L-Form Bacteria and their role in Antimicrobial Resistance

Sylvia Tong

PI: Prof. Paul KS Chan and Prof. Zigui Chen

16th Dec 2021

CUHK, Department of Microbiology

Outline

Introduction

- L-form bacteria
- Urinary tract infections

Potential role of L-form switching in AMR

- Isolation of L-forms in rUTI patients
- Visualization of L-form bacteria
- L-form switching is reliant on antibiotic presence

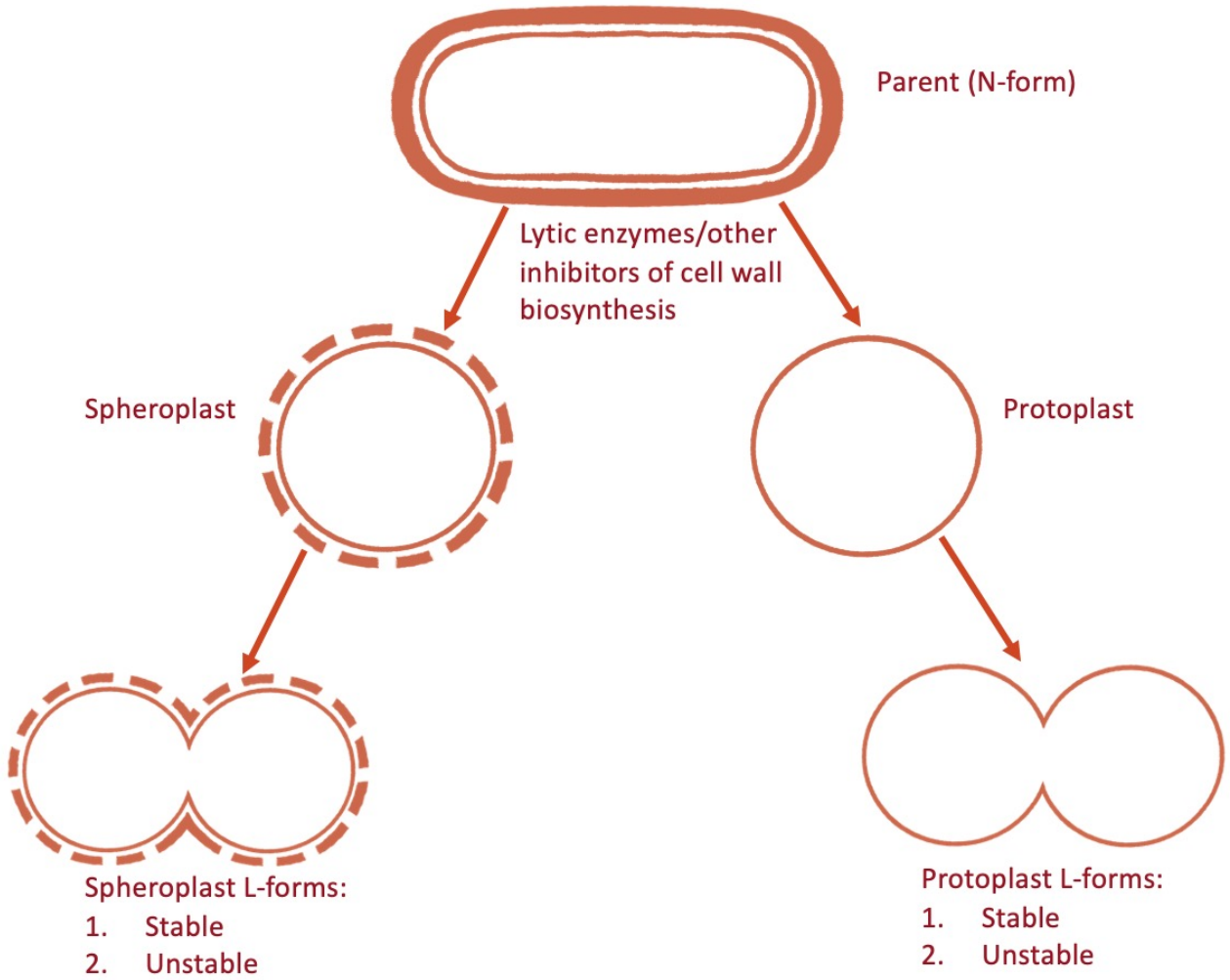
Discussion

- Other bacterial pathogens with L-form mechanisms
- Reassessing routine antibiotics used for UTIs

Conclusions and future directions

L-Form Bacteria

- L-form/L-phase/**cell wall-deficient**
- Discovered in 1935 by Emmy Klieneberger-Nobel at the Lister Institute



L-Forms as a novel Antimicrobial Resistance mechanism

- Cell-wall components recognized by immune system
- Target for immune effectors
- Target for antibiotics

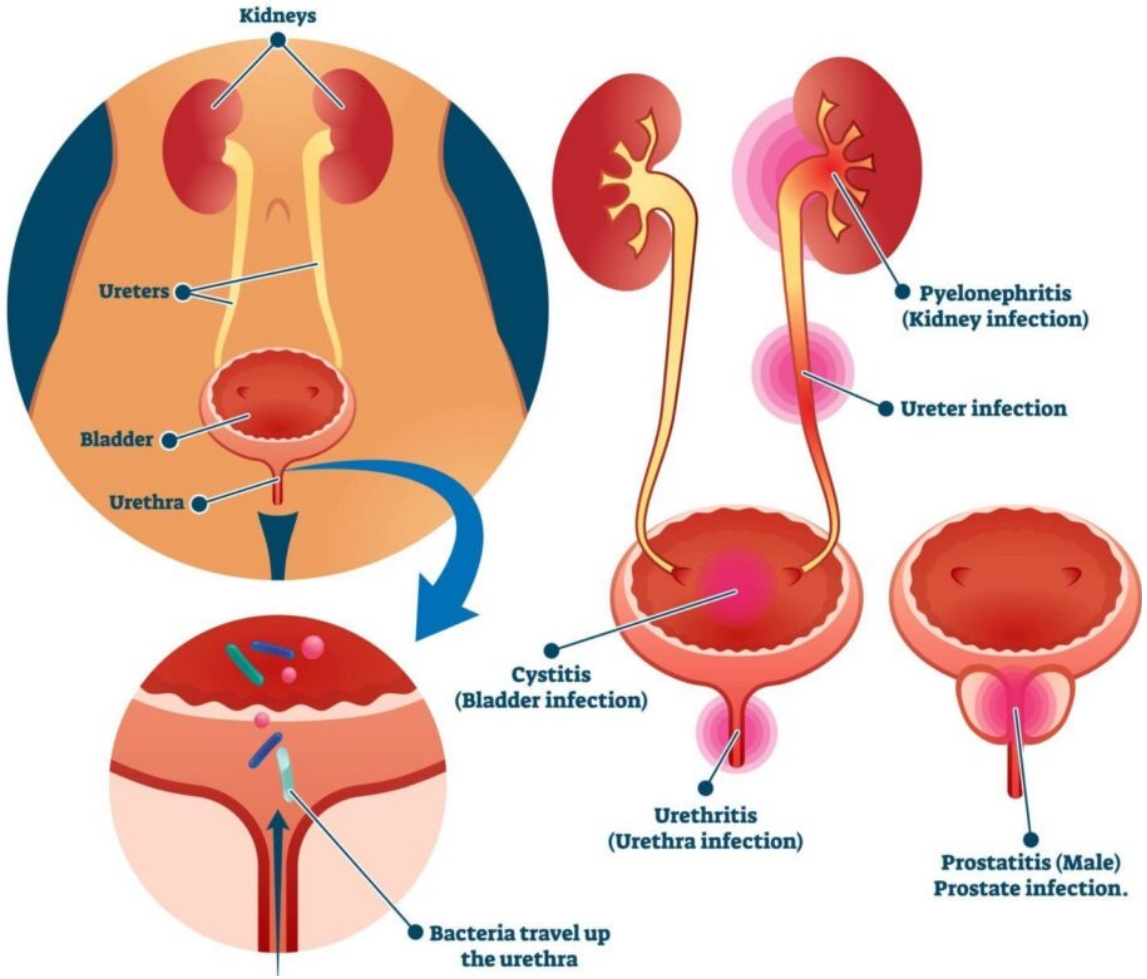
Article | Open Access | Published: 26 September 2019

