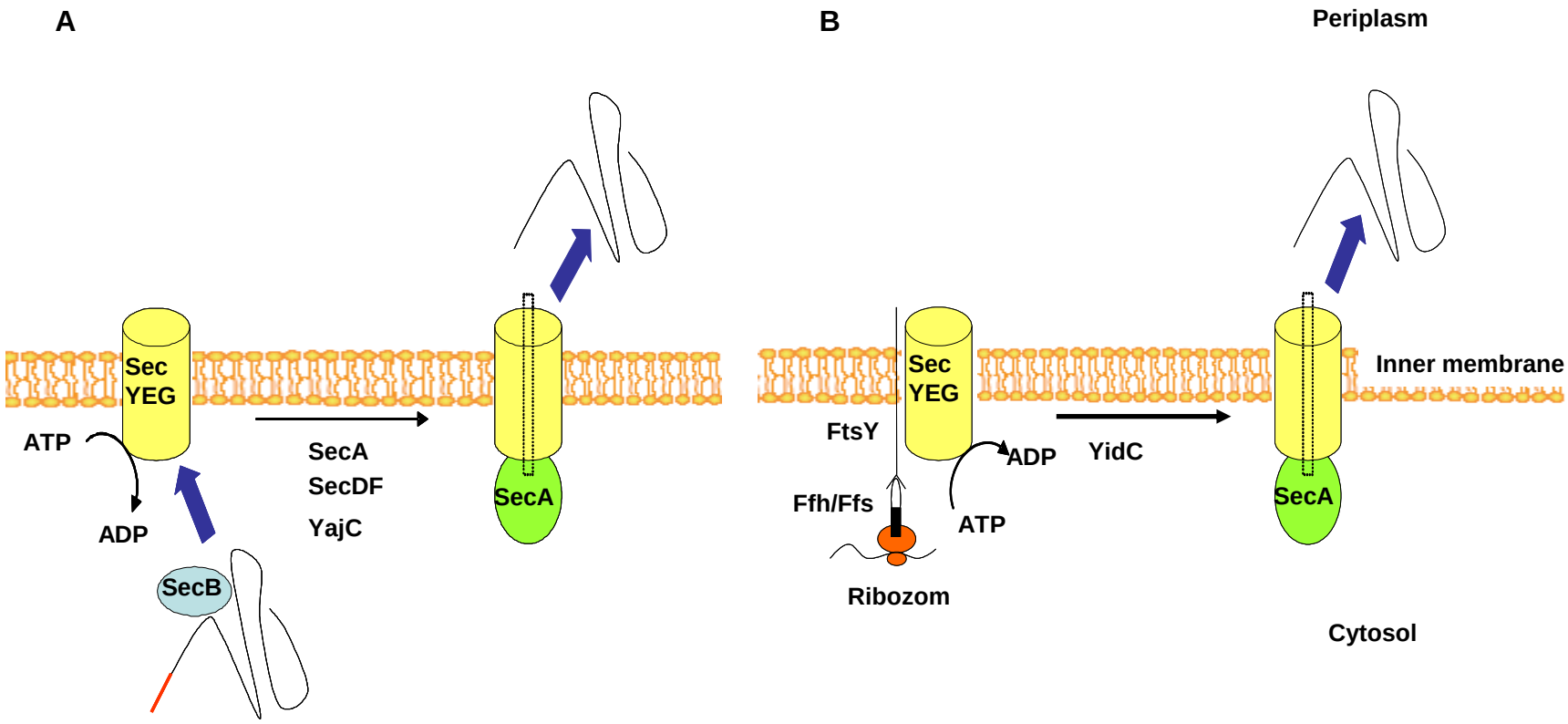
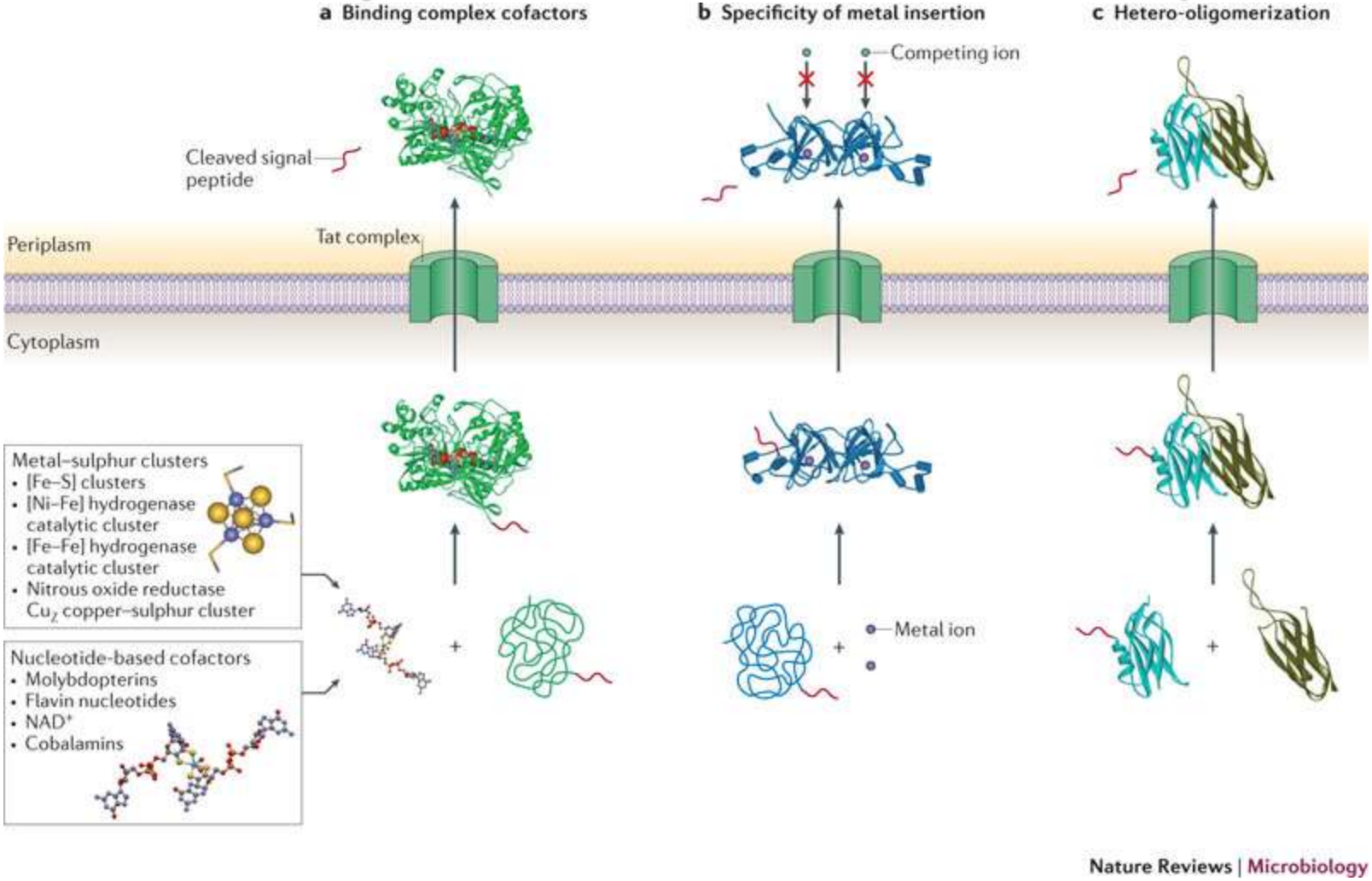
The toxins and S-layer proteins, the capsule material, all need to get out of the cell

How does bacterial secretion work

The general secretory pathway- GSP – type II starts as in G+

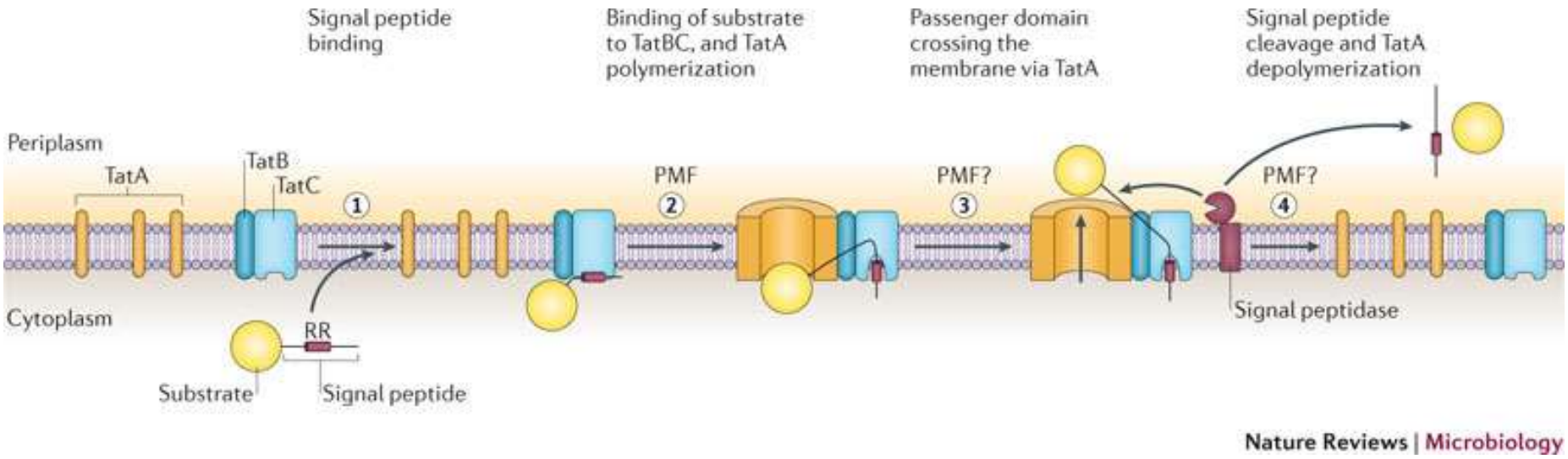


The TAT system for secretion of folded proteins



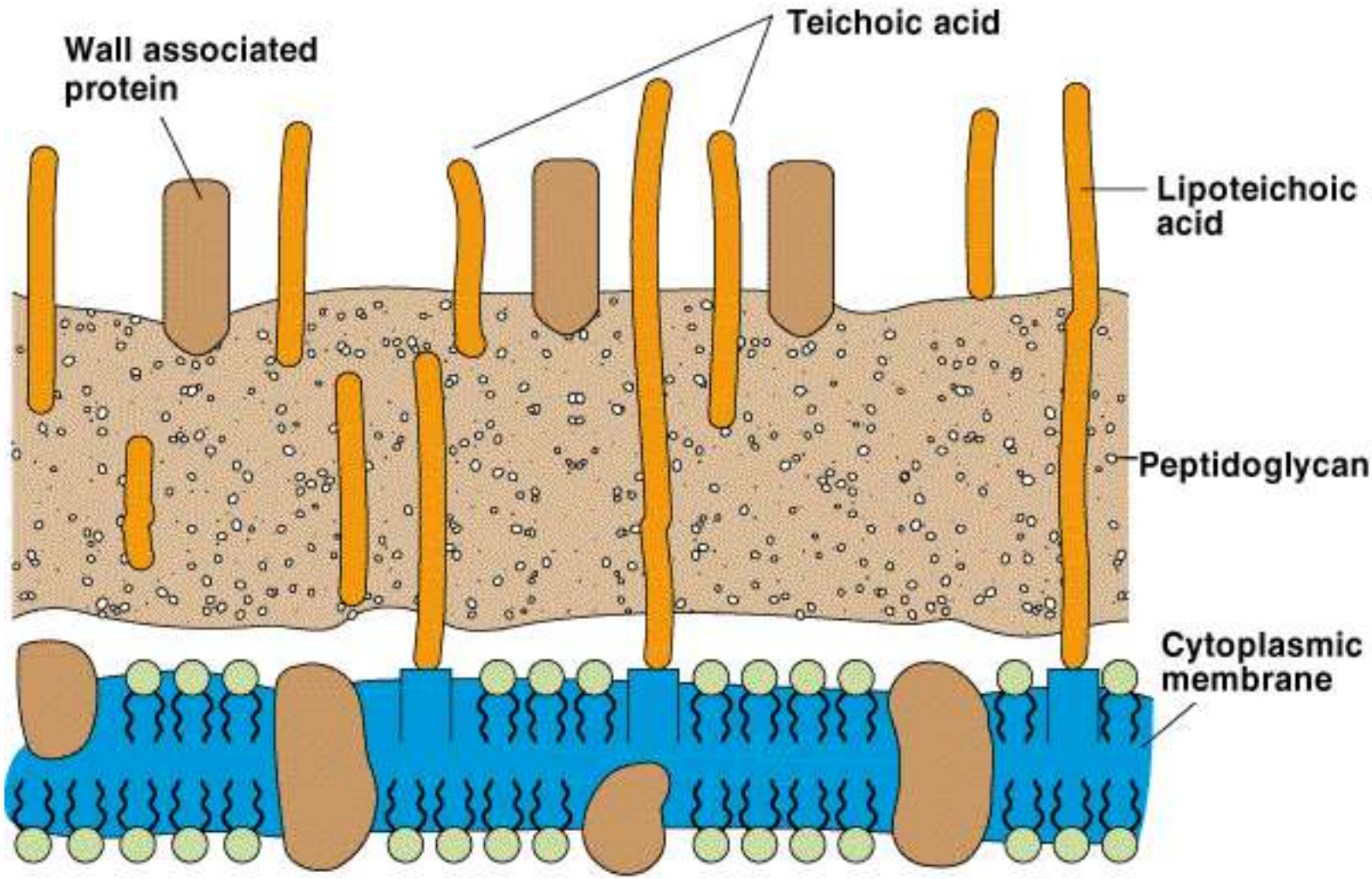
Three reasons that proteins use the twin-arginine translocation (Tat) pathway have been definitively identified. **a** | Tat targeting allows proteins to acquire their cofactor before transport across the cytoplasmic membrane. The example shown is insertion of a molybdopterin cofactor into trimethylamine-*N*-oxide reductase. Only certain cofactors are associated with Tat-mediated transport. These can be broadly divided into metal–sulphur clusters and cofactors containing a nucleotide moiety. **b** | Metal ions compete for binding sites in proteins. Use of the Tat system allows a protein to obtain metal ions under controlled conditions in the cytoplasm. The example shown is the Mn^{2+}-binding periplasmic protein MncA from the cyanobacterium *Synechocystis* sp. PCC 6803; MncA picks up its Mn^{2+} cofactors in the protected cytoplasmic environment to avoid competing ions that are present in the periplasm[37]. **c** | The Tat pathway allows hetero-oligomeric complexes to form in the cytoplasm and then be transported by a signal peptide in just one of the constituent subunits. The example shown here is the SoxYZ complex, which is involved in thiosulphate oxidation[39].

TAT system operation in E. coli

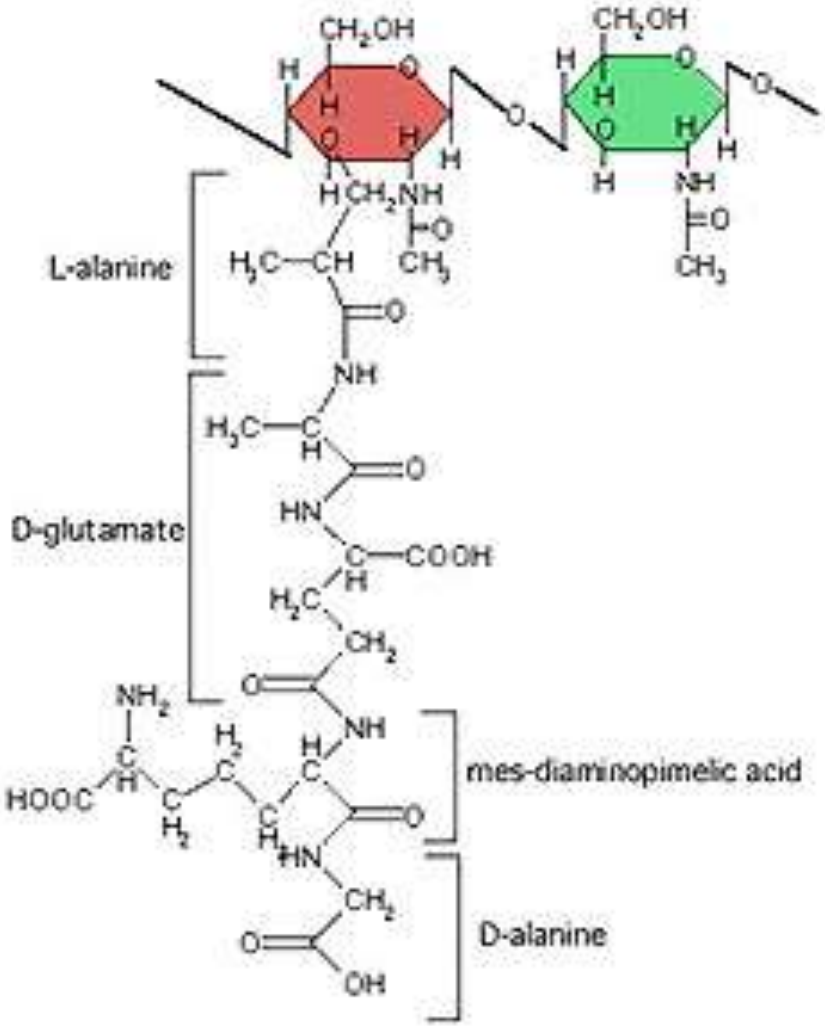


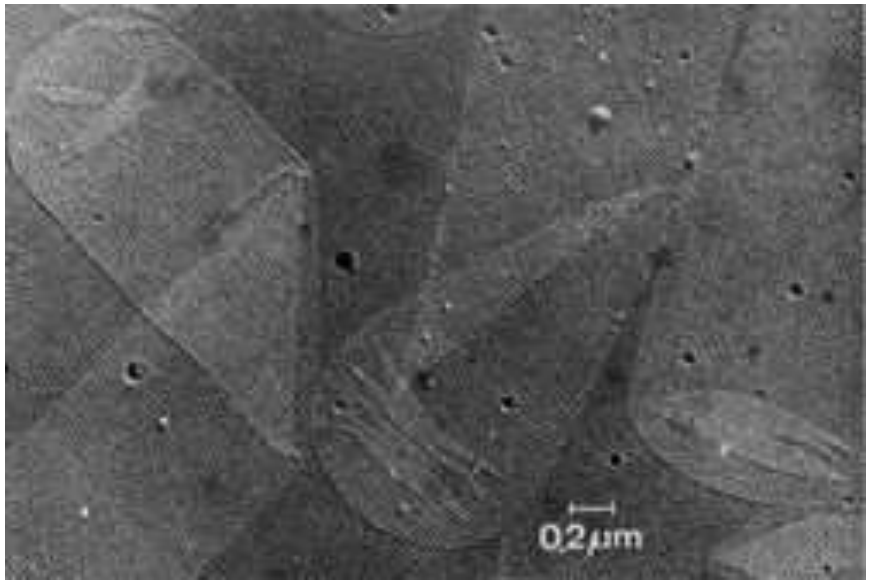
At the start of the cycle, TatB and TatC associate as a complex, whereas TatA is present as dispersed protomers. The TatBC complex contains multiple copies of each protein, but only single copies are depicted, for clarity. The number of subunits in a TatA protomer is uncertain. Step 1: to initiate substrate translocation, the TatBC complex binds the signal peptide of a substrate protein in an energy-independent step; the twin-arginine (RR) consensus motif in the signal peptide is specifically recognized by a site in TatC . The remainder of the signal peptide and the substrate passenger domain are close to TatB. Step 2: in the plant thylakoid Tat system, the proton-motive force (PMF) causes the substrate signal peptide to become more tightly bound to the TatBC complex and less accessible from the cytoplasm. However, the amino-terminus of the signal peptide remains at the cytoplasmic side of the membrane. Some evidence suggests a potentially analogous, but PMF-independent, tightening of TatBC–substrate interactions in the *Escherichia coli* Tat system. TatA protomers are recruited to the TatBC complex and polymerized in a step that depends on the PMF. The resulting TatABC complex is the active translocation site. At this stage, the signal peptide is in contact with all three Tat components[83]. Step 3: the passenger domain of the substrate protein crosses the membrane via the polymerized TatA component, and the signal peptide remains bound to the TatBC complex. It is not known whether this step requires energization by the PMF. Step 4: when the passenger domain has reached the far side of the membrane, the signal peptide is normally proteolytically removed by a signal peptidase at the periplasmic face of the membrane, and TatA dissociates from TatBC and depolymerizes back to free protomers. The fate of the signal peptide following transport is uncertain, so this peptide is arbitarily shown as being released into the periplasmic compartment.

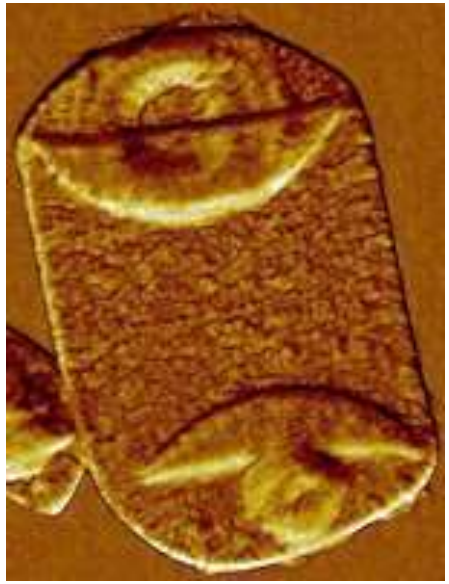
G+ bacteria – secretion is easier due to single membrane???



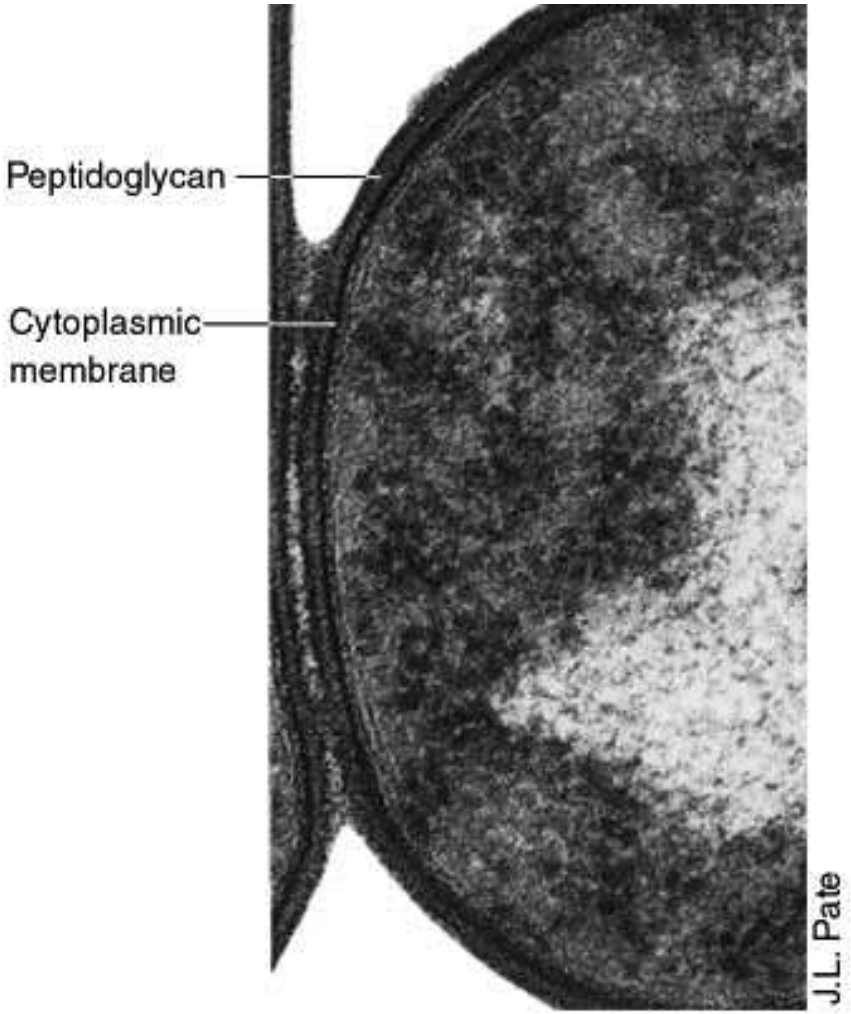
The peptidoglycan

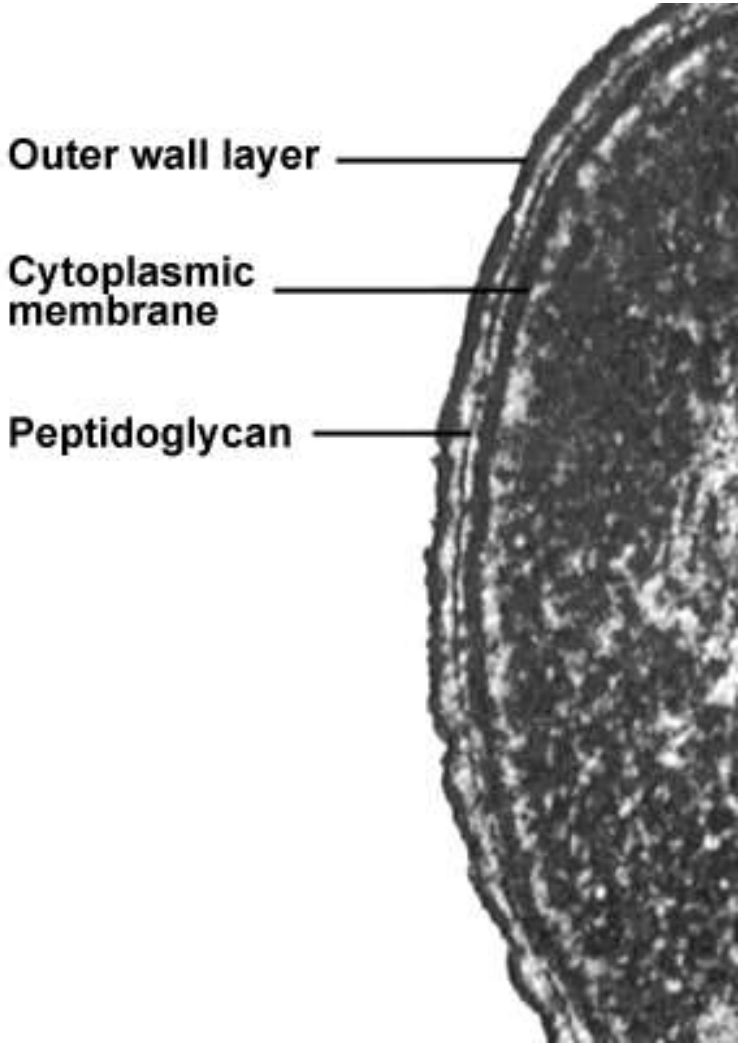




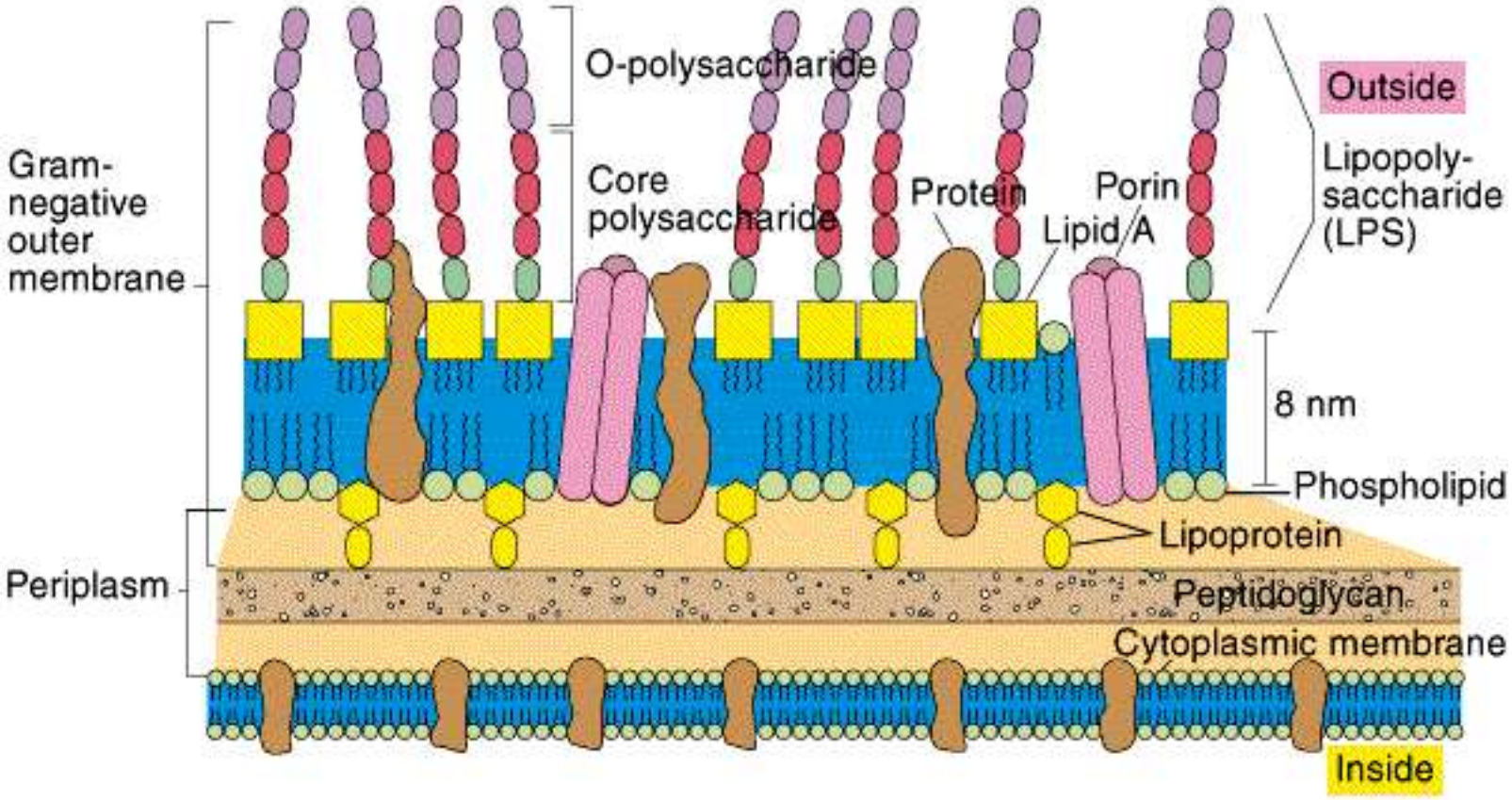


Cell wall of G+ and G- bacteria (TEM)

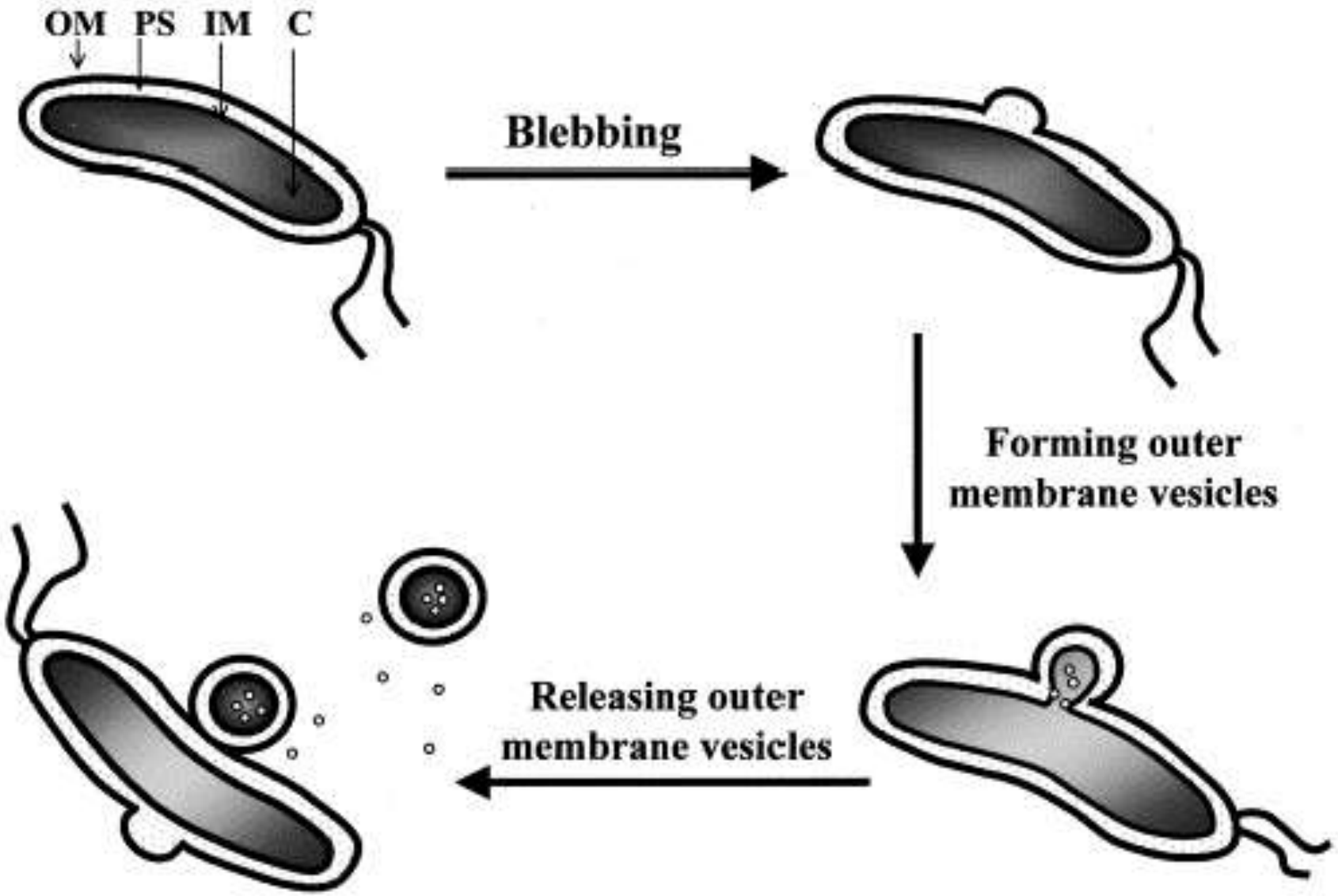




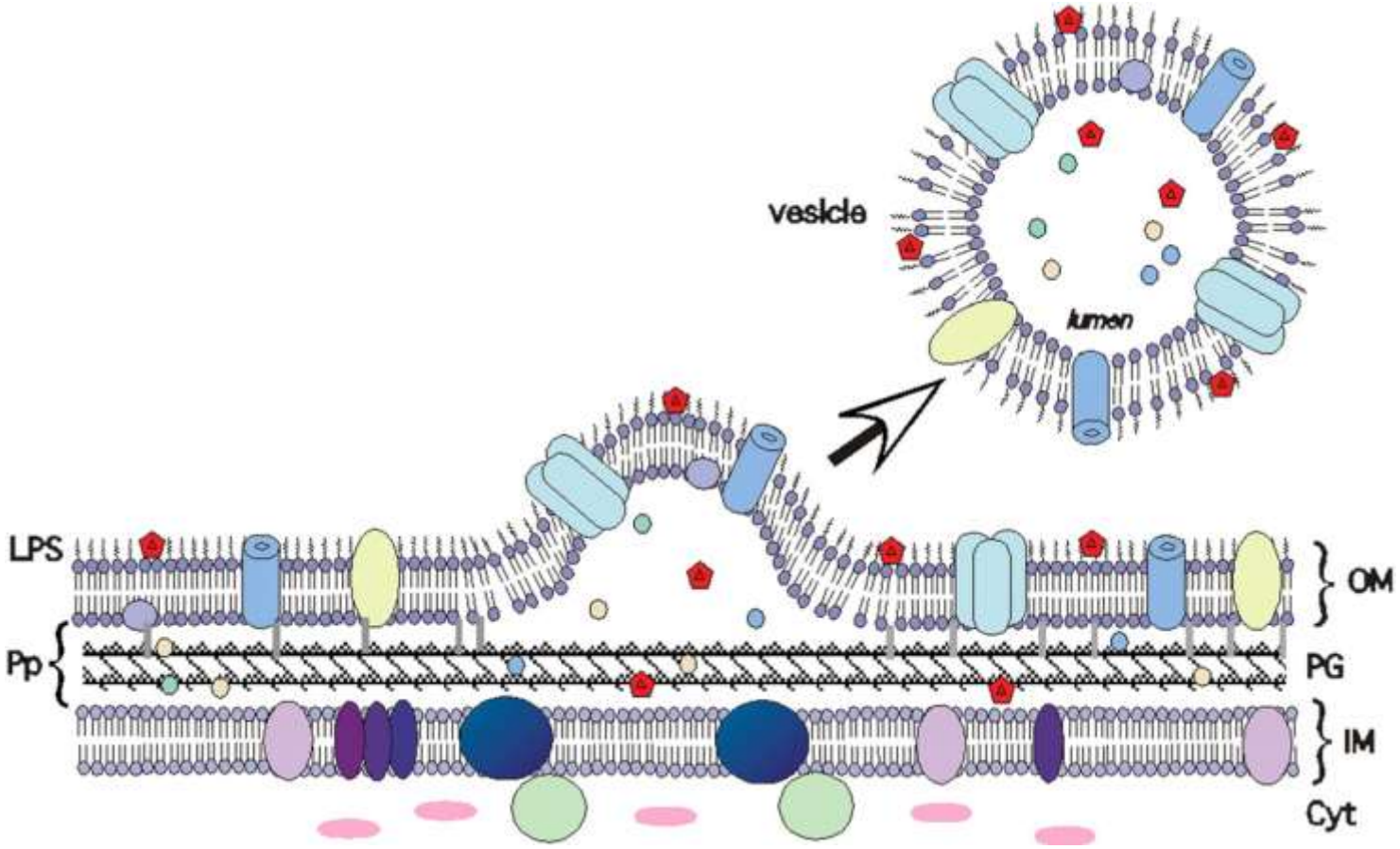
G- bacterial cell wall – a real challenge for protein secretion



