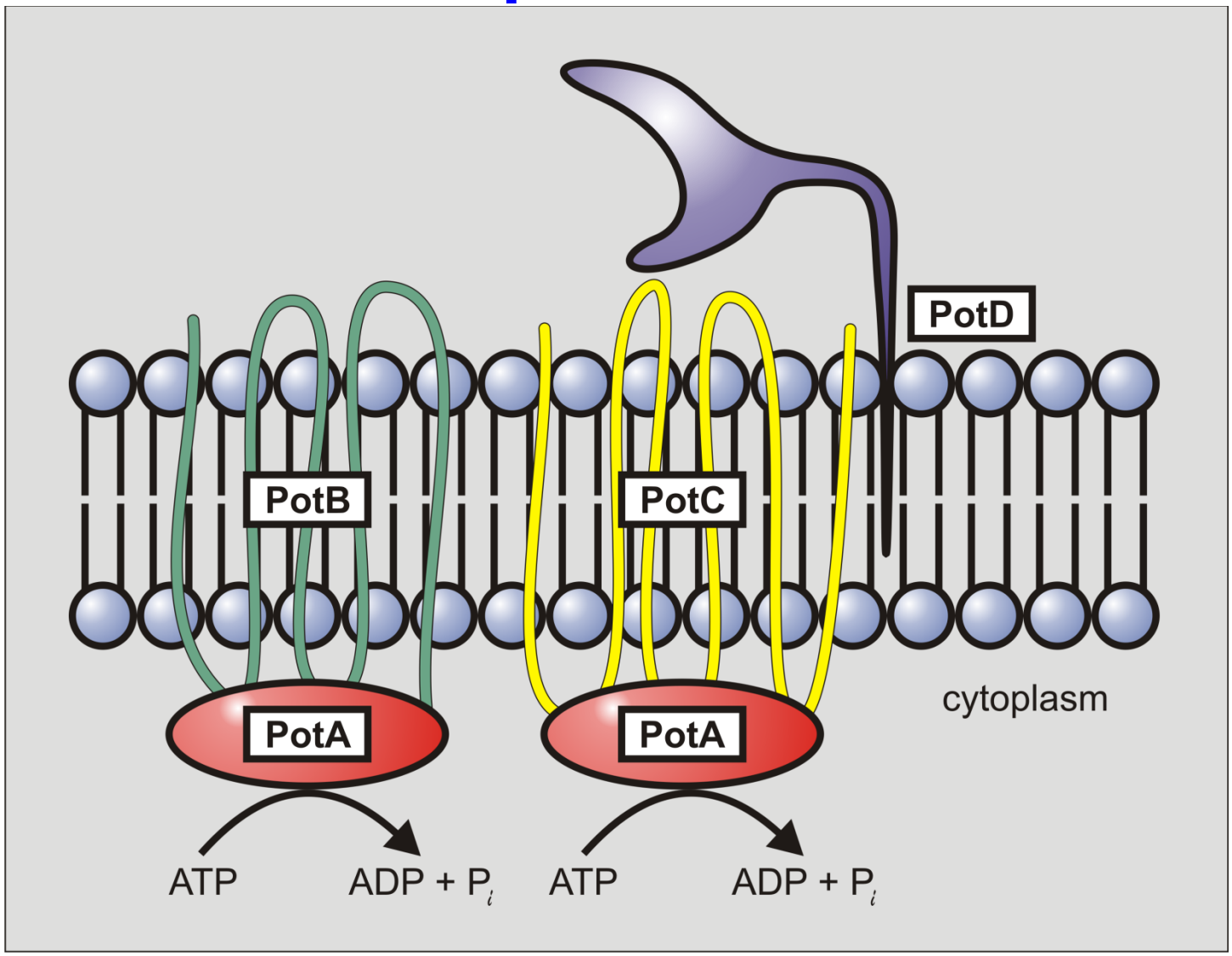
***S. pneumoniae* strain (serotype)**	**Mean fluorescence intensity**
Negative control	2.11
BC51 (6)	91.46
D39 (2)	97.34
EF3030 (19F)	321.97
MW4834 (9)	151.07
TIGR4 (4)	245.04

Shah P and Swiatlo E. Micro.Path (2008), 45(3), 167-72

Streptococcus pneumoniae surface proteins



Proposed model for pneumococcal Pot proteins



Shah P and E. Swiatlo Mol. Micro.(2008) **68** (1), 4-16

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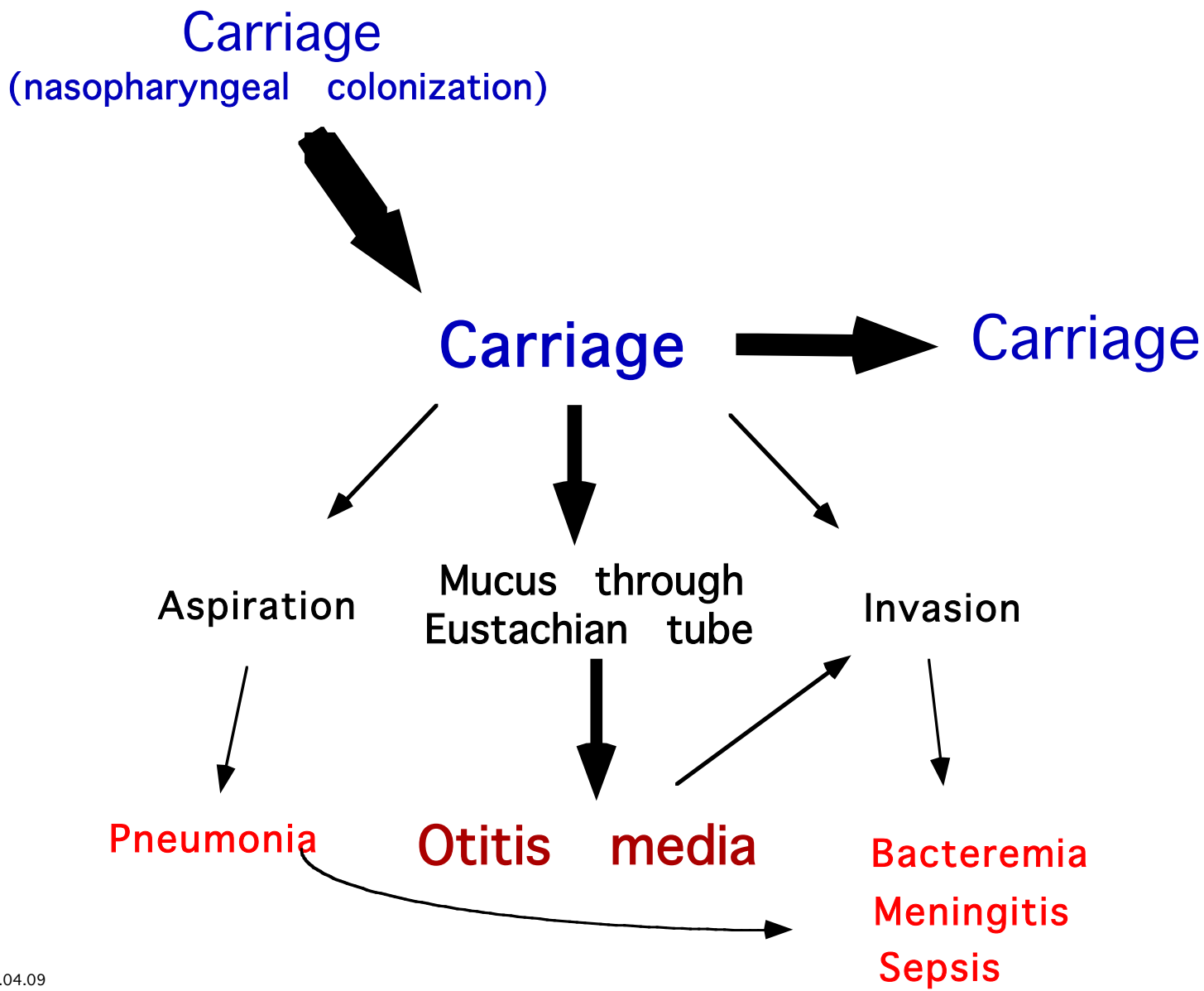
PotD an effective vaccine antigen?

- Present in a significant proportion of virulent pneumococci

Shah P and Swiatlo E. Micro.Path (2008), 45(3), 167-72
Shah P. *et al.* FEMS Microbiol Lett (2006**)** **261**, 235-237
Shah P, Briles DE and Swiatlo E. Exp..Bio. Med (2009), 234(5)

- Immune responses directed against PotD are protective

Pneumococcal Disease



Suitability of PotD as a potential vaccine antigen to prevent *S. pneumoniae* colonization and infection

A) Murine mucosal immunization and challenge model

B) Murine systemic immunization and challenge model

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Murine mucosal immunization and challenge

Mucosal (intranasal) immunization of mice with PotD mixed with Cholera Toxin B (CTB) / CTB alone

Collect serum and saliva for ELISA analysis

Challenge intranasally with either 10^6 cells of serotype 19 strain EF3030 or **10^5 cells** serotype 4 strain TIGR4

Compare pneumococcal colonization of nasopharynx between immunized and control animals

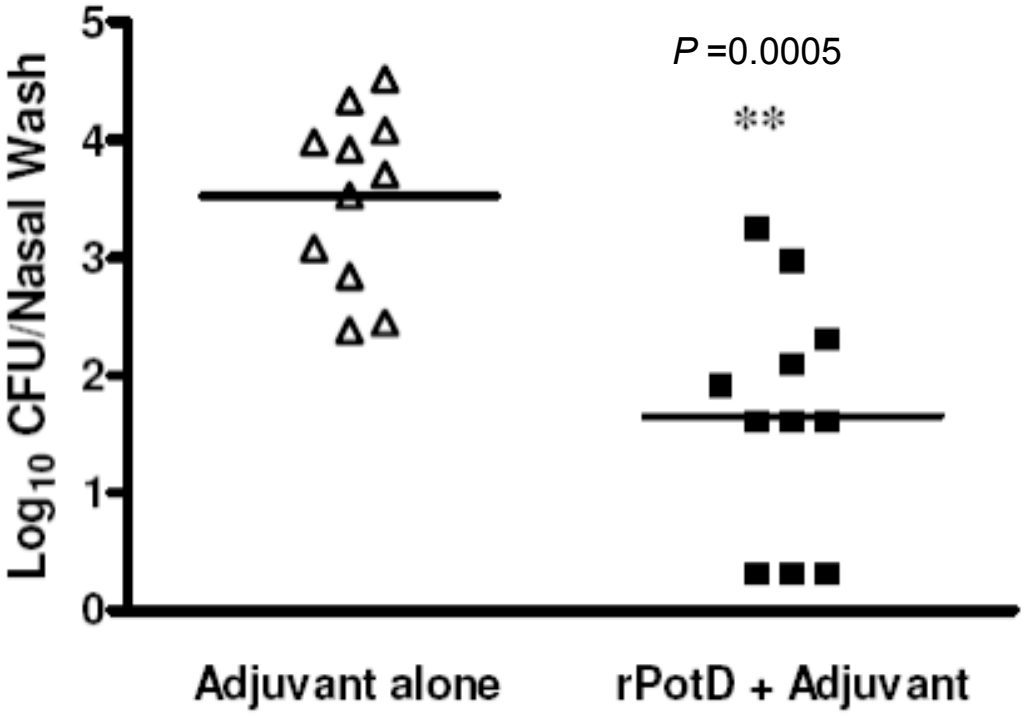
Mucosal immunization with PotD generates specific and high titer immune responses in serum and saliva

Table 1. ELISA titers following mucosal immunizations with rPotD adsorbed to cholera toxin B (CTB) or CTB alone.

Challenge organism	Antigen	Reciprocal endpoint titer (mean ± SEM)	
		IgG- Serum	**IgA- Saliva**
EF3030	10µg rPotD	3125 ± 0	2291 ± 527
	PBS + CTB	< 0.07	458 ± 166
TIGR4	10µg rPotD	2708	2236 ± 447
	PBS + CTB	< 0.07	125 ± 0

Shah P, Briles DE and Swiatlo E. Exp..Bio. Med (2009), 234(5)