Possible role of L-form switching in recurrent urinary tract infection

Katarzyna M. Mickiewicz ✉, Yoshikazu Kawai, Lauren Drage, Margarida C. Gomes, Frances Davison, Robert Pickard, Judith Hall, Serge Mostowy, Phillip D. Aldridge & Jeff Errington ✉

Nature Communications **10**, Article number: 4379 (2019) | Cite this article

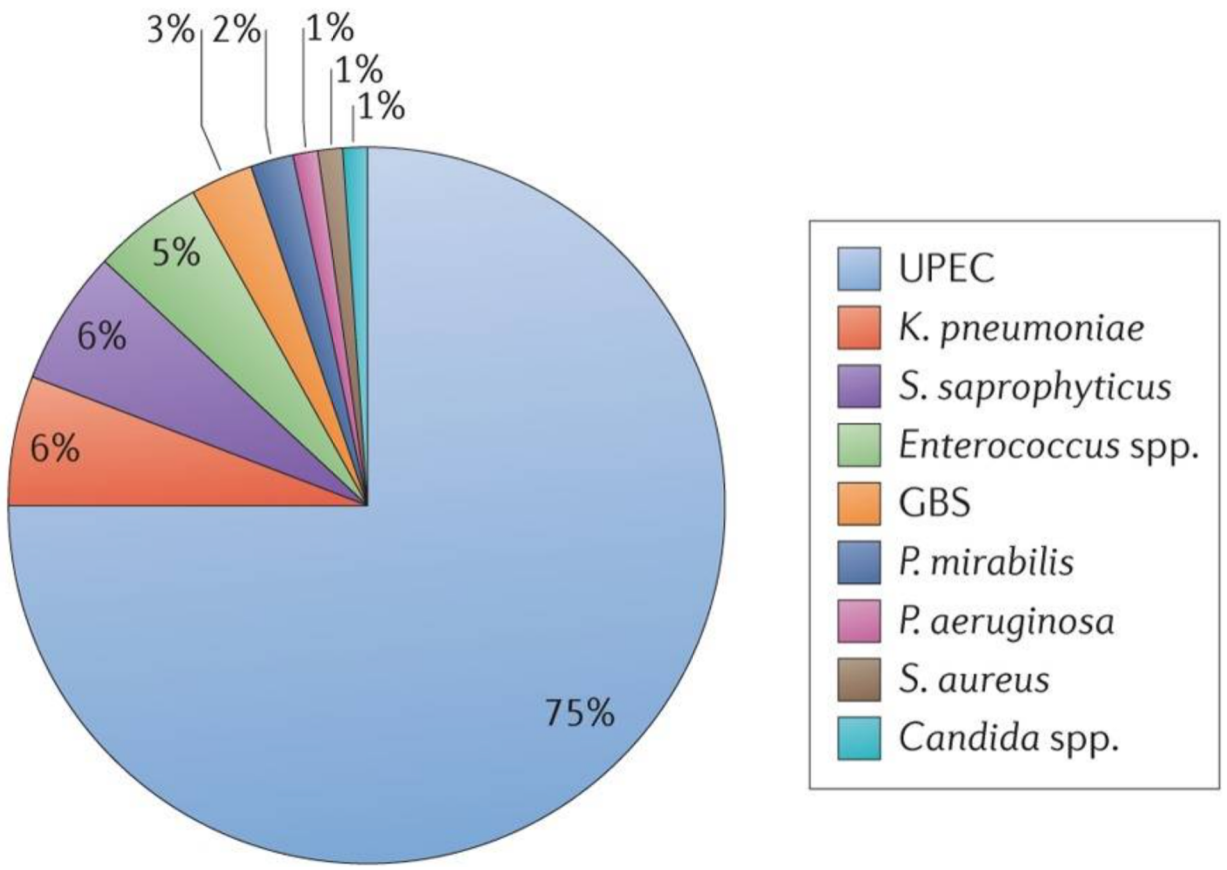
Urinary tract infection (UTI)



- Infection in any part of urinary system
- Most common outpatient infection
- 11% prevalence
  - 20% in >65 women
- 40-60% women will have a UTI in their lifetimes
  - 25% have **rUTI**
- Annual cost of **$1.6 billion** in US

(Sihra et al., 2018, *Nature Reviews Urology*)