Outer membrane vesicles – A decoy target strategy



Outer membrane vesicles – A decoy target strategy



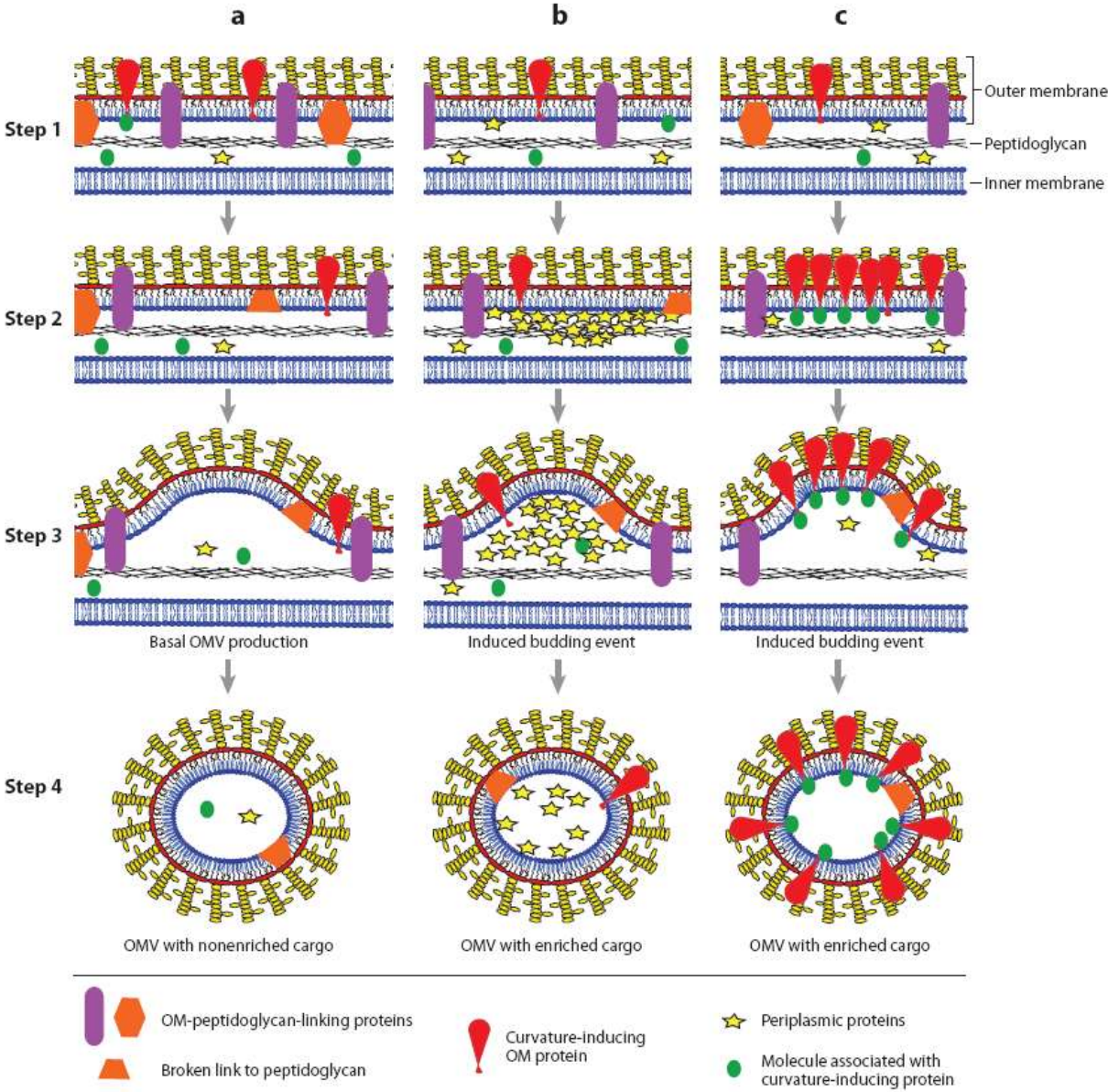


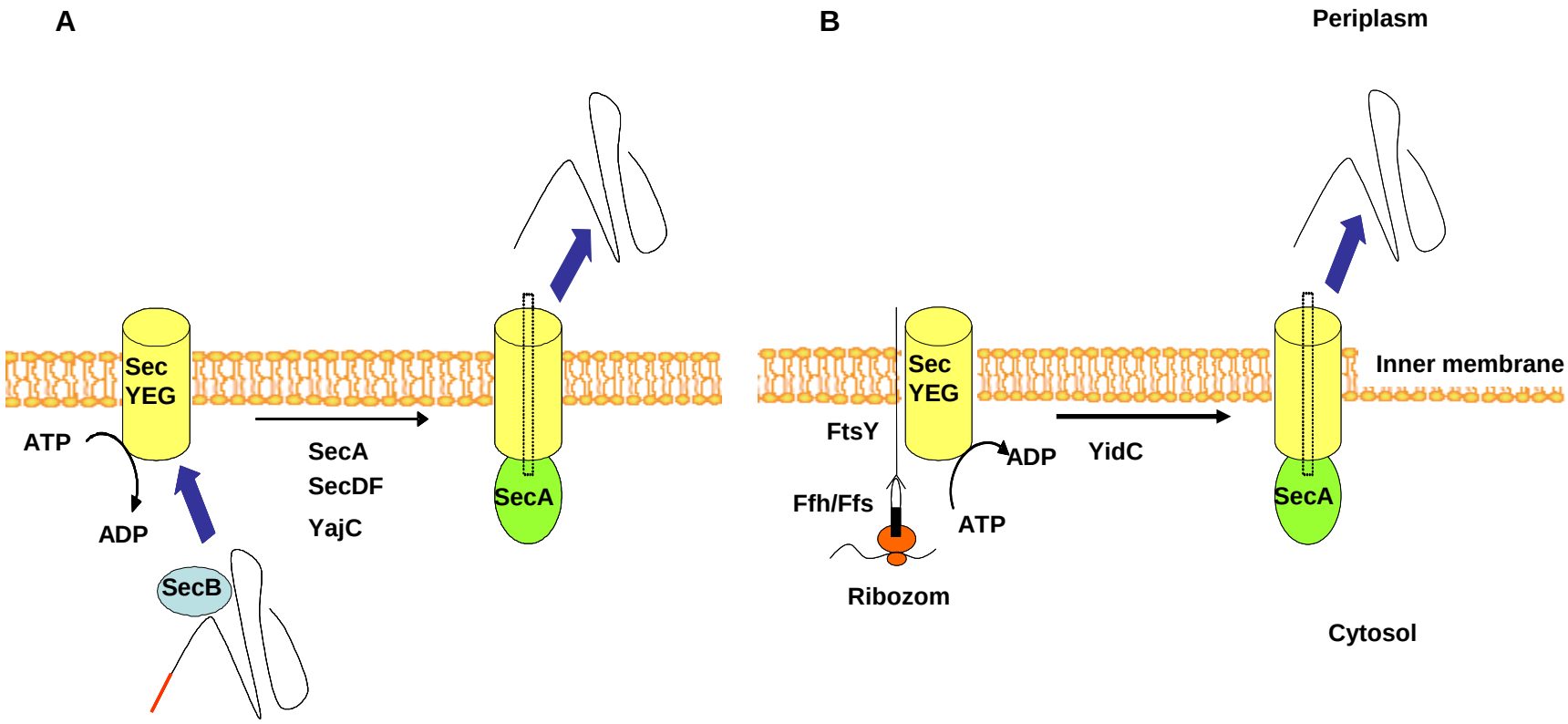
Figure 3

Events in outer membrane vesicle (OMV) biogenesis. Step 1: Unbudded gram-negative envelope. The overall homogenous distribution of envelope proteins, including outer membrane (OM)-peptidoglycan-linking proteins (*purple ovals or orange hexagons*), does not lead to significant areas of OM unlinked from peptidoglycan. Steps 2 and 3: Initial stages of vesiculation. In various areas, links between the OM and the peptidoglycan are lost, either by movement of the linking protein (*purple ovals*) or by breaking the connections directly (*orange half hexagons*). This could be sufficient for basal OMV production (*column a*). Gathering of periplasmic

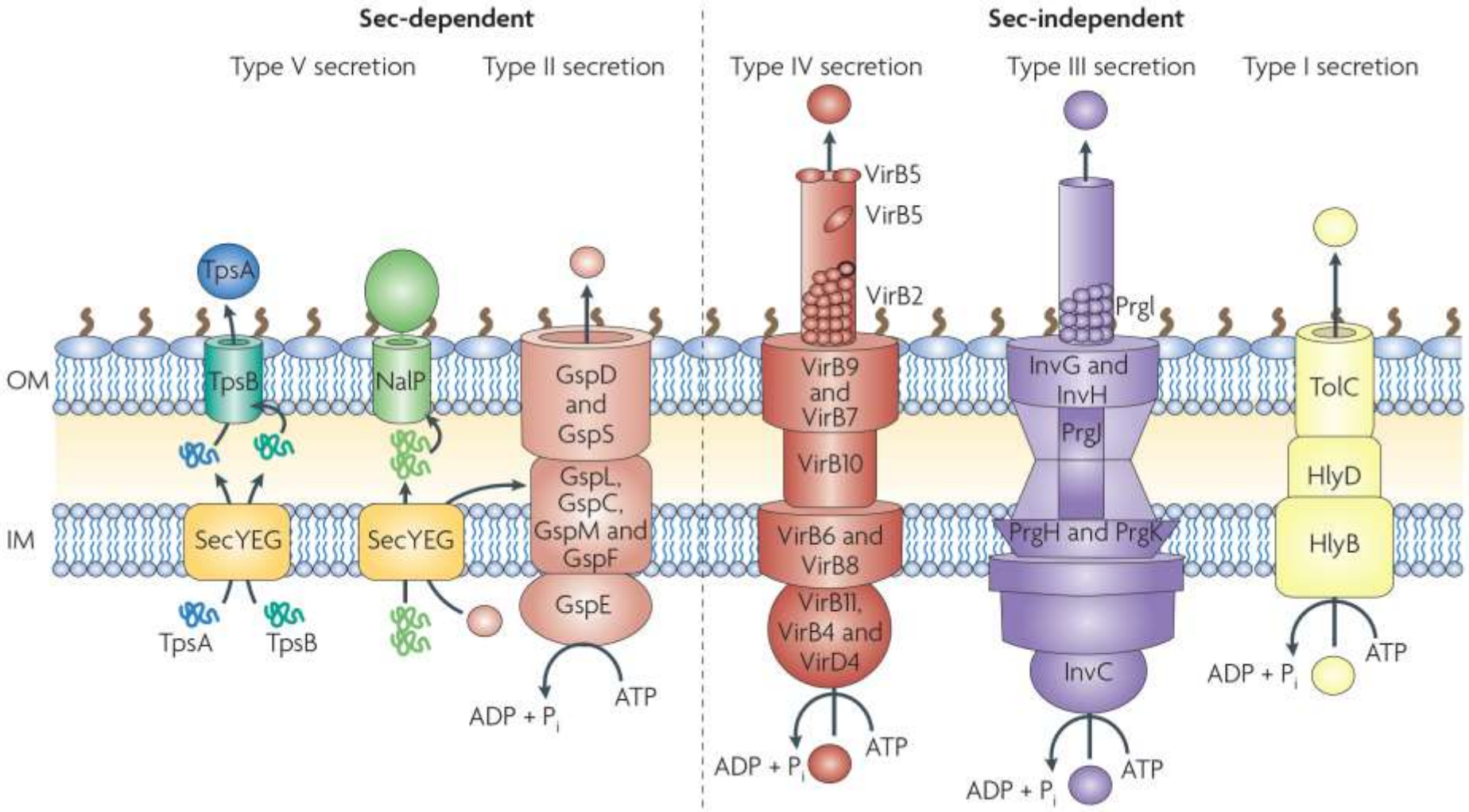
SUMMARY POINTS

1. OMVs are naturally occurring structures derived from the OM of gram-negative bacteria.
2. OMVs are a defined secretory pathway, releasing a cohort of insoluble as well as enclosed or associated soluble molecules with a distinct composition.
3. OMVs can deliver secreted molecules to specific targets in a protected, active, concentrated form.
4. OMVs mediate bacterial envelope stress, survival, colonization, biofilm nucleation and maintenance, virulence, and transformation.
5. Vesiculation is a regulated but poorly defined process that is likely conserved among gram-negative bacteria and shares common principles with outward budding systems of other biological kingdoms.
6. OMV biogenesis results from a heterogenous distribution of envelope components.
7. Vesiculation is probably regulated through the steps that initiate budding, although membrane scission is not necessarily a spontaneous event.

The general secretory pathway- GSP – type II starts as in G+

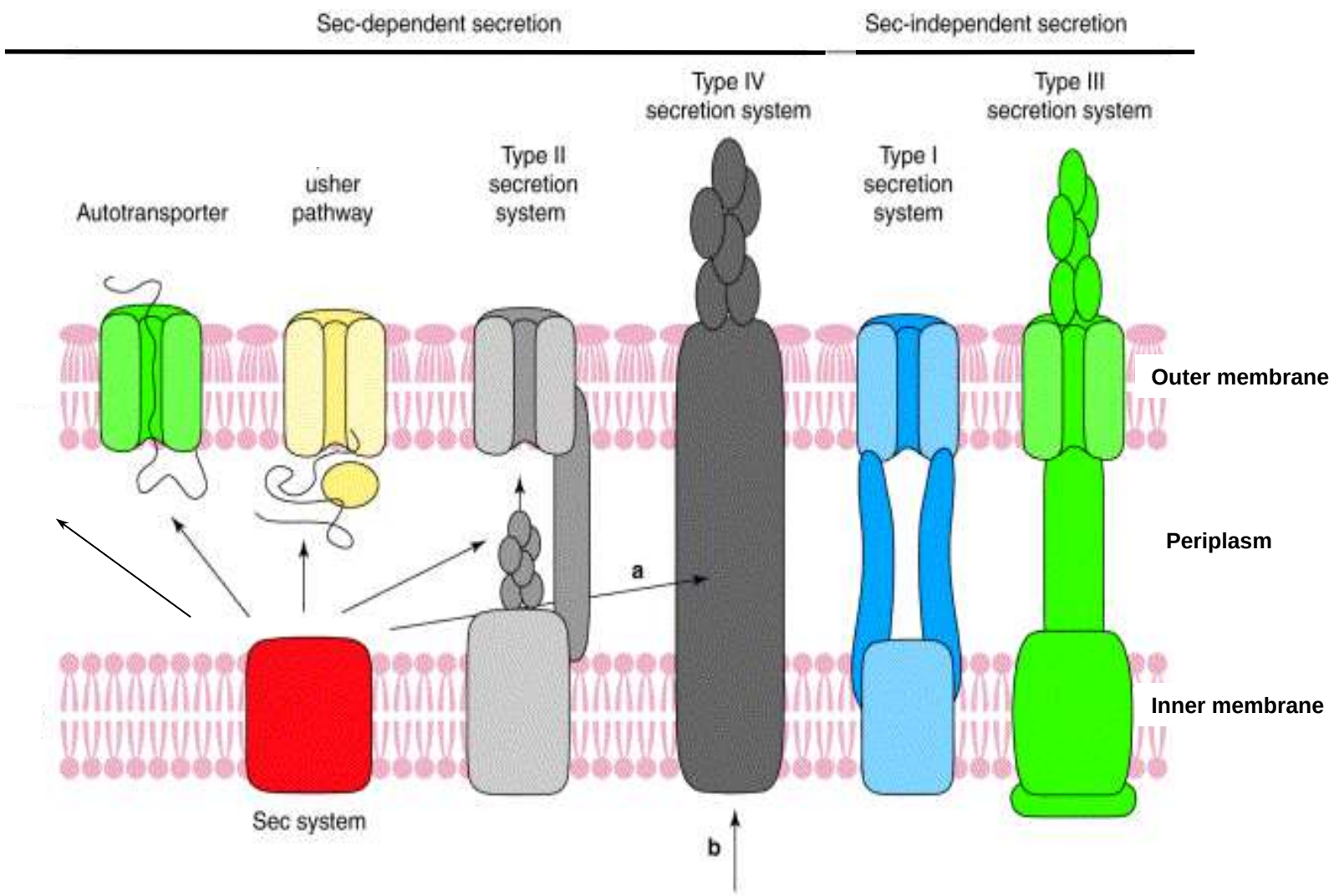


Secretion systems of G- bacteria

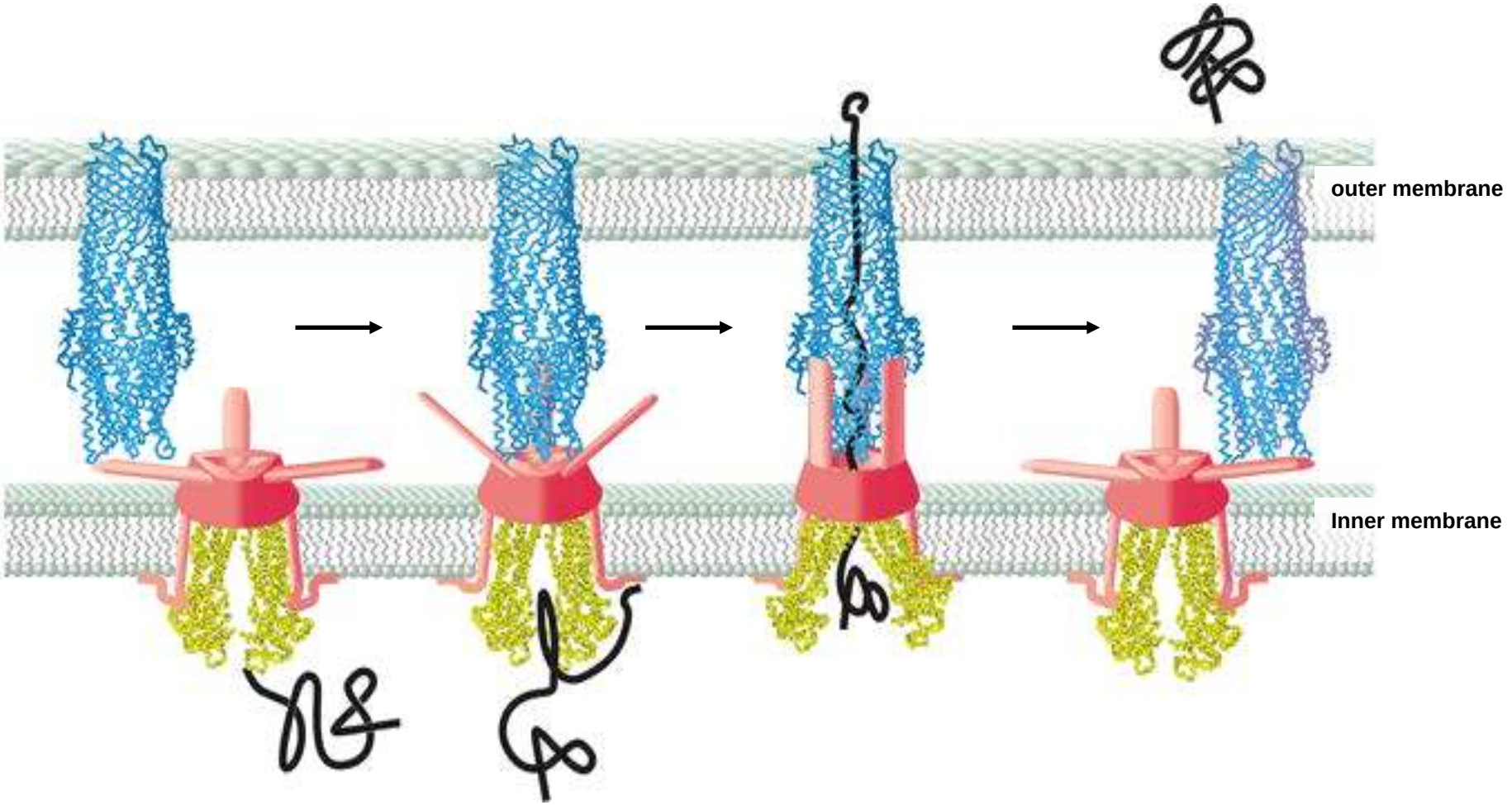


Fronzes et al., Nature Rev. Microbiol. 20

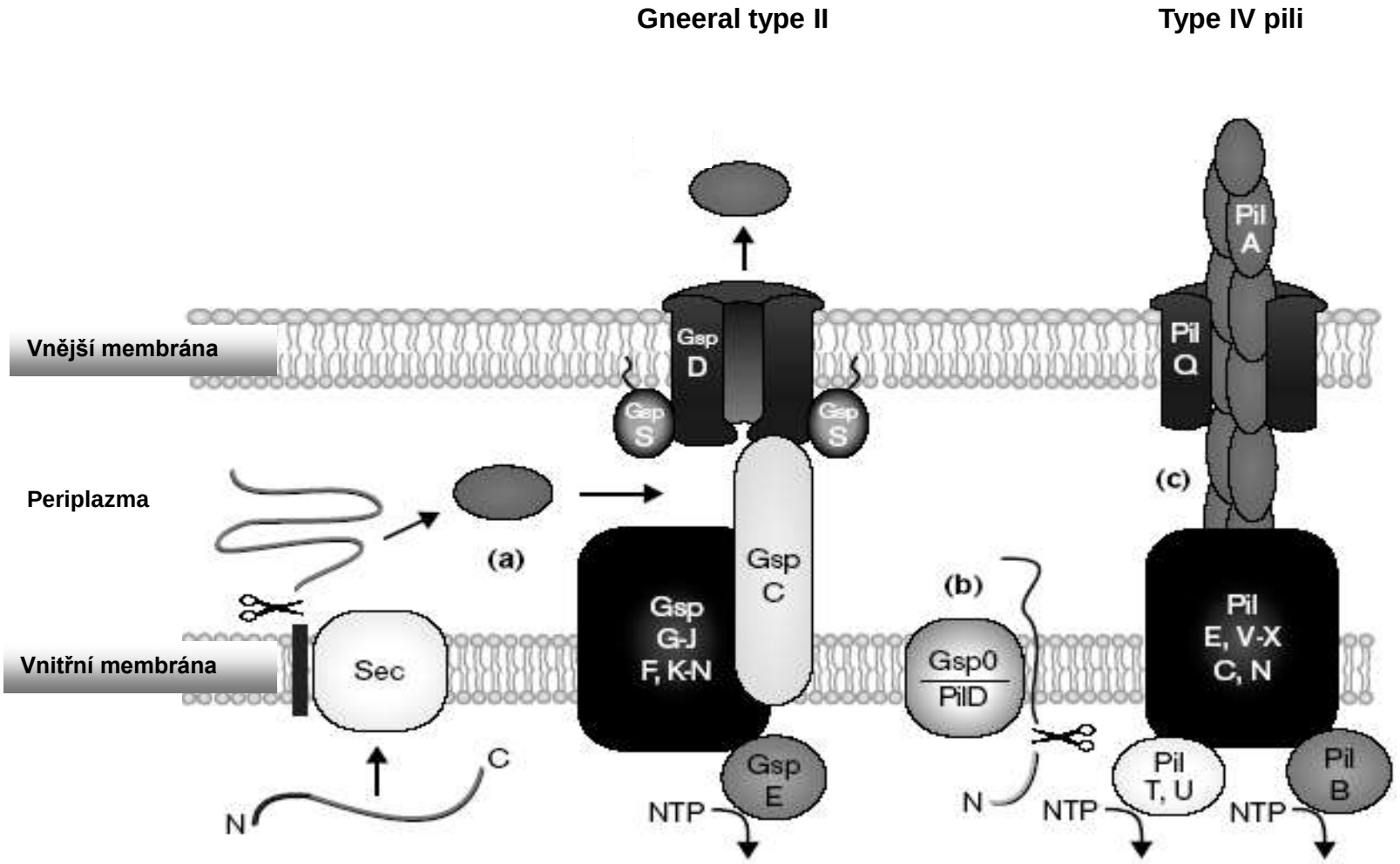
BUT - Secretion of proteins by G- bacteria is a problem…



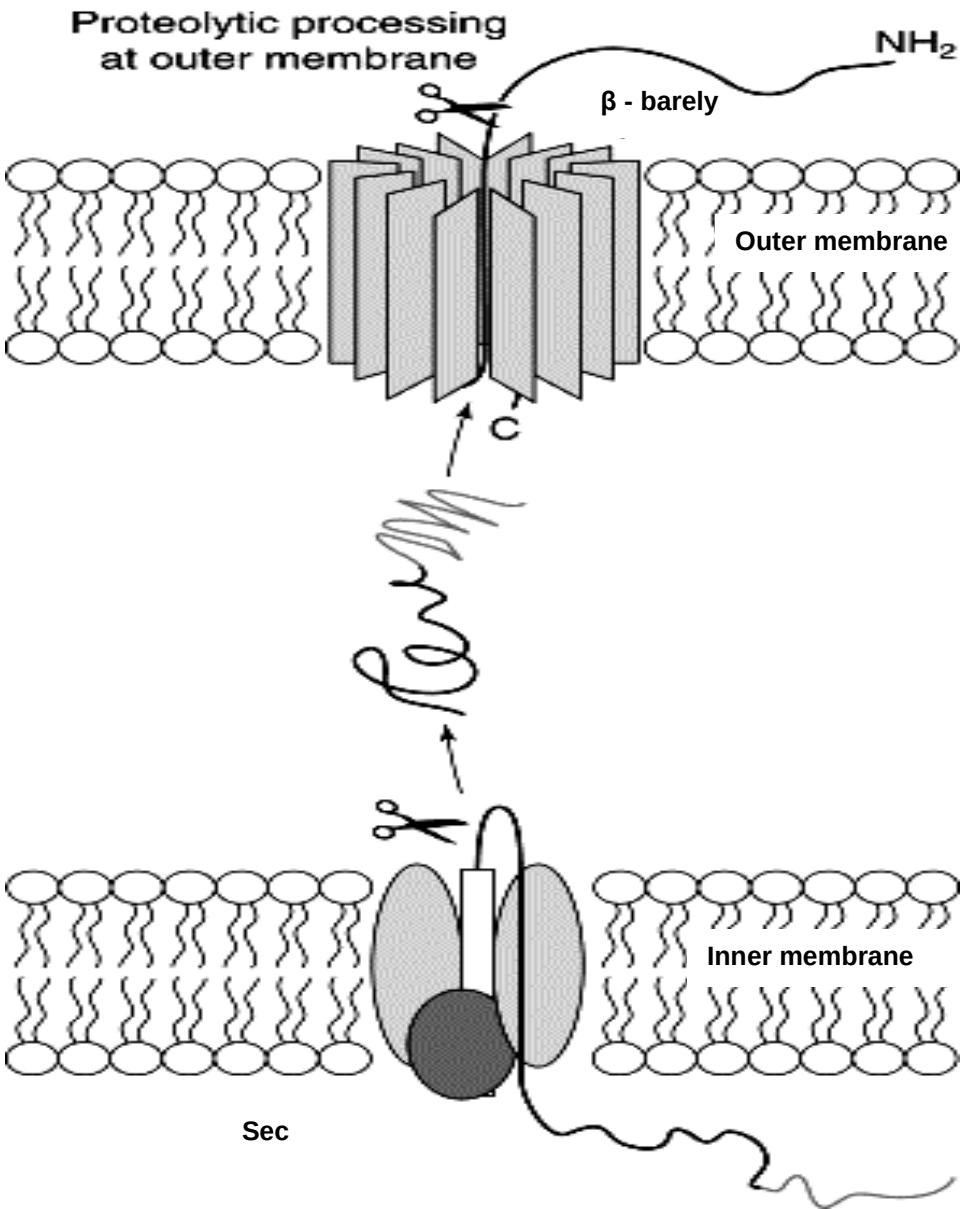
Type I secretion system



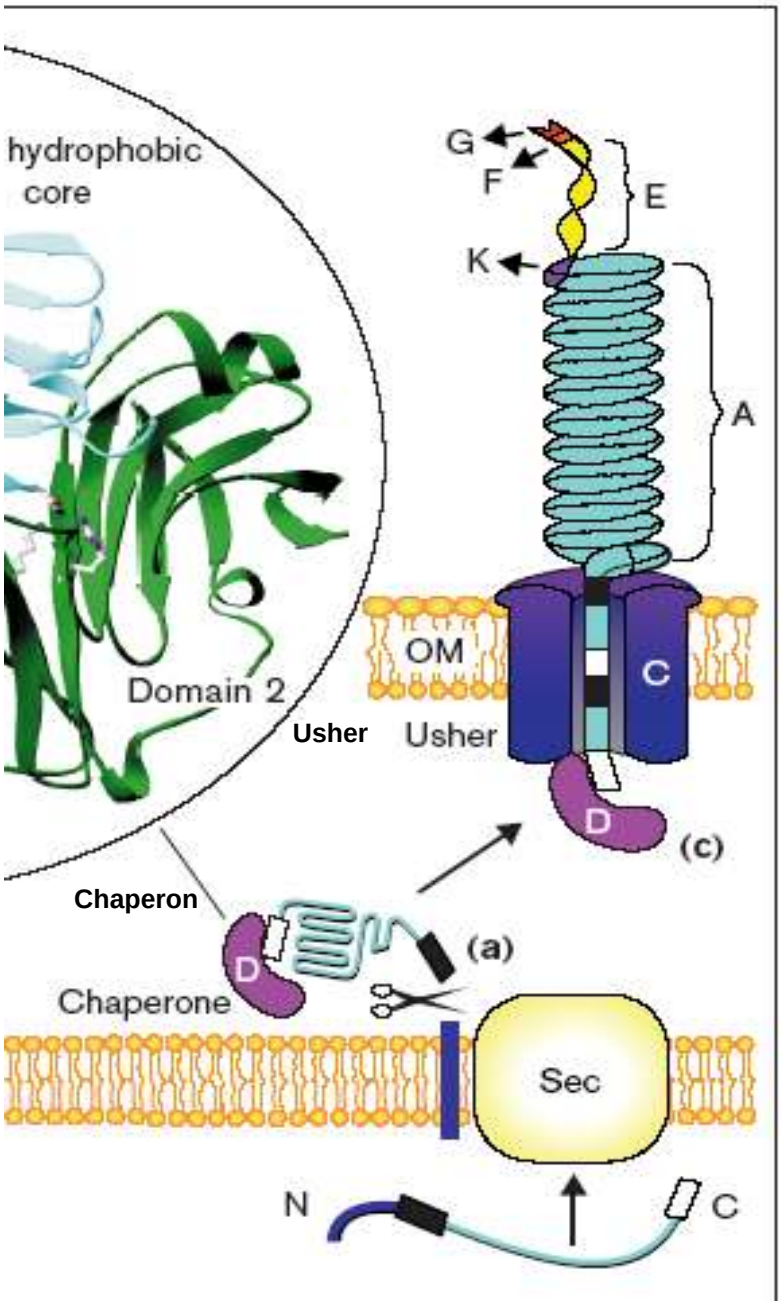
Type II system – the GSP



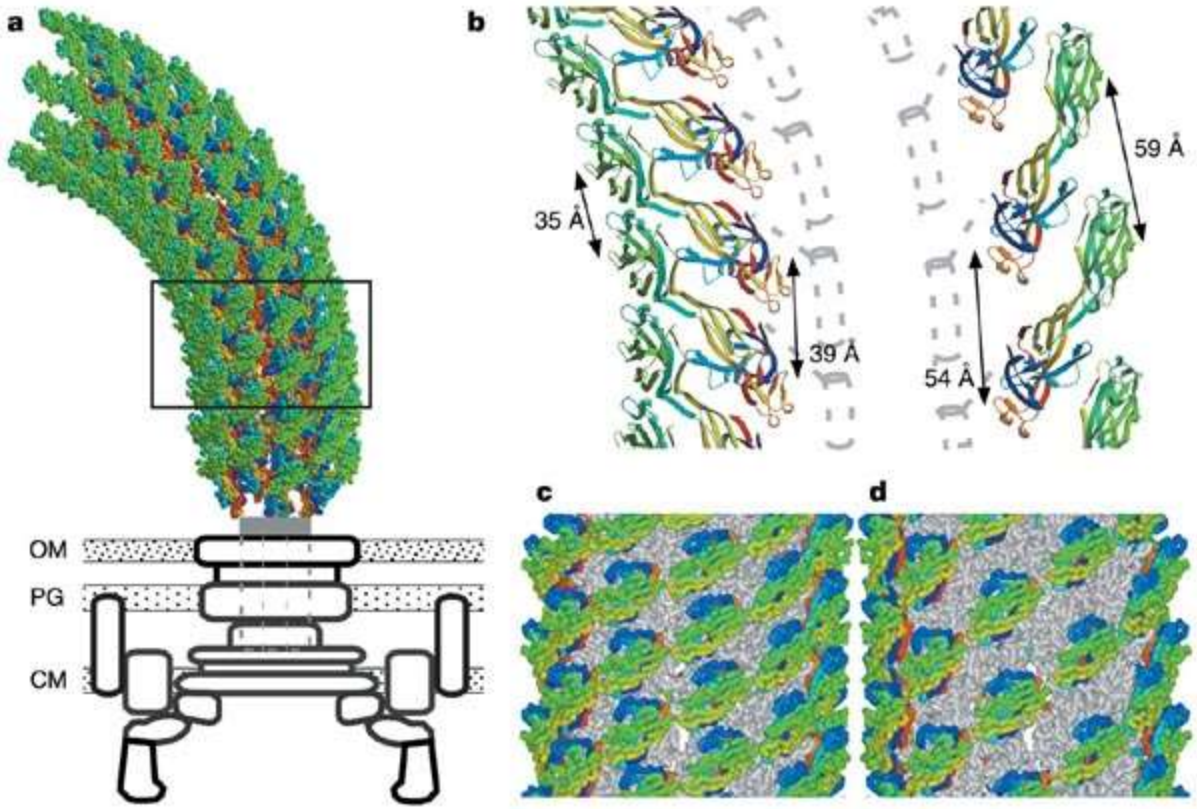
Autotransporters



The chaperone a usher pathway

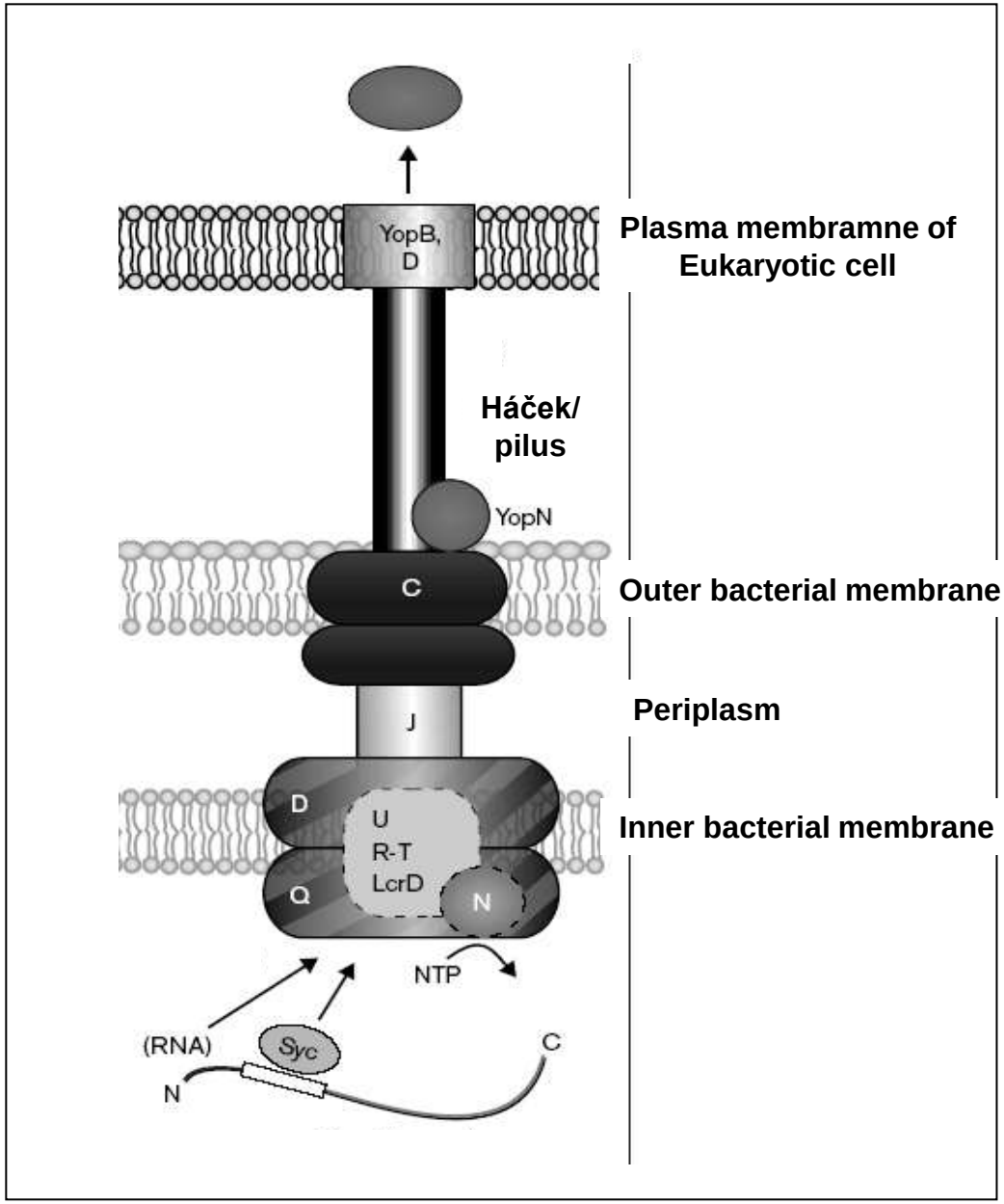


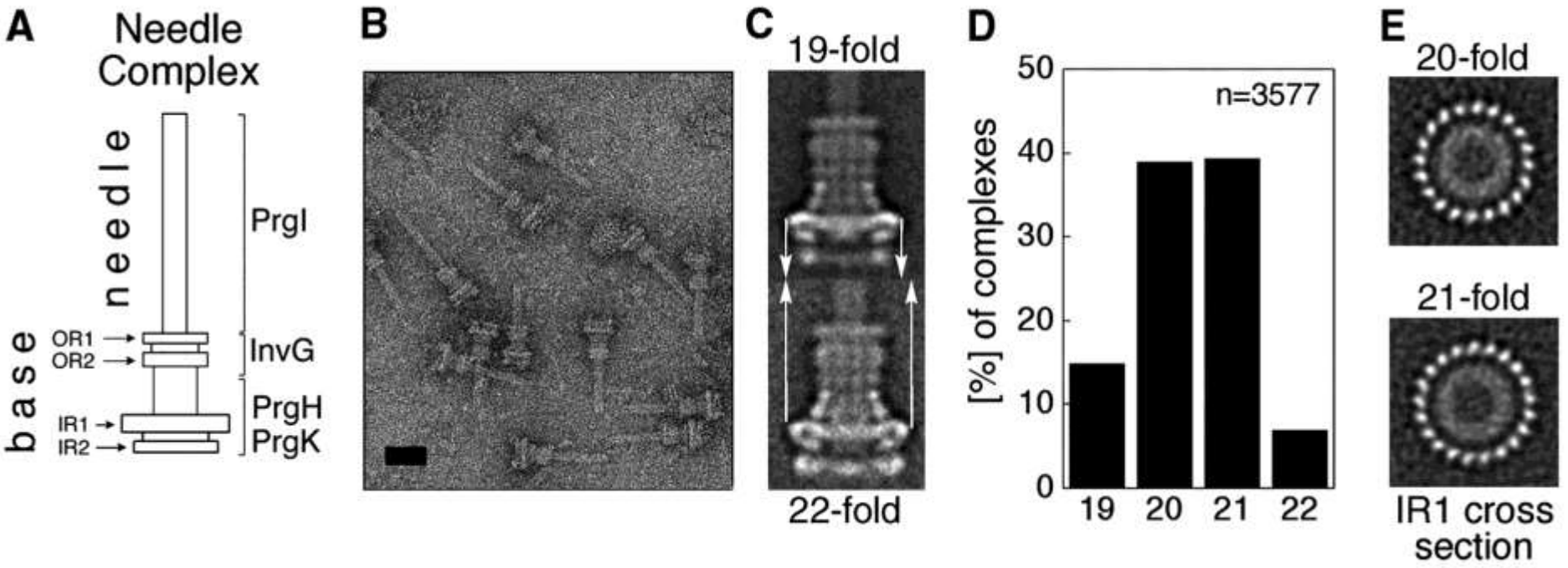
The flagellum pathway



Atomic model of the supercoiled hook. **a**, Atomic model of the coiled hook with a schematic diagram of the basal body spanning the outer membrane (OM) and the cytoplasmic membrane (CM) as well as the peptidoglycan layer (PG). This coiled hook model is part of a supercoiled polyhook with a helical pitch of 950 Å and a diameter of 350 Å. **b**, Magnified image of the coiled hook with the innermost and outermost protofilaments on the left and right, respectively. The inner core domains formed from both terminal chains and the central channel are represented by dotted grey lines. **c**, **d**, Intermolecular packing arrangements of D2 domains on the inner side (**c**) and on the outer side (**d**) of the coiled hook surface. Only domain D2 is colour-coded as in Fig. 1; domain D1 is coloured light grey.