PotD immunization reduces nasopharyngeal colonization by EF3030

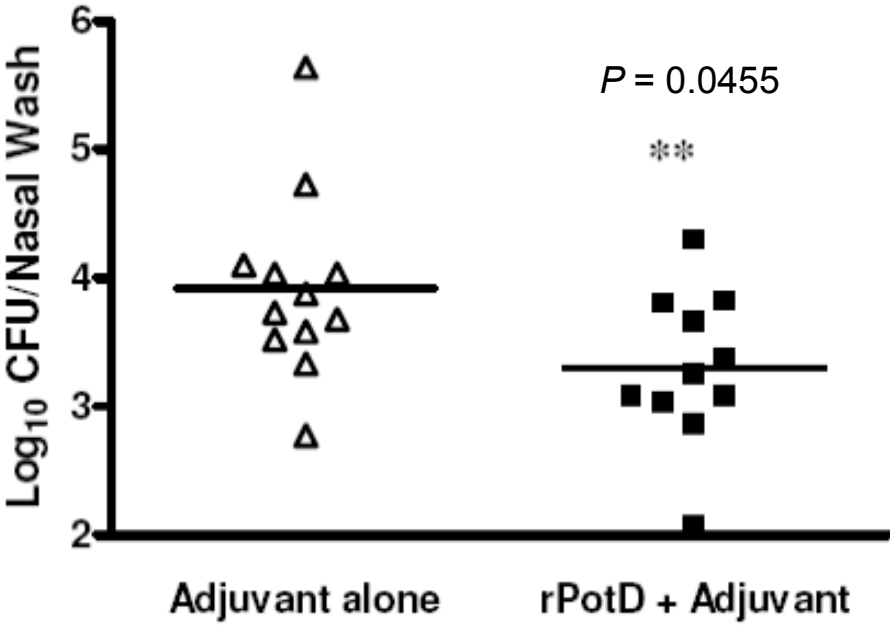


Nasal washes with 1ml PBS- Control vs. Immunized

Challenge dose-5 × 10^6 EF3030 in 20µl LR-Intranasal

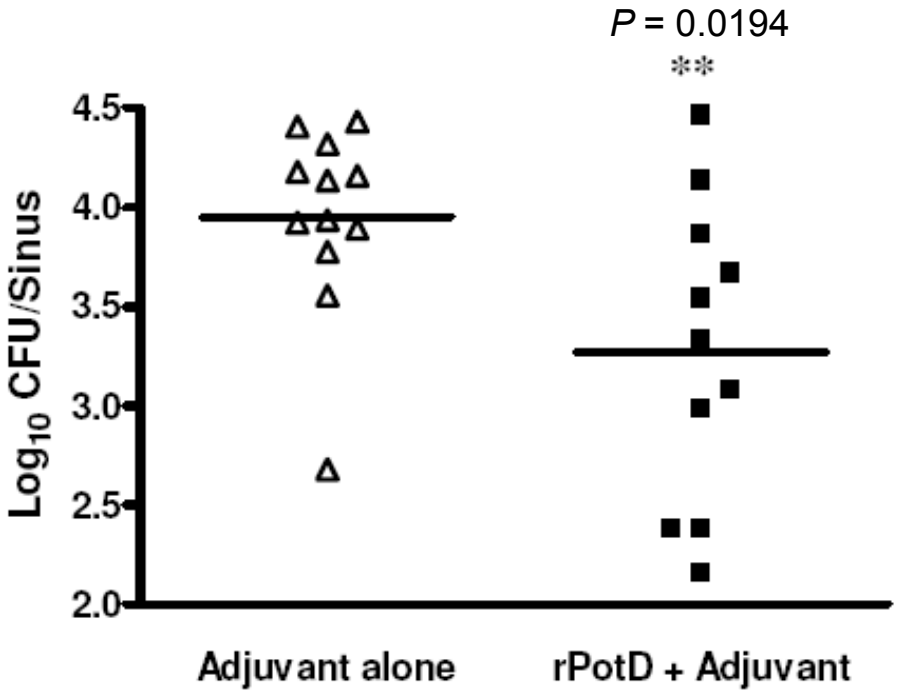
Shah P, Briles DE and Swiatlo E. Exp..Bio. Med (2009), 234(5)

PotD immunization reduces nasopharyngeal colonization by TIGR4



Nasal washes with 1ml PBS- Control vs. Immunized

Challenge dose-4 × 10^5 TIGR4 in 20µl LR



Sinus tissue homogenized in PBS- Control vs. Immunized

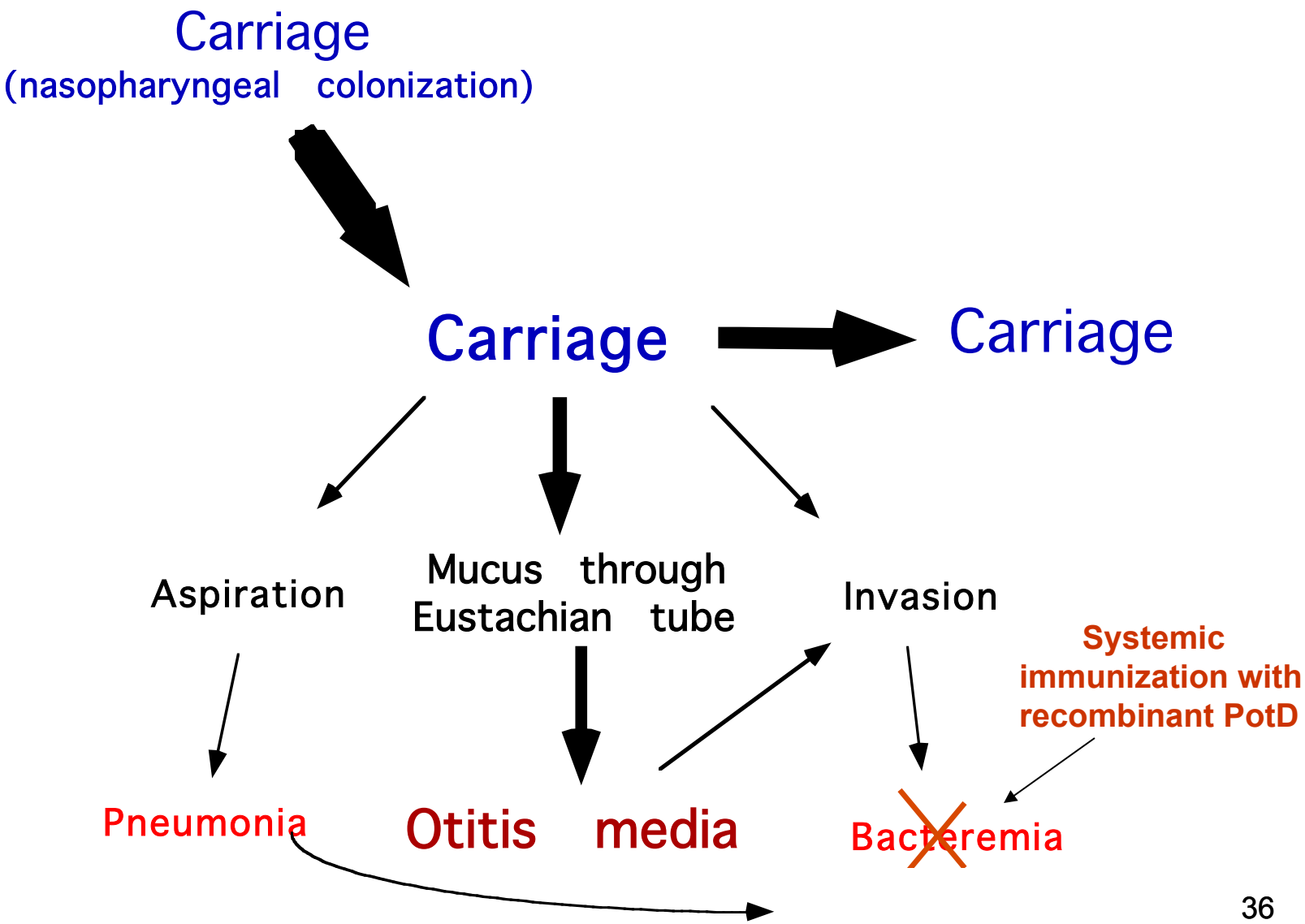
Shah P, Briles DE and Swiatlo E. Exp..Bio. Med (2009), 234(5)

Suitability of PotD as a potential vaccine antigen to prevent *S. pneumoniae* colonization and infection

A) Murine mucosal immunization and challenge model

B) Murine systemic immunization and challenge model

Pneumococcal Disease



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Murine systemic immunization and challenge

Systemic immunization of mice with 5µg PotD in alum / alum alone

↓

Bleed mice prior to challenge to collect serum for ELISA

↓

Challenge systemically (i.v.) with 100X LD_{50} of serotype 3 strain WU2

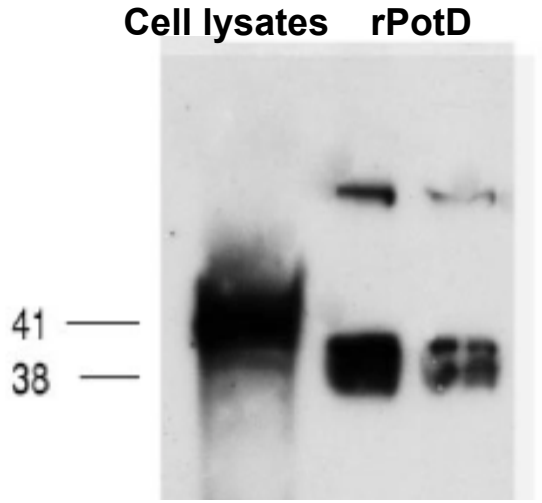
↓

Compare survival of immunized vs. control mice

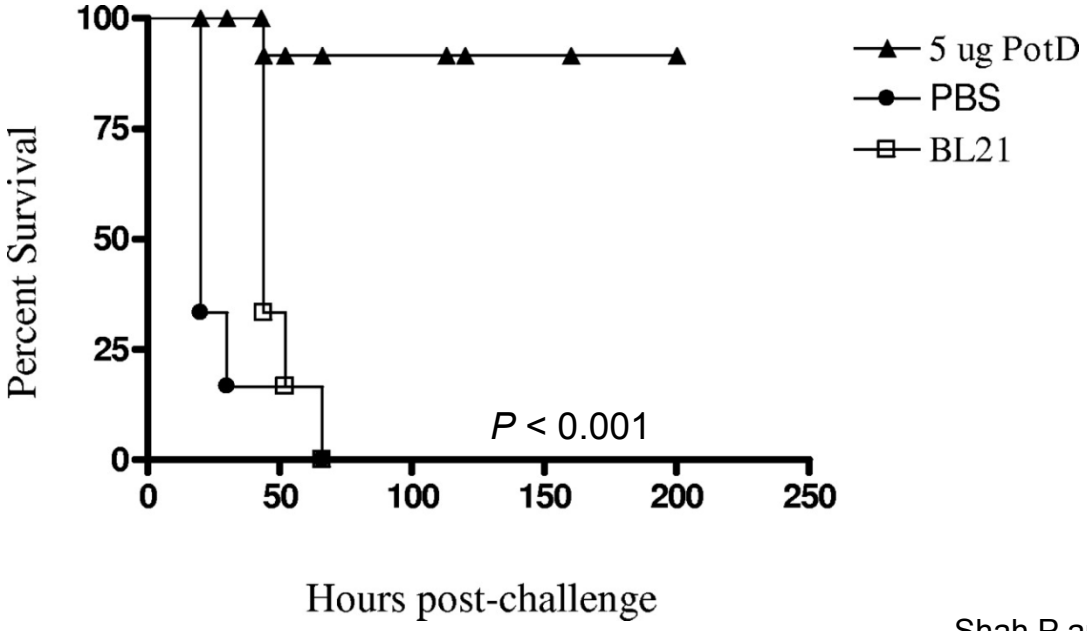
Systemic immunization with PotD is protective against lethal septicemia

TABLE 1. ELISA endpoint dilution titers for mice immunized with recombinant PotD

Group	Reciprocal endpoint titer (mean ± SEM)
Preimmune	235 ± 25
Immunized	
PotD	18,749 ± 3,068
BL21 lysate	1,562 ± 625
PBS	787 ± 150



Immunoblot with mouse serum



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Passive protection with PotD- antiserum

Group	Alive / Dead
1: 100 rabbit anti-PotD antiserum	6 / 0
1: 1000 rabbit anti-PotD antiserum	5 / 1
1: 100 pre-immune antiserum	0 / 6

$P < 0.004$

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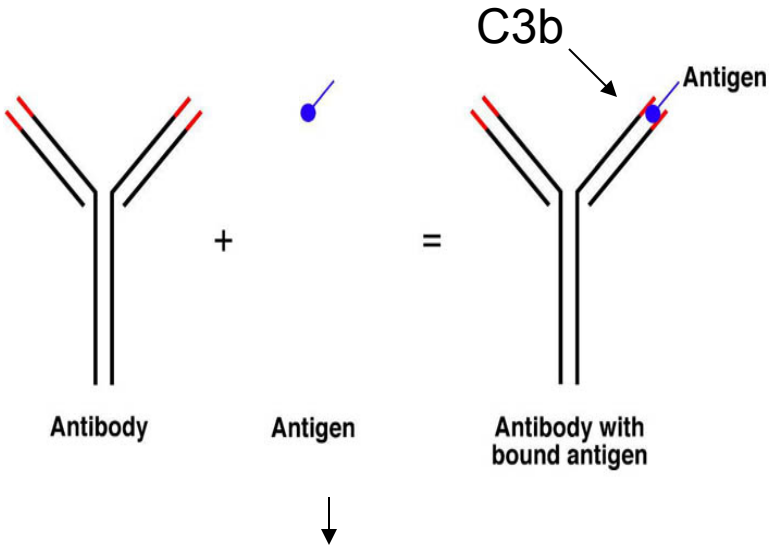
Mode of protection

Clearance



Complement deposition

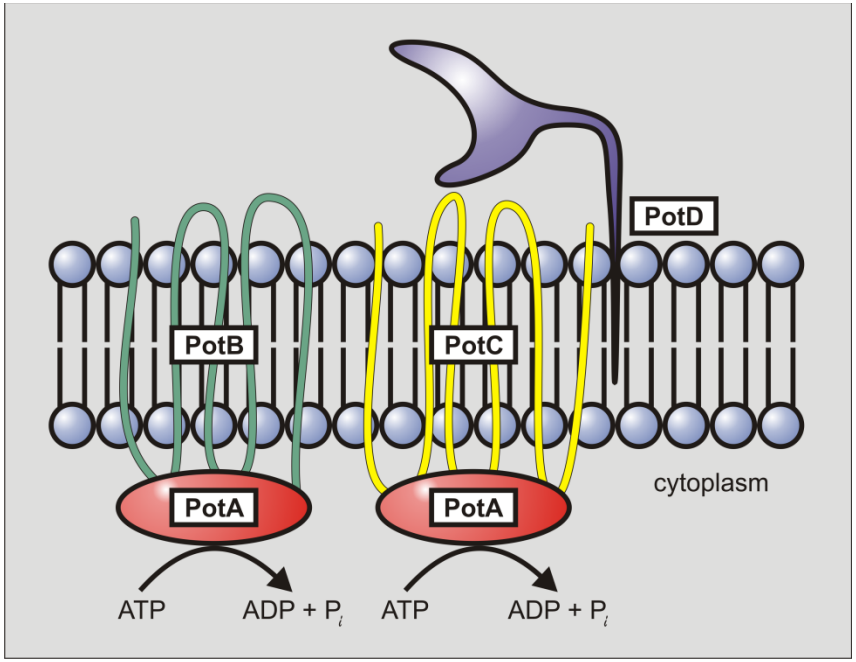
Opsonophgocytosis

Clearance

PspA, PiaU….