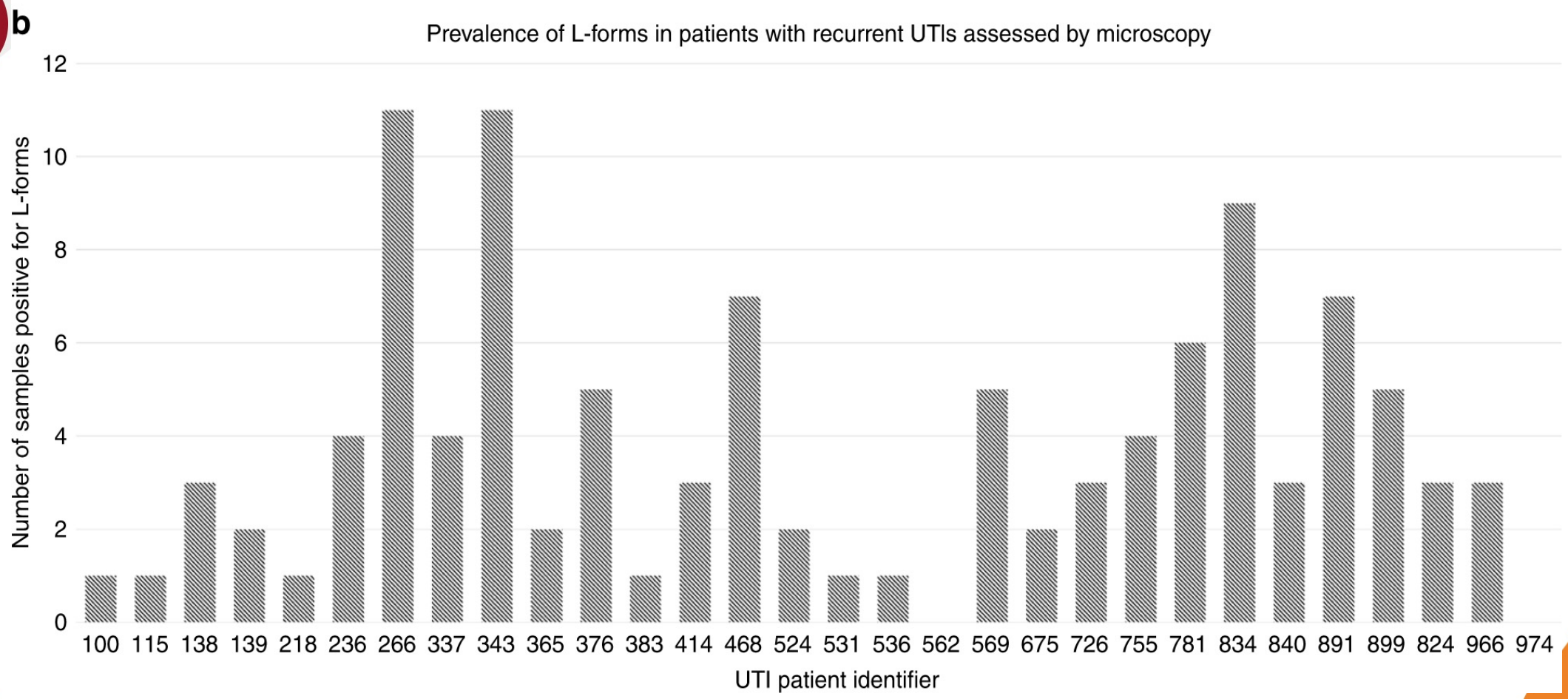
Uropathogenic *Escherichia coli* (UPEC)



- Agents causing UTIs are commensal organisms
- 50-80% UTIs cause by *E. coli*

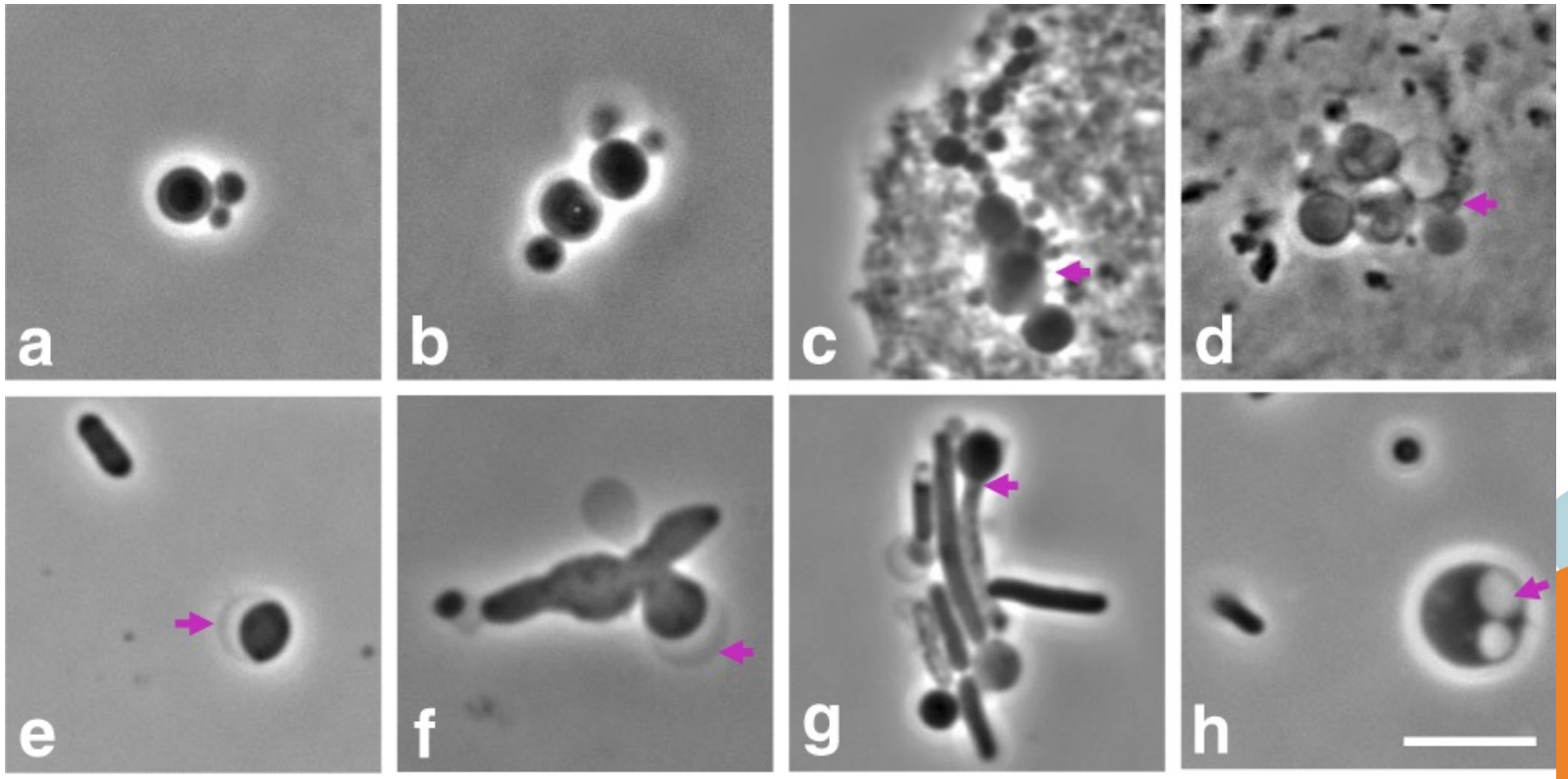
(Flores-Mireles et al., 2015, *Nat Rev Microbiol.*)

b


Prevalence of L-forms in patients with recurrent UTIs assessed by microscopy
Number of samples positive for L-forms
0
2
4
6
8
10
12
100 115 138 139 218 236 266 337 343 365 376 383 414 468 524 531 536 562 569 675 726 755 781 834 840 891 899 824 966 974
UTI patient identifier