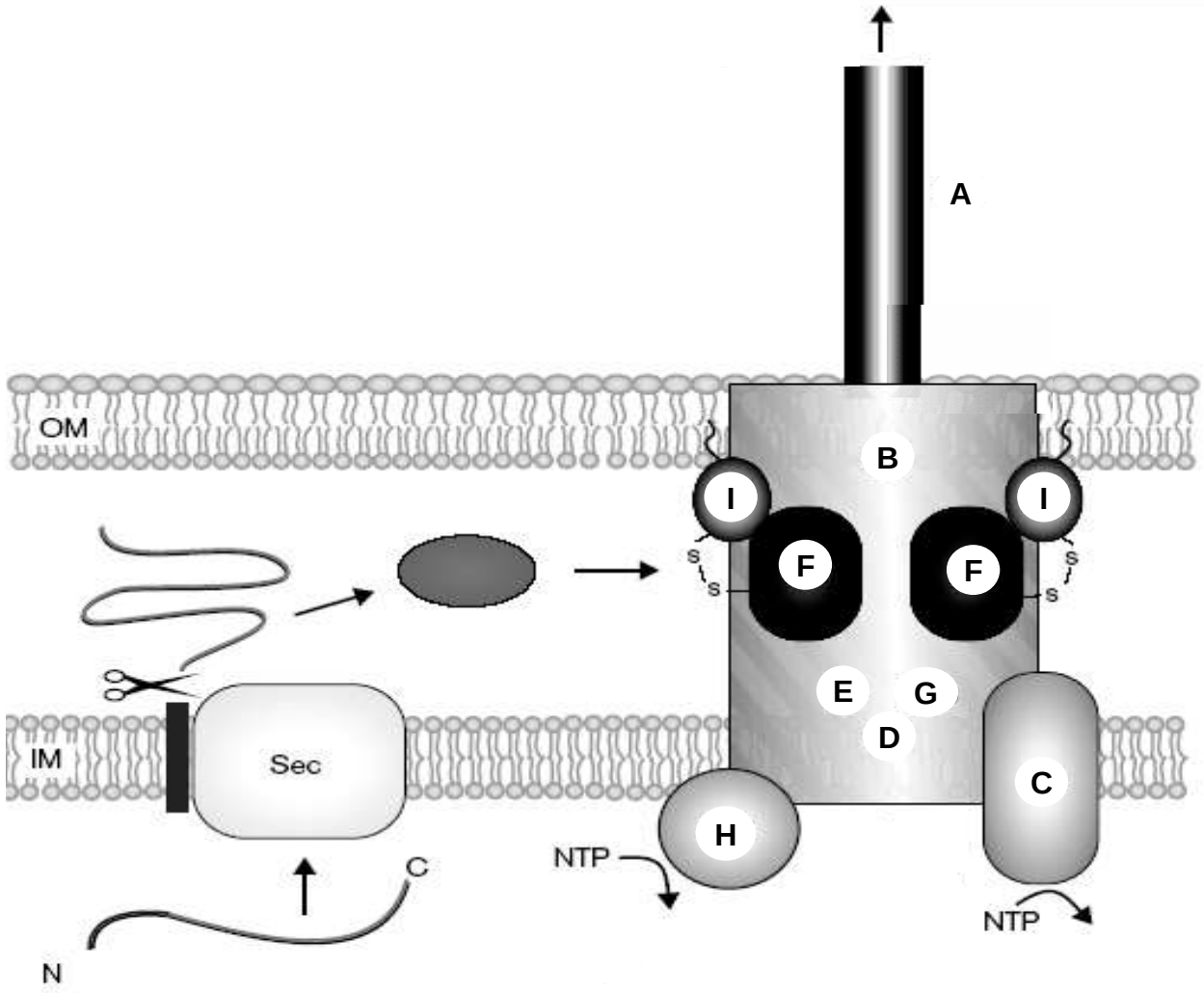
Type III secretion system





The needle complex and the base complex of the TTSS from *S. typhimurium* can adopt different symmetries in vivo. (**A**) Nomenclature of the structural features of the needle complex. The needle complex is divided into two distinctive substructures: the membrane-embedded base and the extracellular needle filament. The base spans the periplasm and is associated with the inner and outer membranes, where ringlike structures are visible in electron micrographs of negatively stained needle complexes (2% phosphotungstic acid, pH 7) (**B**). The outer membrane–associated rings (OR1 and OR2) are composed of the protein InvG, and the inner membrane–associated rings (IR1 and IR2) contain the proteins PrgH and PrgK (*4*). The only protein identified for the needle filament to date is PrgI (*4*). Bar, 30 nm. (**C**) Model-based multireference alignment revealed significant differences in the diameters of the average projections obtained for different rotational symmetries, as indicated by white arrows in the comparison of the IR1 of the 19- and 22-fold particles. (**D**) Distribution of different symmetries in needle complexes isolated from wild-type *S. typhimurium*. The data were generated by examining 3577 particles. (**E**) After sorting of the particles and 3D reconstruction without enforcing any symmetry, the true rotational symmetries could be derived from cross sections through IR1 of the reconstructed needle complexes, as shown for the 20- and 21-fold particles. ***Science, Vol 306 pp 1040-1042 (2004) Marlovits*** et al.

Type IV secretion system

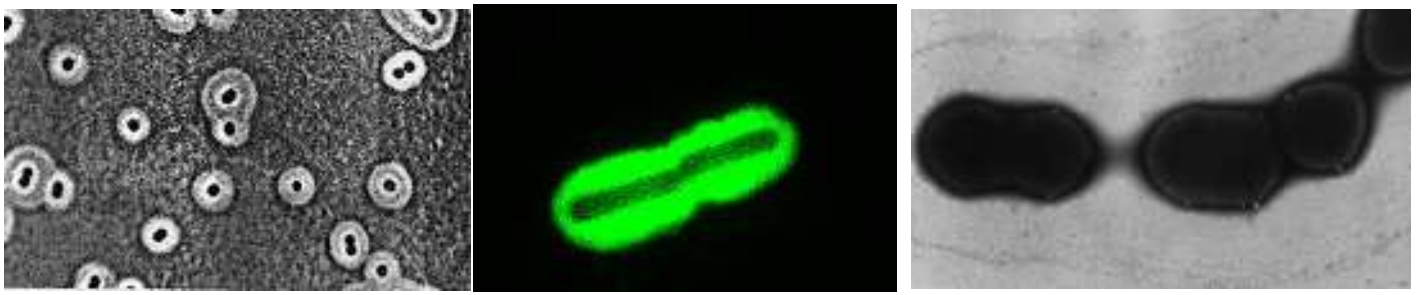


Protective layers and secretion of virulence factors

Avoiding complement killing

- Capsule production
- Alteration of LPS O antigen (main complement target in G- bacteria)
  - C3b binding
  - C5b binding and MAC assembly
  - Sialylation of LPS O antigen – inhibition of C3 convertase *(Neisseria gonorrhoae)*
  - Longer LPS O antigen synthesis – MAC assembly at a distance from membrane…
    - Serum resistant E. coli or Salmonella – not eliminated by MAC
- C5b degrading enzymes eliminating chemotactic signaling

Capsule and S layers



Bacterial capsules visualized by various techniques.
Left. *Streptococcus pneumoniae* -India ink capsule outline (K.Todar);
Middle. *Bacillus anthracis* -fluorescent-tagged antibody (CDC);
Right. *Streptococcus pyogenes* -transmission electron micrograph by Maria Fazio and Vincent A. Fischetti, Ph.D. with permission.

S. pneumoniae capsular material is composed of polysaccharide. The capsule is the pathogen's most important determinant of virulence because it allows the bacterial cells to escape phagocytes in the lung.

The *B.anthracis* capsule is composed of poly-D-glutamic acid. Its capsule is antiphagocytic, and it protects the bacteria from complement- mediated lysis in serum or blood.

The capsule of *S. pyogenes* is composed of hyaluronic acid, the same polymer as found in human connective tissue. The capsule is an antigenic disguise that prevents recognition of the streptococci by phagocytes or the immune system.

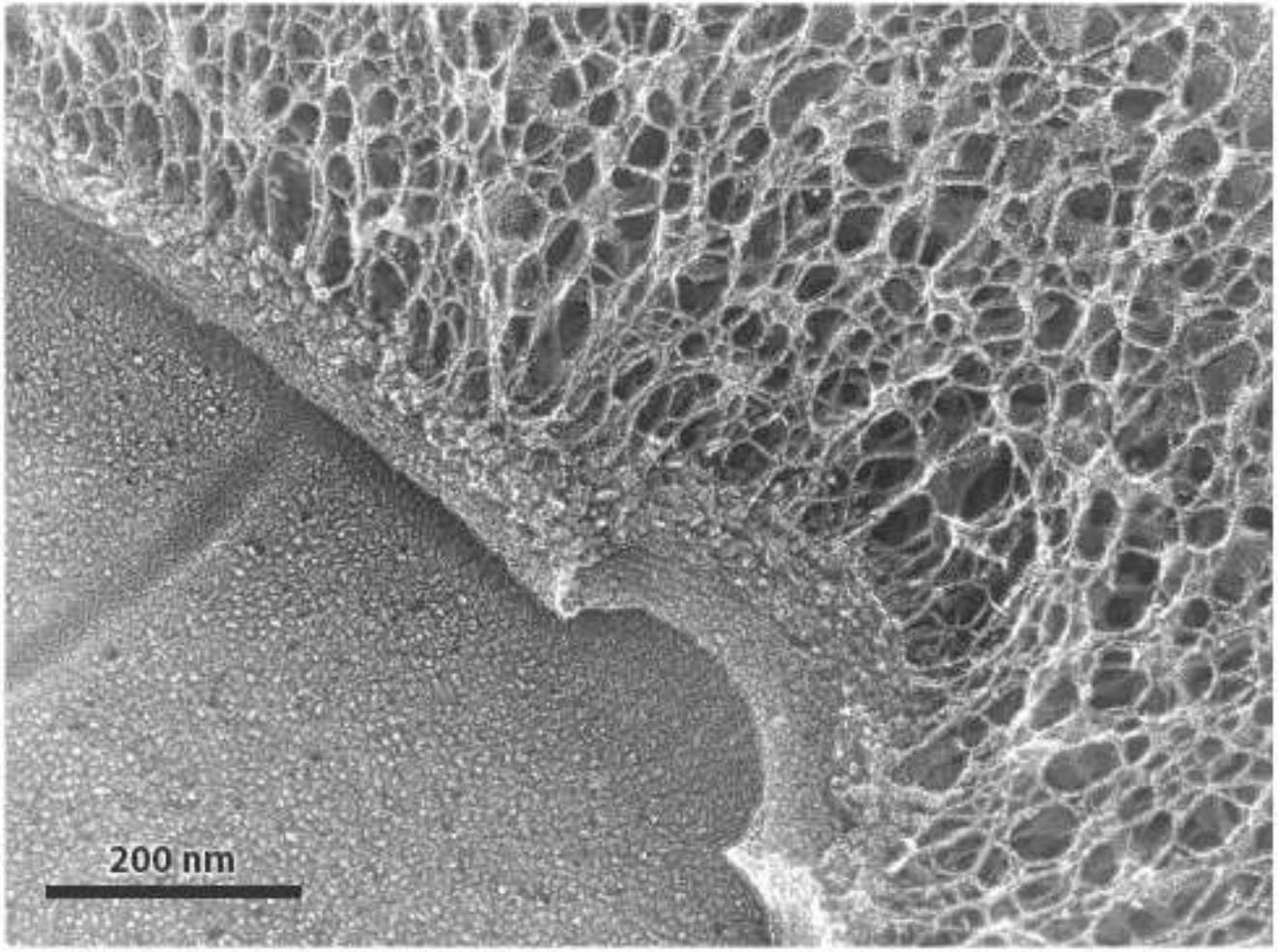
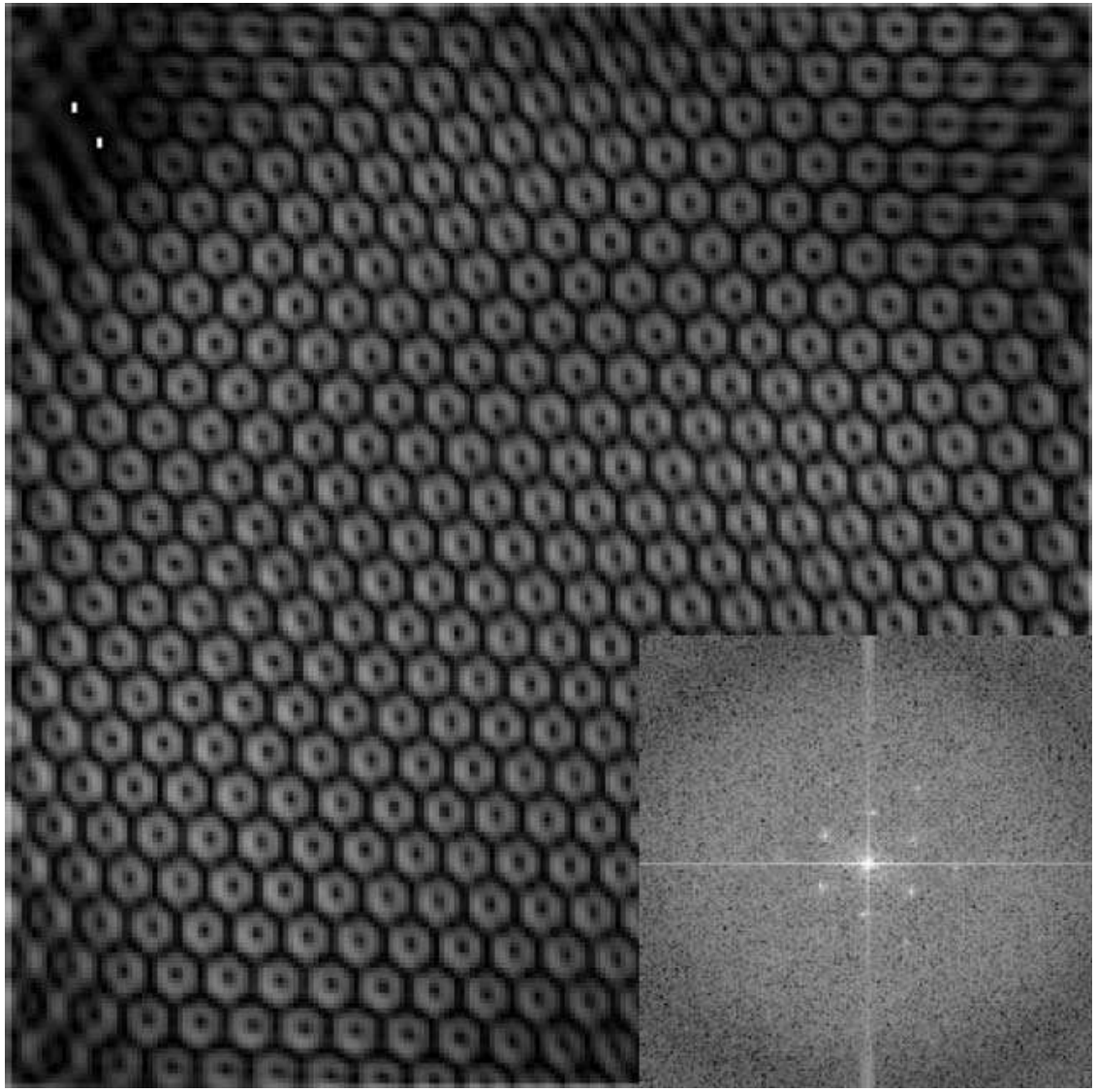


Figure 1

Quick-freeze deep-etch image of the edge of a budding cryptococcal cell. The plasma membrane (*lower left*) is separated by the double-layered cell wall from the capsule meshwork (*upper right*). The wall and capsule surround both the parent cell and the newly emerging bud. Reprinted, with permission, from the cover image associated with Reference 122.

Protective S layers (surface layers)

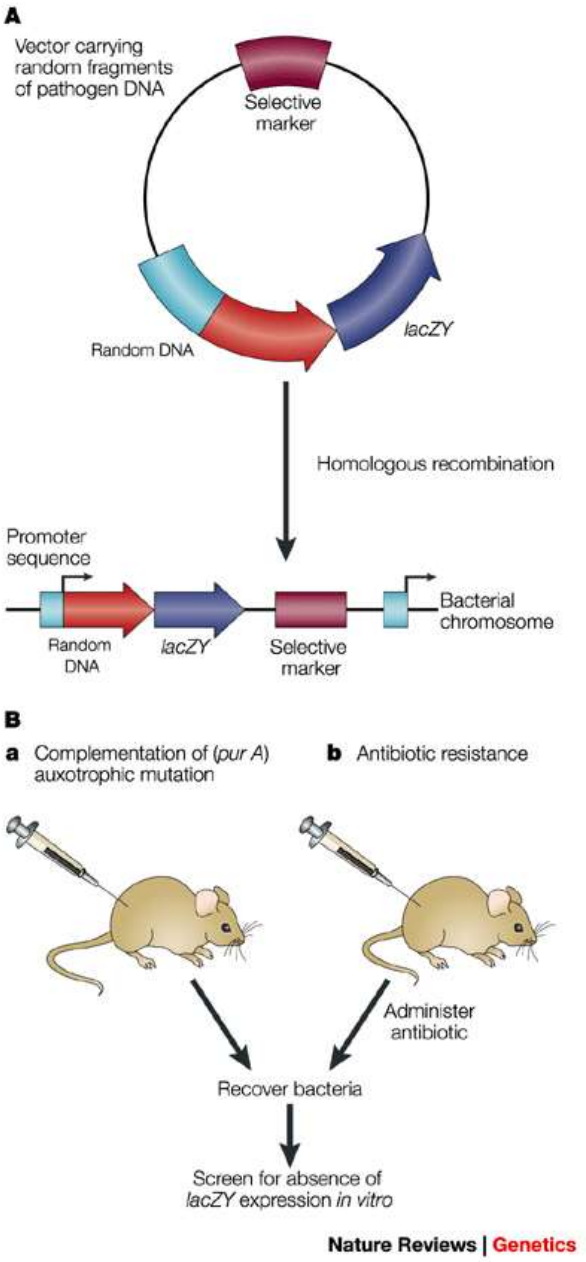


Deinococcus radiodurans is an extraordinarily hardy Gram-positive bacterium that forms reddish-pink colonies. It is the most radiation resistant bacterium known. The relatively large pore size and lattice spacing make S-Layers from this organism ideal for uses where small scale (on the order of ~ 1 μm) patterning is needed.

How to identify genes required for virulence:

Screening for virulence factor genes expressed *in vivo* only…

The *in vivo* expression technology

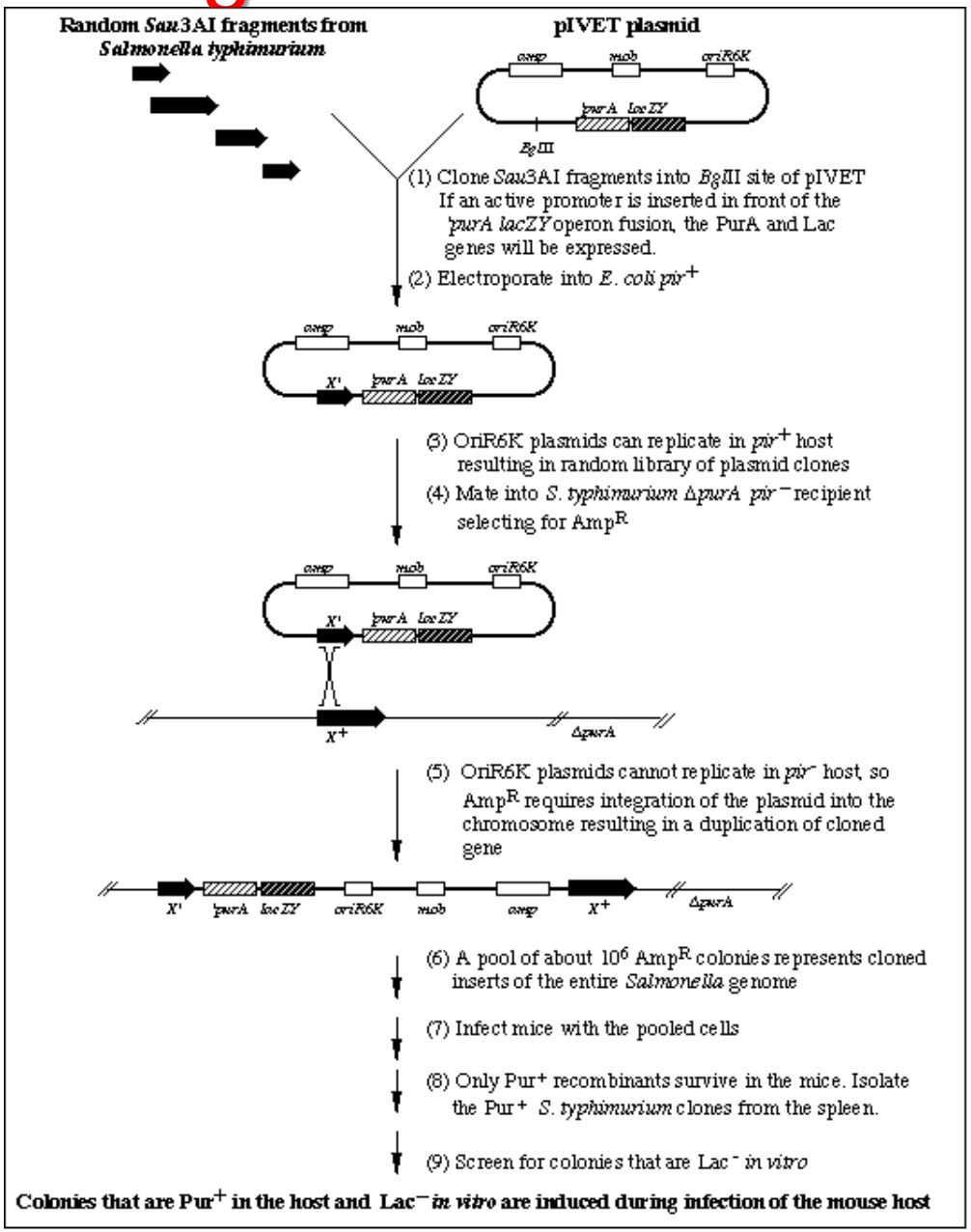


IVET is a promoter trap technology that has been developed to select for bacterial genes that are specifically induced when bacteria infect a host organism.

A | IVET vectors contain a random fragment of the chromosome of the pathogen (red) and a promoter-less gene that encodes a selective marker that is required for survival (burgundy). Random integration of the IVET vector into the pathogen chromosome is performed by insertion–duplication mutagenesis to create a pool of recombinant pathogens (this means that the gene in which the vector has inserted by homologous recombination is not disrupted). Recombinants can be selected using antibiotic resistance, as an additional marker is also on the integrated IVET construct (not shown). Pooled clones are then inoculated into the mouse.

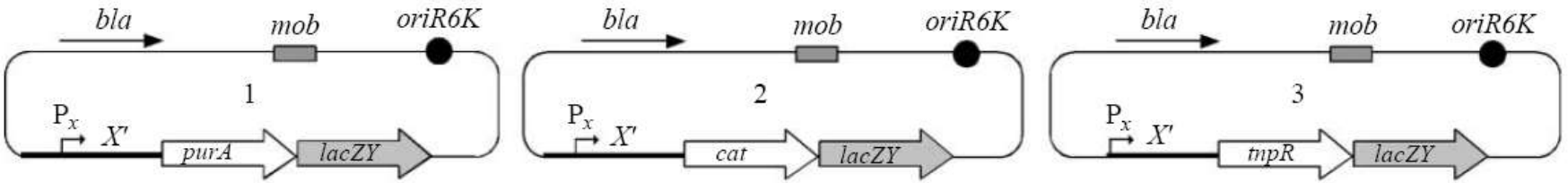
B | The two main types of IVET promoter trap strategies are (a) the complementation of auxotrophic mutation and (b) the expression of antibiotic resistance. Only those bacteria that contain the selective marker fused to a gene that is transcriptionally active in the host are able to survive. After a suitable infection period, bacteria that express the marker are isolated from the spleen or other organs. The inclusion of lacZY gene (blue) allows post-selection screening for promoters that are only active in vivo.

The original variant of *IVET*

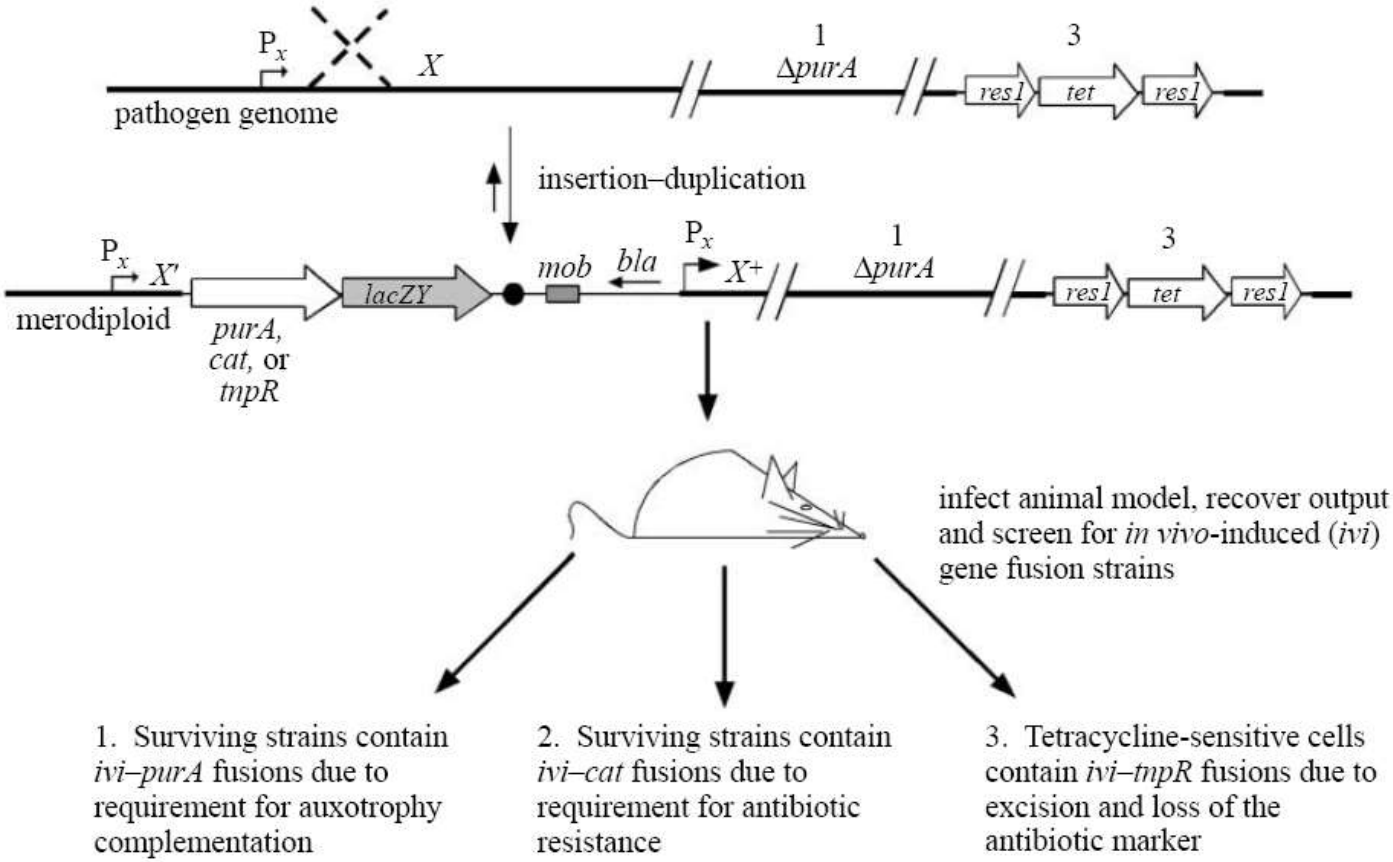


Modified from: Mahan, M., J. Slauch, and J. Mekalanos. 1993. Selection of bacterial virulence genes that are specifically induced in host tissues. Science 259: 686-688.

IVET variants



(gene *X* serves as the sight of homologous recombination into pathogen genome to generate merodiploid)



Legend to figure

Figure 1. Graphic depiction of three variations of IVET. As shown, auxotrophy complementation IVET selections are conducted using fusions to a promoterless *purA* gene (plasmid 1), antibiotic IVET selections are conducted using fusions to a promoterless antibiotic gene such as *cat* (plasmid 2), and recombinase-based *in vivo* expression technology (RIVET) screening is done using a promoterless *tnpR* allele (plasmid 3), which when produced, will cleave a Tc^r gene from elsewhere in the bacterial genome. Reporter gene fusion libraries are constructed by ligating random genomic fragments (designated as gene *X′*) into the IVET vector of choice, followed by transformation into the pathogen of interest. The suicide plasmids then recombine into the chromosome by insertion–duplication creating a merodiploid. In the case of RIVET, a prescreen is required to remove strains harbouring *in vitro* active gene fusions: this is accomplished by selecting for Tc^r, $LacZ^-$ colonies. In all cases, fusion strains are passaged through an appropriate animal model of disease and collected from infected tissues and/or fluids after a period of time. In the case of the antibiotic-based IVET, the antibiotic (in this example, Cm) must be present at sufficient concentrations in animal tissues to select for *in vivo* expression of the gene fusion. Strains containing infection-induced gene fusions to *purA* and *cat* are selected in the host and are subsequently screened for lack of *in vitro* expression on LacZ indicator plates. In contrast, infection-induced gene fusions to *tnpR* are screened for, post-infection, by virtue of Tc^s and lack of expression on LacZ indicator plates.