Opsonophagocytosis assay

Neutralization

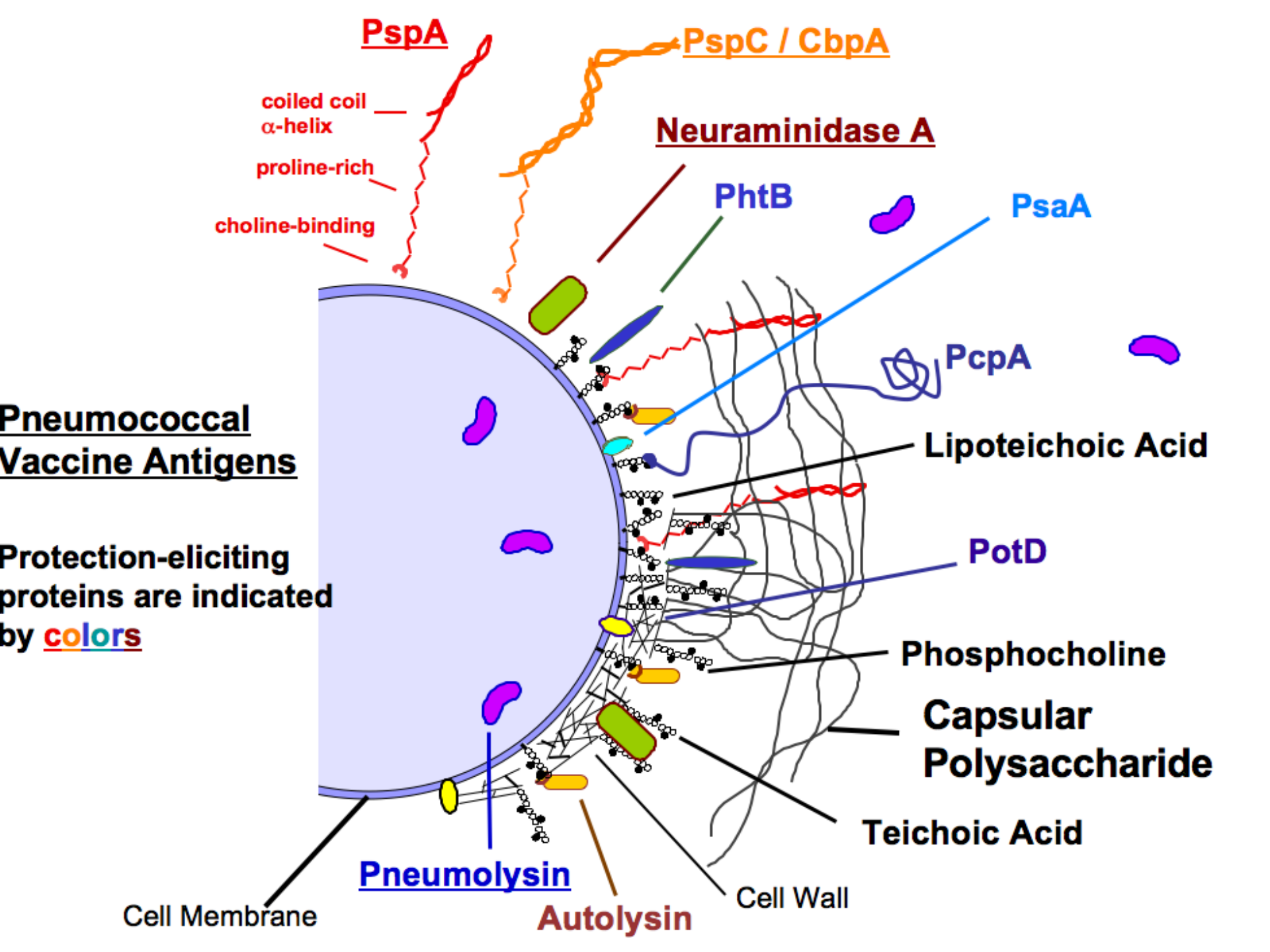


Inhibition of polyamine uptake

Effect on growth/virulence in vivo

STM screen indentified *potB-C* mutants

potABCD mutant


PspA
PspC / CbpA
coiled coil
α-helix
proline-rich
choline-binding
Neuraminidase A
PhtB
PsaA
PcpA
Lipoteichoic Acid
PotD
Phosphocholine
Capsular
Polysaccharide
Teichoic Acid
Cell Wall
Pneumolysin
Autolysin
Cell Membrane
Pneumococcal
Vaccine Antigens
Protection-eliciting
proteins are indicated
by colors

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Summary Section 1

S. pneumoniae **PotD is a secreted and a membrane associated protein**

PotD is expressed across multiple capsular serotypes implicated in pneumococcal disease

Mucosal and systemic immunizations with PotD protective

S. pneumoniae **PotD represents a potential protein-based vaccine candidate.**

Microbial stress and polyamines

- Spermine, spermidine & cadaverine function as free radical scavengers and, in conjunction with SOD, reduce DNA strand breakage by oxygen radicals
- Concentrations of oxygen that are non-toxic to wild-type *E. coli* cells are lethal to polyamine-deficient mutants
- **Role of polyamines and polyamine transport systems in pneumococcal response to stress and infection is unknown**

Hypothesis: Polyamine transport plays a role in pneumococcal stress responses and infection

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Section 2

Role of polyamines and polyamine transport system in *Streptococcus pneumoniae* response to physiological stress and during infection

Hypothesis: Polyamine transport plays a role in pneumococcal stress responses and infection

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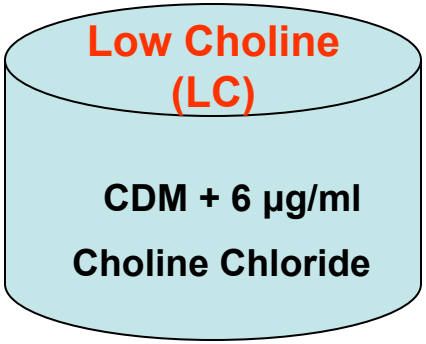
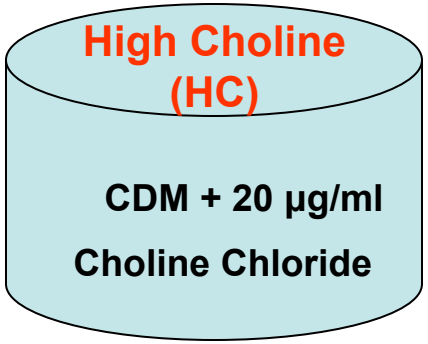
Experimental strategy



or



D39 cells grown to mid-log phase and exposed to:

In vivo Pneumococci

Polyamines

50 µg/ml Putrescine

50 µg/ml Spermidine

Stress

34° C

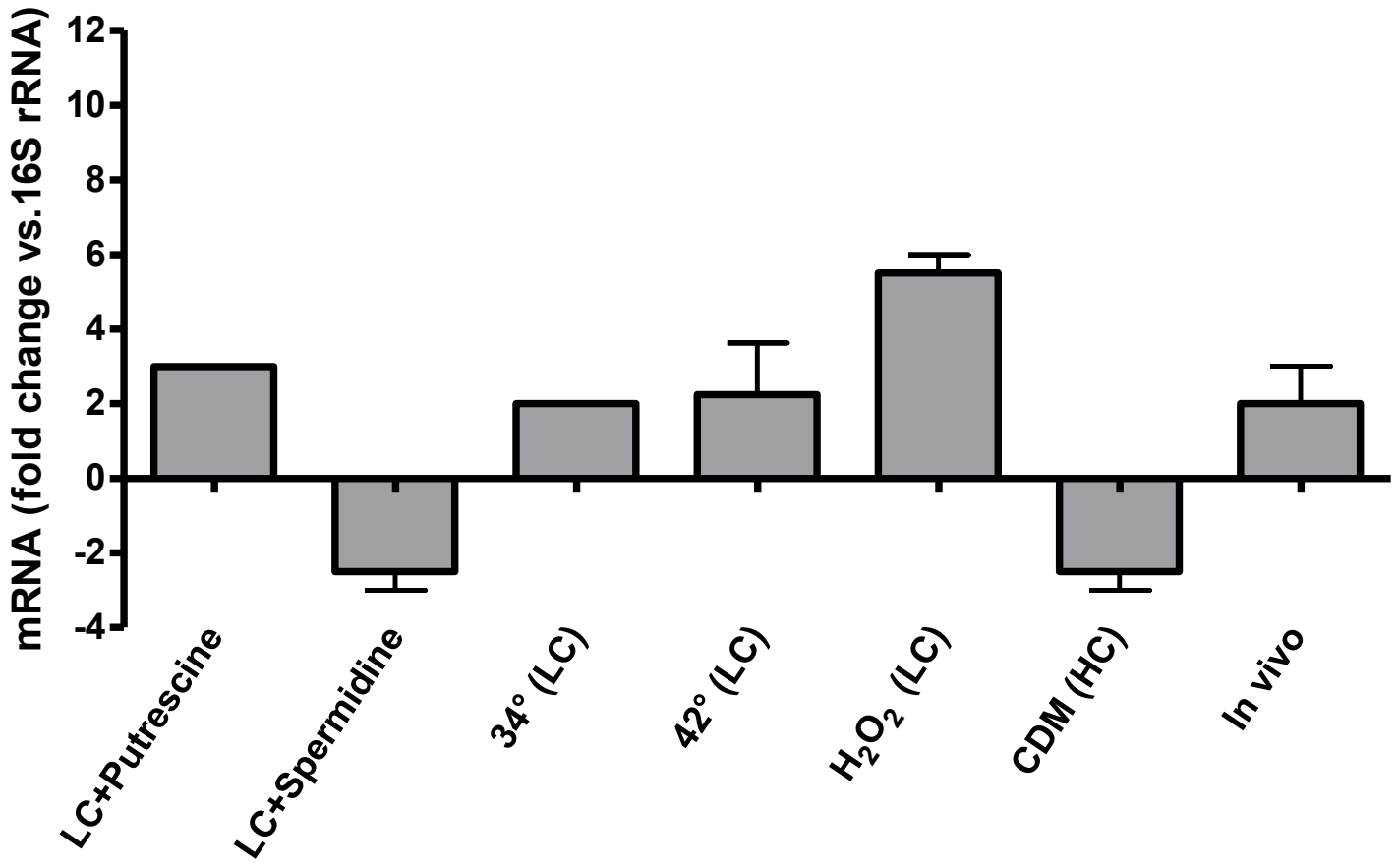
42° C

3mM H_2O_2

Harvest cells at $0D_{600}$0.4 and convert 1µg RNA into cDNA

qPCR analysis with *potD* and *16srRNA* (house-keeping gene) specific primers

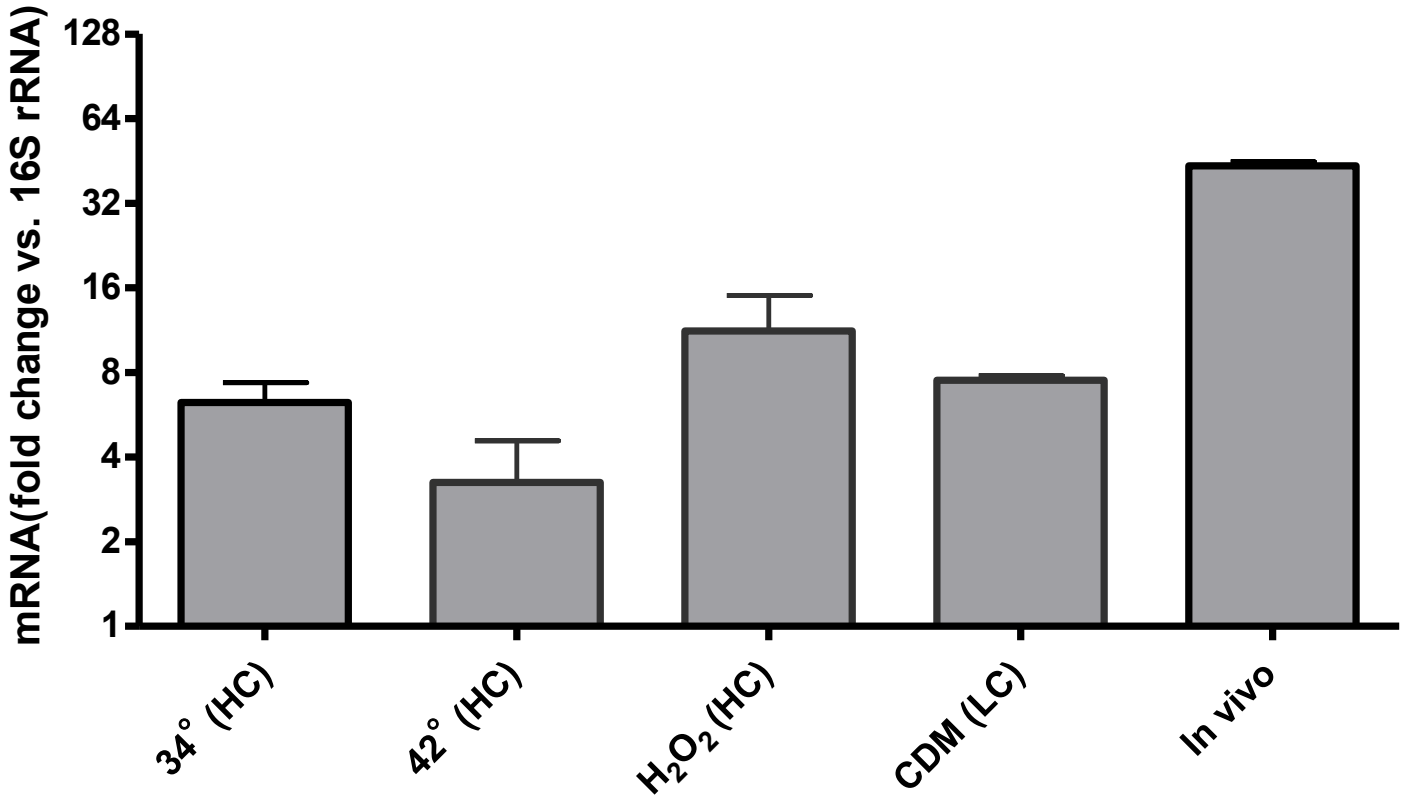
qPCR analysis of *potD* expression



Transcription of *potD* in various growth conditions compared with *potD* transcription in CDM + LC alone at 37°C.