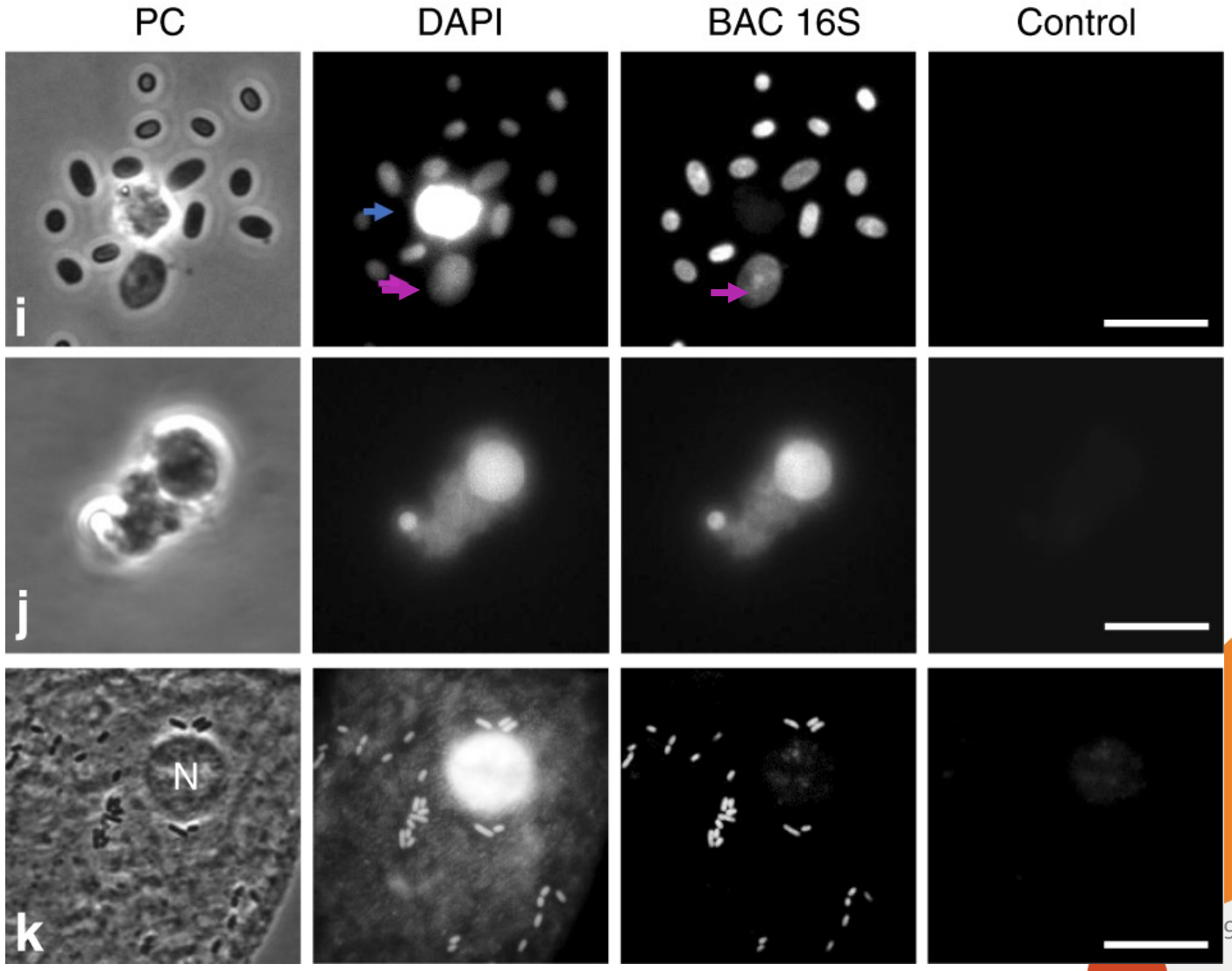
L-Forms found in rUTI urine samples via phase contrast microscopy

- Fresh urine samples phase contrast microscopy



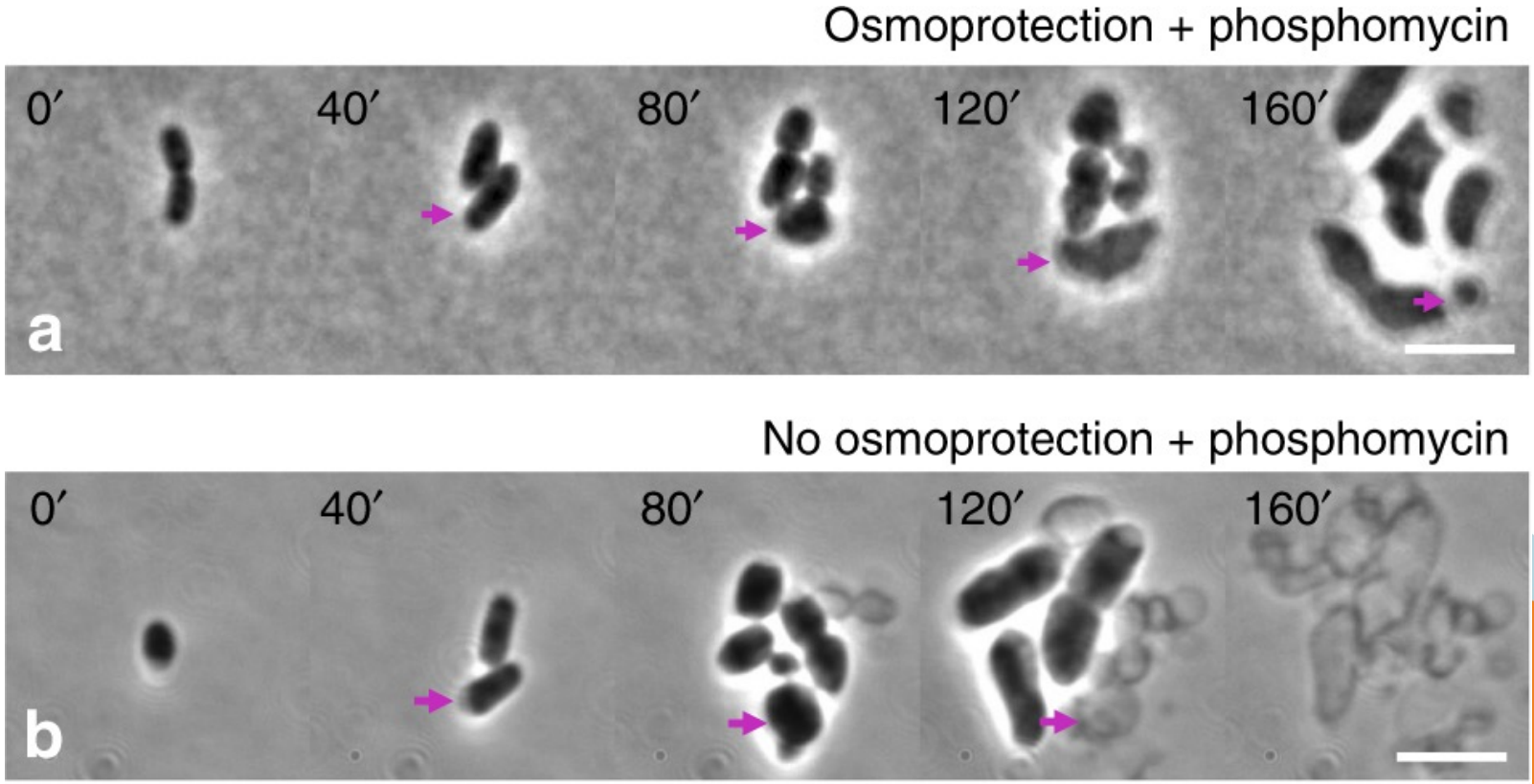
L-Forms found in rUTI patients via fluorescence *in situ* hybridisation (FISH)