Detection and analysis of gene expression during infection by *in vivo* expression technology

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(*dmerrell@opal.tufts.edu, acamilli@opal.tufts.edu*)

Phil. Trans. R. Soc. Lond. B (2000) **355**, 587–599

The RIVET improvement

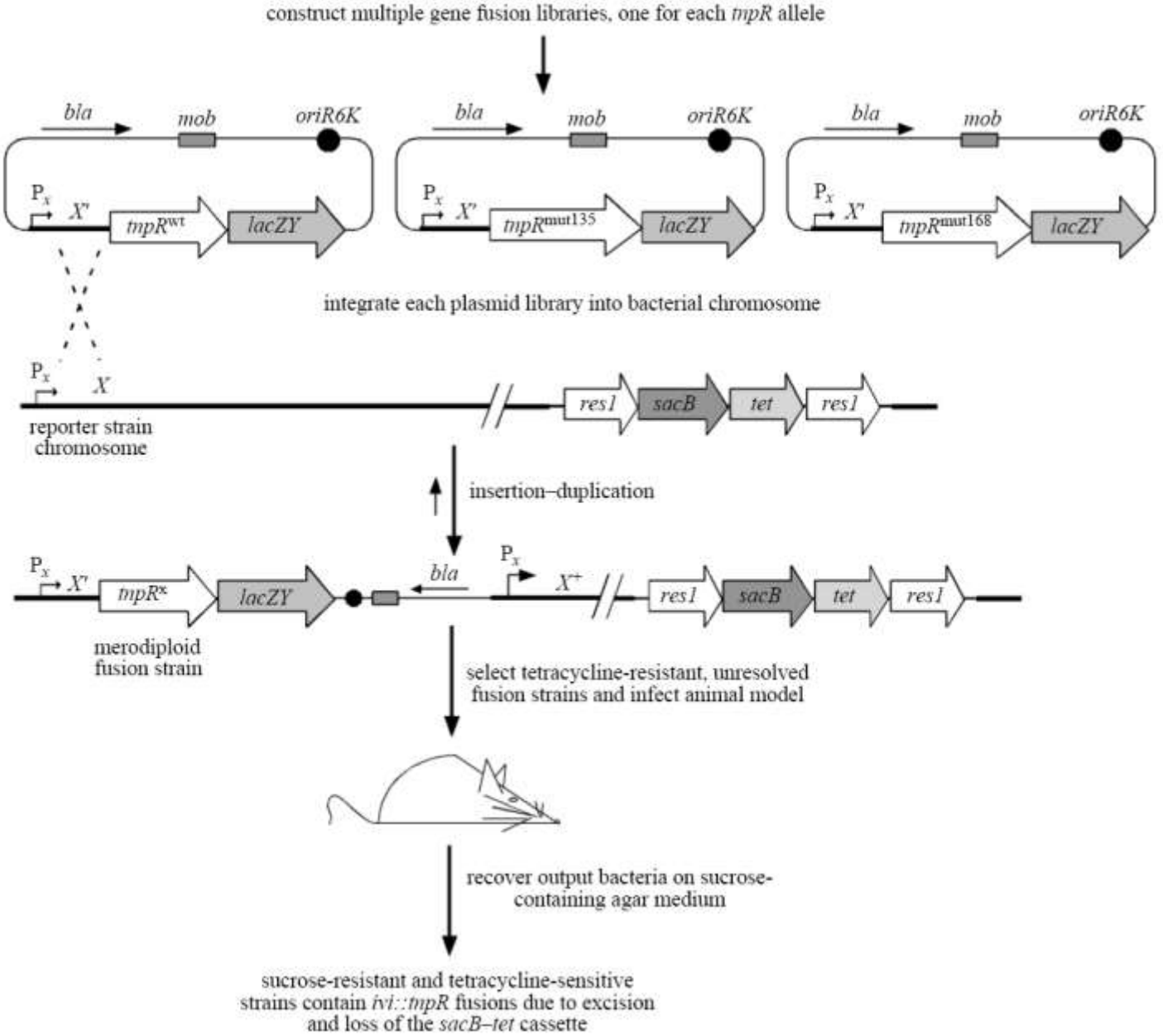


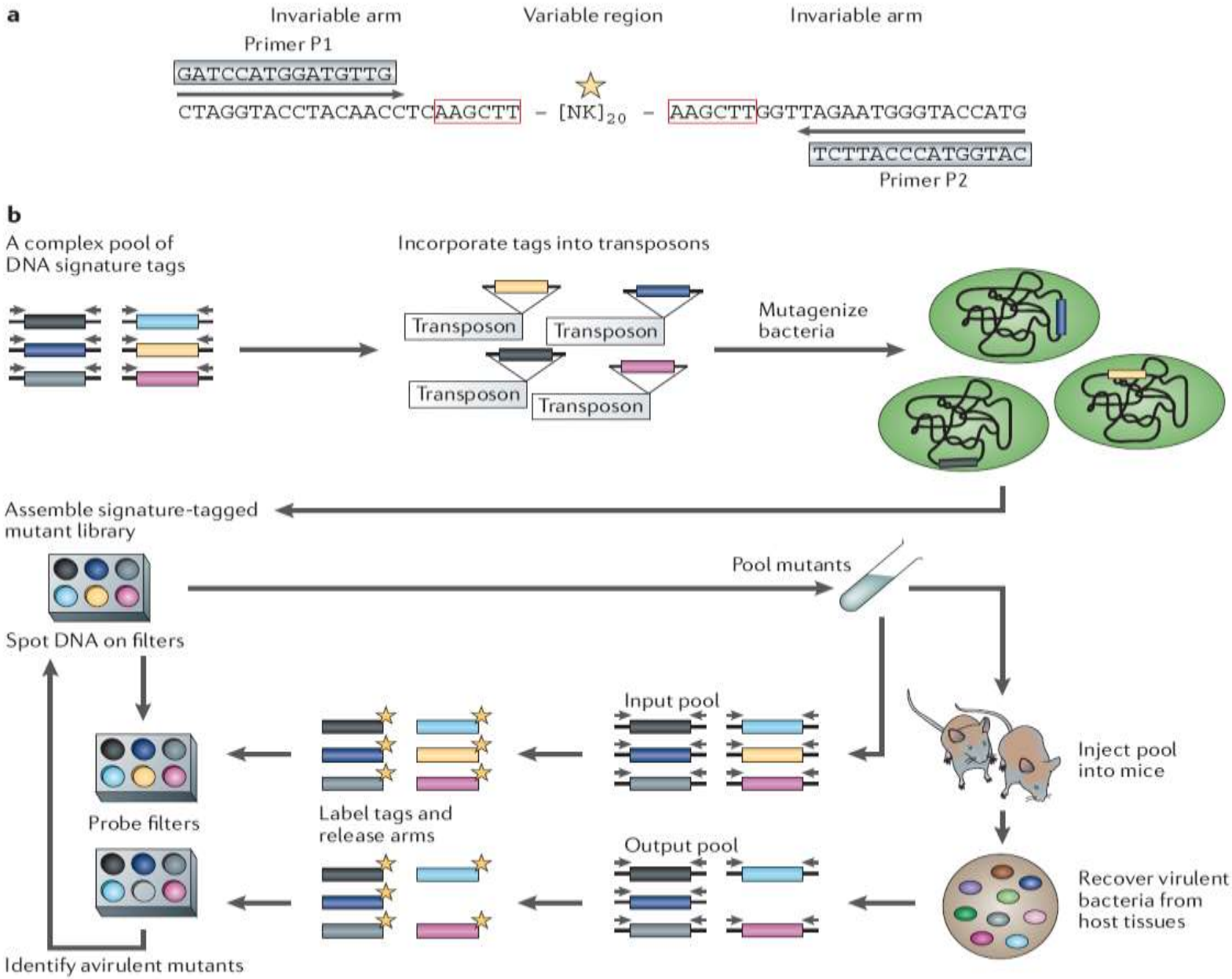
Figure 6. Graphic depiction of a RIVET screen for infection-induced genes. As shown, the screen is conducted using three different promoterless *tnpR* alleles that vary in their translational efficiencies due to different RBSs. Each *tnpR* allele will therefore yield a different class of infection-induced genes: specifically, classes of genes that differ in their basal levels of transcription during *in vitro* growth. Construction of the reporter gene fusion libraries and their subsequent screening is as in the legend to figure 1, with one notable exception: in the present scheme, resolved strains are directly selected from intestinal homogenates through their ability to grow on sucrose-containing agar media.

Signature-tagged mutagenesis: barcoding mutants for genome-wide screens

Mazurkiewicz, P., Tang, C.M., Boone, C. and Holden, D.W. (2006) NATURE REVIEWS **GENETICS:** VOLUME 7, DECEMBER 2006, p. 929

By combining whole-genome microarrays and comprehensive
ordered libraries of mutants, high-throughput functional screens can now be achieved on a genomic scale.

Barcoding an ordered mutant library



Barcoding an ordered mutant library

Figure 1 | **Original signature-tagged mutagenesis of *Salmonella*. a** | Design of a signature tag. Each tag has a unique central sequence of 40 bp ($[NK]_{20}$; N = A, C, G, or T; K = G or T), flanked by invariable arms of 20 bp, which are common to all the tags. These arms allow the sequence tags to be amplified and labelled with radioactive nucleotides (marked with a star) by PCR with primers P1 and P2. Following labelling and before hybridization, the invariant arms are removed by digestion with a restriction enzyme that recognizes sequences (shown in red boxes) between the variable region and the invariable arms. **b** | Signature-tagged mutagenesis screening in mice. A complex pool of tags (shown as coloured rectangles) is ligated to transposons. The tagged transposons are then used to mutagenize bacteria, which are subsequently assembled into a library. Only bacteria with tags that are efficiently amplified by PCR and are not cross-reactive with other tags in hybridization experiments are selected for inclusion in the pool that is used to infect the mice. Genomic DNA is isolated from this pool (input pool) and from the bacteria that are recovered from the animals (output pool). The tags from these two DNA pools are amplified and radiolabelled to create probes for hybridization. DNA from the colonies of the mutant library that hybridize to the probes from the input pool but not to the probes from the recovered pool represent mutants with attenuated virulence.

Methods for generating pools of tagged mutants

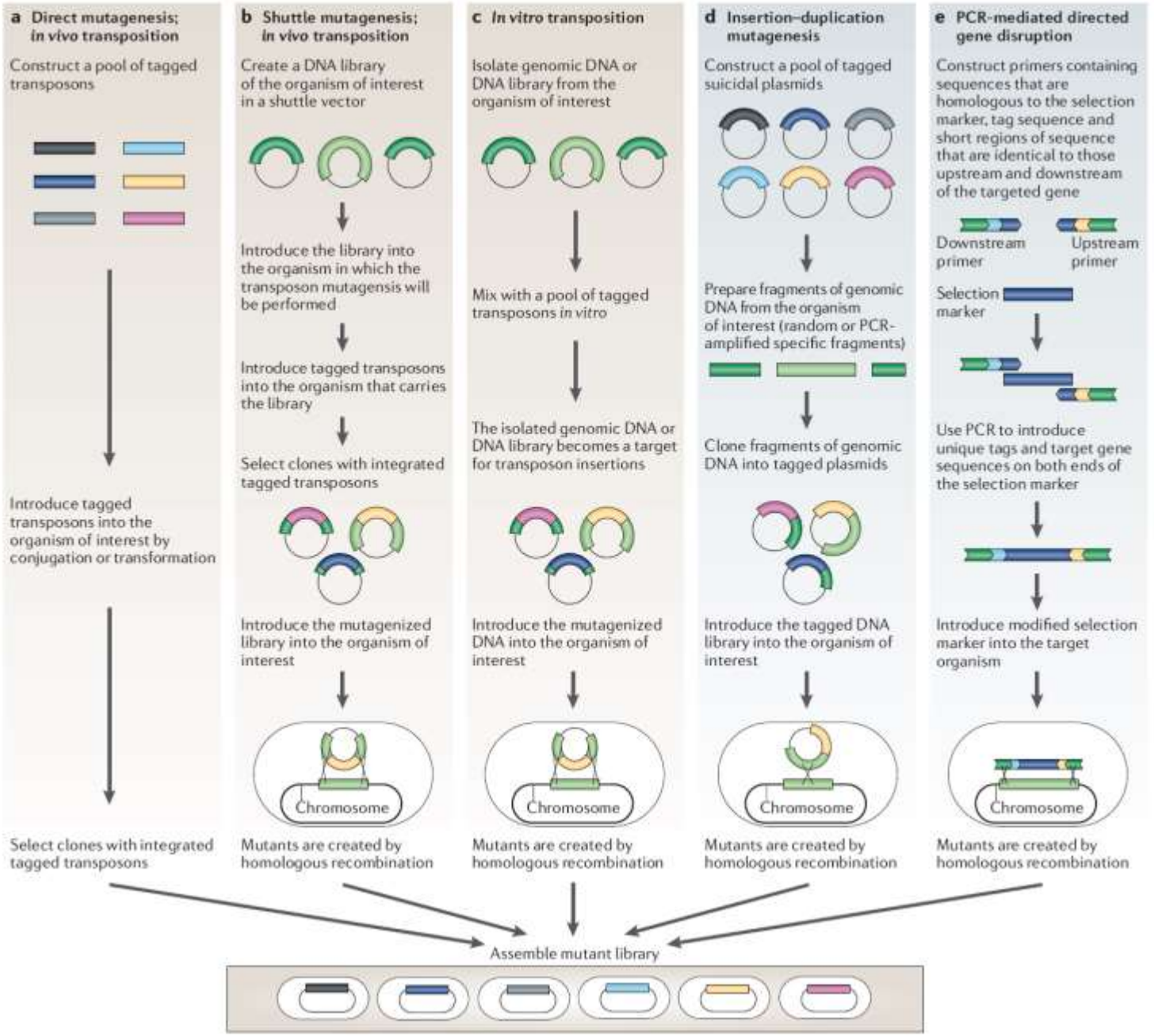


Figure 2 | **Methods for generating pools of tagged mutants.** Panels **a**, **b** and **c** show methods that involve random transposition. The methods in panels **d** and **e** use homologous recombination. DNA tags are represented by different coloured segments, whereas the targeted sequences are shown in green. Tags can be introduced into the genome by direct *in vivo* transposition of the target organism (panel **a**). Alternatively, transposition can be carried out on the target DNA library *in vivo*, in an organism for which an efficient transposition method exists (panel **b**), or *in vitro*, on isolated DNA (panel **c**). In the methods depicted in panels **b** and **c**, the mutagenized DNA is subsequently reintroduced into the target organism to allow the incorporation of tags into the chromosome by homologous recombination. Insertion–duplication mutagenesis (panel **d**) involves ligating small fragments (random or specific) of target DNA into a pool of tagged plasmids. The plasmids are then introduced into the microorganism of interest, where they integrate into the genome. In PCR-mediated gene disruption (panel **e**), PCR is carried out to amplify a selectable marker, which is flanked with short sequences (~50 bp) that are identical to those immediately downstream and upstream of the targeted gene. When introduced into a cell, the resulting PCR product that incorporates the marker can replace the targeted gene by homologous recombination. For more information on techniques of transposon-based mutagenesis see REF. 81.

Detection of signature tags

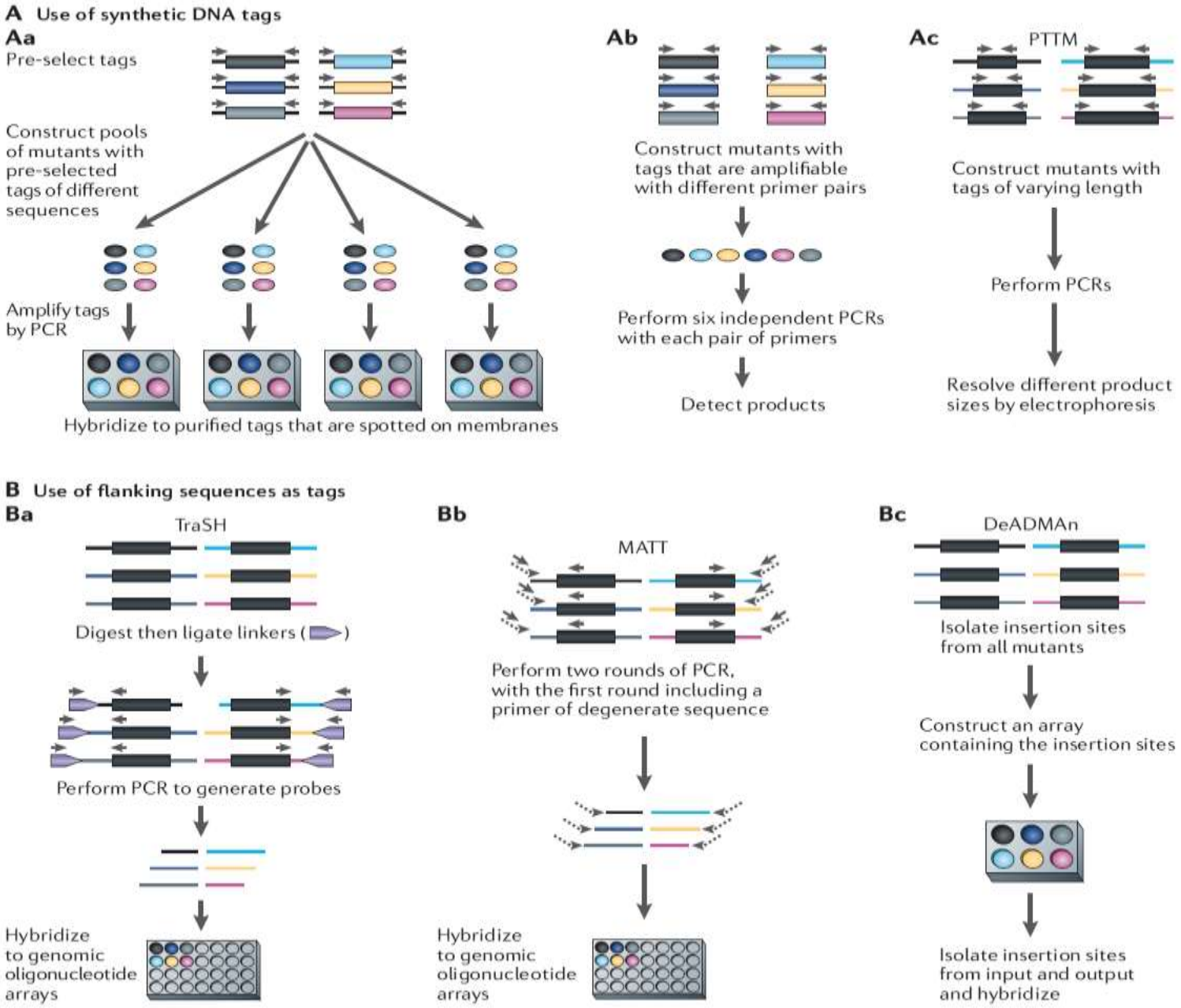


Figure 3 | **Methods for the detection of signature tags.** Several techniques have been designed that incorporate synthetic DNA tags (**A**) or that take advantage of flanking sequences (**B**). **A** | Tags that are efficiently and specifically amplified and labelled can be pre-selected and used repeatedly to generate separate pools of mutants (coloured ovals in part **Aa**). Membranes can then be constructed with purified tags or the plasmids that harbour them. The detection of tags can be carried out without the need for hybridization. Tags can be amplified in multiple PCRs, each containing a different primer pair for a specific tag (**Ab**), or in polymorphic tag-length transposon mutagenesis (PTTM, panel **Ac**), tags of different length are amplified with a single primer pair, giving rise to products of various sizes. **B** | Probes that are generated from the flanking sequences can be used to hybridize to genomic microarrays. In transposon site hybridization (TraSH; panel **Ba**), flanking sequences are amplified by ligating linkers to digested genomic DNA from pools of mutants. In microarray tracking of transposon mutants (MATT; panel **Bb**), flanking sequences are amplified by arbitrary PCR (which involves two rounds of PCRs, with the first round including a primer of degenerate sequence (dashed arrow) and a transposon specific primer (solid arrow)). In designer arrays for defined mutant analysis (DeADMAn; panel **Bc**), the sequences that flank each mutation are isolated and assembled onto an array, which is then used for subsequent hybridizations.

Examples of insight gained through STM

Salmonella

Signature-tagged mutagenesis (STM) of *Salmonella typhimurium* led to the discovery of a specialized type 3 secretion (T3S) system, which is encoded by a horizontally acquired pathogenicity island called SPI2. The T3S system transfers over 15 virulence proteins across the vacuolar membrane that encloses intracellular bacteria. At least some of the virulence proteins are involved in controlling vacuolar membrane dynamics through regulation of molecular motors[74], and together they enable intracellular bacterial replication during infection. A strain of *Salmonella typhi* that lack the SPI2 T3S system shows considerable promise as a new live attenuated vaccine for typhoid fever[75].

Mycobacterium tuberculosis

The lung is the predominant organ that is affected by *M. tuberculosis*, and pulmonary infection causes around two million deaths each year (see the World Health Organisation web site). Two separate STM studies demonstrated that a complex cell wall lipid, phthiocerol dimycocerosate (DIM), is necessary for survival of the bacterium in the lung, but not in other tissues such as the spleen[76,77]. Mutations that attenuate virulence were found to cluster in a locus that is necessary for the biosynthesis and export of this lipid, which is produced mainly by pathogenic members of the *Mycobacterium* genus. One of the affected genes (*lppX*) encodes the first member of a new family of lipoproteins that function as carriers of lipophilic molecules across the mycobacterial cell envelope[78]. DIM production evidently protects *M. tuberculosis* from the toxic effects of nitric oxide that are produced by macrophages[79].

Shigella flexneri

Shigella flexneri is the leading cause of bacillary dysentery worldwide, and causes disease after invading epithelial cells. The invasion is dependent on the activity of a T3S system that delivers cell bacterial effectors into the host, eliciting dramatic rearrangements of the cytoskeleton. Detailed analysis of colonization-defective mutants identified those with minor alterations in the composition of the outer-membrane molecule lipopolysaccharide (LPS)[80]. The work revealed an intimate relationship between the ability of the LPS molecule to protect the pathogen from innate immune responses, and to allow correct presentation of the T3S system at the bacterial surface. The balance is achieved through conformational changes in the LPS molecule, which are induced by glucosylation[80].

Defining interactions of host and pathogen genes: differential STM

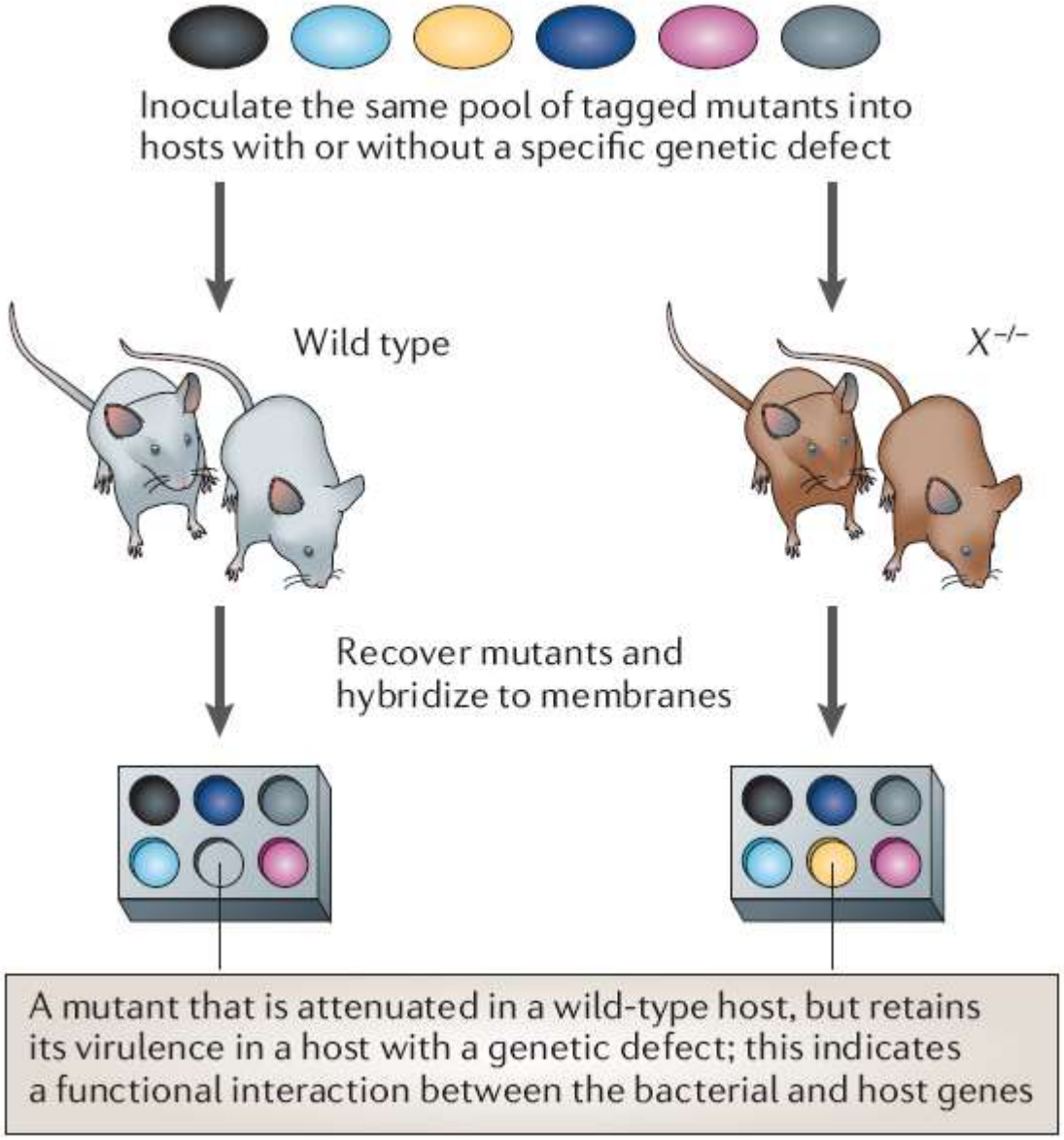
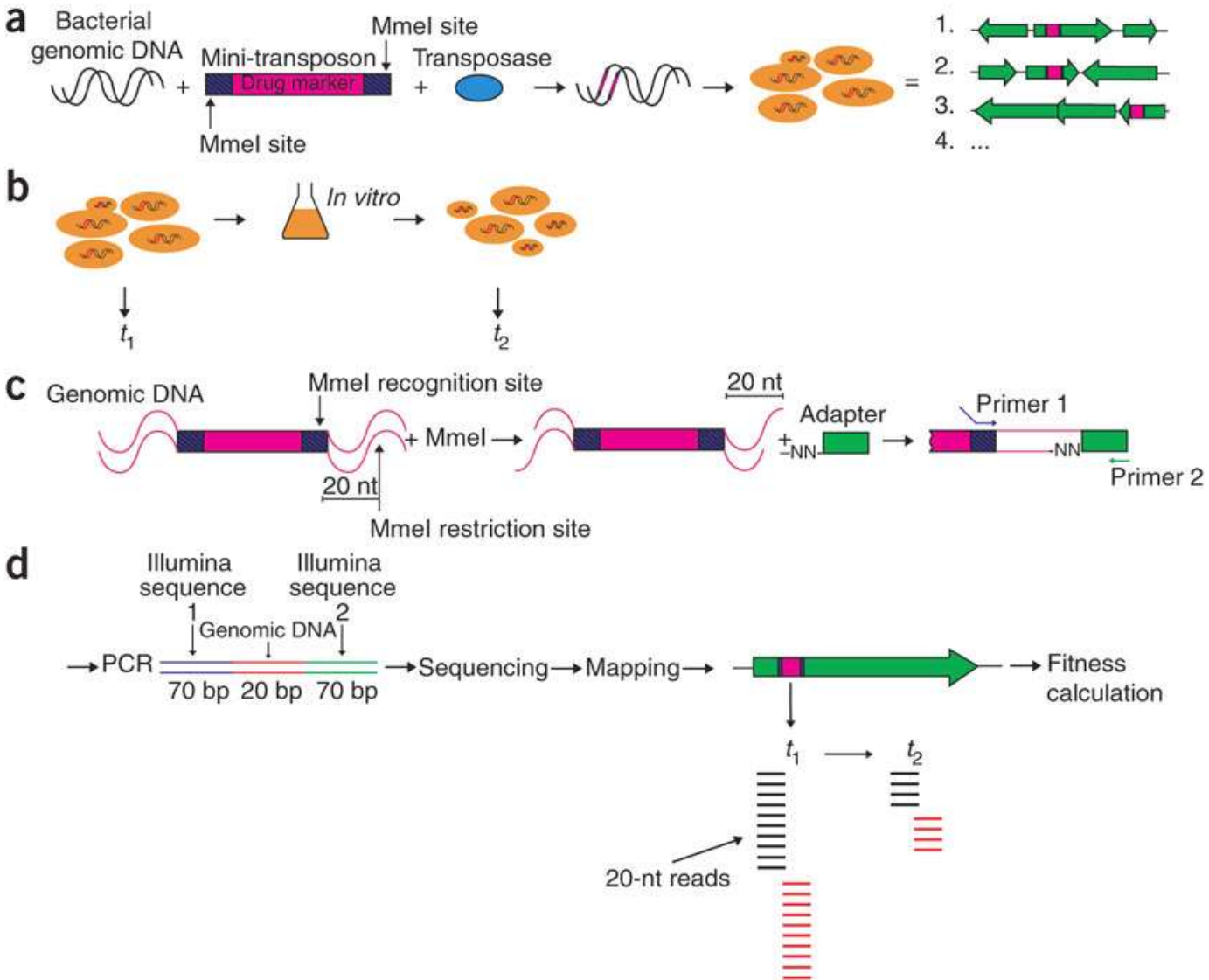


Figure 4 | **Differential STM screen.** The same set of mutants is inoculated into wild-type animals, and those with a specific genetic defect ($X^{-/-}$). Counter-immune mutants are attenuated in a wild-type host, but retain their virulence in the knockout animals. This indicates that there is an interaction between the product of the gene that is affected in the counter-immune mutant and the function that is lost in the knockout animal.

Tn-Seq



(**a**) A gene disruption library is constructed by first transposing the mini-transposon *magellan6*, which contains an Mme restriction site within each inverted repeat, into bacterial genomic DNA *in vitro* and then transforming a bacterial population with the transposed DNA. The result is a bacterial pool in which each bacterium contains a single transposon insertion in its genome. (**b**) DNA is isolated from a portion of the bacterial pool (t_1) and another portion is used to seed a culture on which selection is performed, then DNA is isolated again from recovered bacteria (t_2). (**c**) DNA from both time points is digested with MmeI. (**d**) A PCR amplification was performed to obtain a 160-bp sequence with 20 bp of bacterial-specific DNA flanked by Illumina-specific sequences, which enable sequencing. After sequencing, different samples are identified based on barcode sequence, and the 20-bp reads are mapped to the genome and are counted for each insertion, thus allowing fitness to be calculated.
Van Opijnen et al.Nature Methods **6**, 767 - 772 (2009) doi:10.1038/nmeth.1377

Tn-Seq variants/improvements

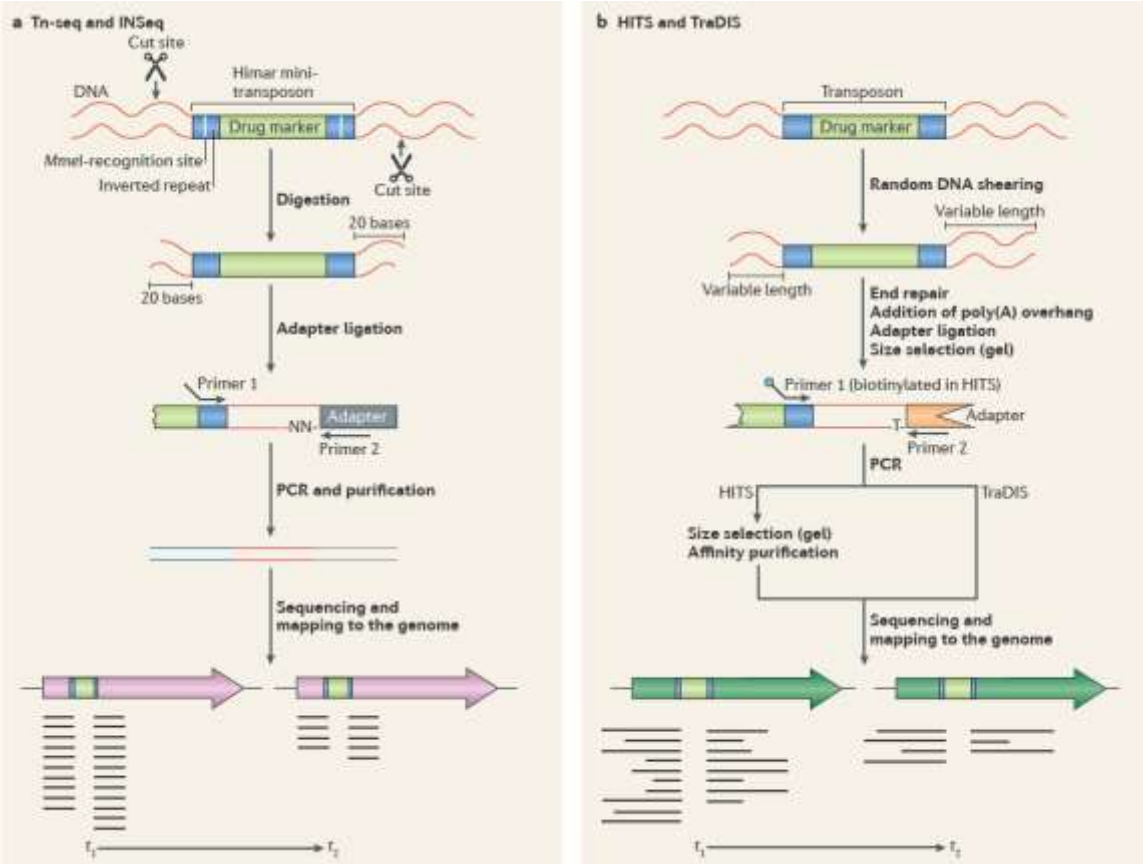


Figure 1 | **Four methods of massively parallel sequencing of transposon insertions.** Each of four methods of transposon sequencing is illustrated, starting from pooled genomic DNA from the transposon insertion library and ending with sequencing of the left and right transposon junctions. The number of sequences (reads) for each junction can differ between the start of the experiment (t_1) and the end (t_2), after a selection has been carried out on the library of transposon insertion mutants. In both examples shown, the transposon insertion mutation decreases fitness during growth under the conditions tested, as indicated by there being fewer reads at the end of the experiment than at the start. **a** | The Tn-seq (named for transposon sequencing) and insertion sequencing (INSeq) methods are highly similar, but INSeq includes a PAGE gel purification step following adaptor ligation and PCR, whereas Tn-Seq includes an agarose gel purification at this point. A recent study[66] introduced additional steps to the original INSeq protocol: a linear-PCR step using a biotinylated primer and subsequent purification of the product with magnetic streptavidin beads were added following adapter ligation. These steps reduce both the amount of sample and the amount of enzymes needed. Although they make the protocol more laborious, the results suggest that these modifications increase the sensitivity of the technique. **b** | The high-throughput insertion tracking by deep sequencing (HITS) and transposon-directed insertion site sequencing (TraDIS) methods are more similar to each other than to Tn-seq and INSeq: after shearing of the DNA, the DNA ends are repaired, and a poly(A) tail is added. However, the methods diverge after the PCR step; in HITS, the PCR products undergo size selection (on a gel) and affinity purification before sequencing, whereas in TraDIS, the PCR products are sequenced directly.