Shah P and Swiatlo E. Micro.Path (2008), 45(3), 167-72

qPCR analysis of *potD* expression



Transcription of *potD* in various growth conditions compared with *potD* transcription in CDM + HC alone at 37°C.

Shah P and Swiatlo E. Micro.Path (2008), 45(3), 167-72

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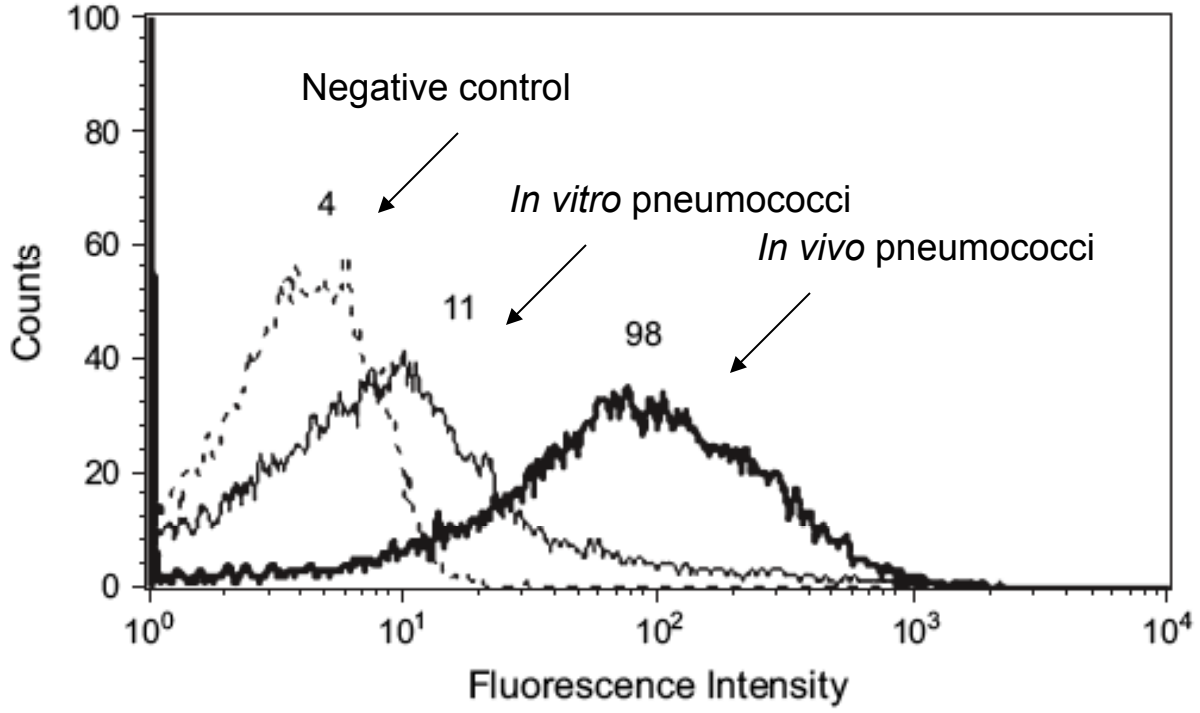
Flow cytometric analysis of PotD expression

Condition		Mean Fluorescence Intensity ± SEM
Negative control		**2.11 ± 0.41**
Low Choline		**21.03 ± 1.59**
Low Choline + Putrescine		49.14 ± 2.59
Low Choline	34°C	51.81 ± 4.38
Low Choline	42°C	45.49 ± 2.91
Low Choline	H_2O_2	37.55 ± 3.50
High Choline		**22.94 ± 1.15**
High Choline	34°C	53.01 ± 6.59
High Choline	42°C	59.55 ±12.20
High Choline	H_2O_2	38.67 ± 7.36
In vivo		**94.00 ± 2.00**

Shah P and Swiatlo E. Micro.Path (2008), 45(3), 167-72

FAC analysis of pneumococcal PotD expression during murine septicemia



Representative flow cytometric analysis of PotD expression on *Streptococcus pneumoniae* D39 cells collected from the blood of systemically infected mice. Dashed line-D39 cells stained with the secondary antibody alone (negative control); thin line-D39 cells grown in CDM; thick line-D39 cells collected from infected mice. Data are shown as fluorescence intensity of the total cell population, and the geometric mean of each peak is indicated.

Shah P and Swiatlo E. Micro.Path (2008), 45(3), 167-72

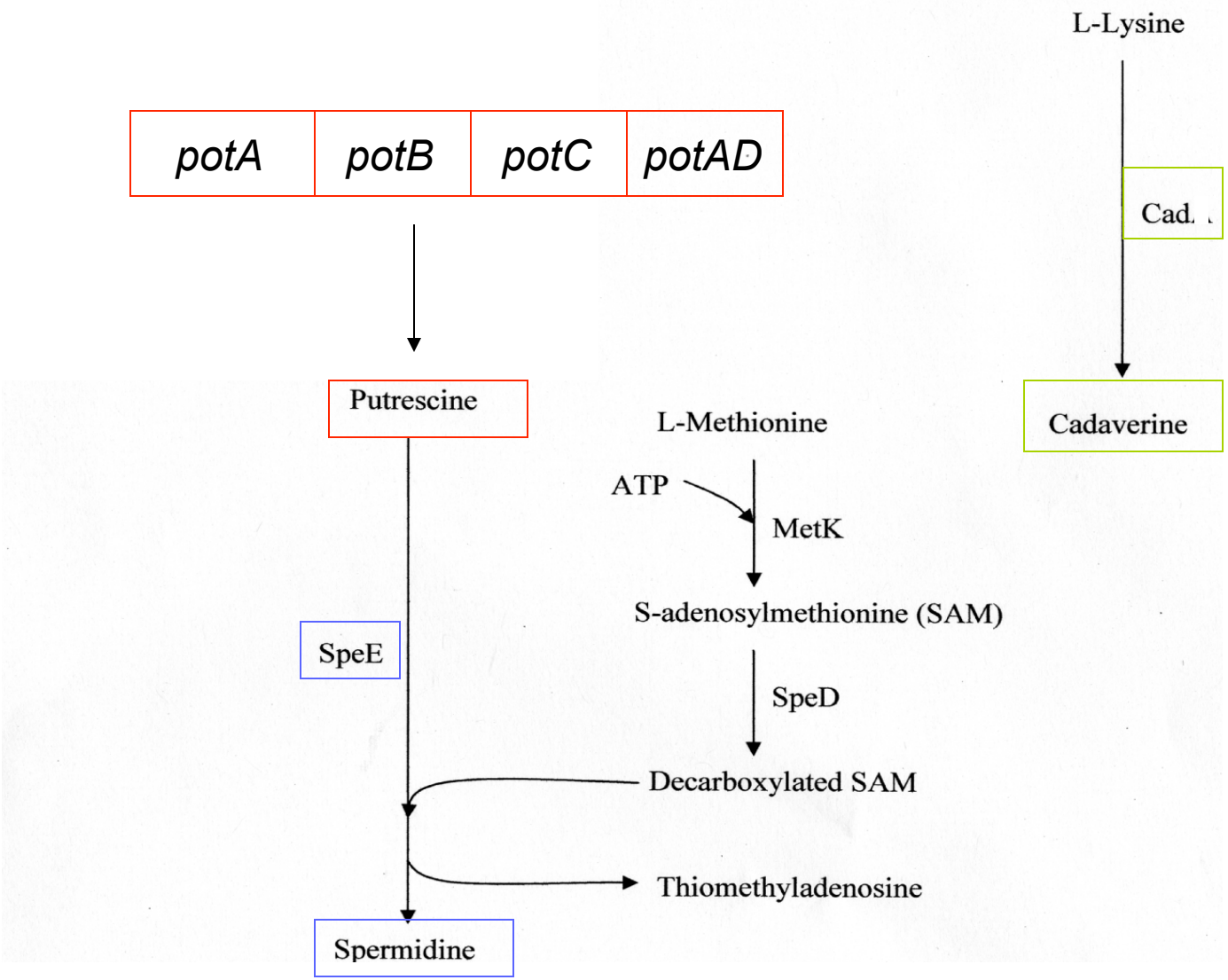
Summary Section 2

- Choline, putrescine, and spermidine modulate expression of pneumococcal PotD

- *S. pneumoniae* PotD expression is upregulated in response to temperature, oxidative stress and during infection

- Polyamine acquisition may be an important adaptive response in pneumococci during growth and virulence in various host imposed micro-environments

Proposed polyamine biosynthesis and transport pathways in pneumococcus



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Rationale

- *potB&C*- STM-Polissi- 1999- ⇓pneumonia
- PotD- Protective immunogen and stress responses
- *cad*- STM-Camilli- 2002- ⇓pneumonia

Hypothesis: Polyamines play important roles in pneumococcal fitness *In vivo*

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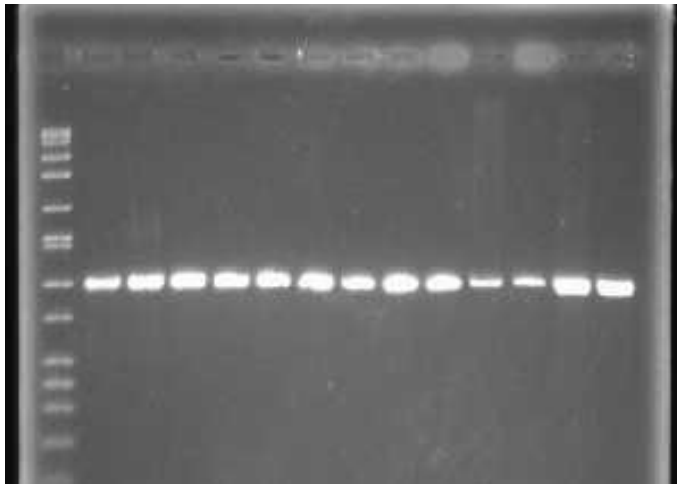
Section 3

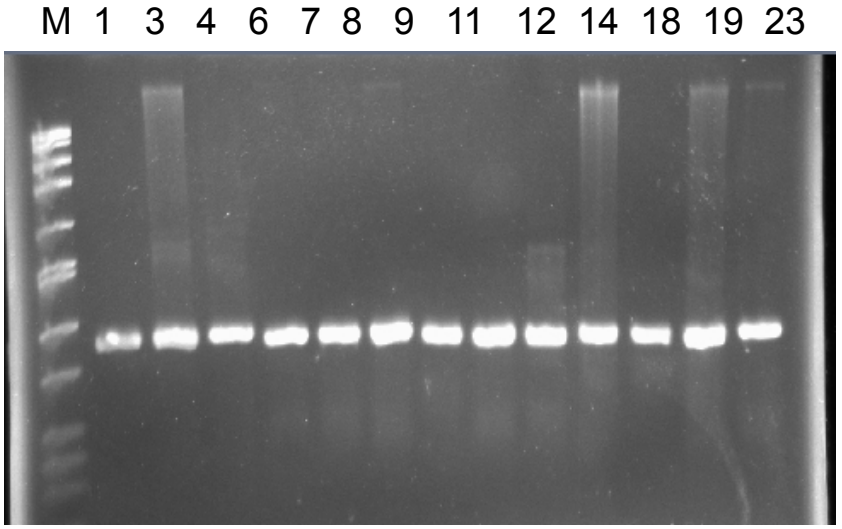
POLYAMINE BIOSYNTHESIS AND TRANSPORT CONTRIBUTE TO THE *IN VIVO* FITNESS OF THE PNEUMOCOCCUS