- fixed and stained **DAPI** and fluorescent DNA probe against bacterial 16S rRNA (**BAC 16S**)



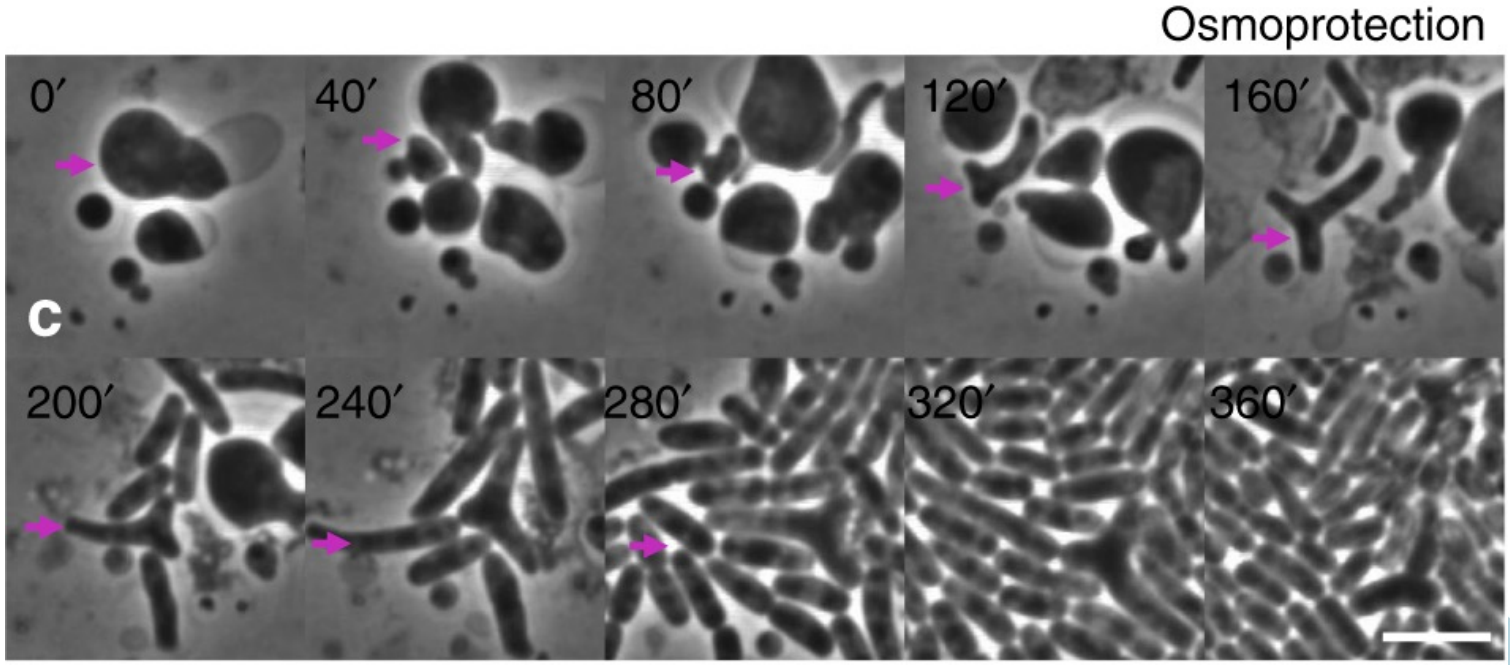
E. coli switches between L-form and walled states

- *E. coli t*ime-lapse microscopy in presence of **phosphomycin**,
  - with and without osmoprotection