Hiding in host cells in order to escape complement, antibodies, and phagocytes

cytosol of eukaryotic cells is the heaven for bugs…

Facultatively intracellular bacteria:

-*Mycobacterium tuberculosis, leprae, kansasii, smegmatis*

-*Mycoplasma hominis, buccale, orale, salivarum,fer,entas, genitalum*
Ureaplasma, Anaeroplasma

-*Mycoplasma pneumoniae* - ,

-*Chlamidia trachomatis, psittaci, pecorum*

- ulcerative lesions– lymphoadenopaties

- trachomas

-Paratrachomas

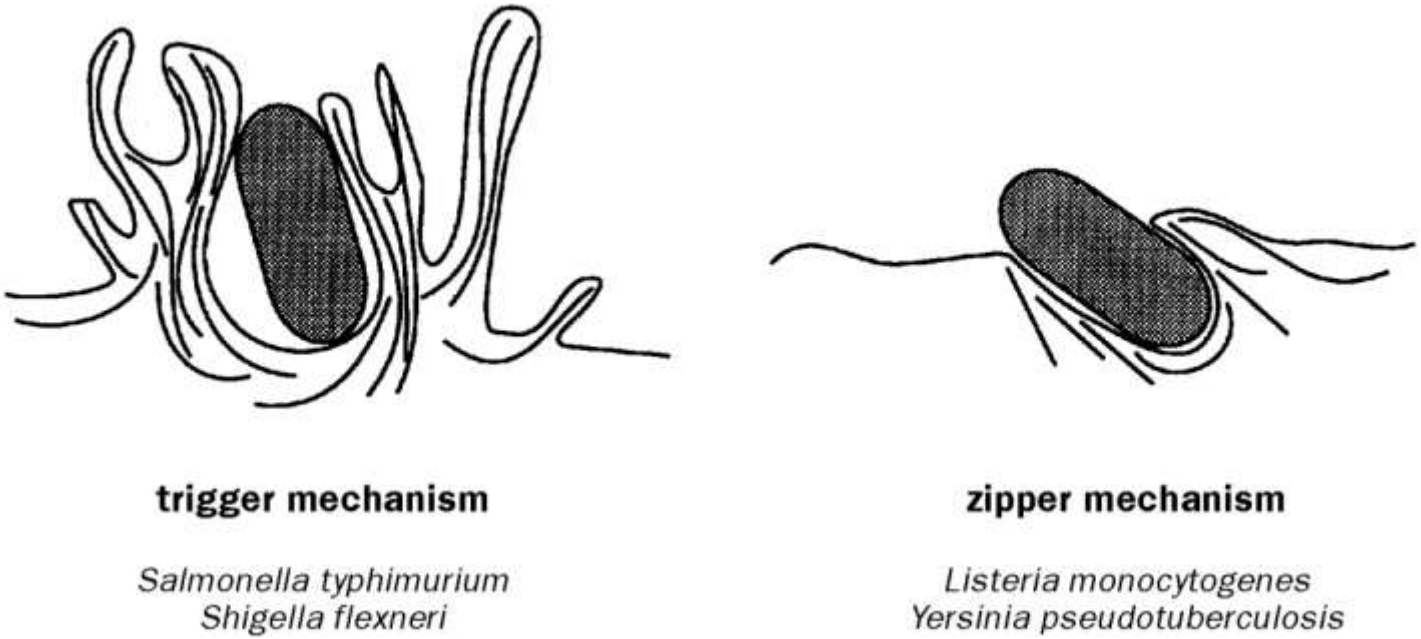
-*Bartonella baciliformis, henselae*

-*Ricketsia typhi, prowazeki, ricketsii*

-*Shigella, Samonella, Francisella, Listeria ….*

There is limited access of antibodies into eukaryotic cytosol:

- almost no antibodies
- food ‘ad libitum’
- host needs to kill infected cells with the bacteria

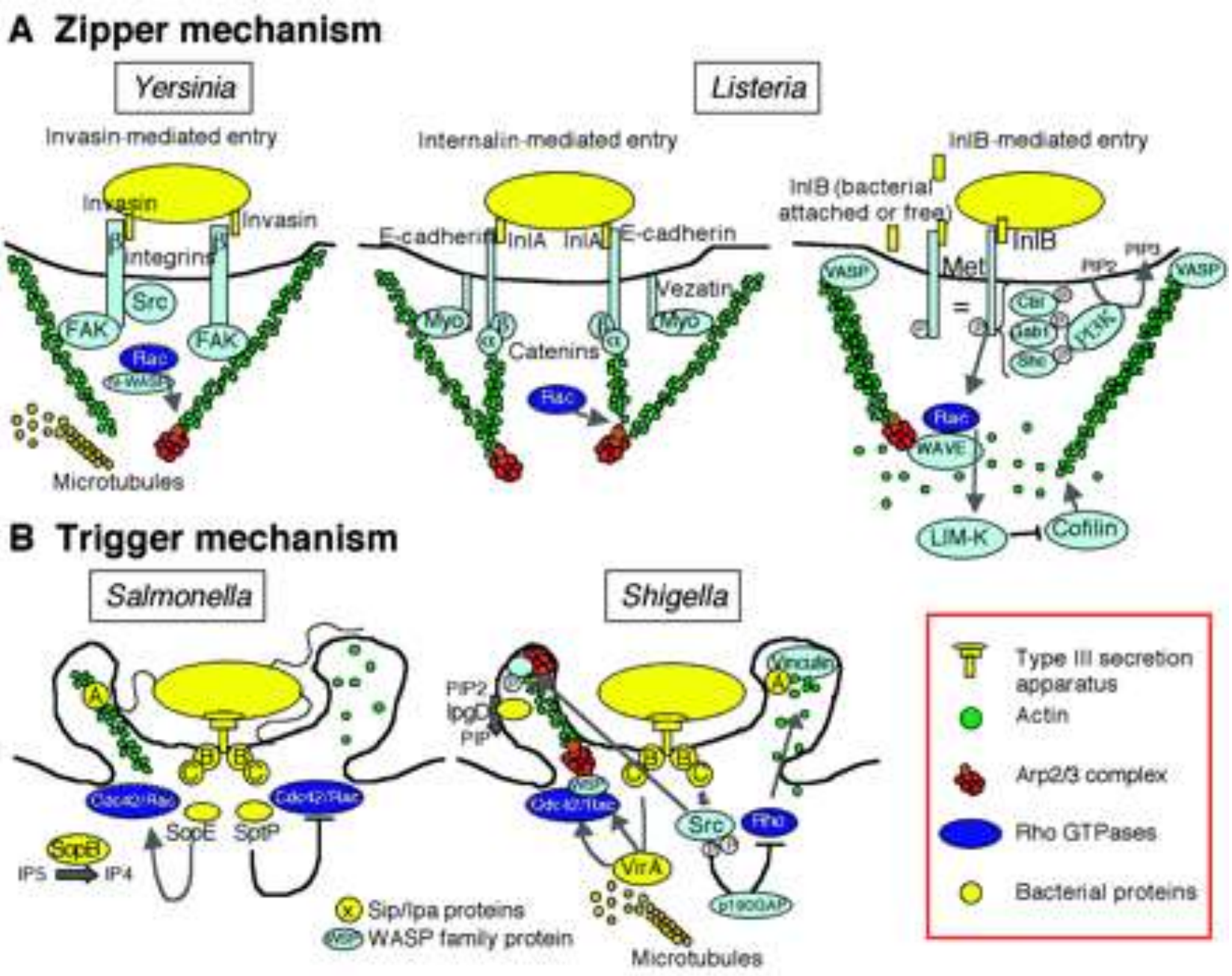


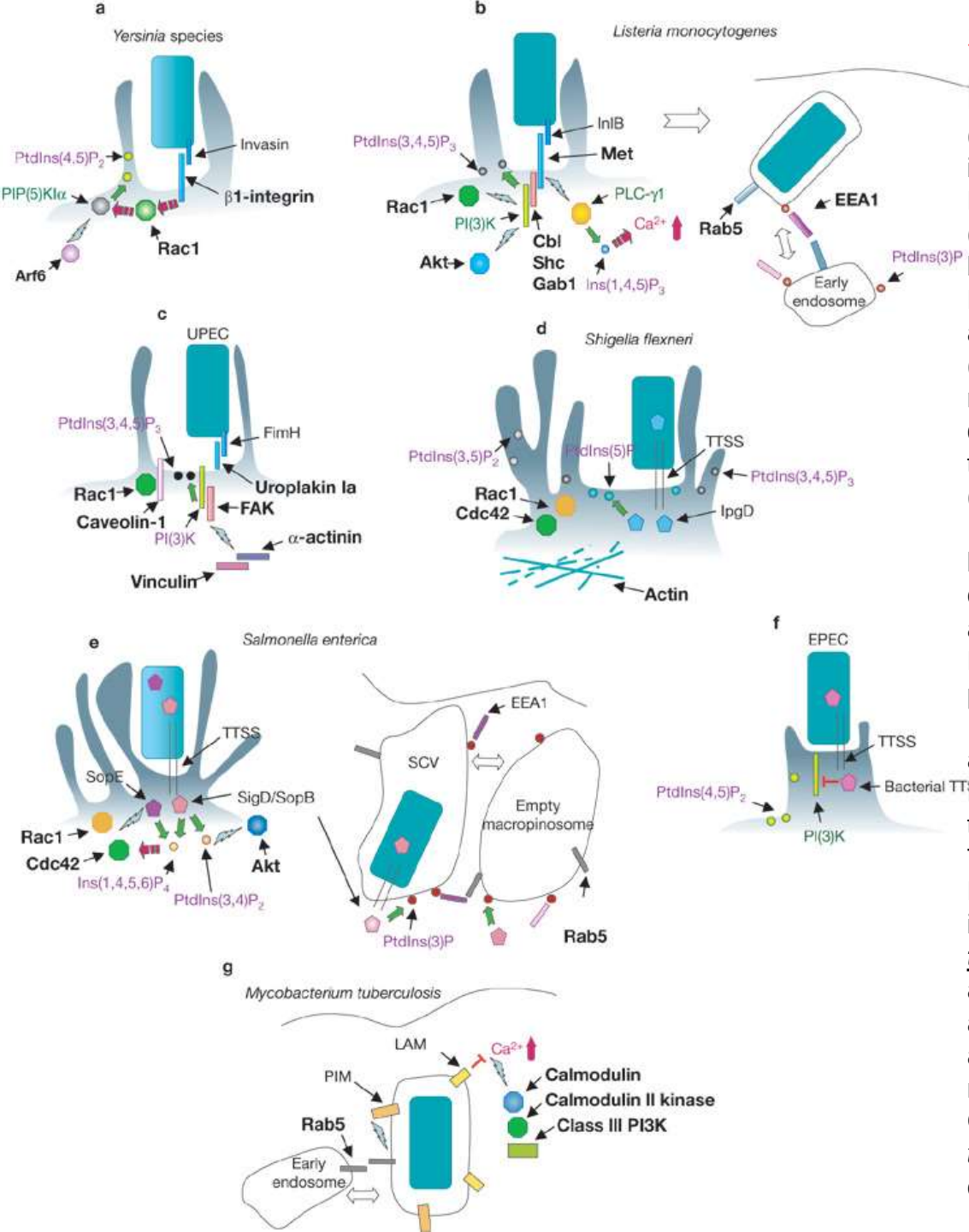
trigger mechanism

Salmonella typhimurium
Shigella flexneri

zipper mechanism

Listeria monocytogenes
Yersinia pseudotuberculosis

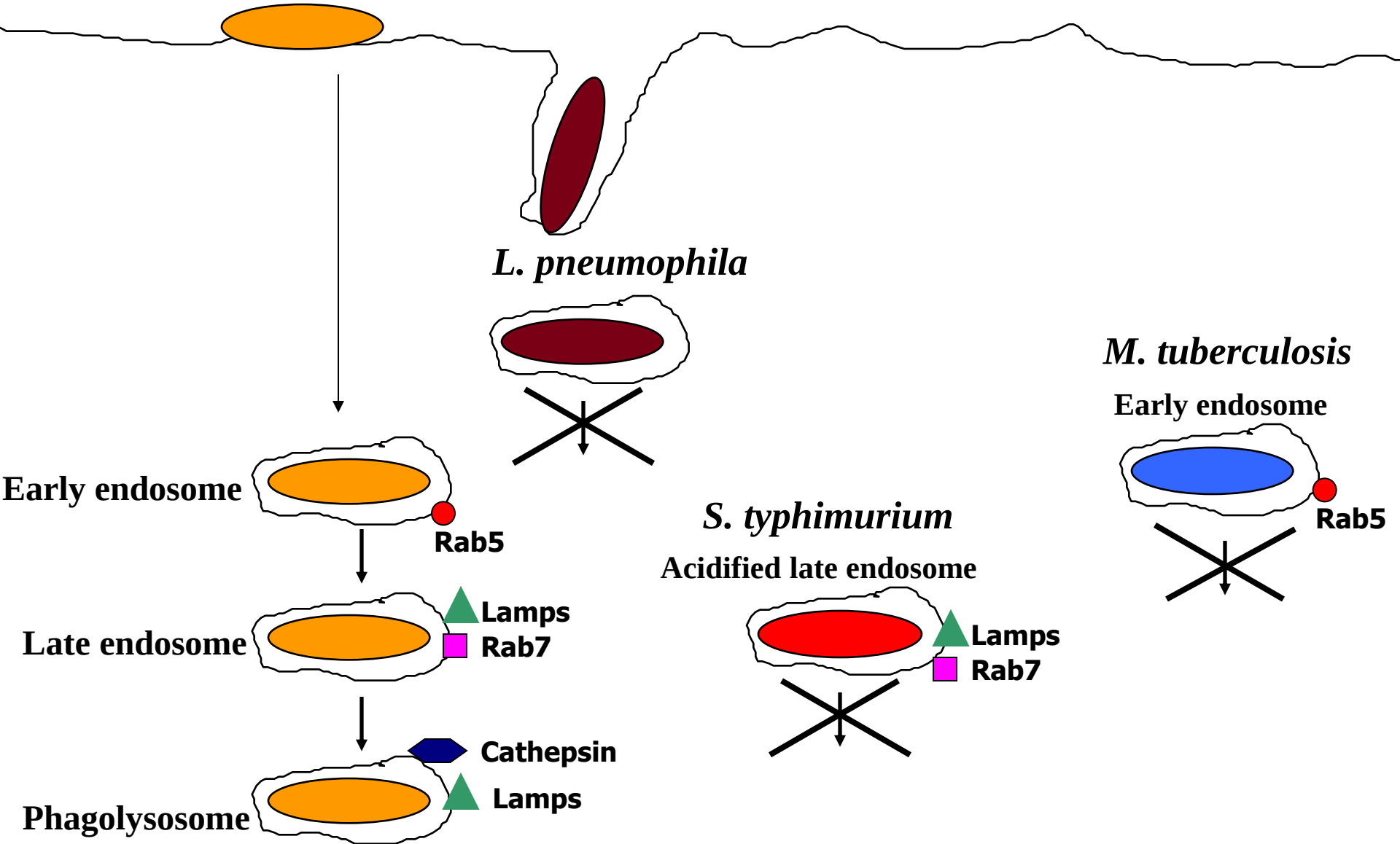

A Zipper mechanism
Yersinia
Invasin-mediated entry
Invasin
Invasin
β1 integrins
Src
FAK
FAK
Rac
N-WASP
Microtubules
Listeria
Internalin-mediated entry
E-cadherin
InlA
InlA
E-cadherin
Vezatin
Myo
Myo
Catenins
Rac
InlB-mediated entry
InlB (bacterial attached or free)
InlB
Met
VASP
PIP2
PIP3
VASP
Cbl
Gab1
Shc
PI3K
Rac
WAVE
LIM-K
Cofilin
B Trigger mechanism
Salmonella
SopB
IP5
IP4
SopE
SptP
Shigella
PIP2
IpgD
PIP
Vinculin
Src
Rho
VirA
p190GAP
Microtubules
Sip/Ipa proteins
WASP family protein
Type III secretion apparatus
Actin
Arp2/3 complex
Rho GTPases
Bacterial proteins

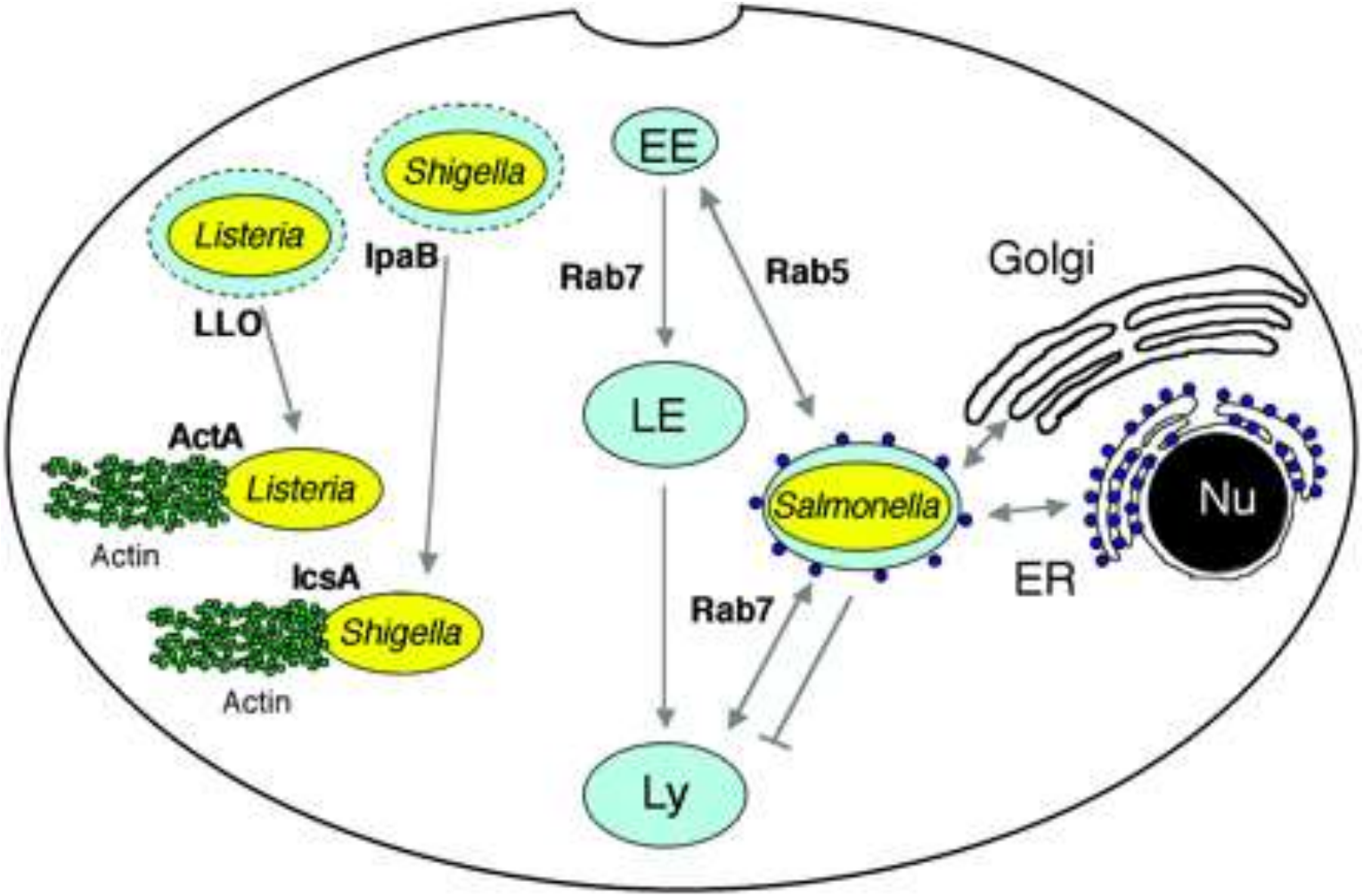


Signalling pathways exploited by bacterial pathogens.

(**a**) ***Internalization of Yersinia species.*** Interaction of invasin with 1-integrins induces Rac1 recruitment and PIP(5)K(1) translocation to the bacterial entry site; (**b**) ***Cellular invasion by L. monocytogenes***. (1) InlB binds to Met, inducing Gab/ Cbl/Shc and class I PI(3)K recruitment; Rac1 is then activated, controlling actin dynamics during entry; (**c**) ***UPEC entry into bladder cells***. The FimH receptor is uroplakin Ia, which has a short cytoplasmic domain and interacts with unknown molecules to trigger entry; (**d**) ***Internalization of S. flexneri.*** The TTSS effector IpgD dephosphorylates PtdIns(4,5)P2 into PtdIns(5)P leading to membrane detachment from the underlying cytoskeleton, facilitating Rac1 and Cdc42 ruffling activity; (**e**) *S. enterica* invasion of target cells. (1) the Rac1/Cdc42 exchange factor SopE and the phosphatidylinositol phosphatase SigD/SopB promote Ins(1,4,5,6)P4 generation and indirect Cdc42 activation; (**f**) ***Phagocytosis inhibition by EPEC***. Bacteria multiply extracellularly on top of pedestals; translocation to the host cell cytoplasm of an unknown TTSS effector leads to inactivation of class I PI(3)K, PtdIns(4,5)P2 accumulation and blockage of bacterial internalization. (**g**) ***Internalization of M. tuberculosis.*** The bacterial phosphatidylinositol analogue LAM inhibits intracellular Ca2+ rise, blocking a calmodulin–calmodulin kinase II pathway that activates class III PI(3)K; simultaneously, the mycobacterial phosphoinositide PIM activates a GTPase (probably Rab5) inducing fusion of the *M. tuberculosis*-containing compartment with early endosomes.

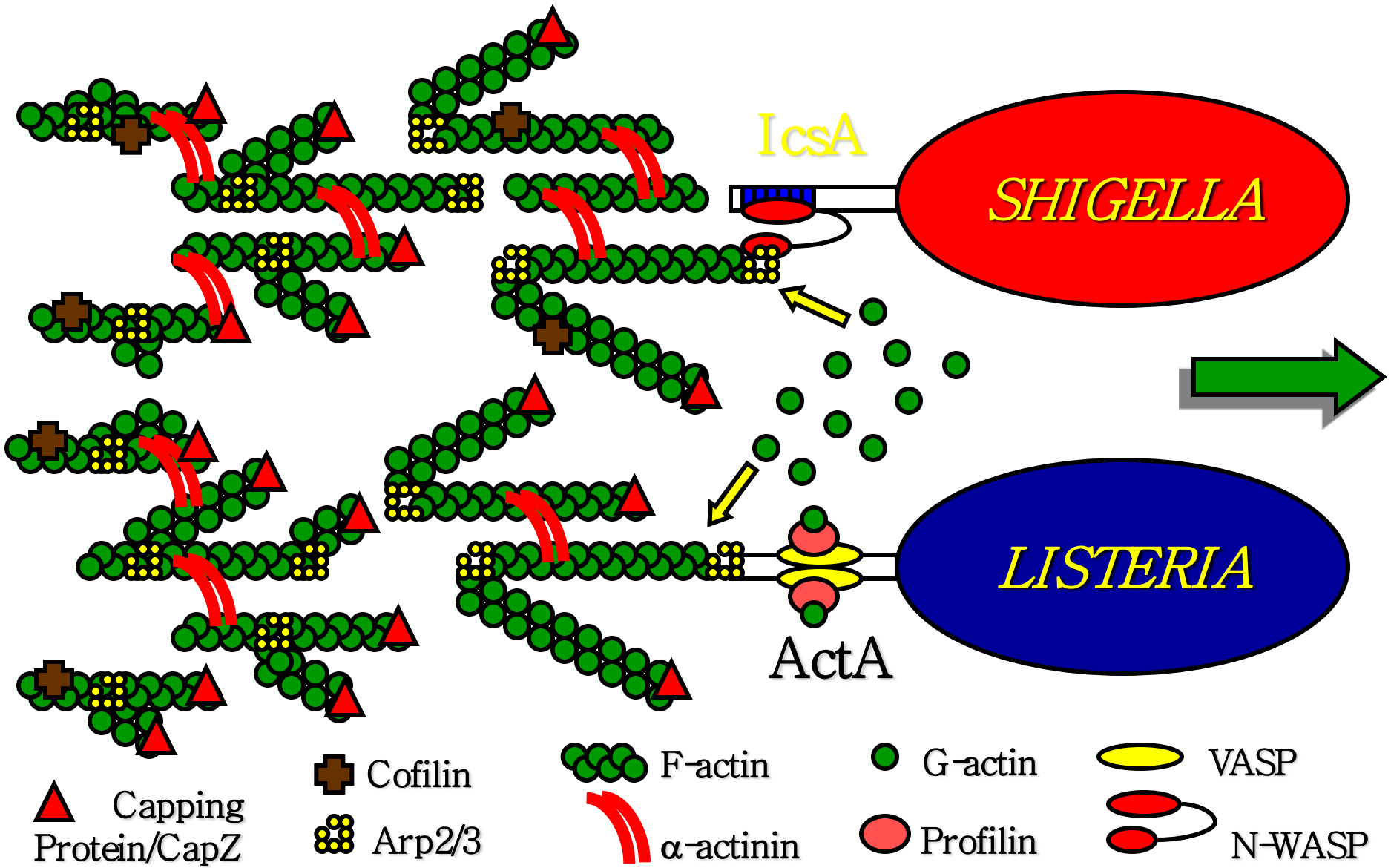
Vacuolar intracellular pathogens

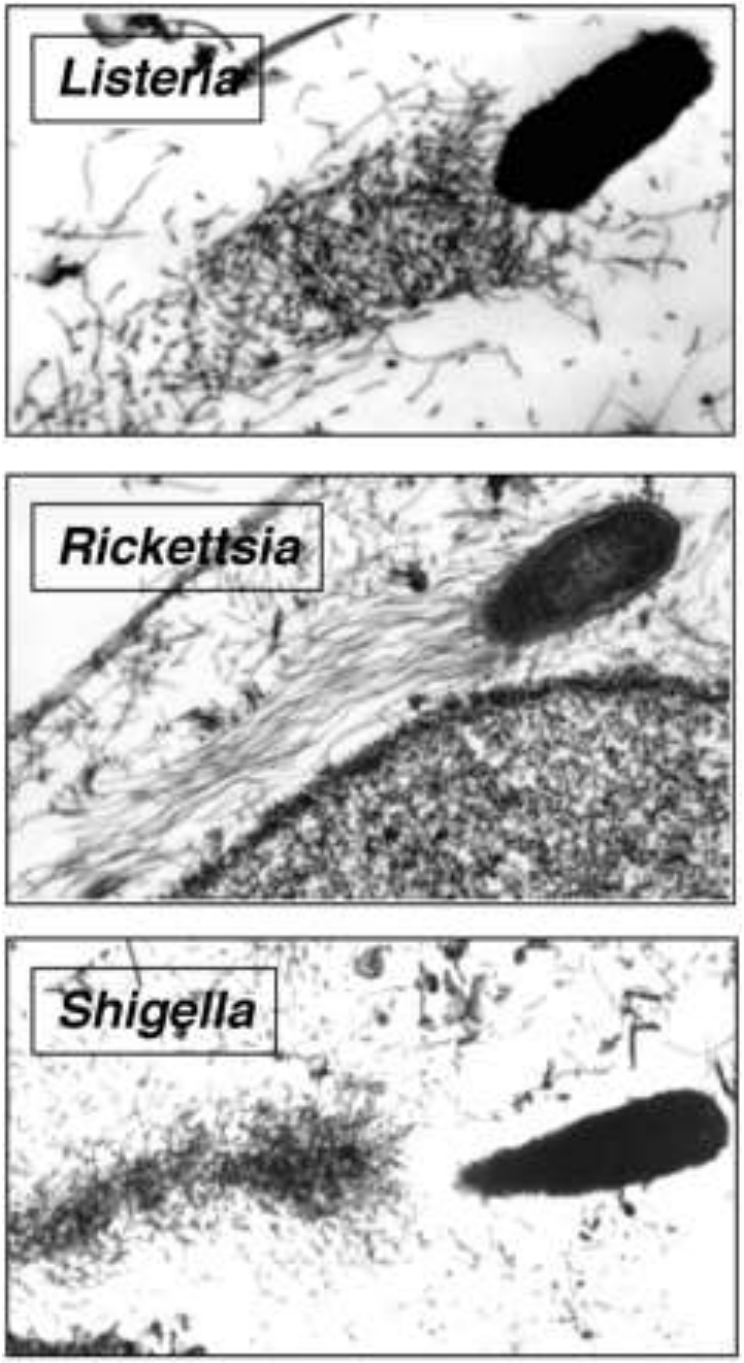




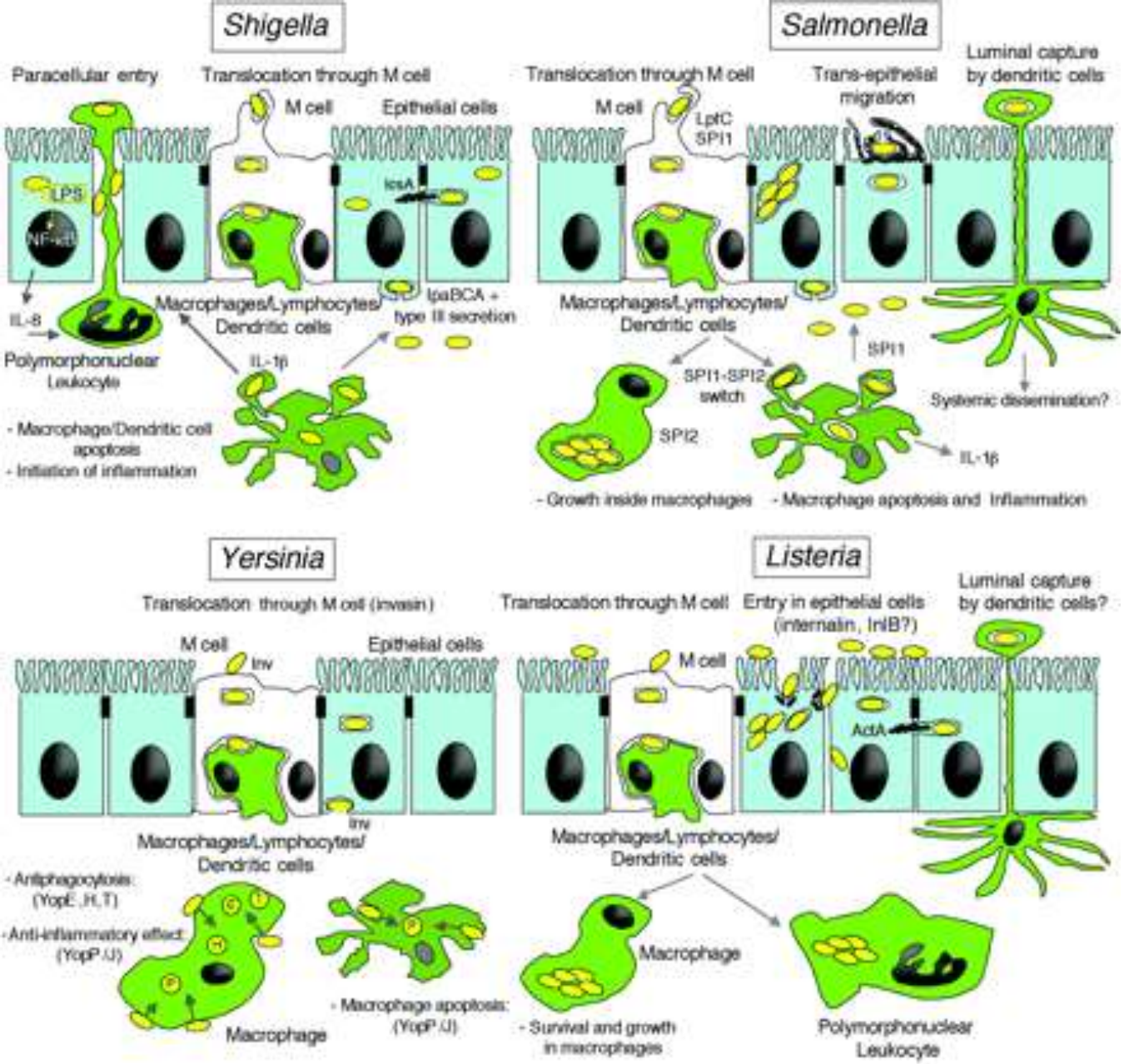
Intracellular life-styles. Schematic representation of the *Salmonella*-containing vacuole (see text). *Listeria* and *Shigella* lyse the vacuole and move in the cytosol by an actin-based motility process mediated by ActA or IcsA/VirG, which interact with Arp2/3 or N-WASP and Arp2/3, respectively. EE: early endosome; LE: late endosome; Ly: lysosome; ER: endoplasmic reticulum

ACTIN-DEPENDENT MOTILITY OF BACTERIA DISRUPTING THE ENDOCYTIC VACUOLE AND ESCAPING INTO THE CYTOPLASM



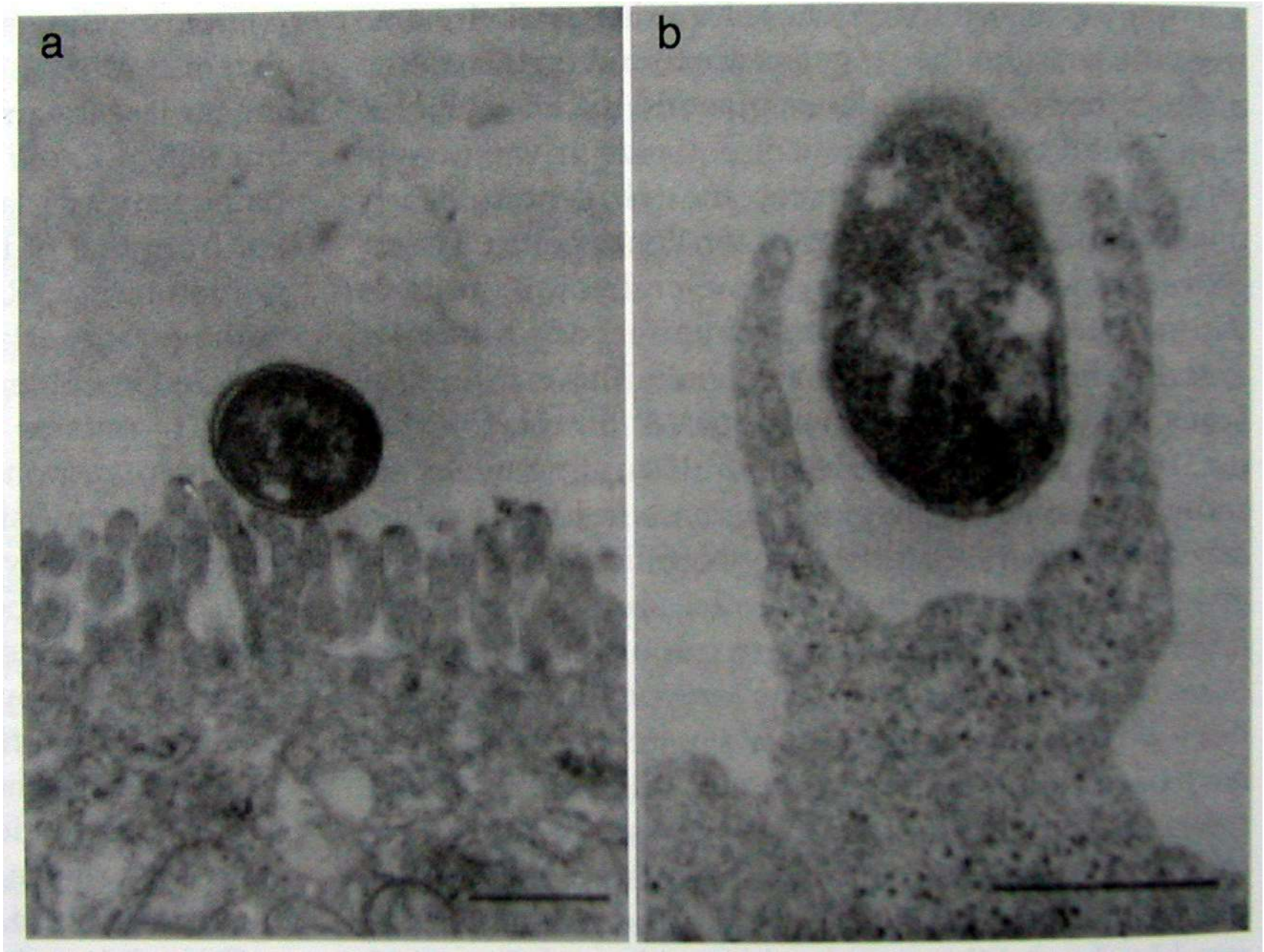


Actin-based motility of *Listeria, Rickettsia*, and *Shigella.* Electron micrographs of actin tails labeledwith fragment S1 of myosin

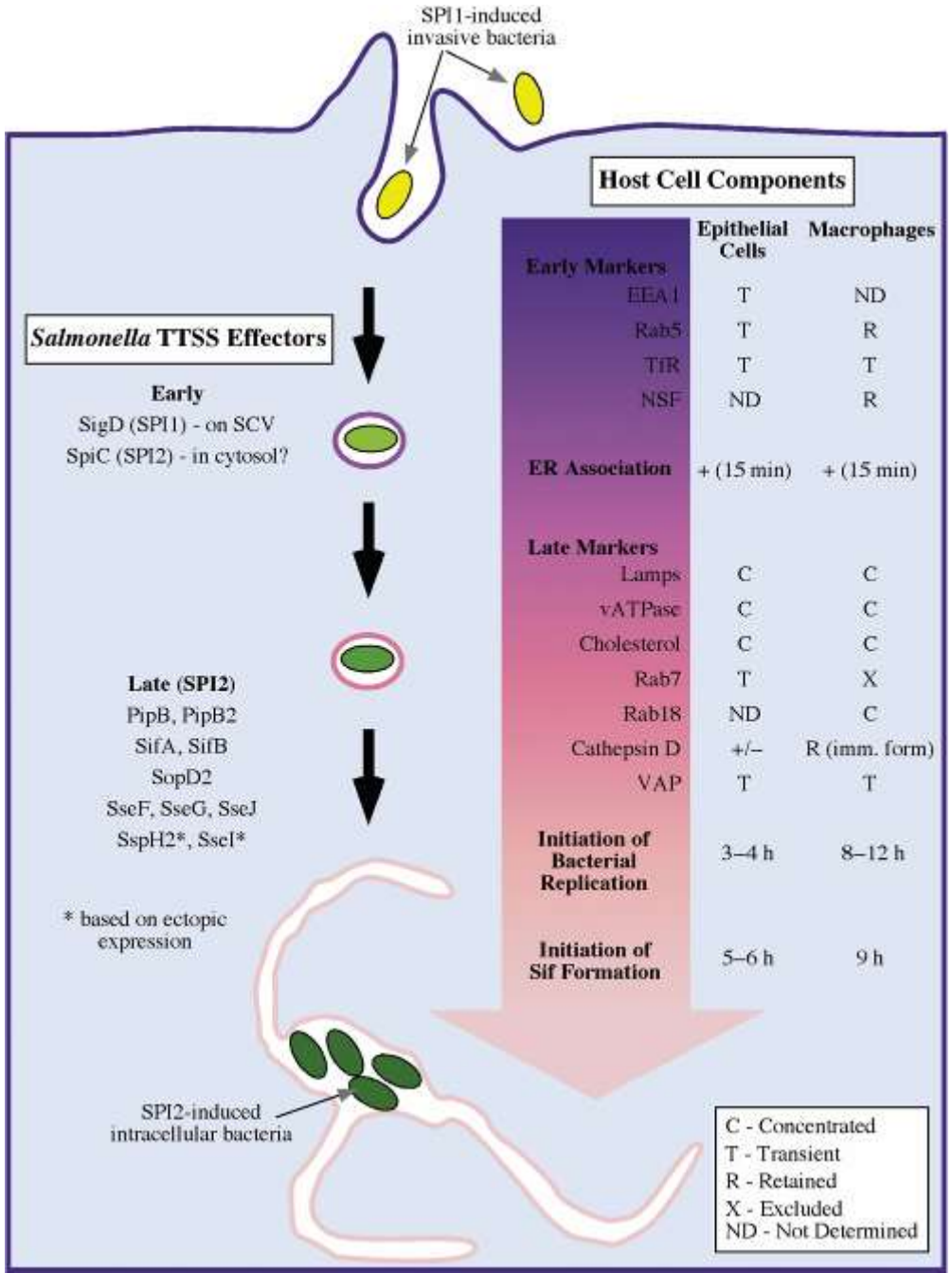


The invasive strategies of enteroinva-sive pathogens. Intestinal epithelial cells (IECs) maintain a physical barrier against commensal flora, although specialized sites such as the follicle-associatedepithelium (FAE) allow constant sampling of the luminal flora through M cells.
Invasive pathogens take advantage of this route to cross the epithelial barrier. Once translocated, bacteria must survive attack by macrophages. The four bacterial species considered have solved this issue differently: ***L. monocytogenes*** are phagocytosed but escape into the cytoplasm, and thus avoid being killed in lysosomal compartments. *Yersinia* adopt an antiphagocytic strategy by intracellular injection of YopE, H, and T that inactivate the actin cytoskeleton. ***Shigella*** not only cause apoptosis of macrophages and monocytes, thus ensuring their own survival, but also trigger early mucosal inflammation through the release of mature IL-1ß and IL-18, which disrupts epithelial impermeability and facilitates bacterial spread at a distance. Finally, ***Salmonella*** remodel their phagosomes, thus avoiding its transition to a lysosome and creating an intracellular niche that allows their efficient replication.