Hypothesis: Polyamines play important roles in pneumococcal fitness *In vivo*

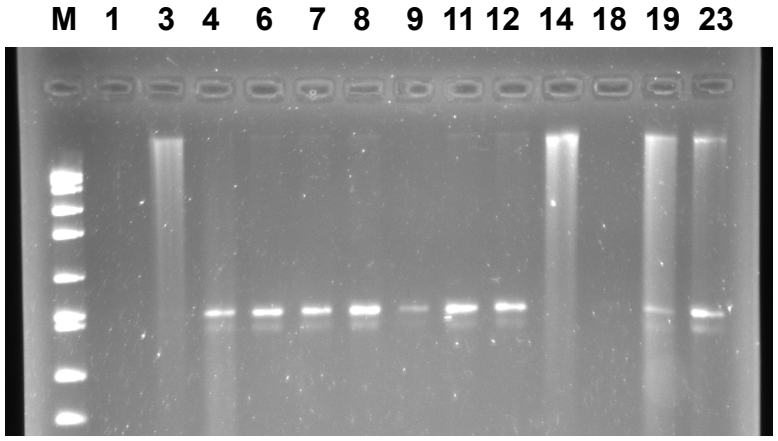
Distribution of polyamine biosynthesis and transport genes in different capsular serotypes



potD



speE



cad

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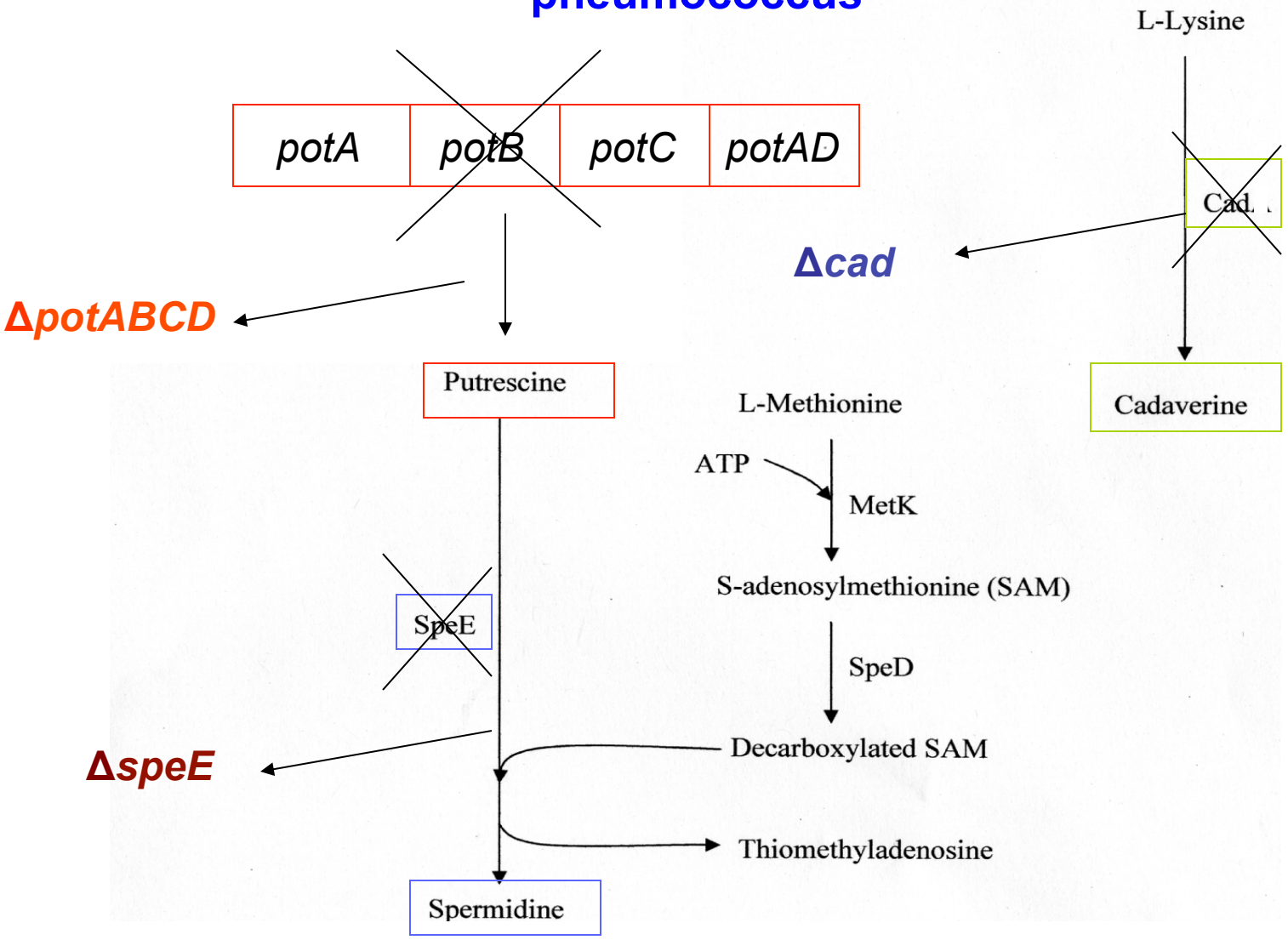
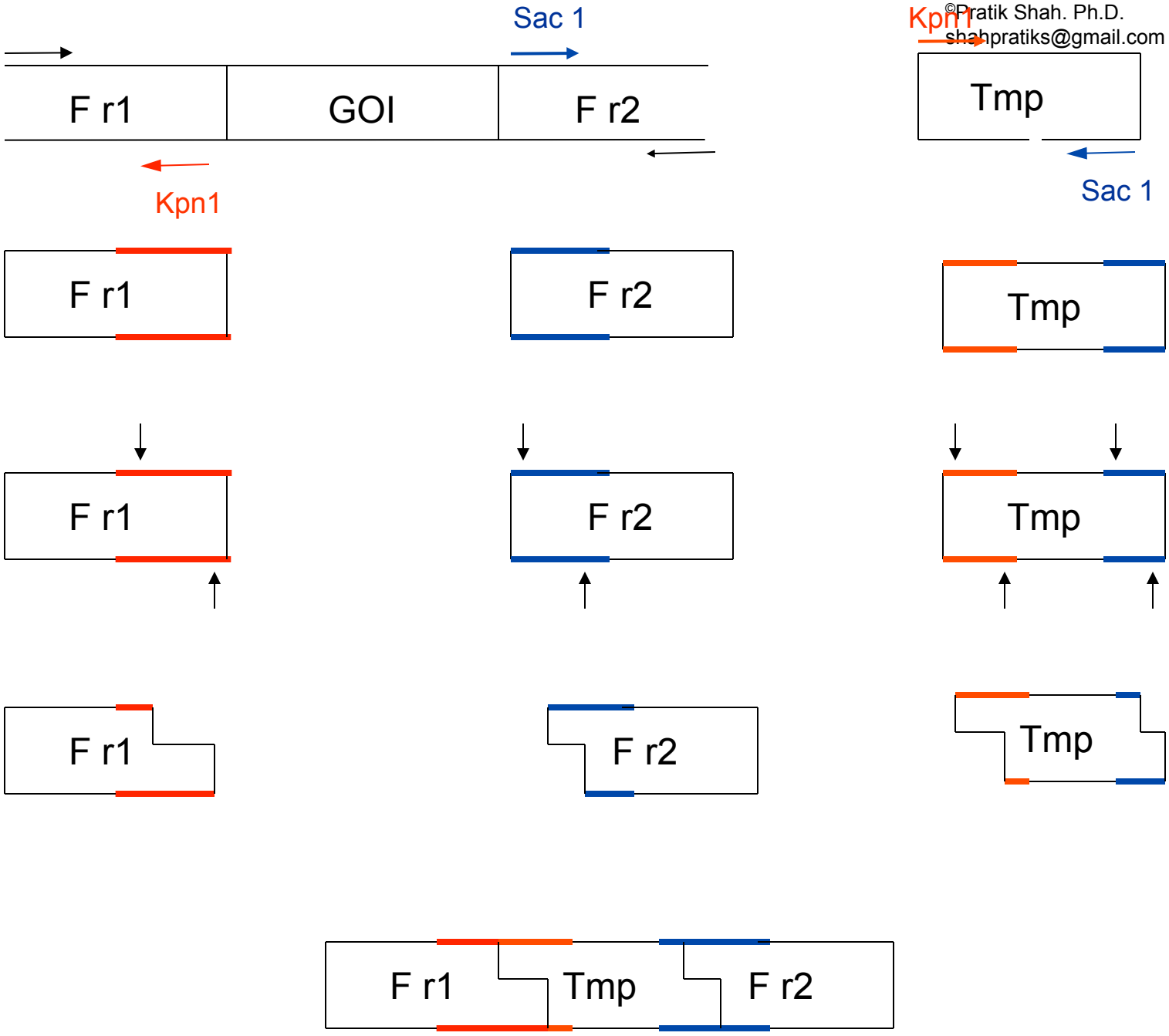
Proposed polyamine biosynthesis and transport pathways in pneumococcus





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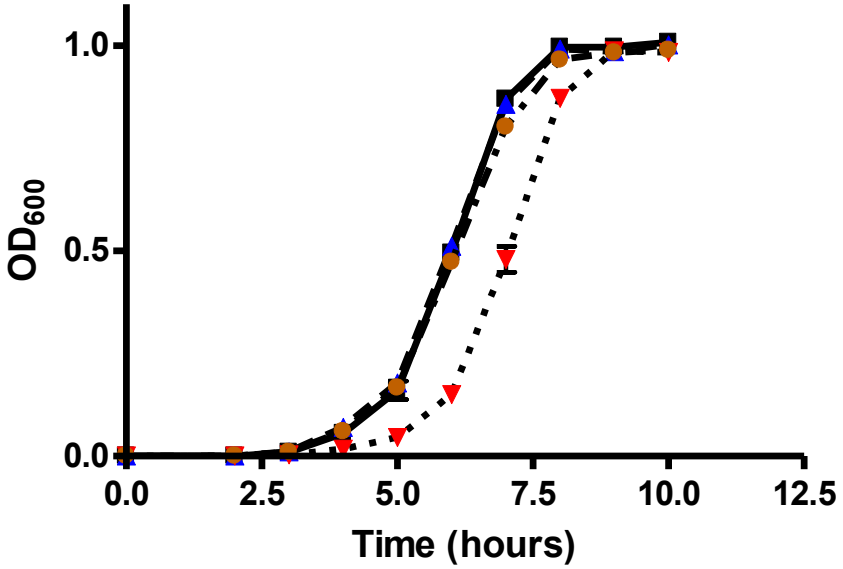
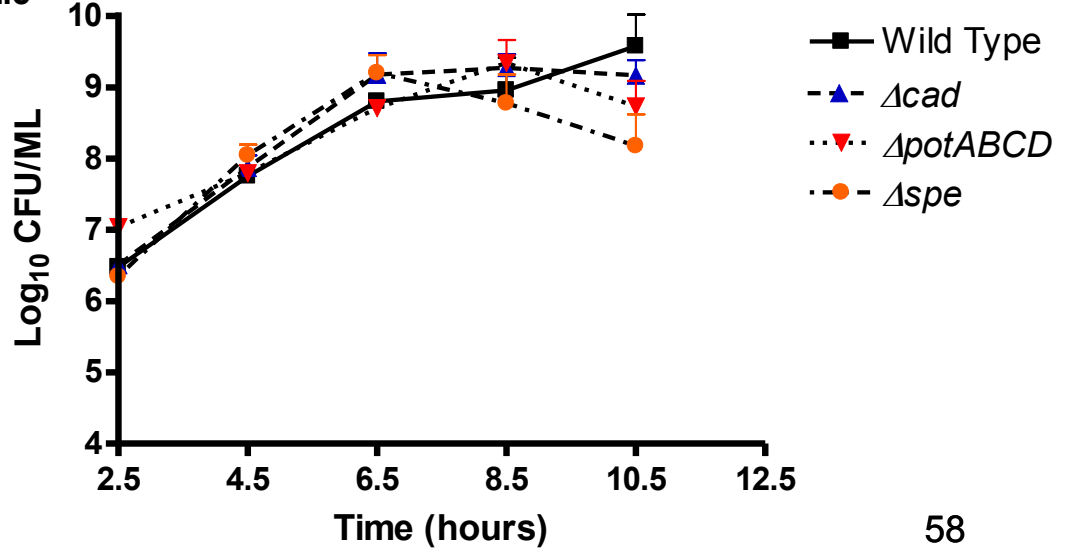
Mutant Strains

Mutant	Polyamine Deficiency
ΔpotABCD	Putrescine/Spermidine
Δcad	Cadaverine
ΔspeE	Spermidine

In vitro growth





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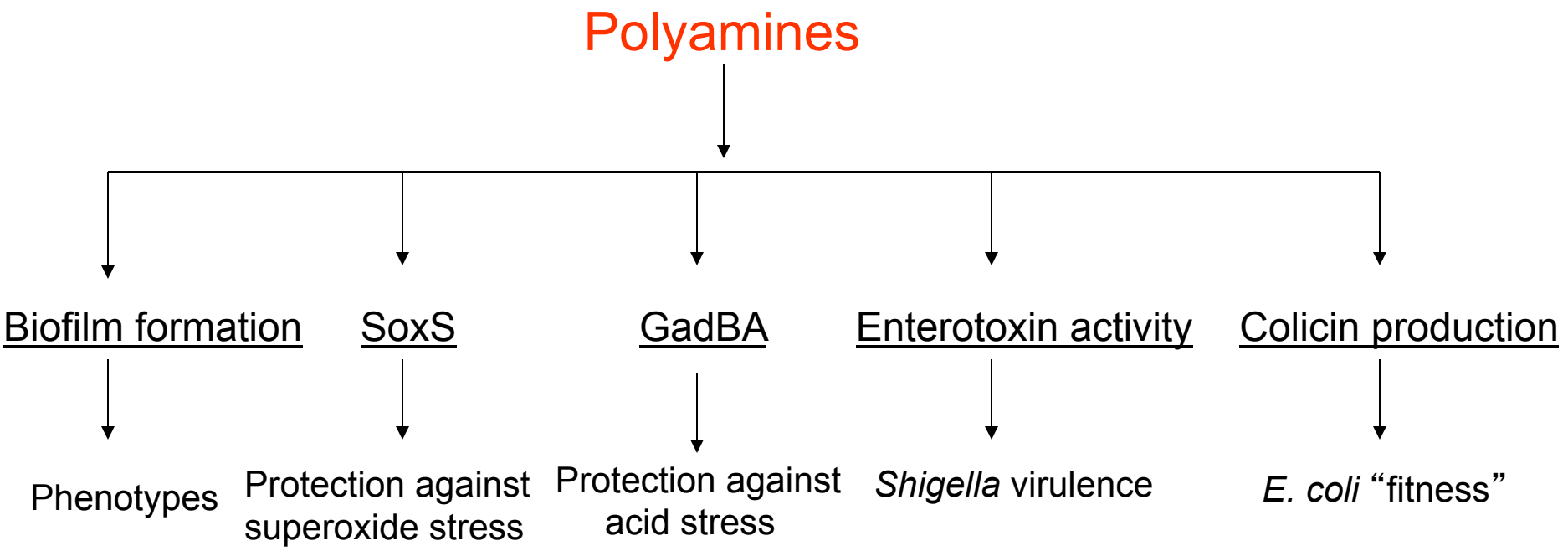
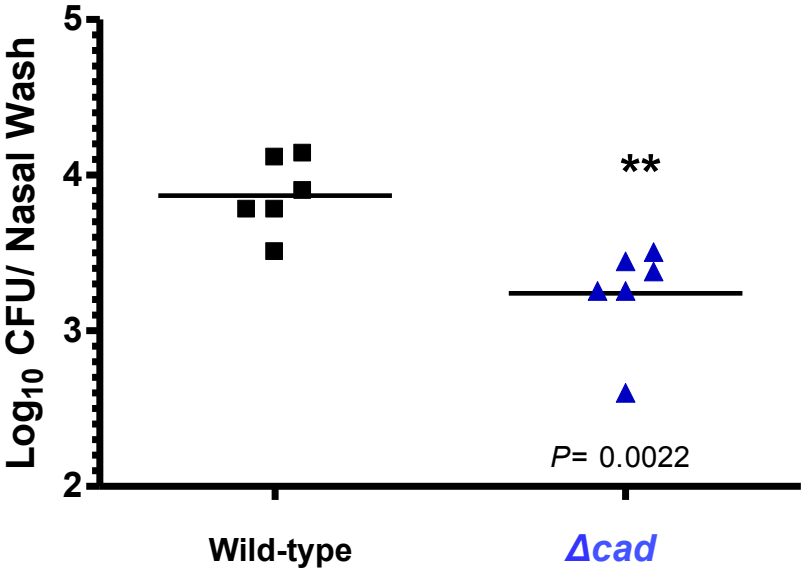
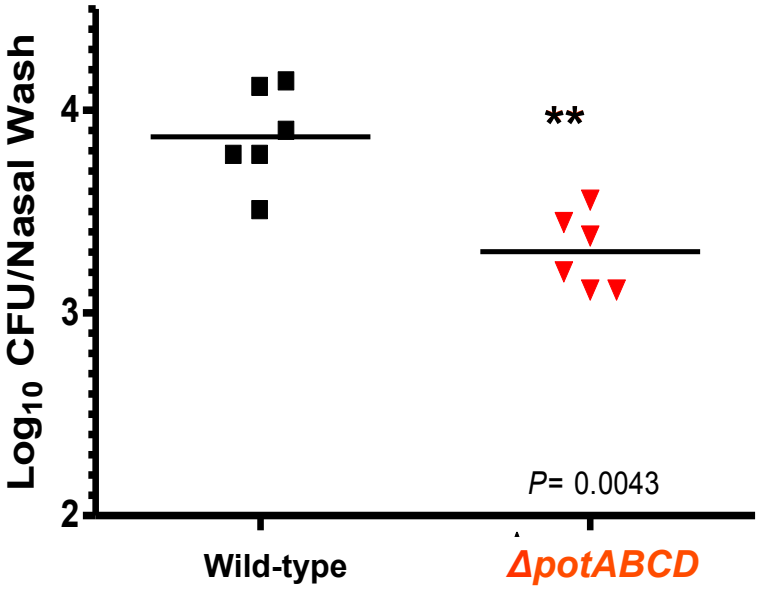
Polyamines as "regulators" of multiple gene expression cascades