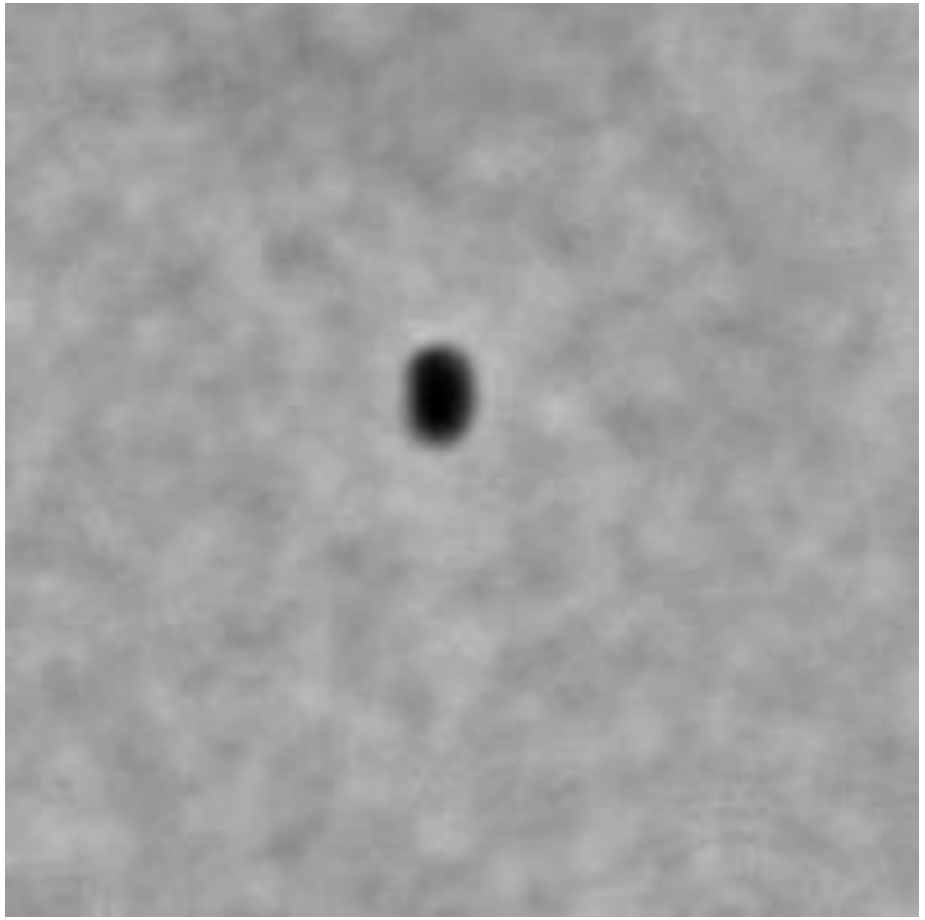


L-form *E. coli* reverts to walled states following phosphomycin removal

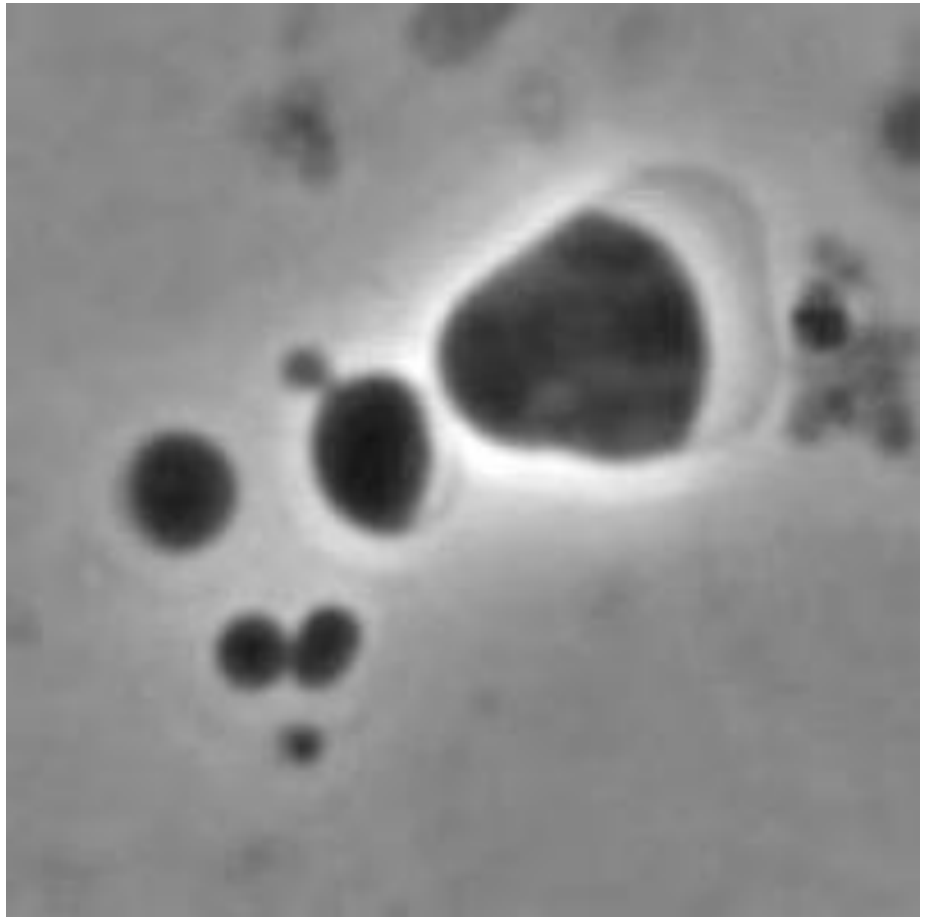
- 5 h time lapse
  - 40 min increments
- L-form *E. coli* time-lapse microscopy in removal of phosphomycin



L-form switching time-lapse



*E. coli t*ime-lapse microscopy in presence of phosphomycin.



*E. coli t*ime-lapse microscopy in absence of phosphomycin

Summary: L-form transition allows bacteria to evade antibiotic challenge in rUTIs

- L-form switching is a clinically relevant phenomenon that may contribute to the recurrence of infection in rUTI patients

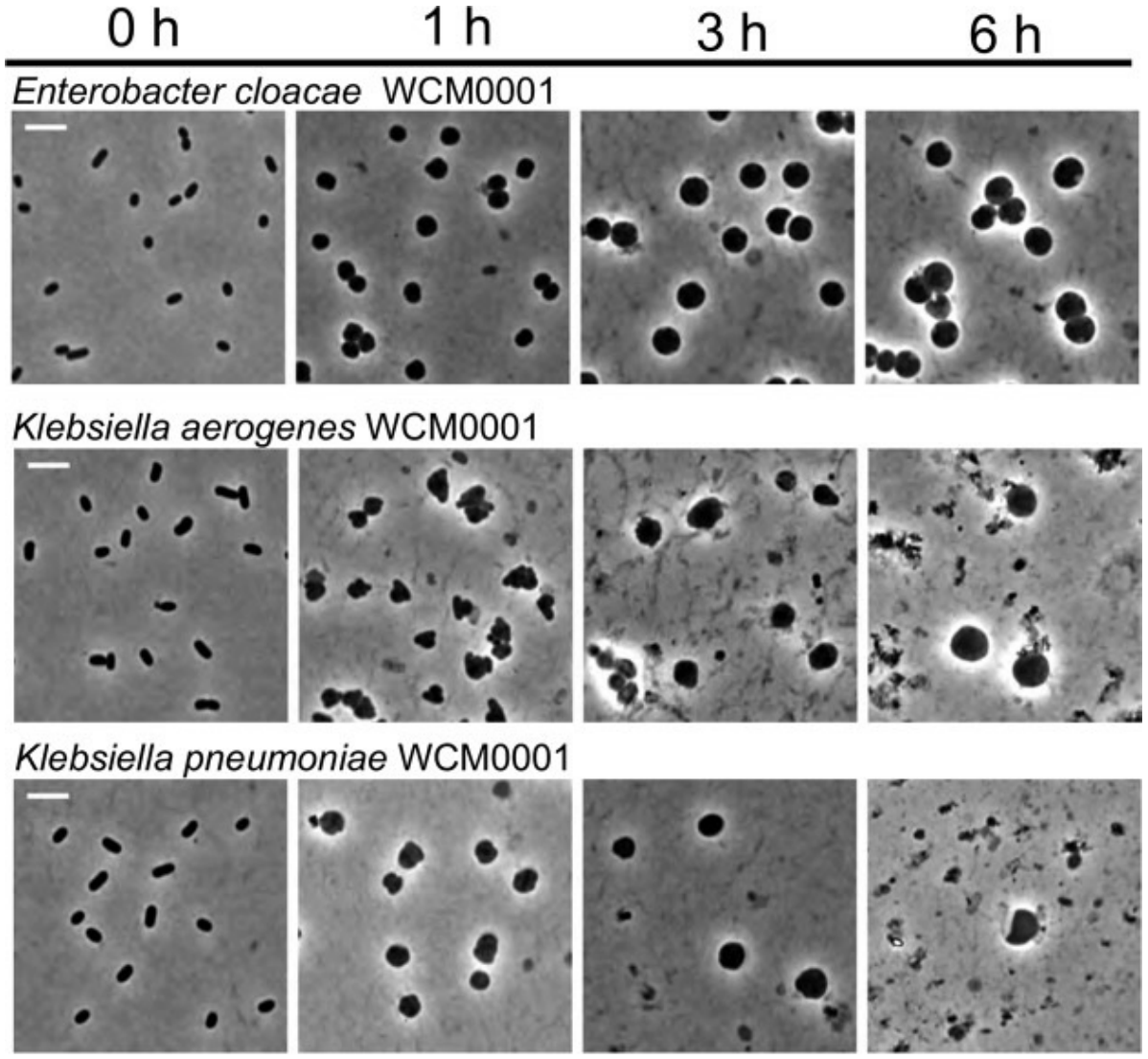
Can other bacteria become L-forms?