The ‘Kiss and swallow’ of Salmonella…



Salmonella uses the T3SS effectors for enforcing uptake into host cells



The genus *Listeria*

Listeria monocytogenes

Listeria ivanovii

Listeria innocua

Listeria seeligeri

Listeriabwelshimeri

Listeria grayi

Listeria monocytogenes

foodborne pathogen

Transmission:	**dairy products, meat, vegetables, fish**
Disease:	**meningitis, encephalitis, septicemia, abortions, neonatal infections**
Population at risk:	**elderly, newborns, immuno-comprimised, pregnant women**
Mortality rate:	**30%**

Concern for public health **Problem for food industry**

Listeria monocytogenes

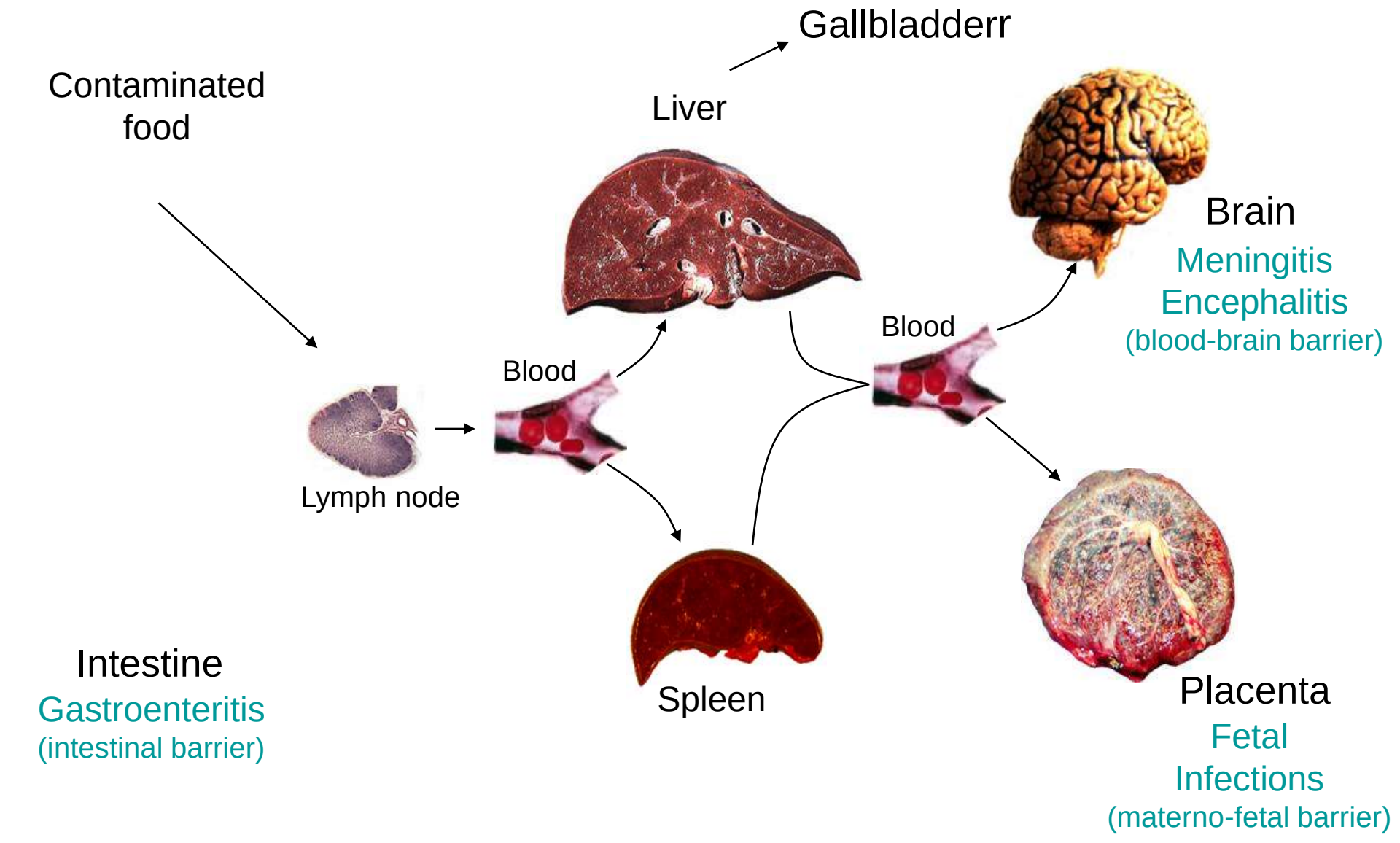
intracellular pathogen

Crosses 3 barriers: **the intestinal barrier**
the blood-brain barrier
the placental barrier

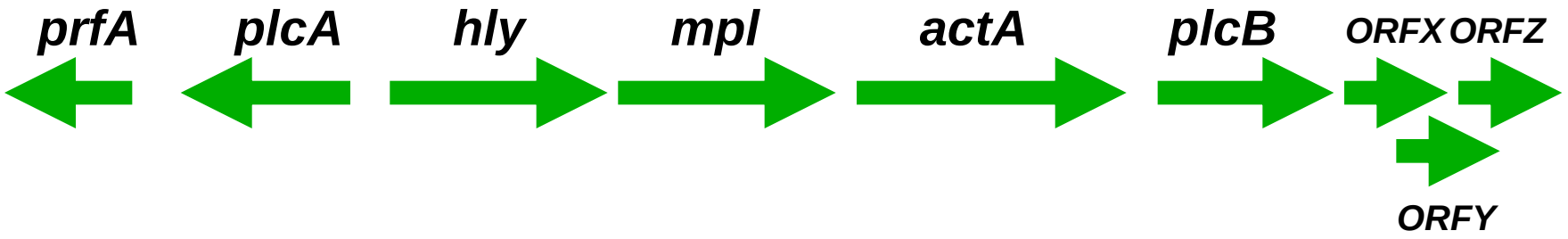
Exploits mammalian cell functions:

during entry
intracellular movement
cell to cell spread

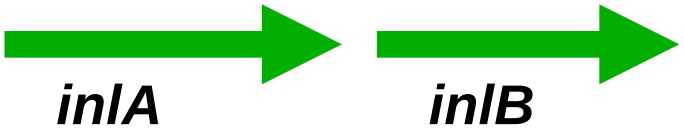
Successive steps of human listeriosis



The *Listeria monocytogenes* virulence gene regulon



PrfA



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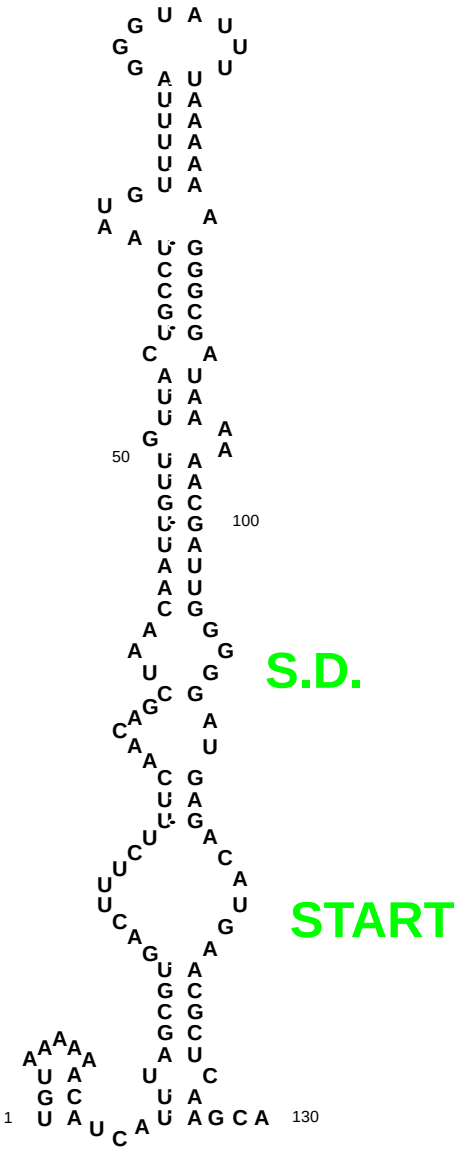
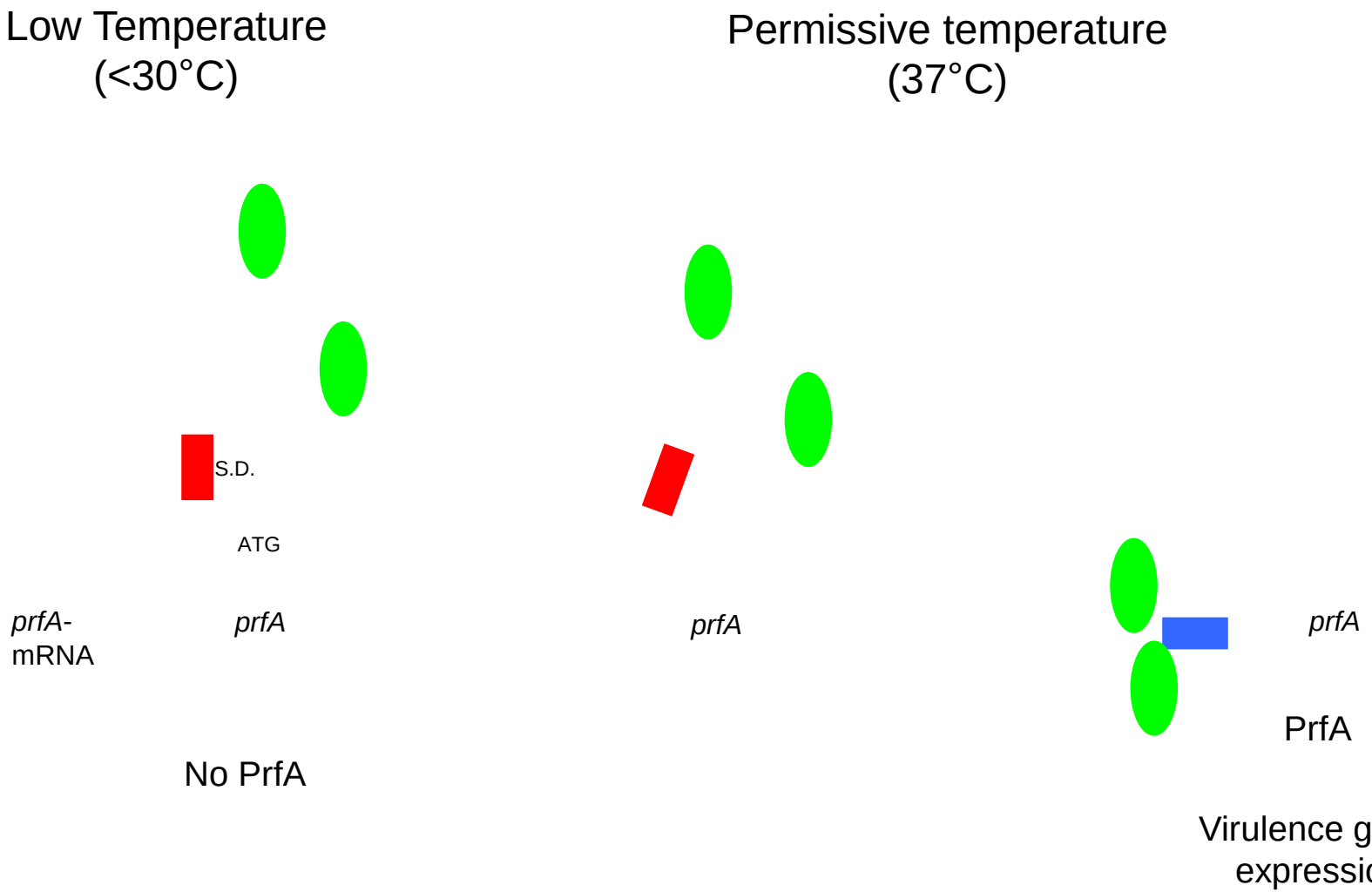
The 5’ UTR region of *prfA* can adopt a secondary structure

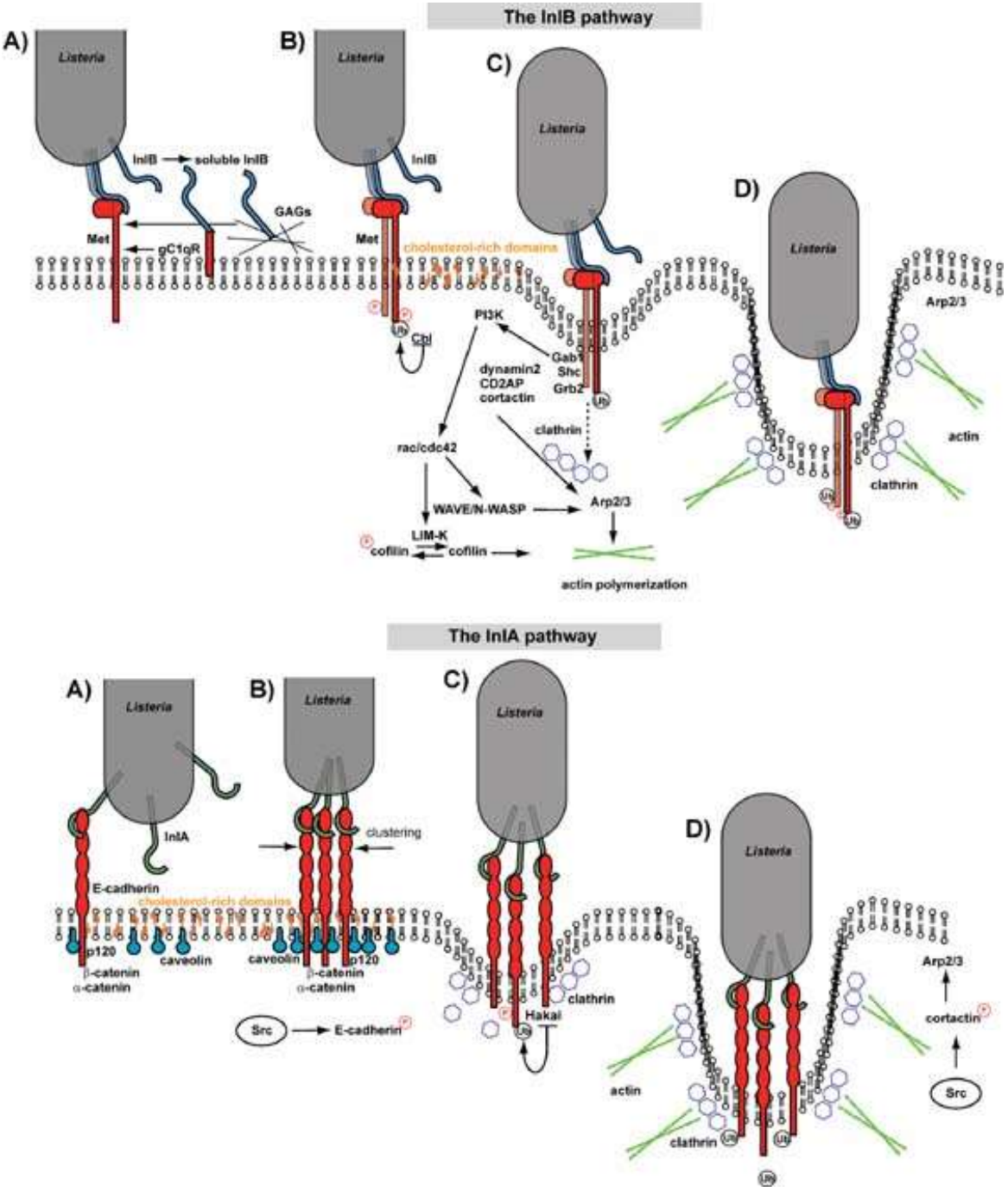


Thermoregulation of virulence genes by a RNA thermosensor



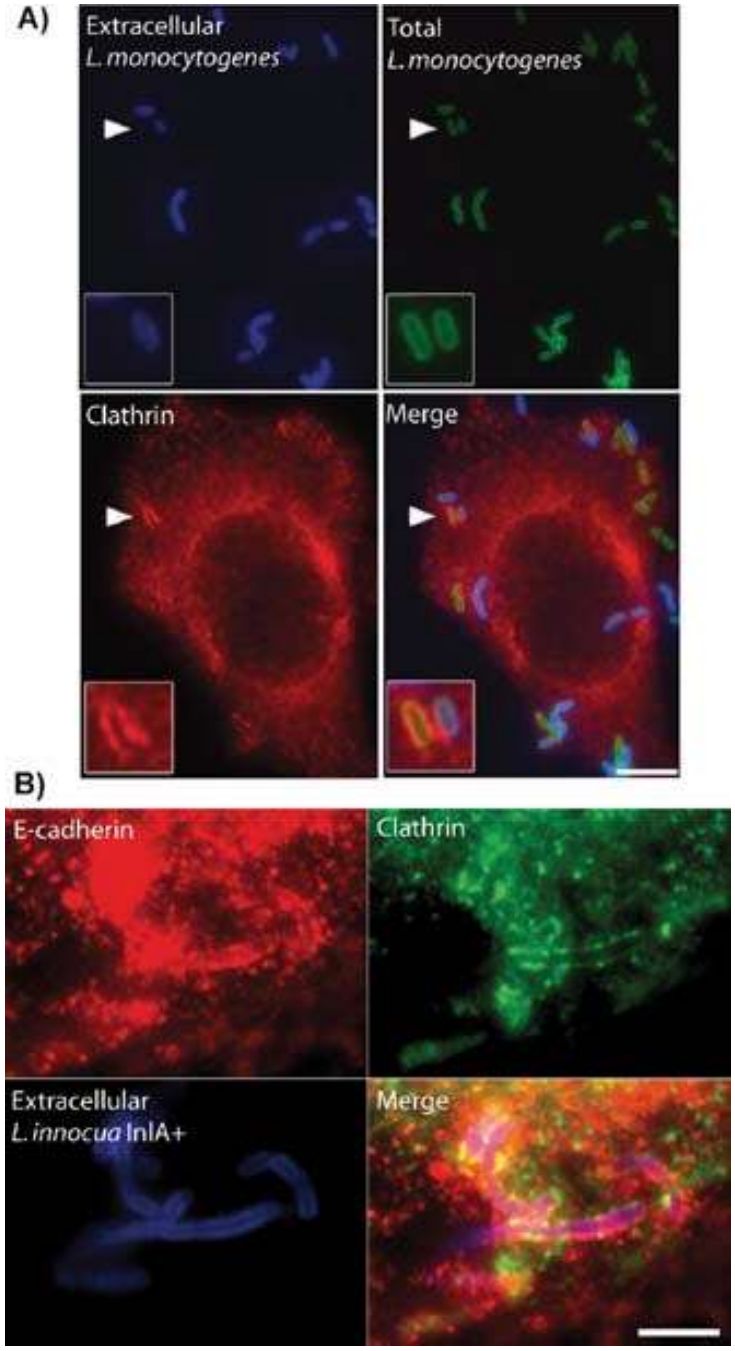
Take home messages

- *Listeria monocytogenes* is an invasive bacterium that spreads from cell to cell using an actin-based motility process
- Virulence genes are co-regulated by PrfA whose expression is controlled by an RNA thermosensor



Signaling pathways triggered by *L. monocytogenes* during invasion of host cells.

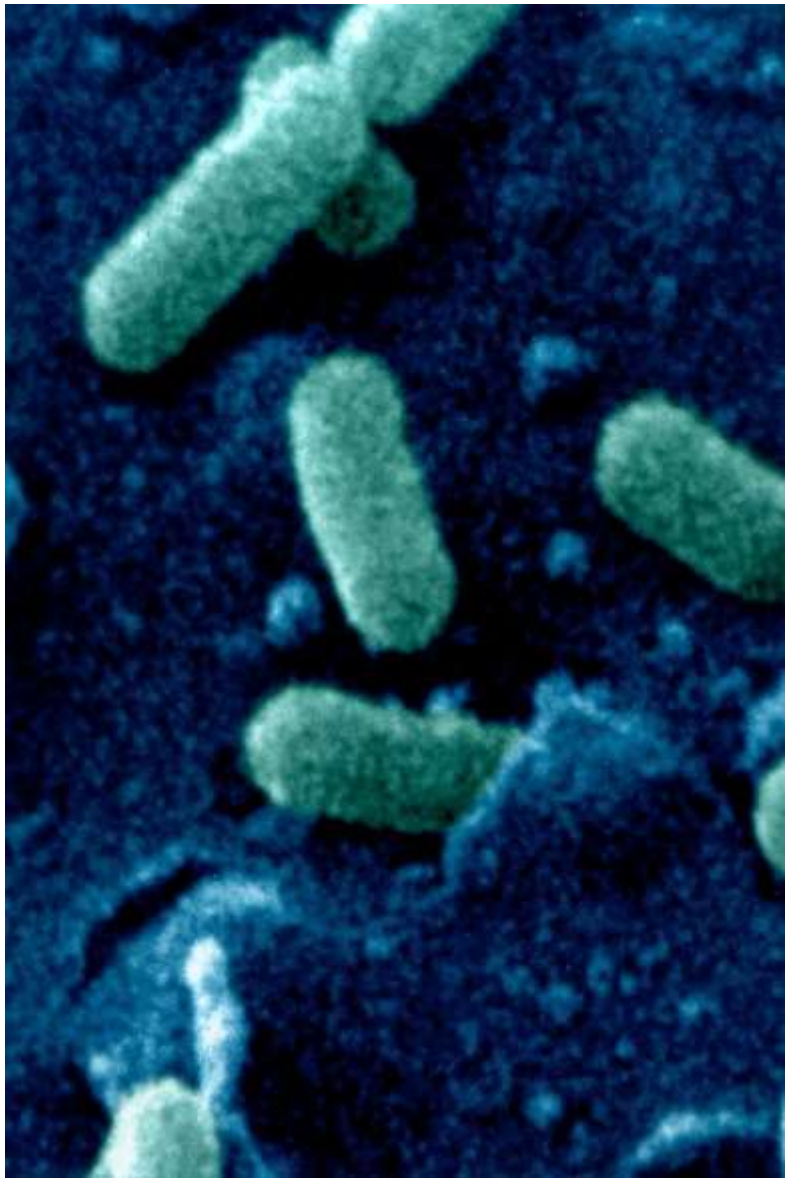
Upper panel: Entry of *L. monocytogenes* via the InlB/Met interaction. **(A)** The bacterial surface protein InlB can interact through its C-terminal domain with the gC1q-R protein and with extracellular glycosaminoglycans (GAGs), but it is the binding of the tyrosine receptor kinase Met by the InlB N-terminal domain that efficiently triggers bacterial internalization. **(B)** Activation of Met leads to its autophosphorylation and recruitment/phosphorylation of Cbl, an ubiquitin ligase required for Met ubiquitination. **(C)** Ubiquitinated Met promotes the recruitment to the bacterial entry site of the clathrin endocytic machinery. Other protein adaptors recruited by Met, including Gab1, Shc, Grb2, activate the type I PI 3-kinase p85/p110 for the production of PIP3 within cholesterol-rich domains, which in turn is involved in the activation of Rac and/or Cdc42 (depending on the cell type), activation of Wave and/or N-Wasp, and Arp2/3 for actin polymerization. Dynamin, which is recruited by the endocytosis machinery to the bacterial entry site, can interact with cortactin, which in turn can participate to the activation of the Arp2/3 complex and actin polymerization. The LIM kinase controls the phosphorylation state of cofilin to regulate actin depolymerization. **(D)** Interactions between clathrin and polymerizing actin triggers the invagination of the bacteria in a partially clathrin-coated compartment. Lower panel: Entry of *L. monocytogenes* via the InlA/E-cadherin interaction. **(A)** The bacterial protein InlA interacts with its lipid raft-associated receptor E-cadherin to promote the recruitment to the bacterial entry site of several members of the catenin family including p120, β- and -catenin. **(B)** Caveolin-1 participates in the clustering of E-cadherin and in the activation of Src, which in turn phosphorylates E-cadherin. **(C)** E-cadherin phosphorylation triggers the ubiquitination of the receptor by the ubiquitin-ligase Hakai which is required for the subsequent recruitment of clathrin. **(D)** Src is also involved in the phosphorylation of cortactin, which promotes Arp2/3 complex activation and actin polymerization.

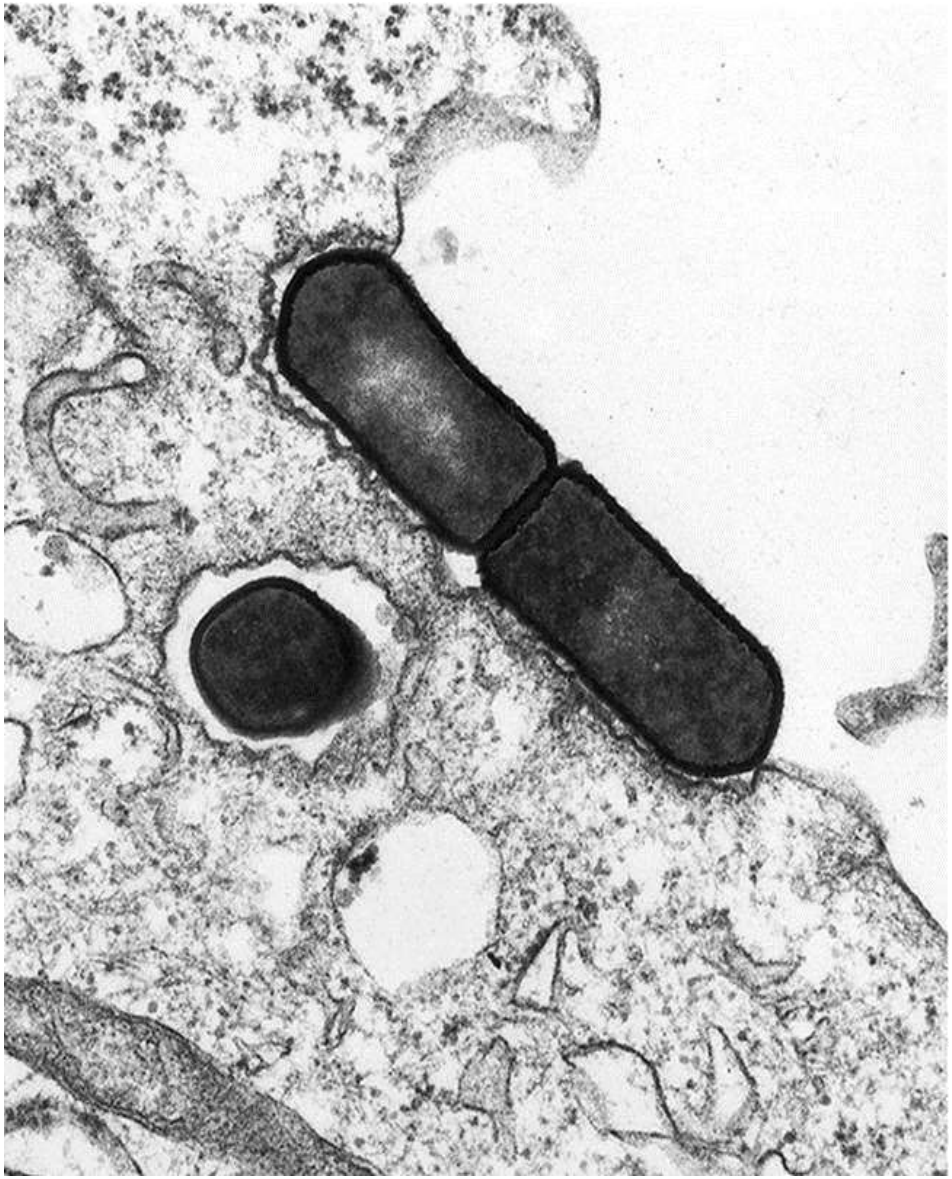


Recruitment of clathrin to the entry sites of *L. monocytogenes* and *L. innocua*$^{InlA+}$.

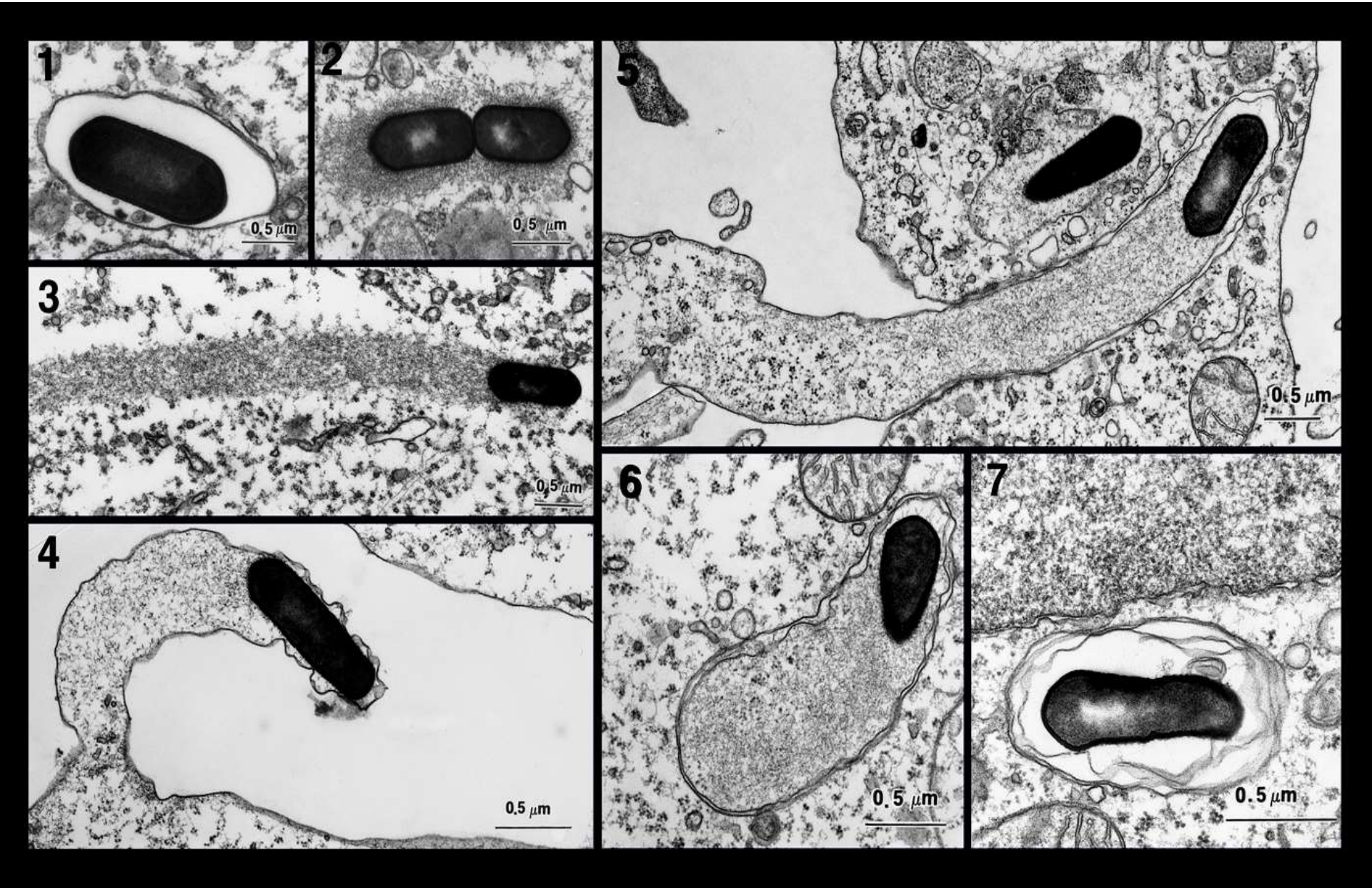
(A) HeLa cells were infected for 5 min with a *L. monocytogenes* recombinant strain expressing InlB covalently attached to the bacterial cell wall. Immunofluorescence in non-permeabilized cells firstly allows labeling of extracellular bacteria (shown in blue); labeling of clathrin (red) and total bacteria (green) was performed subsequently after permeabilization. The area indicated by the arrowhead is magnified to better show how clathrin is recruited to the bacterial entry site. Bar: 6 µm. Adapted from Ref. 22**(B)** HeLa cells were transfected with wild-type E-cadherin and incubated with *L. innocua* expressing InlA for 45 min. Immunofluorescence was performed to label transfected E-cadherin (red), endogenous clathrin (green), and *L. innocua*$^{InlA+}$ (blue).

Entry into non phagocytic cells



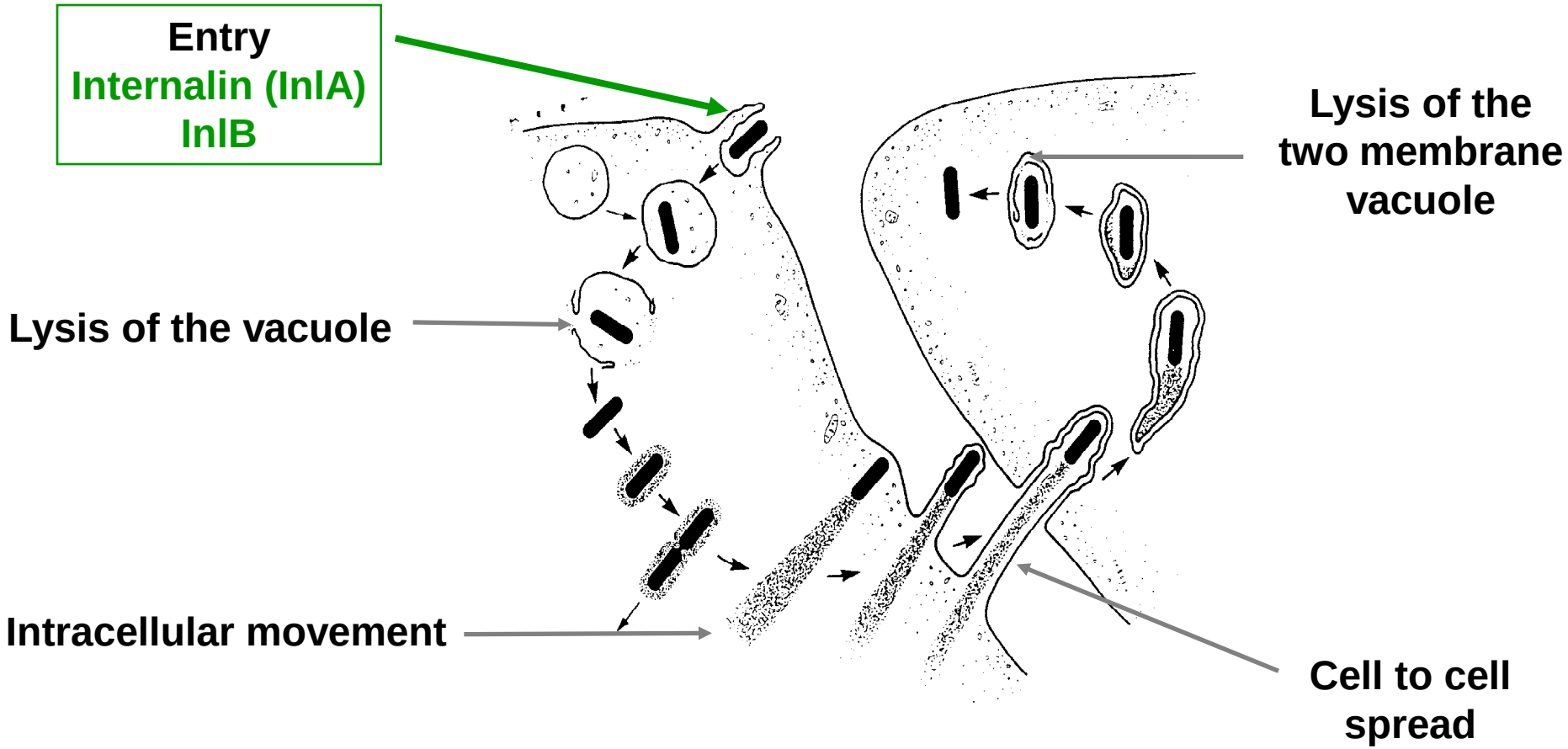


Successive steps of the cell infectious process



Successive steps of the cell infectious process

bacterial factors

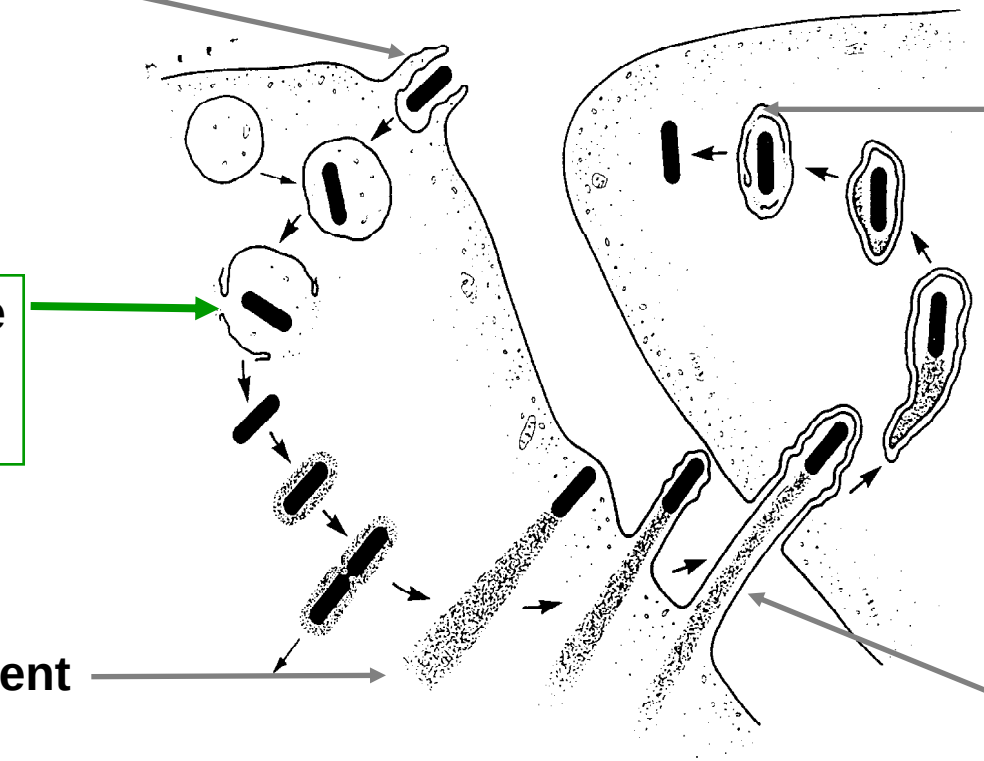


Adapted from L.G. Tilney and D.A. Portnoy J.Cell.Biol. (1989) 109, 1597

Successive steps of the cell infectious process

bacterial factors

Entry
Internalin (InlA)
InlB



Lysis of the two membrane vacuole

Lysis of the vacuole
LLO

Intracellular movement

Cell to cell spread

Adapted from L.G. Tilney and D.A. Portnoy J.Cell.Biol. (1989) 109, 1597

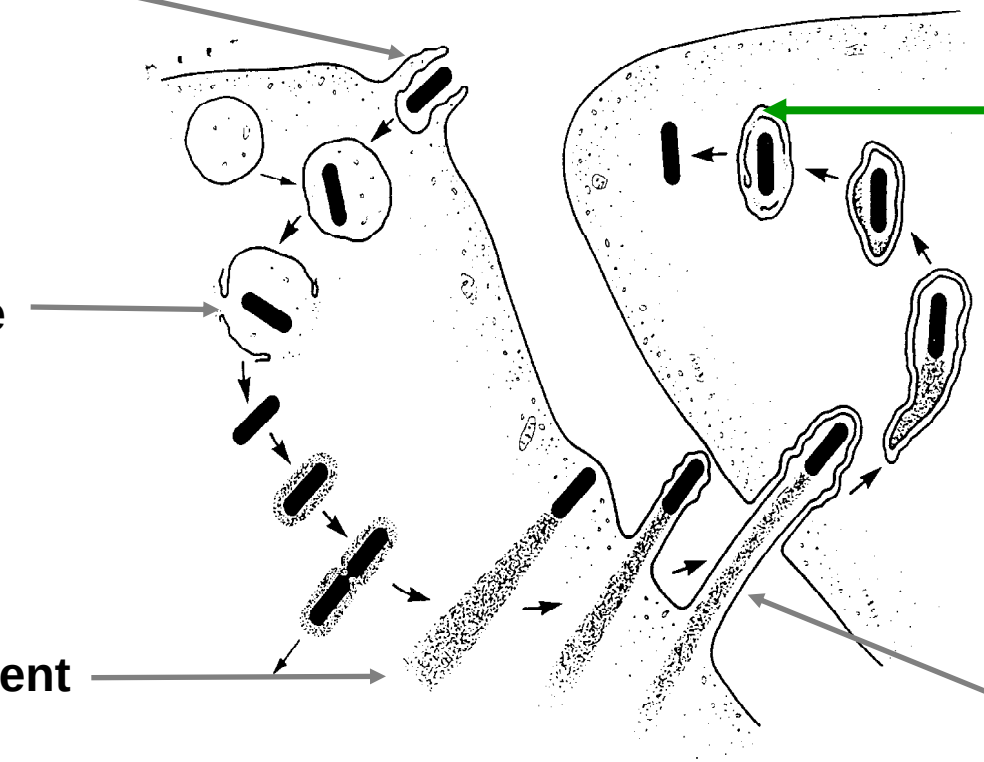
Successive steps of the cell infectious process

bacterial factors

Entry
Internalin (InlA)
InlB

Lysis of the vacuole
LLO
(PI-PLC)