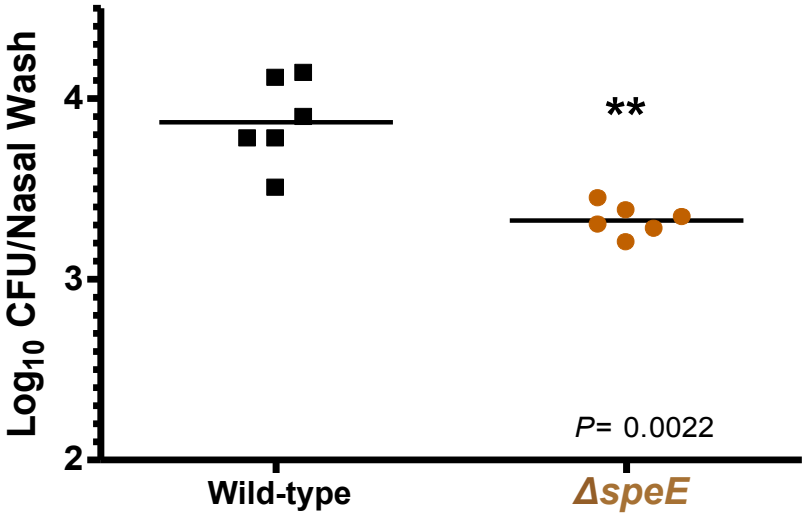


Polyamines may regulate expression of pneumococcal virulence

Nasopharyngeal colonization

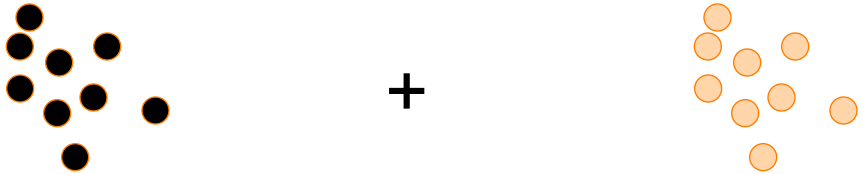




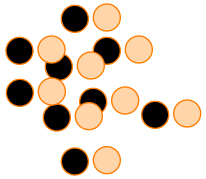


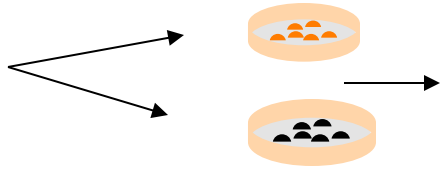
Challenge dose-4 × 10^5 TIGR4 in 20µl of LR-Intranasal

Equal no. of mutant and wild-type cells



Mix



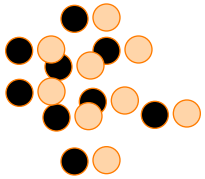


Confirm

Infect

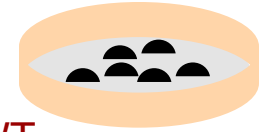
$$CI = \frac{\text{No. of } \textit{mutants}}{\text{No. of WT}}$$

Recover

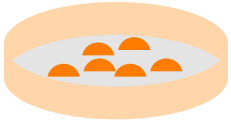


CI < 1 (attenuated)

Plate



Tmp^r + WT



Tmp^r

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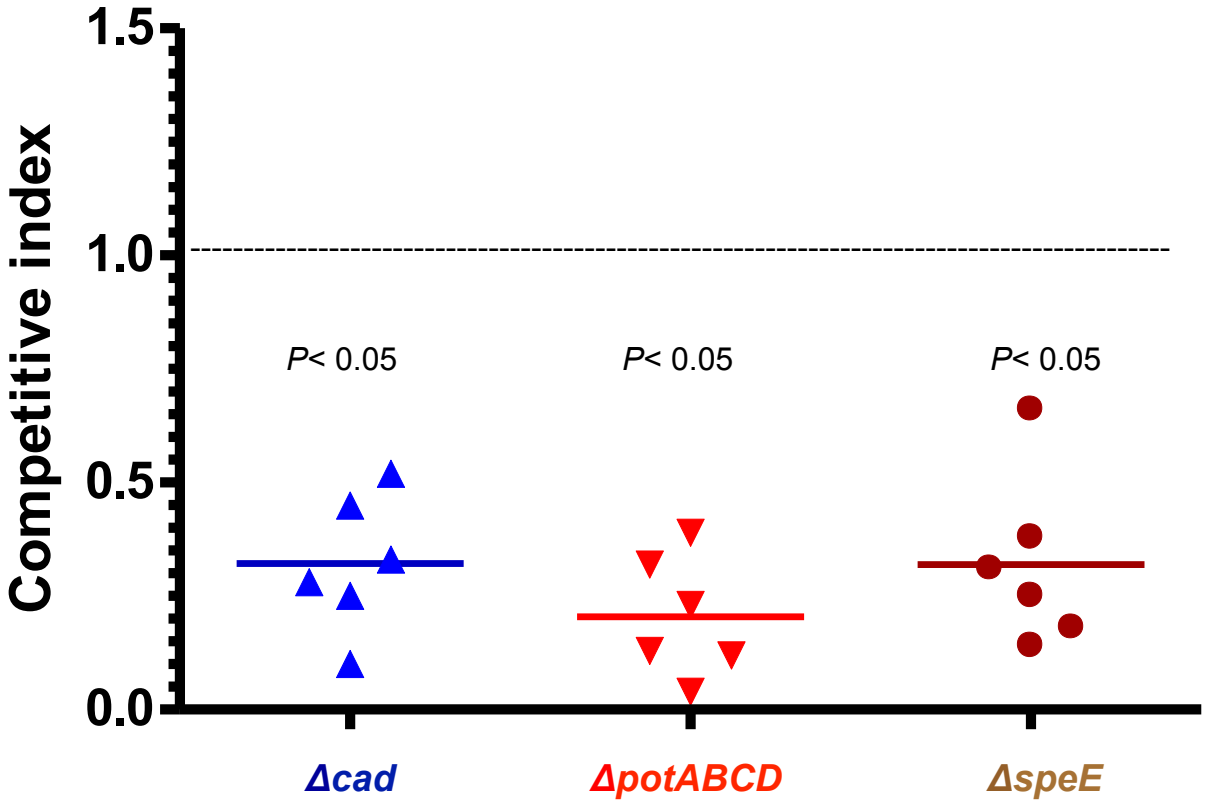
Nasopharyngeal colonization: Competitive Index



Challenge dose-4 × 10^5 TIGR4 in 20µl of LR-Intranasal

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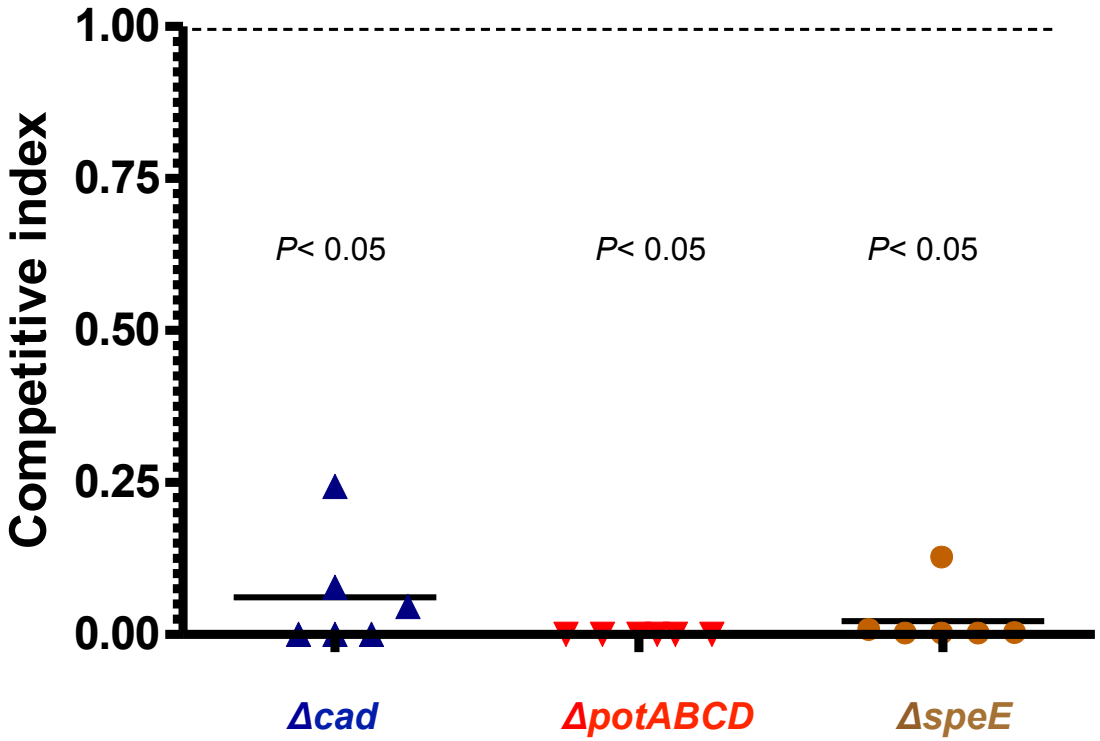
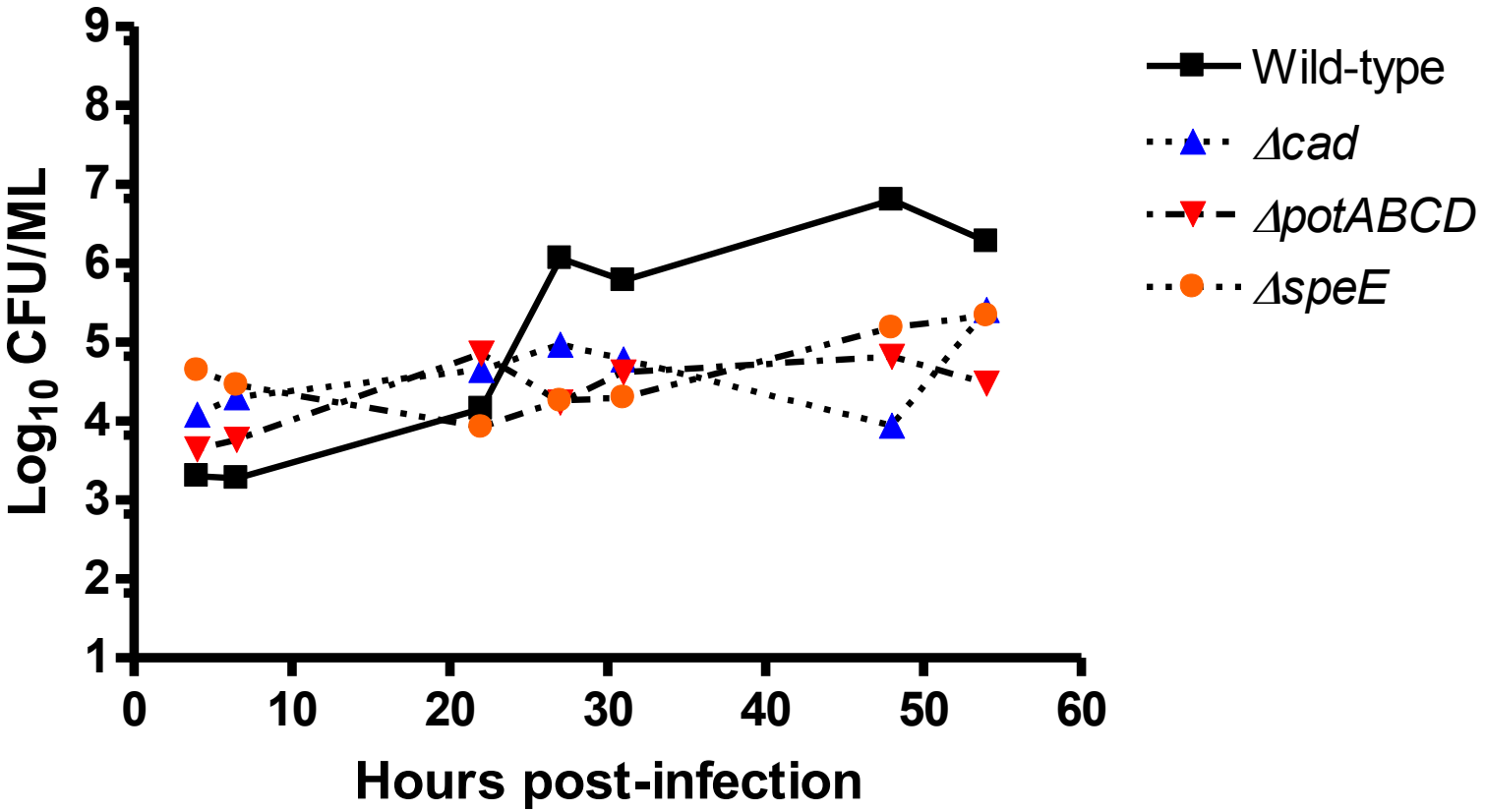
Pneumonia: Competitive Index