Infectious agent	Example on ability to switch to L-form
Klebsiella pneumoniae	Cross et al., **Spheroplast-Mediated Carbapenem Tolerance in Gram-Negative Pathogens**. Antimicrob Agents Chemother. 2019 Aug 23;63(9):e00756-19. doi: 10.1128/AAC.00756-19. PMID: 31285232; PMCID: PMC6709500.
Proteus mirabilis	Tölg et al., **Dependence of induction of enterobacterial AmpC beta-lactamase on cell-wall peptidoglycan, as demonstrated in Proteus mirabilis and its wall-less protoplast L-form**. J Gen Microbiol. 1993 Nov;139(11):2715-22. doi: 10.1099/00221287-139-11-2715. PMID: 8277255.
Pseudomonas aeruginosa	White et al., **Cell wall characteristics of Pseudomonas aeruginosa and its carbenicillin-induced L-form.** Acta Biol Acad Sci Hung. 1978;29(1):67-74. PMID: 112813.
Enterococcus spp.	Kawai et al., **Crucial role for central carbon metabolism in the bacterial L-form switch and killing by β-lactam antibiotics.** Nat Microbiol. 2019 Oct;4(10):1716-1726. doi: 10.1038/s41564-019-0497-3. Epub 2019 Jul 8. PMID: 31285586; PMCID: PMC6755032.

Can other bacteria become L-forms?

- Cross et al., **Spheroplast-Mediated Carbapenem Tolerance in Gram-Negative Pathogens**.

- Overnight cultures of isolates were subcultured into prewarmed BHI+ liquid medium containing 10 µg/ml meropenem



(Cross et al., 2019, *Antimicrob Agents Chemother*.)

Antibiotics used for UTIs

	Antibiotic treatment	Target
First Line	Nitrofurantoin	Bacterial ribosomal proteins
First Line	Amoxicillin-clavulanic acid	Cell wall synthesis
First Line	Phosphomycin	Cell wall synthesis
Second Line	Cefuroxime	Cell wall synthesis
Second Line	Levofloxacin	DNA gyrase
Second Line	Ciprofloxacin	DNA gyrase
Second Line	Sulfamethoxazole -trimethoprim	Folic acid pathway

- resistance of UPEC to antimicrobial agent ranges from 14.6% to 60%
- Antibiotic sensitive bacteria could L-form switch and persist
  - Unable to be found by current routine testing
- Understanding the mechanism of L-form switching can inform clinician choice of treatment

(chp.gov.hk, ”Guidance notes for uncomplicated cystitis”)

Conclusions and future directions

L-forms provide a source of bacterial survivors during cell wall specific antibiotics

- Continue proliferating during treatment
- Revert to walled state

L-form switching may be an underappreciated mechanism of antibiotic tolerance in chronic infections

Further understanding of L-forms may inform clinicians on better choices of treatment

Further understanding of mechanisms of L-form switching may allow development of a rapid test

Thank you for listening.
Any questions?

Supplementary slides

References

1. Allan, E.j., J. Jass, L.e. Phillips, J.w. Costerton, and H.m. Lappin-Scott. “A Novel Method for Differentiating L-Form Bacteria from Their Parental Form Using the Hucker Gram Staining Technique.” *Letters in Applied Microbiology* 15, no. 5 (1992): 193–96. https://doi.org/10.1111/j.1472-765X.1992.tb00761.x.
2. “Bacterial L-Forms - PubMed.” Accessed December 10, 2021. https://pubmed.ncbi.nlm.nih.gov/19426852/.
3. Cross, Trevor, Brett Ransegnola, Jung-Ho Shin, Anna Weaver, Kathy Fauntleroy, Michael S. VanNieuwenhze, Lars F. Westblade, and Tobias Dörr. “Spheroplast-Mediated Carbapenem Tolerance in Gram-Negative Pathogens.” *Antimicrobial Agents and Chemotherapy* 63, no. 9 (September 2019): e00756-19. https://doi.org/10.1128/AAC.00756-19.
4. Errington, Jeff, Katarzyna Mickiewicz, Yoshikazu Kawai, and Ling Juan Wu. “L-Form Bacteria, Chronic Diseases and the Origins of Life.” *Philosophical Transactions of the Royal Society B: Biological Sciences* 371, no. 1707 (November 5, 2016): 20150494. https://doi.org/10.1098/rstb.2015.0494.
5. Fabijan, Aleksandra Petrovic, Muhammad Kamruzzaman, David Martinez-Martin, Carola Venturini, Katarzyna Mickiewicz, Neftali Flores-Rodriguez, Jeff Errington, and Jonathan R. Iredell. “L-Form Switching Confers Antibiotic, Phage and Stress Tolerance in Pathogenic Escherichia Coli,” June 21, 2021. https://doi.org/10.1101/2021.06.21.449206.
6. Flores-Mireles, Ana L., Jennifer N. Walker, Michael Caparon, and Scott J. Hultgren. “Urinary Tract Infections: Epidemiology, Mechanisms of Infection and Treatment Options.” *Nature Reviews. Microbiology* 13, no. 5 (May 2015): 269–84. https://doi.org/10.1038/nrmicro3432.
7. Glover, William A., Yanqin Yang, and Ying Zhang. “Insights into the Molecular Basis of L-Form Formation and Survival in Escherichia Coli.” *PLOS ONE* 4, no. 10 (October 6, 2009): e7316. https://doi.org/10.1371/journal.pone.0007316.
8. Kawai, Yoshikazu, Romain Mercier, Ling Juan Wu, Patricia Domínguez-Cuevas, Taku Oshima, and Jeff Errington. “Cell Growth of Wall-Free L-Form Bacteria Is Limited by Oxidative Damage.” *Current Biology* 25, no. 12 (June 15, 2015): 1613–18. https://doi.org/10.1016/j.cub.2015.04.031.
9. Kawai, Yoshikazu, Katarzyna Mickiewicz, and Jeff Errington. “Lysozyme Counteracts β-Lactam Antibiotics by Promoting the Emergence of L-Form Bacteria.” *Cell* 172, no. 5 (February 22, 2018): 1038-1049.e10. https://doi.org/10.1016/j.cell.2018.01.021.
10. Mercier, Romain, Yoshikazu Kawai, and Jeff Errington. “Excess Membrane Synthesis Drives a Primitive Mode of Cell Proliferation.” *Cell* 152, no. 5 (February 28, 2013): 997–1007. https://doi.org/10.1016/j.cell.2013.01.043.
11. Öztürk, Recep, and Ahmet Murt. “Epidemiology of Urological Infections: A Global Burden.” *World Journal of Urology* 38, no. 11 (November 2020): 2669–79. https://doi.org/10.1007/s00345-019-03071-4.
12. Park, Ju Yi, Kyung Ok Ko, Jae Woo Lim, Eun Jeong Cheon, and Jung Min Yoon. “Increase in Aminotransferase Levels during Urinary Tract Infections in Children.” *Pediatric Gastroenterology, Hepatology & Nutrition* 16, no. 2 (June 2013): 89–94. https://doi.org/10.5223/pghn.2013.16.2.89.
13. Jack Westin. “Presence Of Cell Wall In Bacteria - Classification And Structure Of Prokaryotic Cells - MCAT Content,” March 7, 2020. https://jackwestin.com/resources/mcat-content/classification-and-structure-of-prokaryotic-cells/presence-of-cell-wall-in-bacteria.
14. Sihra, Néha, Anna Goodman, Rhana Zakri, Arun Sahai, and Sachin Malde. “Nonantibiotic Prevention and Management of Recurrent Urinary Tract Infection.” *Nature Reviews Urology* 15, no. 12 (December 2018): 750–76. https://doi.org/10.1038/s41585-018-0106-x.
15. Society, Microbiology. “Investigating the Possible Role of L-Form Switching in Recurrent Urinary Tract Infections.” Accessed November 26, 2021. https://microbiologysociety.org/our-work/75th-anniversary-a-sustainable-future/antimicrobial-resistance-amr/antimicrobial-resistance-amr-case-studies/l-form-switching-in-urinary-tract-infections.html.