Intracellular movement
ActA

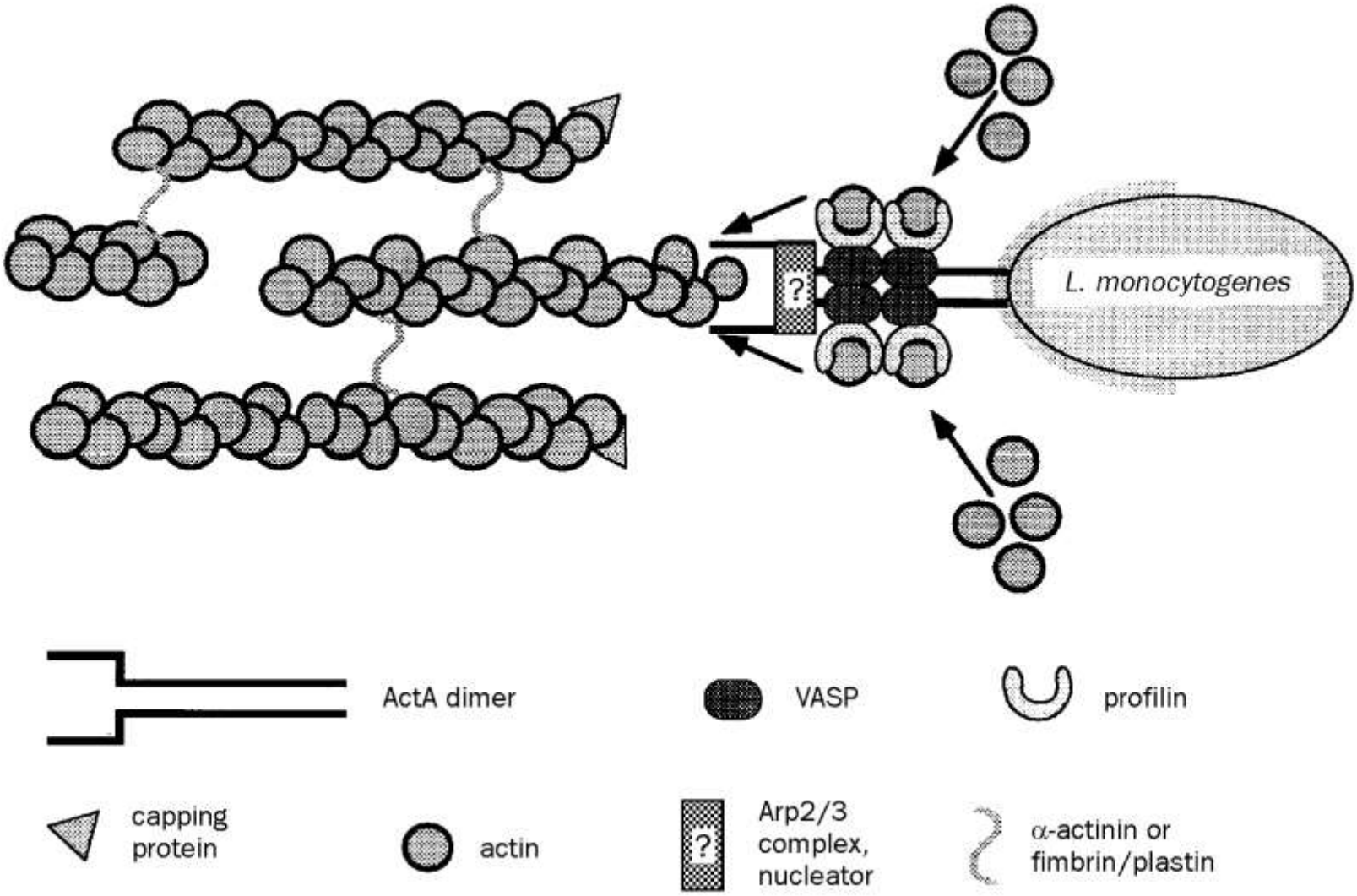


Lysis of the two membrane vacuole
Lecithinase
LLO

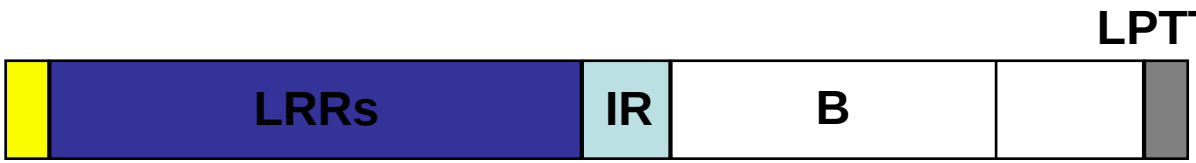
Cell to cell spread
ActA

Adapted from L.G. Tilney and D.A. Portnoy J.Cell.Biol. (1989) 109, 1597

The ‘propulsion jet’ motor of Listeria



Internalin (InlA)



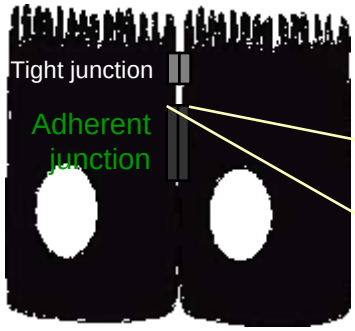
- *L. innocua* and *E. faecalis* expressing internalin are invasive
- Internalin-coated beads are invasive

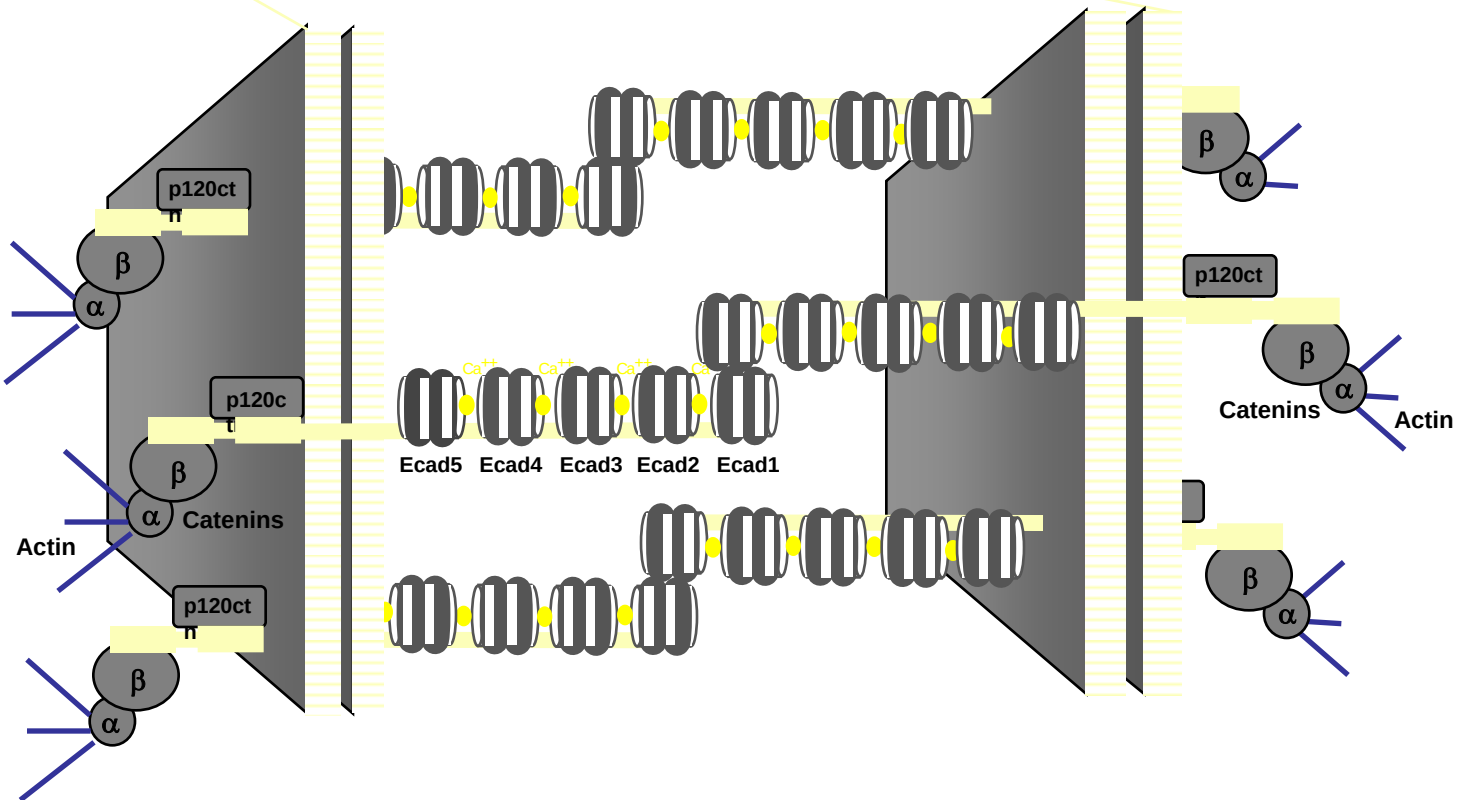
Internalin is sufficient for entry

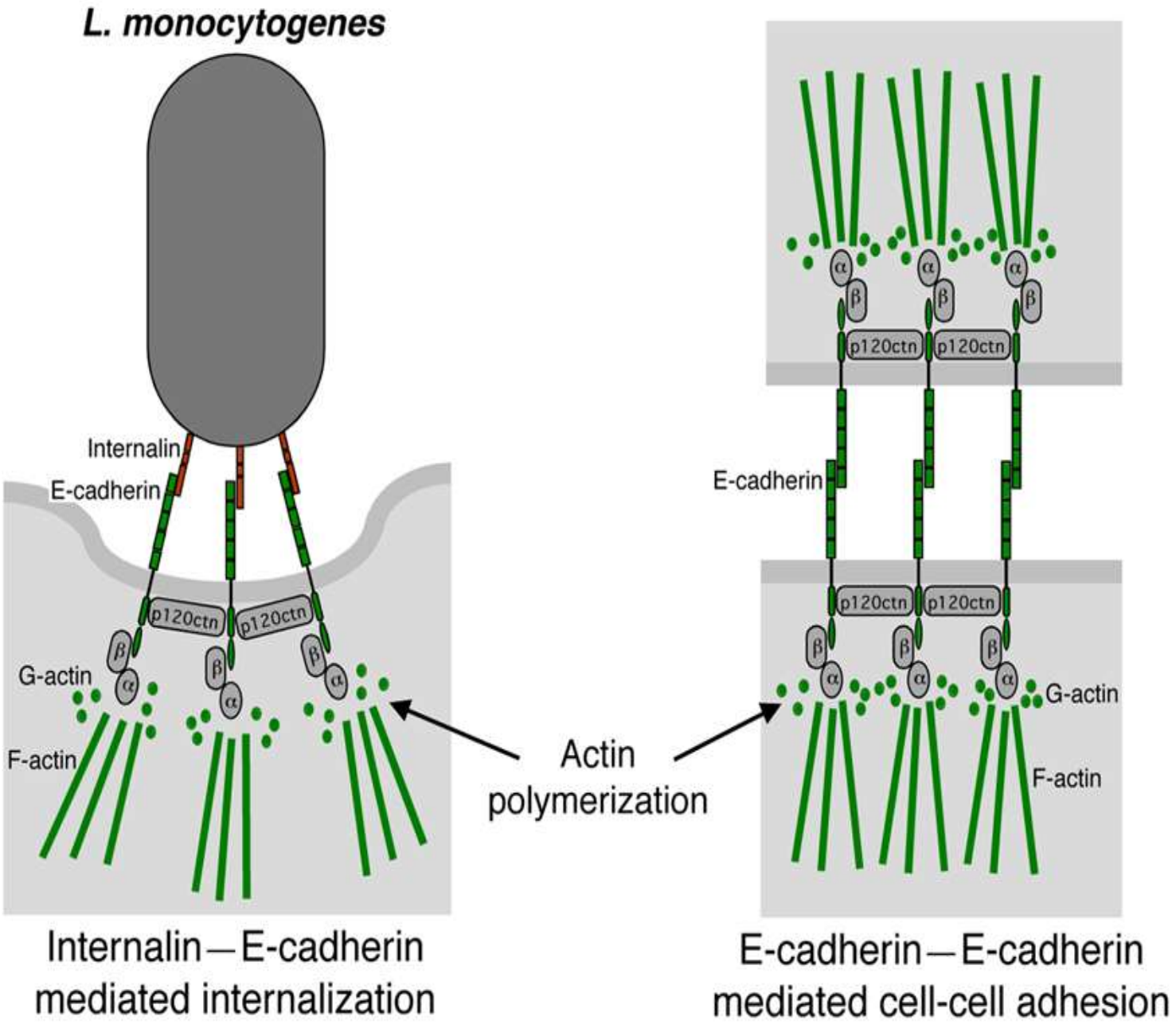
The receptor has been purified by affinity chromatography:

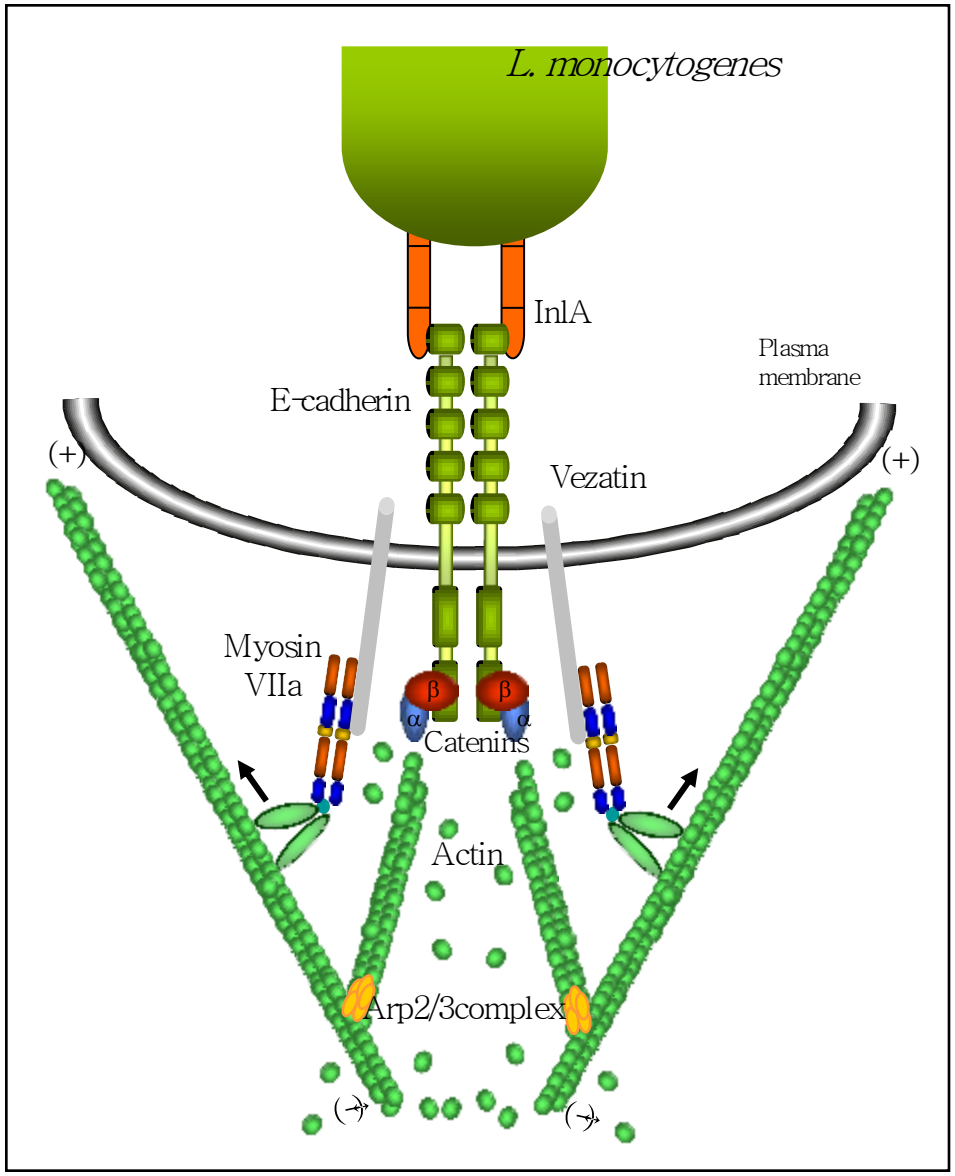
E-cadherin

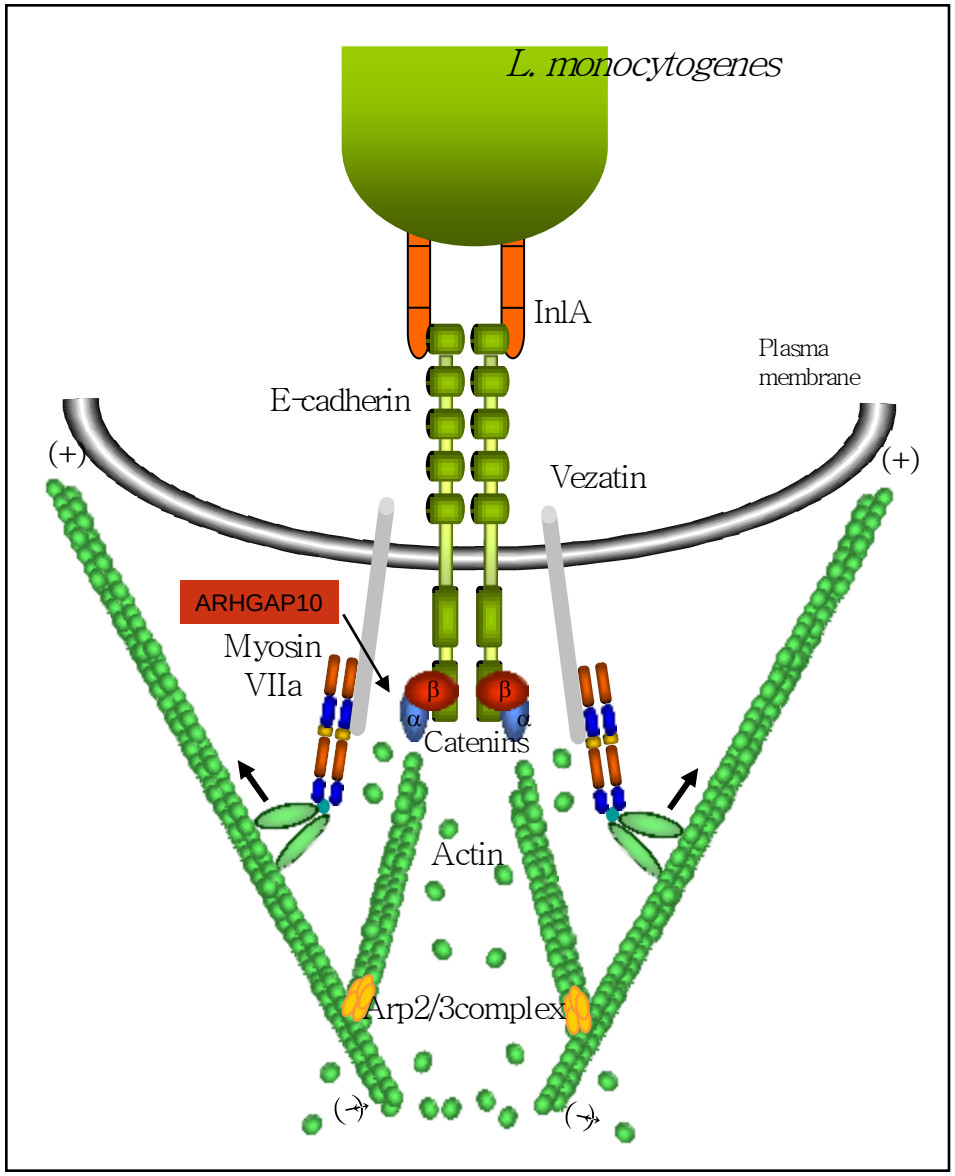
E-cadherin homophilic interactions





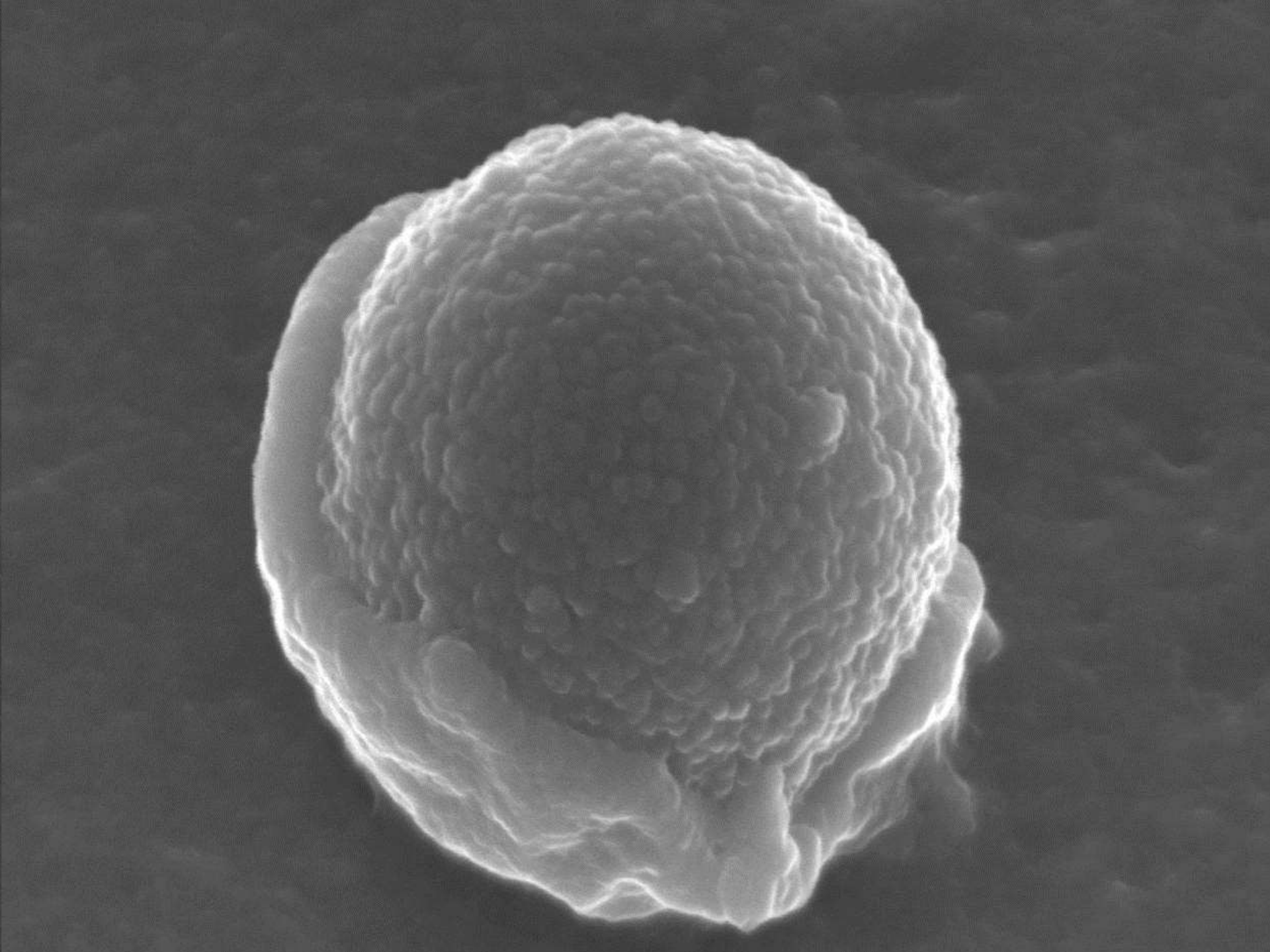

L. monocytogenes
Internalin
E-cadherin
p120ctn
p120ctn
G-actin
F-actin
Actin
polymerization
E-cadherin
p120ctn
p120ctn
G-actin
F-actin
Internalin—E-cadherin
mediated internalization
E-cadherin—E-cadherin
mediated cell-cell adhesion


L. monocytogenes
InlA
Plasma membrane
E-cadherin
(+)
(+)
Vezatin
Myosin VIIa
β
β
α
α
Catenins
Actin
Arp2/3complex
(-)
(-)


L. monocytogenes
InlA
Plasma membrane
E-cadherin
(+)
Vezatin
(+)
ARHGAP10
Myosin VIIa
β
β
α
α
Catenins
Actin
Arp2/3complex
(-)
(-)

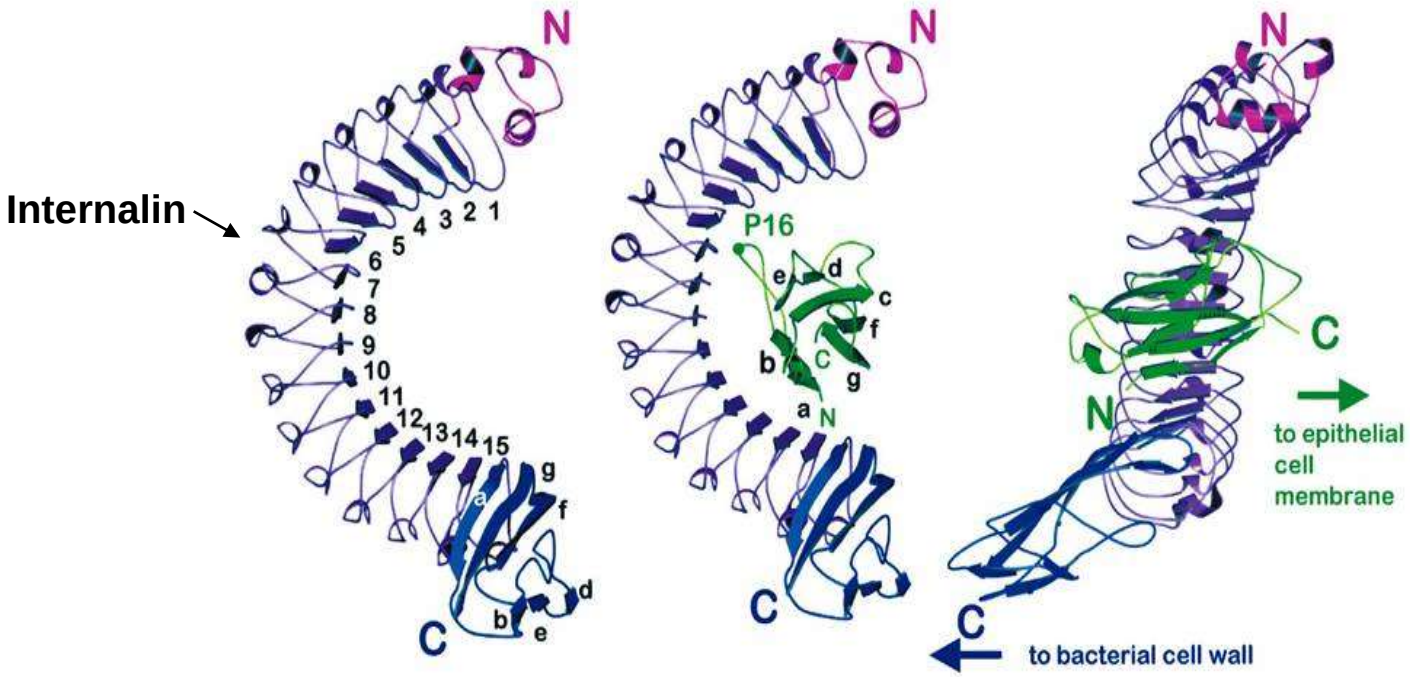
InlB, the second invasion protein

Two receptors



This species/host specificity is due to the nature of a single amino acid:

Pro16 in human E-cadherin *vs* Glu16 in murine E-cadherin



W.D. Schubert *et al*. 2002

Conclusions

-Internalin is critical for targeting and crossing the placental barrier in humans

- crossing is due to a *bona fide* ligand-receptor interaction

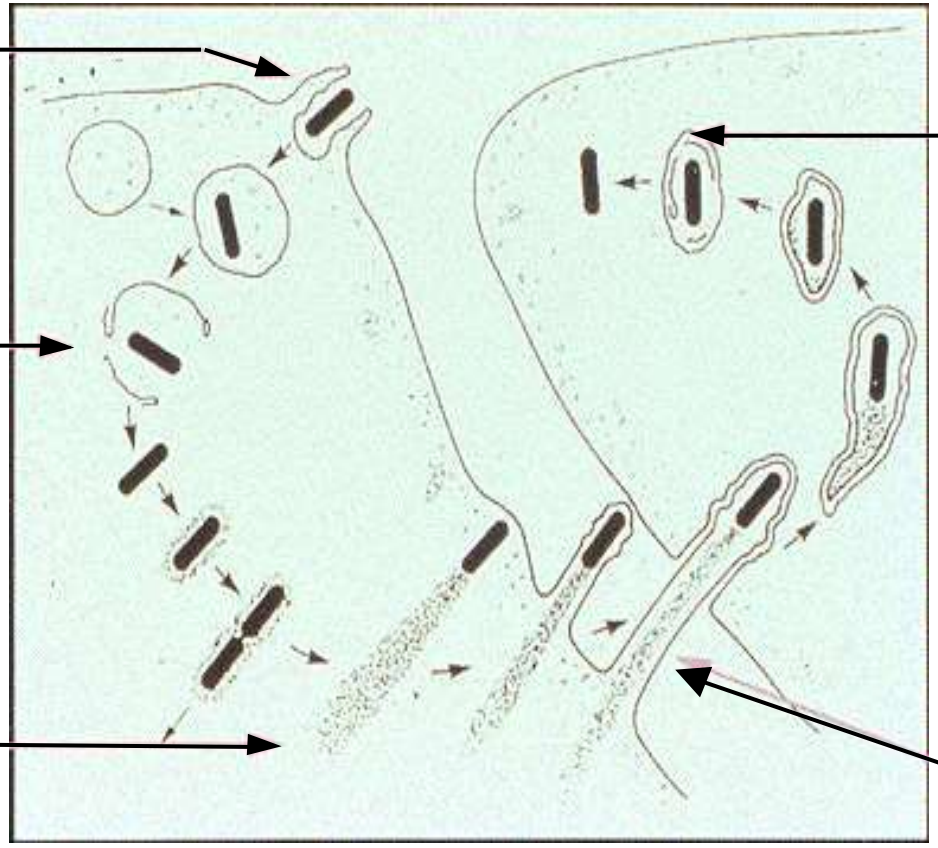
- Listeriosis is not solely due to “pregnancy-associated immunodeficiency”

-*Listeria* deploys the same strategy to cross the placental and intestinal barriers

- Does *Listeria* use internalin to cross the blood brain barrier ?

MVEC : endothelial cells with epithelial characteristics (tight junctions)
Choroid plexus : epithelial cells…

Different steps of the infection of mammalian cells



Entry
InlA, InlB

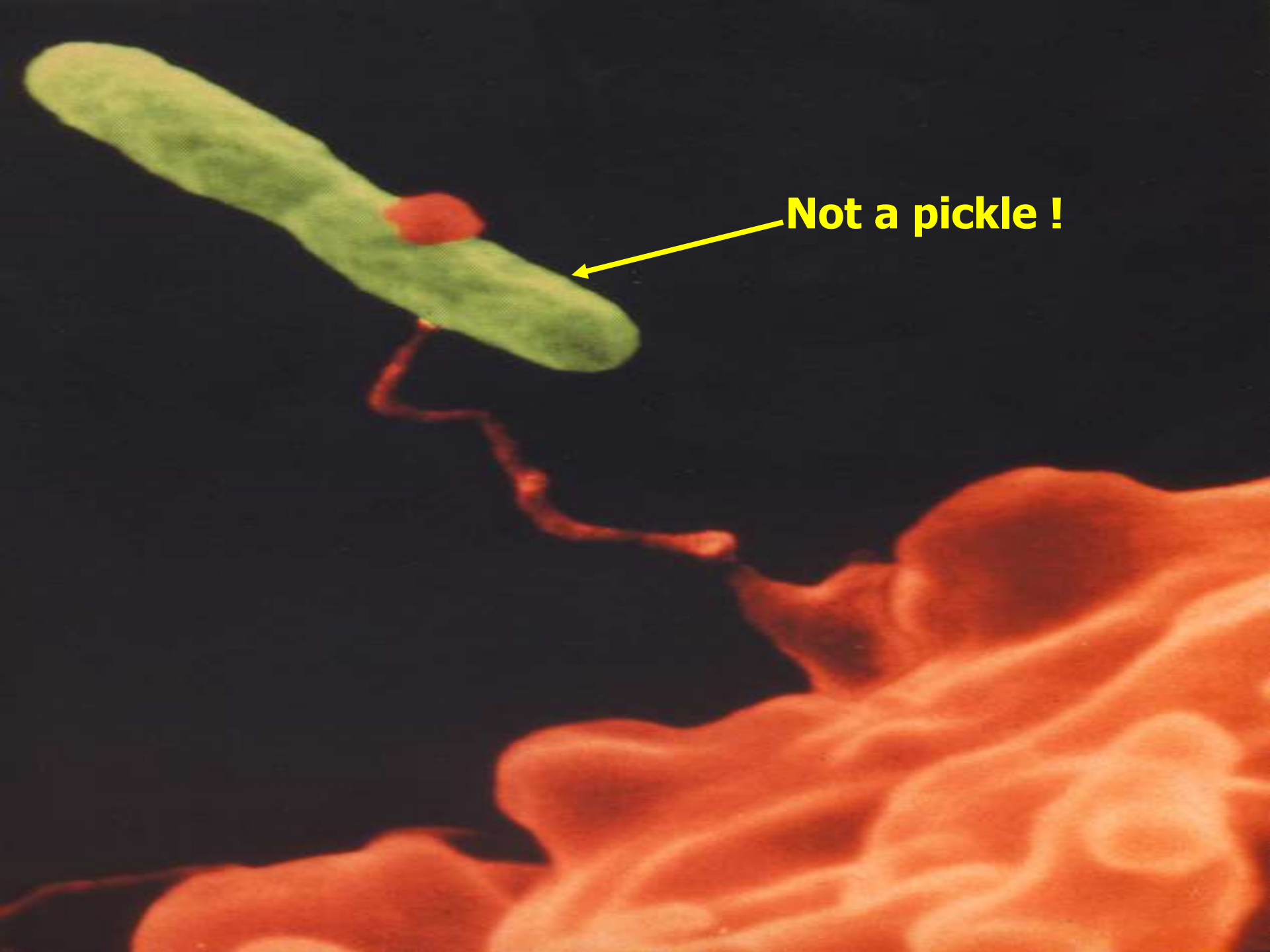
Lysis of the vacuole
LLO

Intracellular movement
ActA

Lysis of the double membrane
Lecithinase

Cell to cell spread
ActA

adapted from L.G. Tilney and D.A. Portnoy, J Cell Biol. (1989) 109,


Not a pickle !