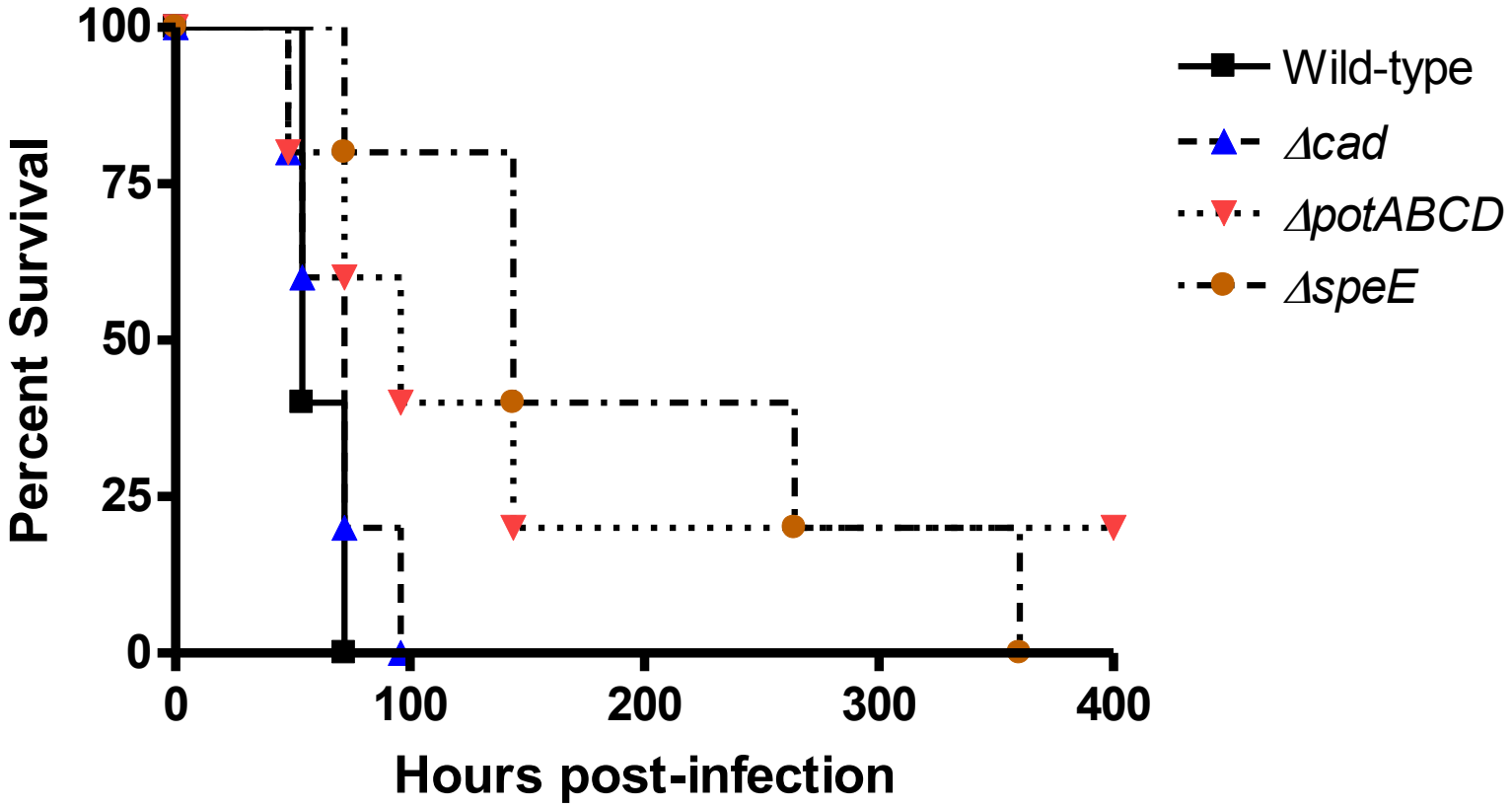
Challenge dose-4 × 10^5 TIGR4 in 40µl of LR (anaesthetized mice)-Intranasal

Bacteremia-Replication



I.v. infection with mutant or wild-type TIGR4

Bacteremia-Lethal



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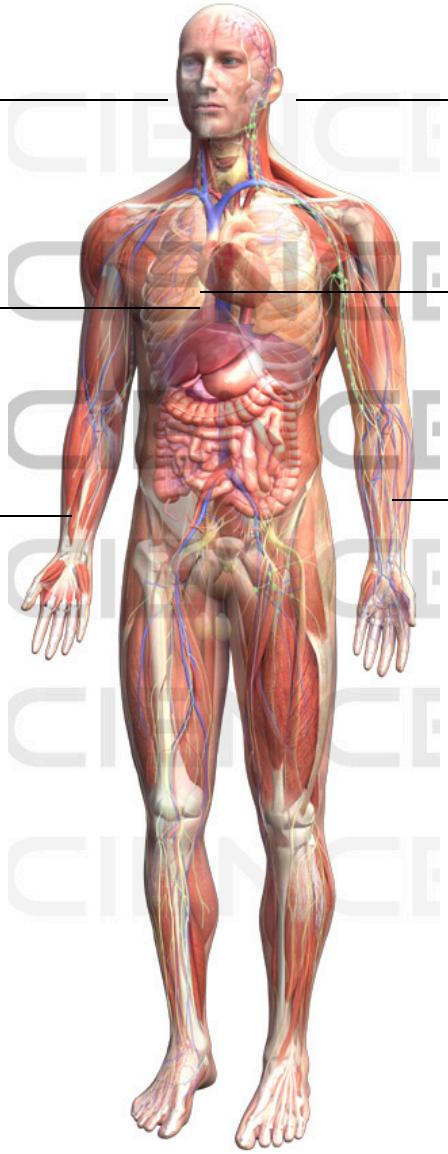
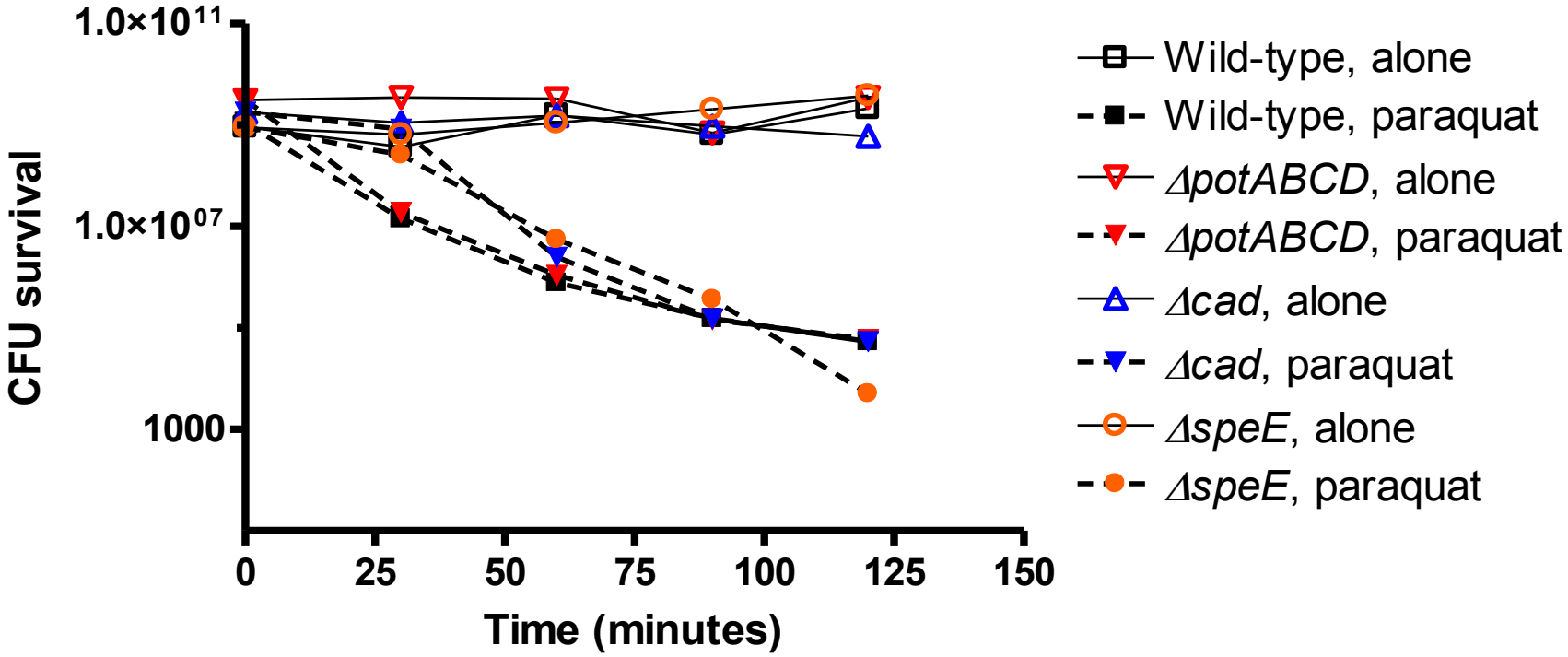


Availability of polyamines regulates virulence?

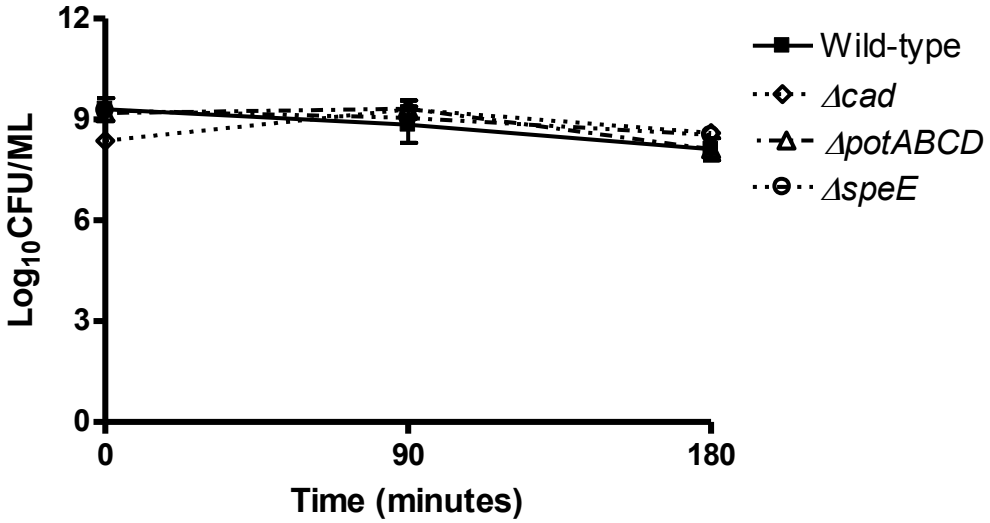
Mechanism of attenuation?

Oxidative stress-No difference in survival



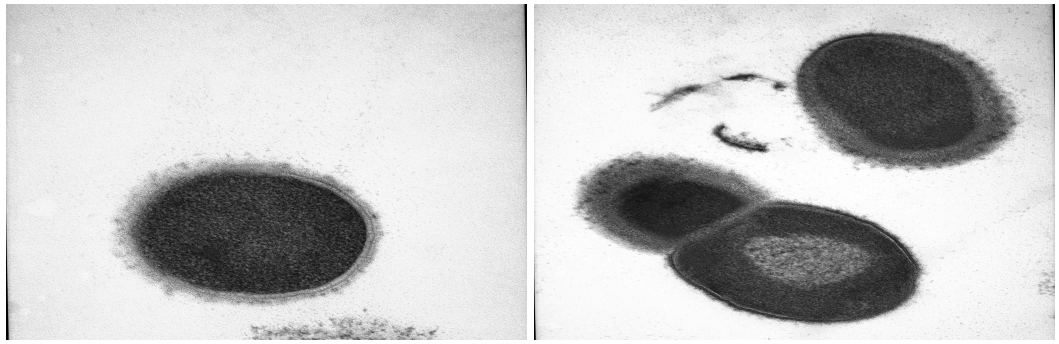
Mechanism of attenuation?