Legionella pneumophila

* Accounts of ~15% of cases of pneumonia.

* The hallmark of the disease is intracellular replication in alveolar cells.

* High risk groups: Immunocompromised and Smokers.

* 15-50% fatality rate.

* Environmental organism/parasite of amoebae

History of *Legionella*

1976 **Outbreak of severe pneumonia among attendees of the 56th Legionnaires's convention in Philadelphia**

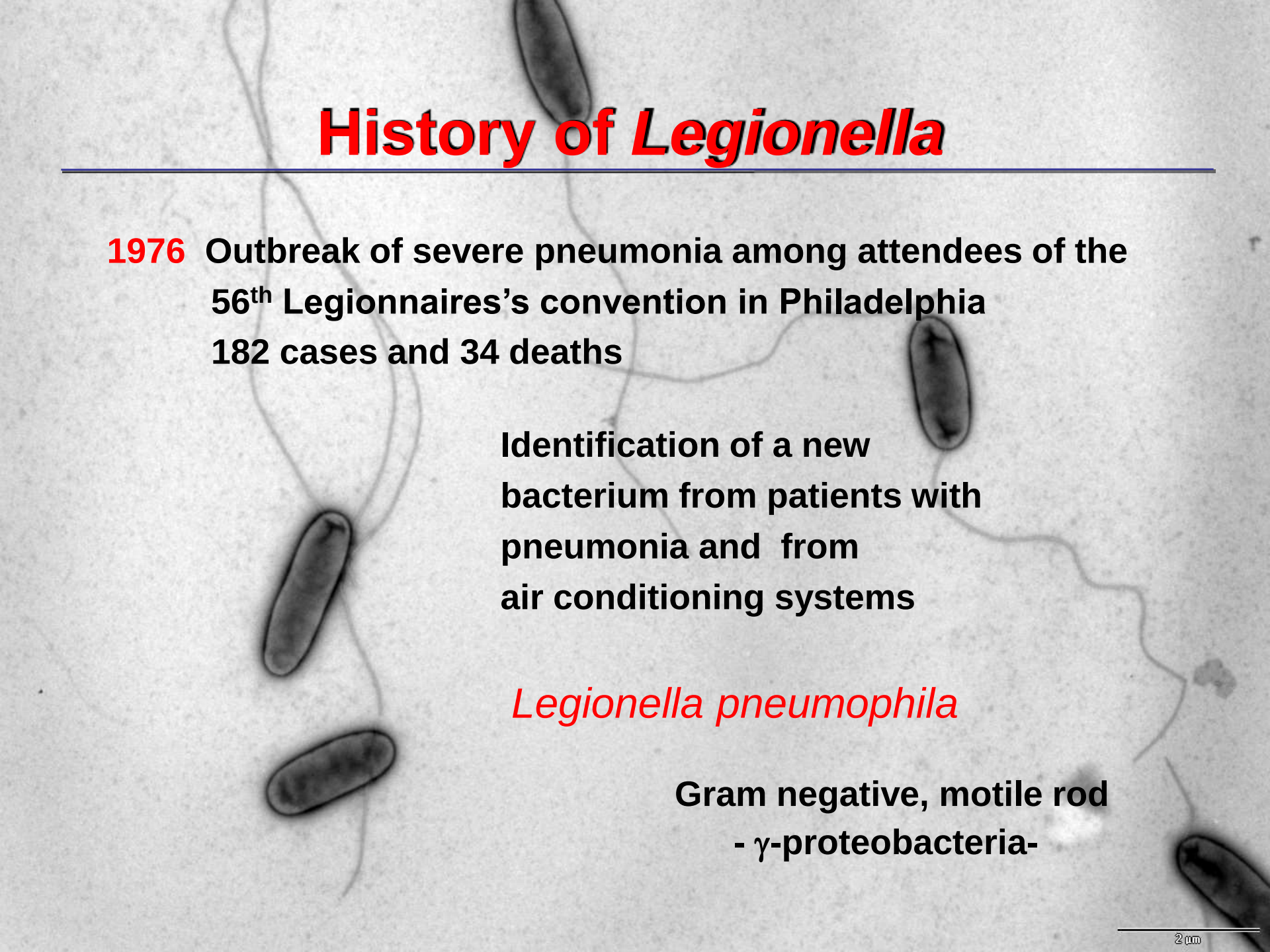
182 cases and 34 deaths

Identification of a new bacterium from patients with pneumonia and from air conditioning systems

Legionella pneumophila

Gram negative, motile rod

- γ-proteobacteria-



The genus *Legionella*

Legionella pneumophila

Legionella longbeachae

Legionella anisa

Legionella micdadei

Legionella hackeliae

Legionella dumoffii

Legionella gratiana ….. **42 species**

Legionella pneumophila - legionellosis

transmitted by inhalation of infected aerosols

Transmission:	air conditioning systems, cooling towers, showers, spa, decorative water curtains and foutains,thermal installations
Disease:	self-limited febrile respiratory illness in healthy young people (‘Pontiac fever’) severe pneumonia with systemic complications in older or immune compromised (‘Legionnaire’s disease’)
Population at risk:	smokers, elderly, immuno-comprimised persons, men
Mortality rate:	20-30%

Ecology of *Legionella*

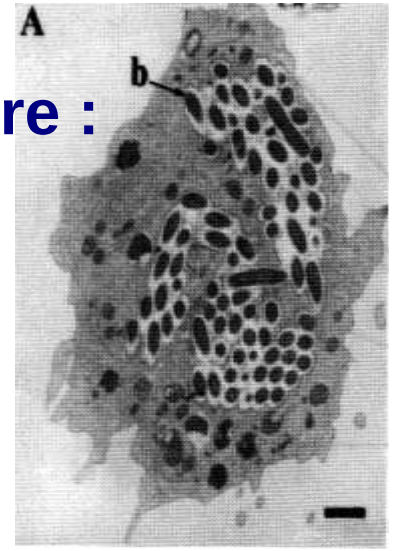
Reservoir principally aquatique:

Natural environnements: lakes, rivers

Artificial environnements: showers and taps, air conditioning systems, humidifiers, cooling tours, thermal installations, fountains …

Multiplication favoured at higher temperature :

warm water reservoirs up to 50°C



Intracellular bacterium:

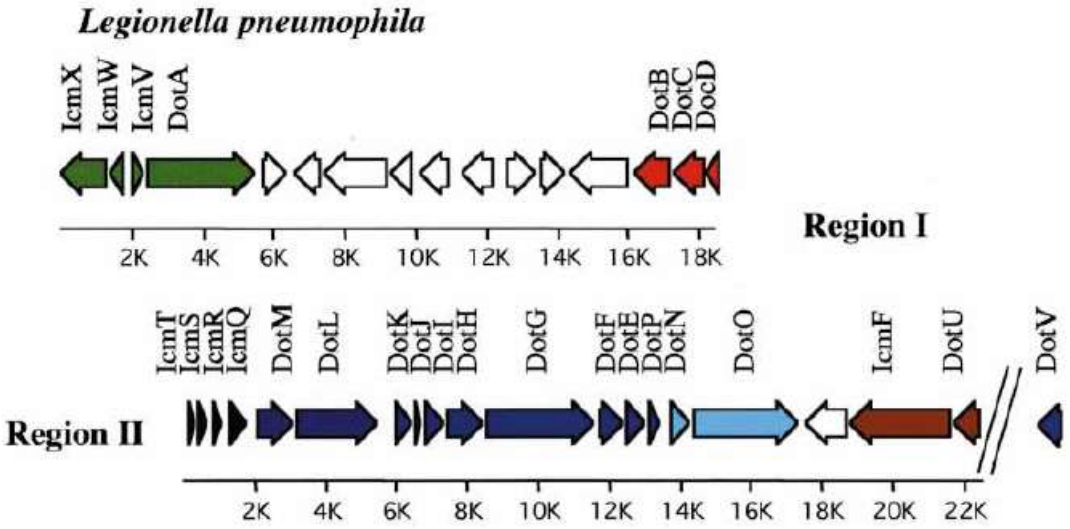
Ecologic niche : free-living amoebae (*Acanthamoeba castellani, Hartmanella vermiformis*, **+++)** and ciliated protozoa

Intracellular infectious cycle of *L. pneumophila*

Dot/Icm type IV secretion system

Dot: defective organelle trafficking
Icm: intracellular multiplication [defect]

Assembles pore through which proteins can pass

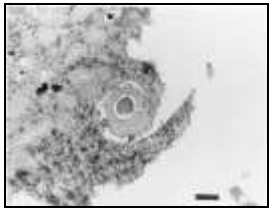


Modification of the phagosomal membrane

↓

Inhibition of endocytic maturation

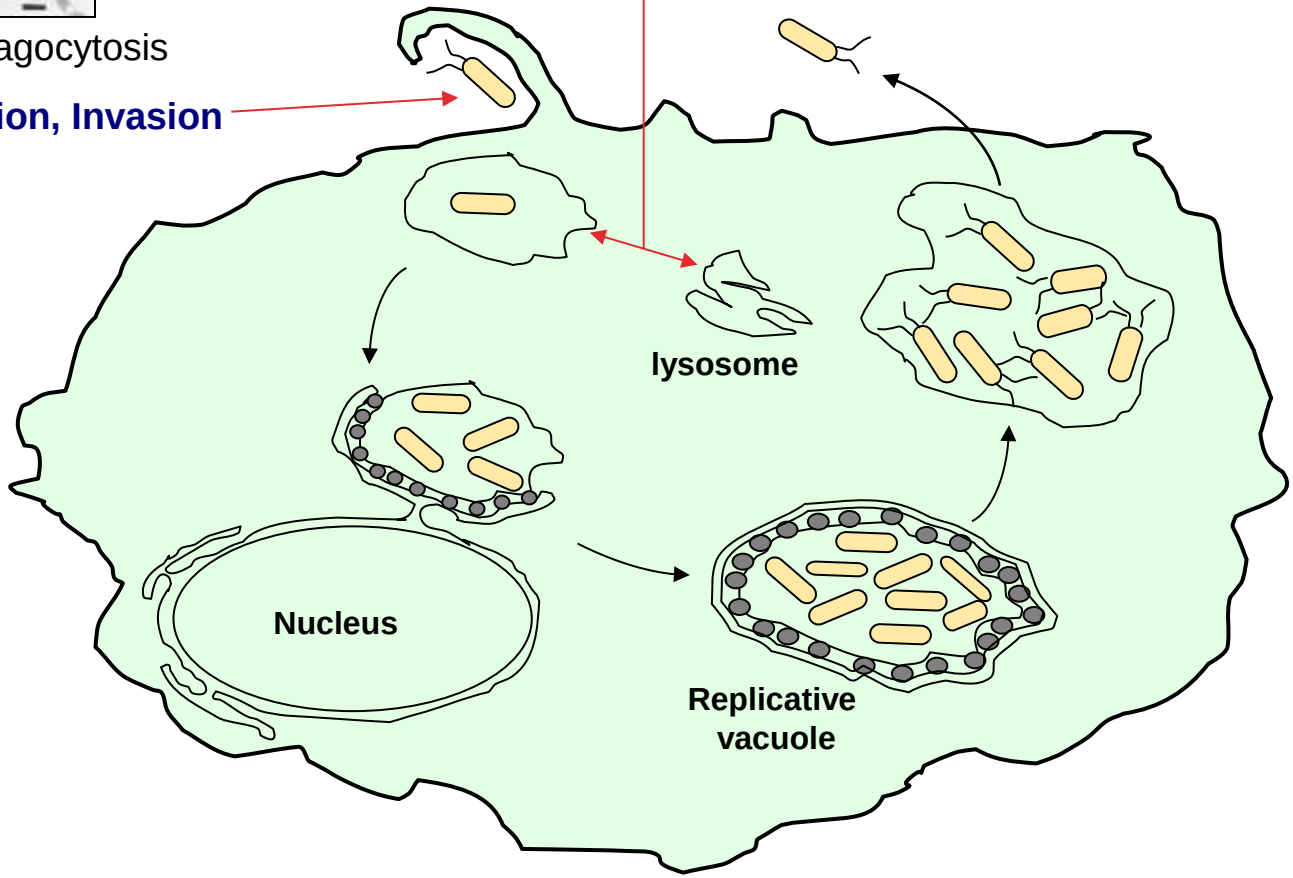
Intracellular life cycle of *L. pneumophila*



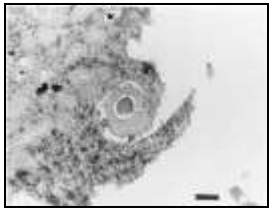
Coiling phagocytosis

Inhibition of phagosome lysosome fusion (type IV secretion system Dot/Icm)

Adhesion, Invasion



Intracellular life cycle of *L. pneumophila*



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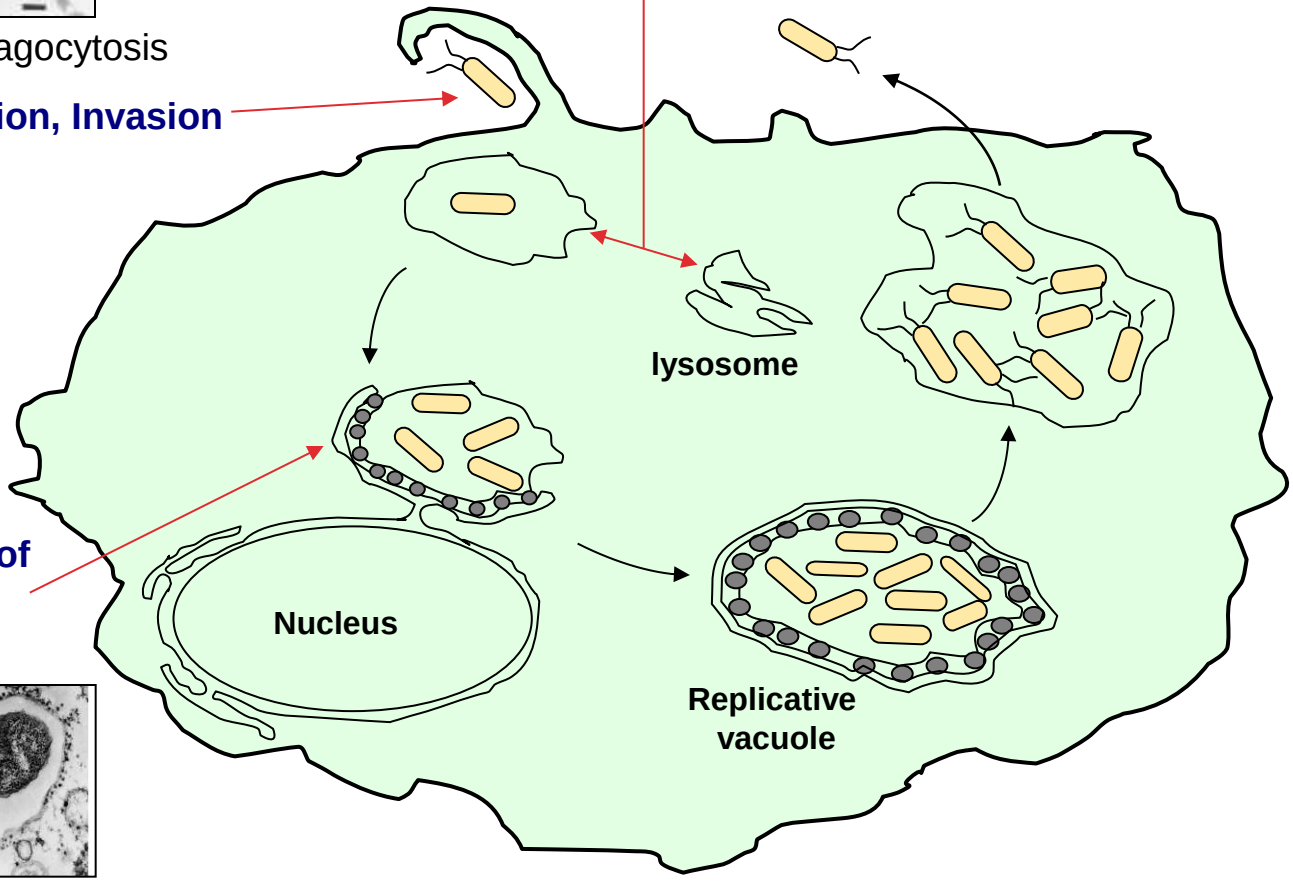
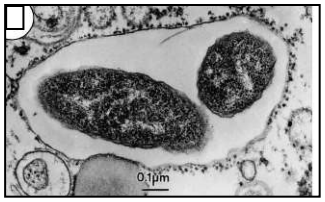


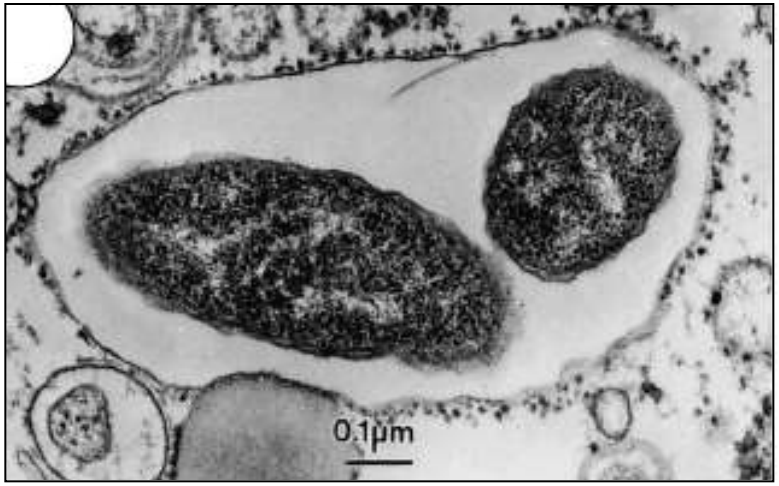
Recruitment of the ER (RalF)



Recruitment of ARF

Intracellular life cycle of *L. pneumophila*

Recruitment of the endoplasmic Reticulum (ER)

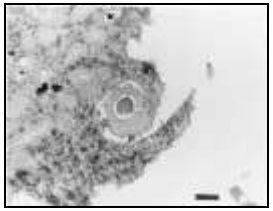


RalF : substrate of the Dot/Icm system allows the recruitment of ARF to the phagosome

ARF : Host protein involved in the transport of ER vesicles to the phagosome membrane
(eukaryotic ADP ribosylation factor)

Nagai *et al*, Science, 2002

Intracellular life cycle of *L. pneumophila*



Coiling phagocytosis

Inhibition of phagosome lysosome fusion (type IV secretion system Dot/Icm)

Adhesion, Invasion

Stringent response –virulence factors Transmissive phase

lysosome

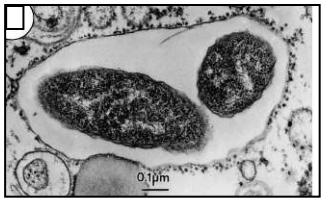
Autophagy Fusion with lysosome

Recruitment of the ER (RalF)

Nucleus

Replicative vacuole

Acid tolerant ↓virulence factors Replicative phase



Recruitment of ARF

Intracellular infectious cycle of *L. pneumophila*

Stringent response

Amino acid depletion

↓

RelA activation

↓

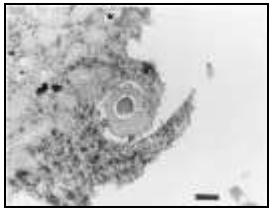
ppGpp accumulation

↓

Regulation cascade

	Abundant nutrients **Replicative phase**	**Limiting nutrients** **Transmission phase**
Virulence factors	↓	↑
Motility	-	+
Stress resistance	-	+
Resistance to biocide, antibiotics	-	+
Cytotoxixity	-	+
Evade lysosome	-	+

Intracellular life cycle of *L. pneumophila*



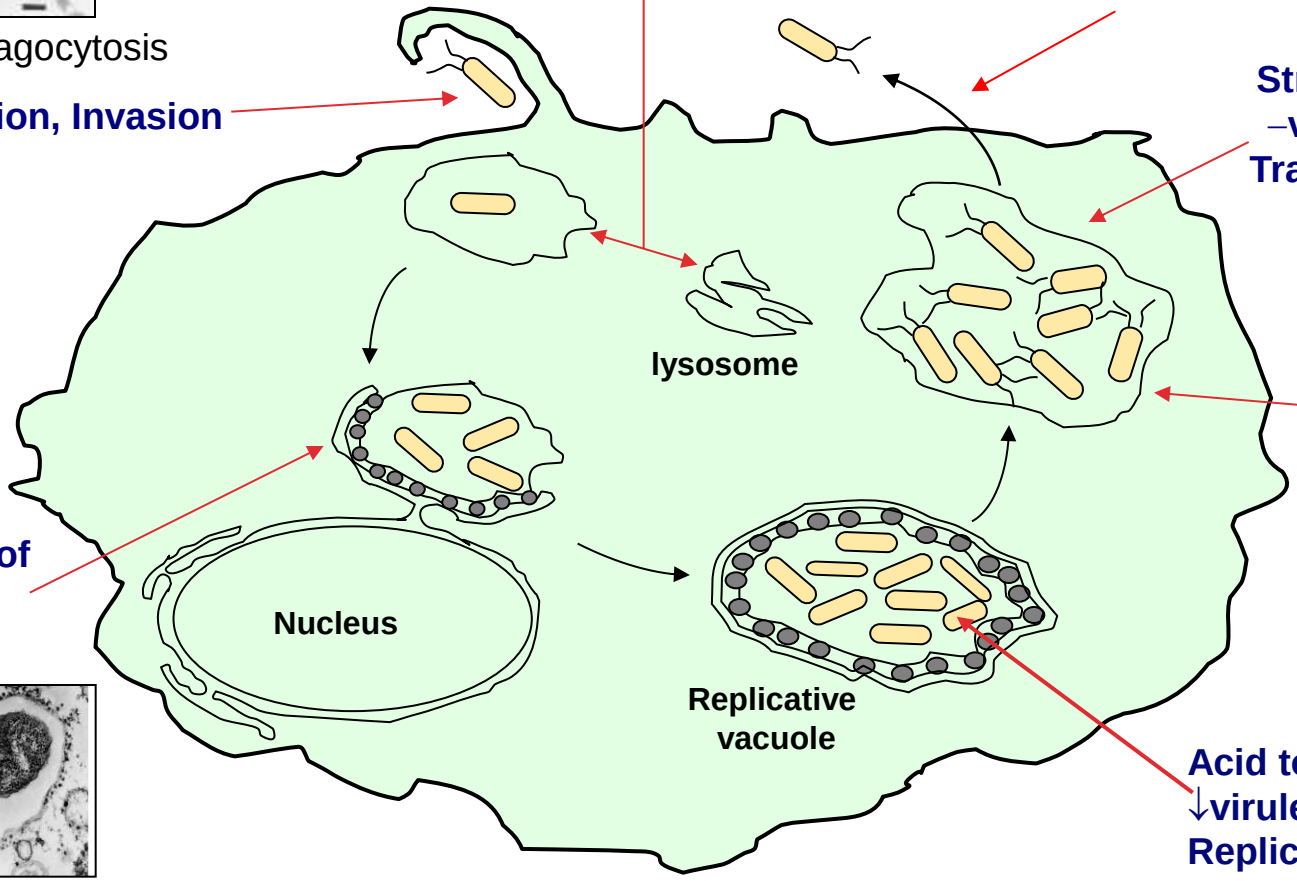
Coiling phagocytosis

Inhibition of phagosome lysosome fusion (type IV secretion system Dot/Icm)

Evasion (cytotoxin)

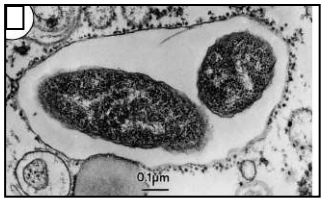
Adhesion, Invasion

Stringent response –virulence factors Transmissive phase



Autophagy Fusion with lysosome

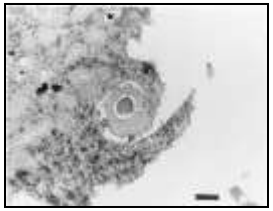
Recruitment of the ER (RalF)



Recruitment of ARF

Acid tolerant ↓virulence factors Replicative phase

Intracellular life cycle of *L. pneumophila*



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Evasion (cytotoxin)

Adhesion, Invasion

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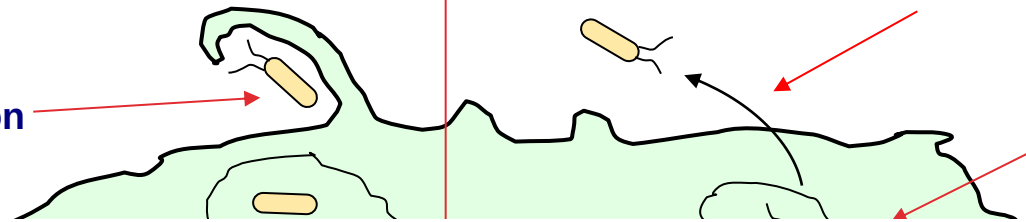


How does *Legionella pneumophila* subvert host functions, enter, survive, replicate and evade amoebae or alveolar macrophages?

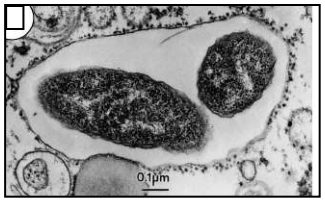
Autophagy Fusion with lysosome

Recruitment of the ER (RalF)

Nucleus

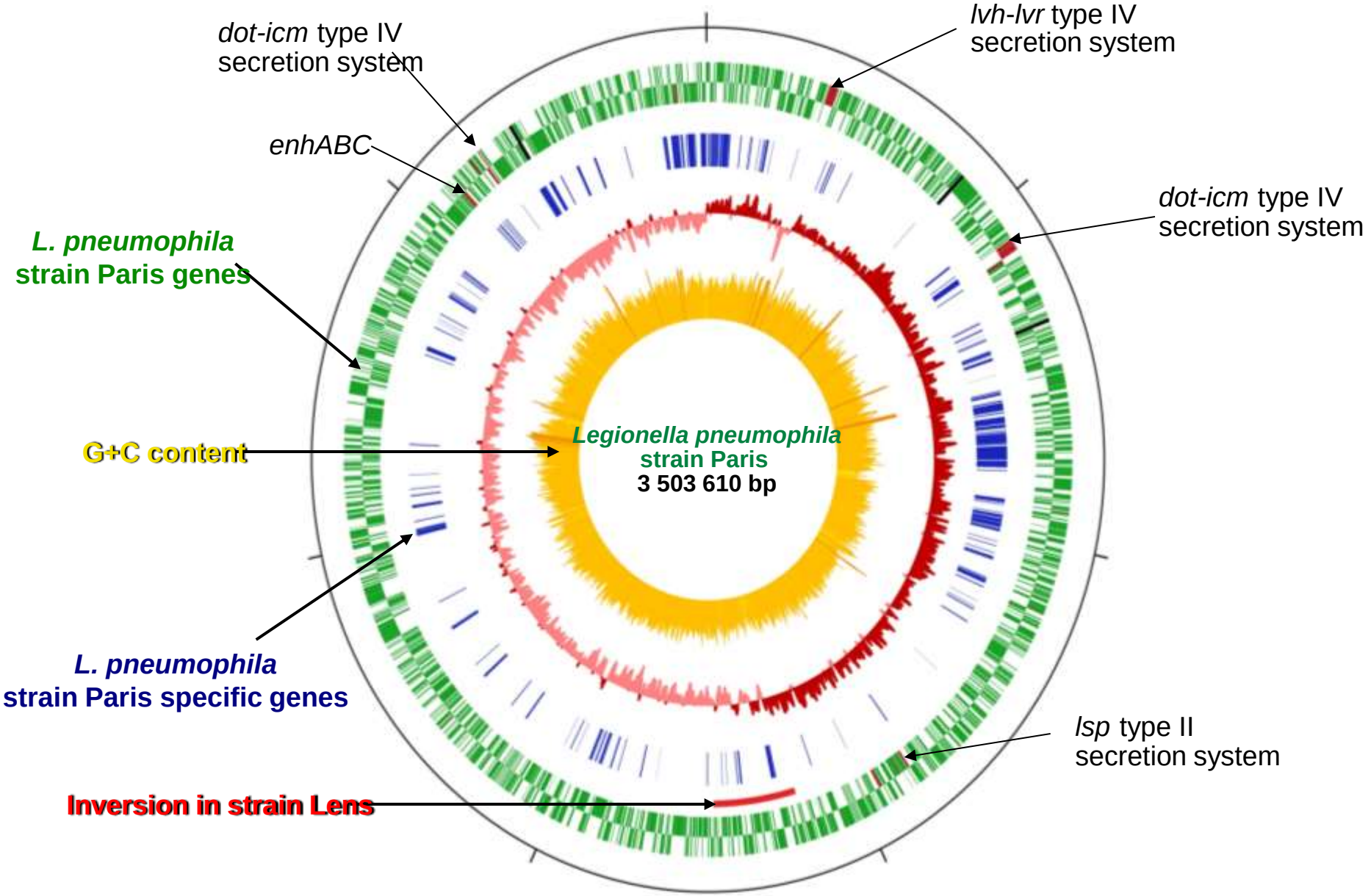
Replicative vacuole

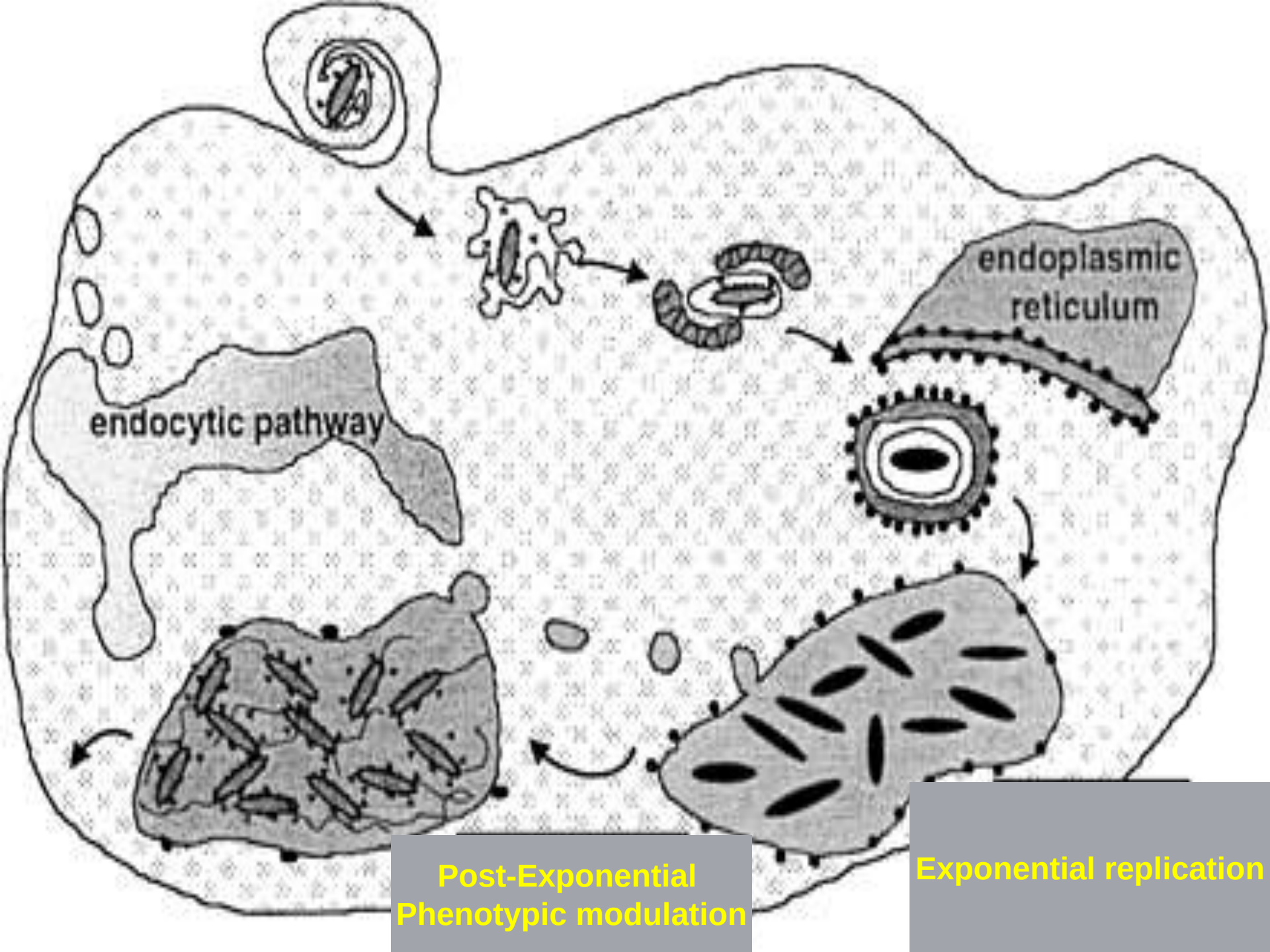
Acid tolerant ↓virulence factors Replicative phase

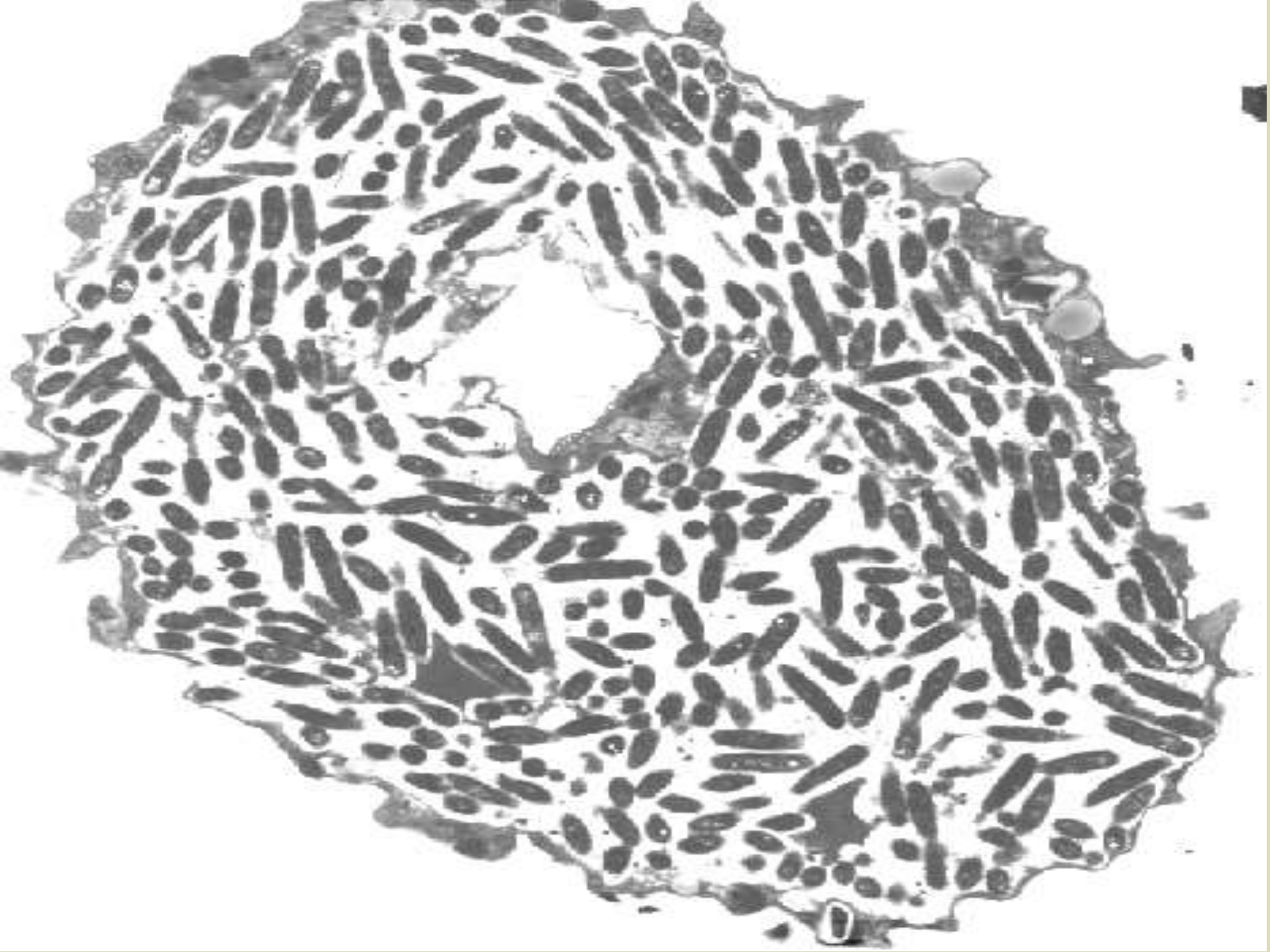


Recruitment of ARF

L. pneumophila genome map




endoplasmic
reticulum
endocytic pathway
Post-Exponential
Phenotypic modulation
Exponential replication



Type IV Secretion System