Acid Stress (exposure to low pH)- No difference in survival

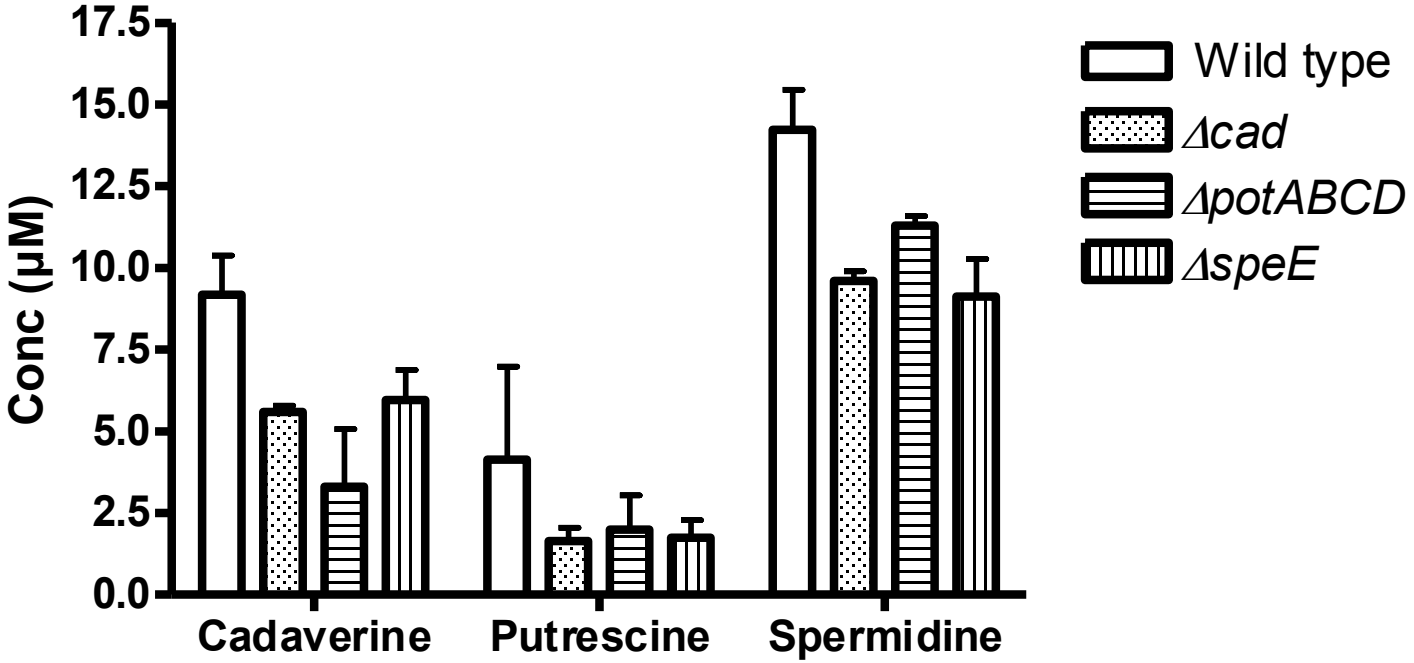


No difference in capsule thickness

WT Δ



Intracellular polyamine levels



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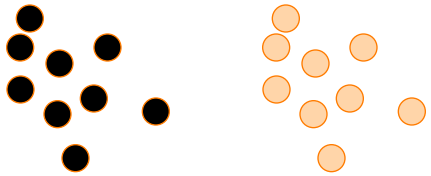
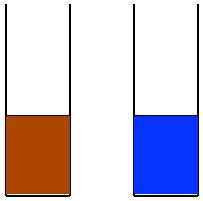
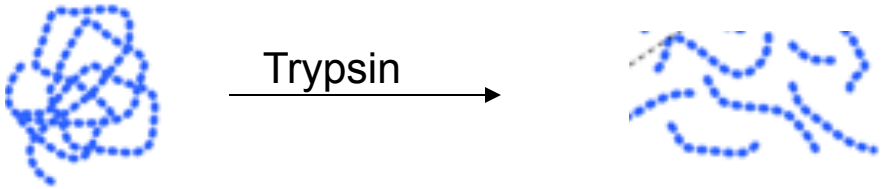
Large-scale proteomics

Equal no. of *ΔpotABCD* and wild-type cells (THY)



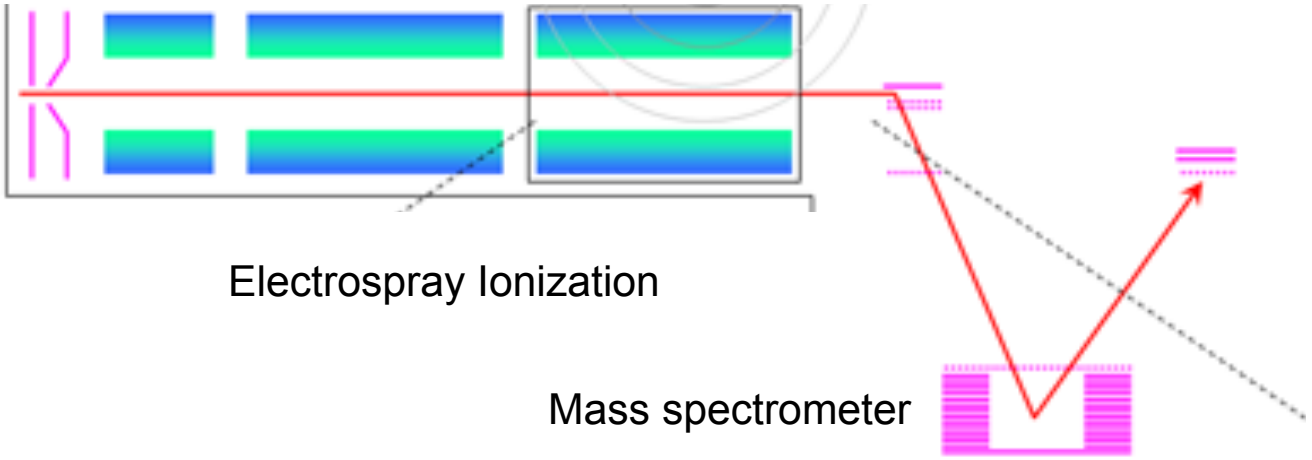


Sample fractionation



Proteins

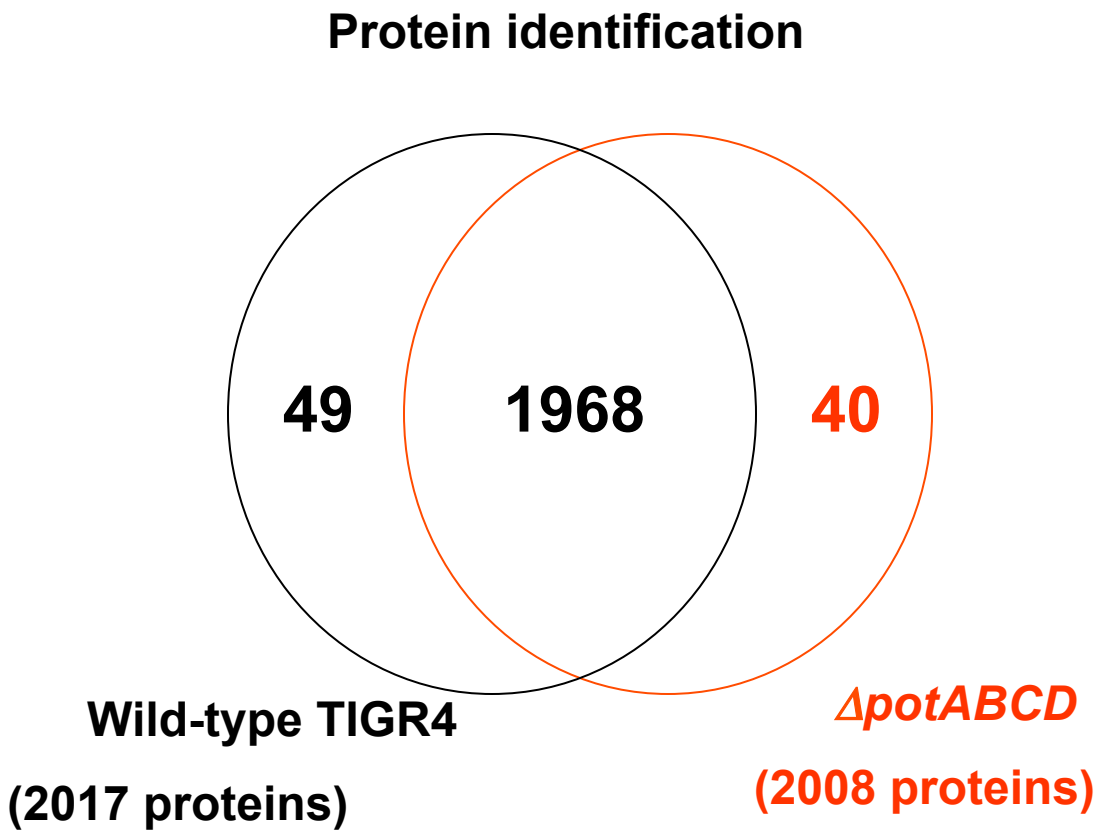
Peptides



Electrospray Ionization

Mass spectrometer

Tandem mass spectrometry analysis



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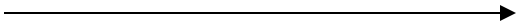
Proteins upregulated in *ΔpotABCD*

Amino acid biosynthesis

Carbohydrate metabolism

+



Polyamines

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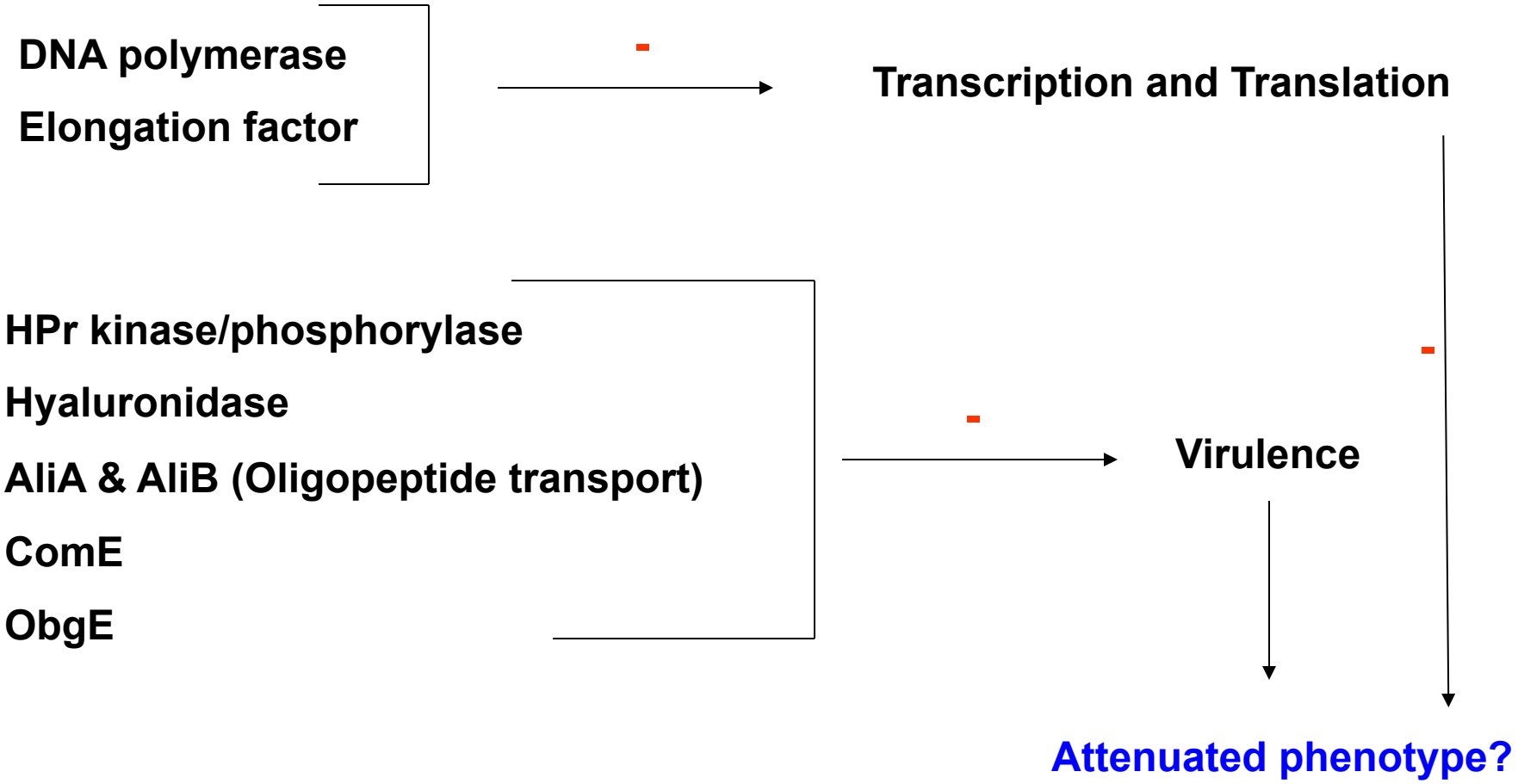
Proteins downregulated in *ΔpotABCD*



1698–1703 | PNAS | February 5, 2008 | vol. 105 | no. 5

A direct link between carbohydrate utilization and virulence in the major human pathogen group A *Streptococcus*

Samuel A. Shelburne III*, David Keith*, Nicola Horstmann†, Paul Sumby‡, Michael T. Davenport*, Edward A. Graviss§, Richard G. Brennan†, and James M. Musser‡¶

Sucrose metabolism contributes to *in vivo* fitness of *Streptococcus pneumoniae*

Ramkumar Iyer[1] and Andrew Camilli[2]*

CodY in *Staphylococcus aureus:* A regulatory link between metabolism and virulence gene expression

Mycobacterial persistence requires the utilization of host cholesterol
***PNAS* | March 18, 2008 | vol. 105 | no. 11 | 4376-4380**

Polyamines and Pneumococci: Partners in Crime?