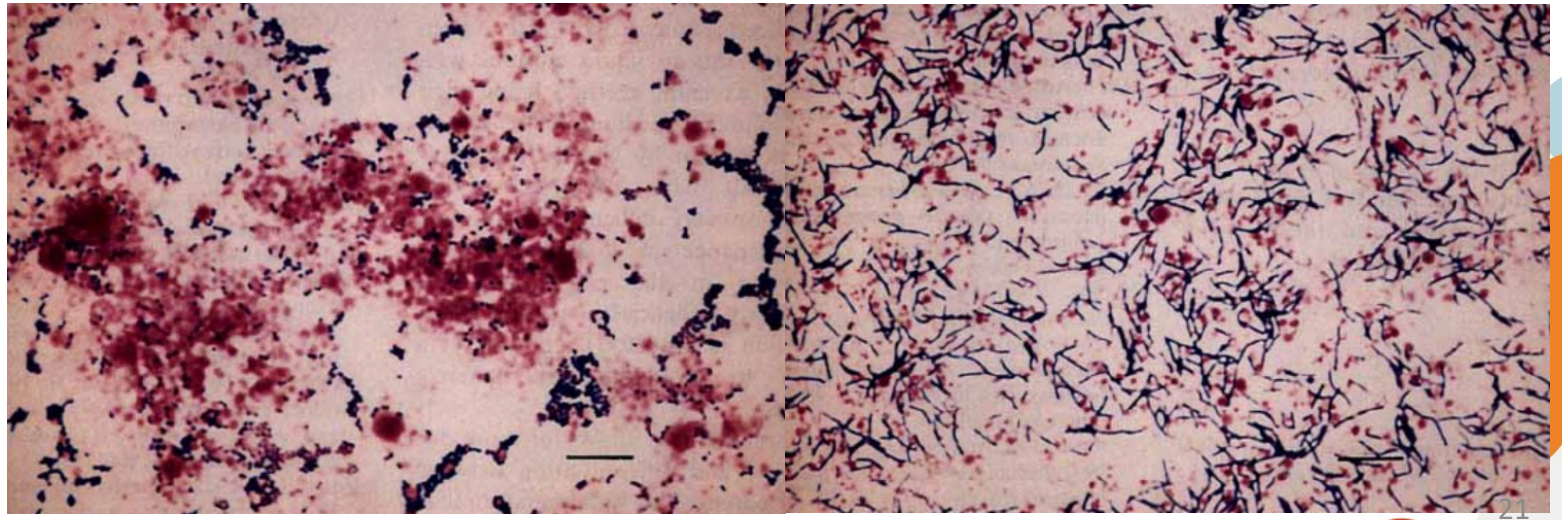
Methods for identifying L-forms?

- Modified Gram stain
- Requires culturing with L-form media before fixing and staining
- L-forms are red due to counterstain

A novel method for differentiating L-form bacteria from their parental form using the Hucker Gram staining technique

E.J. ALLAN, J. JASS*, L.E. PHILLIPS*, J.W. COSTERTON† &
H.M. LAPPIN-SCOTT* *Department of Agriculture, University of Aberdeen, 581 King Street, Aberdeen, AB9 1UD, *Department of Biological Sciences, Hatherly Laboratories, University of Exeter, EX4 4PS, UK and †Department of Biological Sciences, University of Calgary, Calgary, Alberta, T2N 1N4, Canada*

DST/10: received 3 June 1992 and accepted 15 June 1992

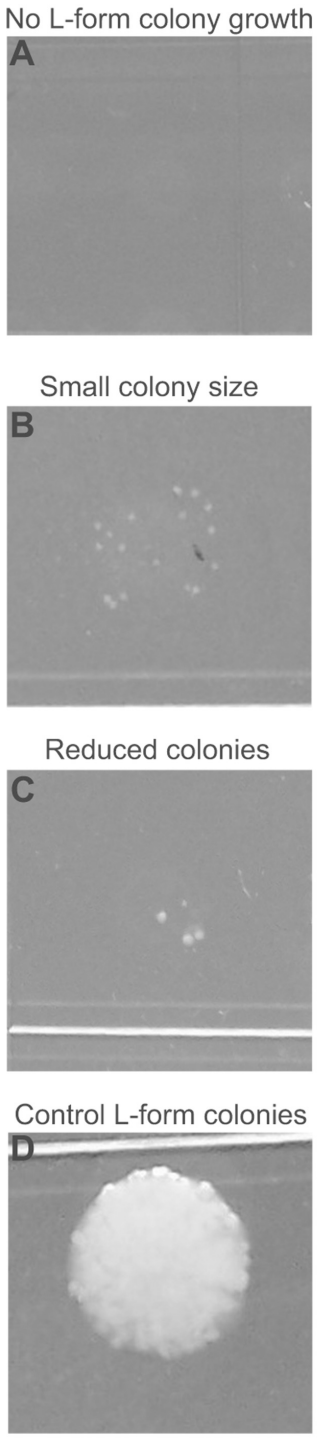


(Allan et al., 1992, *Letters in Applied Microbiolo.*)

Methods for identifying L-forms?

- Glover et al., (2009) Insights into the **Molecular Basis of L-Form Formation and Survival in *Escherichia coli***
- **csB**, **ruvA**, **fur**, and **smpA** that are essential in the formation or survival of L-form colonies
- Pathways involved:
  - Cell envelope stress
  - DNA repair
  - Iron homeostasis
  - Drug efflux/ABC transporters
  - Outer membrane biogenesis

(Glover et al., 2009, *PLOS one*)



Group 1 mutants (24)

rcsC, rcsB, rcsF cpsB, wcaA, wcaF, wcaI, wza, wzb, wzc, wzxC, gmd, galU, manA, smpA, yfgL, ruvA, recG, fur, yhdP, acrA, acrB, yrbC, mrcB

Group 2 mutants (18)

yjbG, dnaT, recB recC, efp, galE, wcaJ, priA, fes, dedD, zwf, ubiE, ubiF, lpxM, yraO, smpB, damX, ychM

Group 3 mutants (10)

wcaL, fcl, vacJ, yrbD, yrbE, rffD, ompA, recA, fis, cpxA

BW25113 parent

Methods for identifying L-forms?

- **L-form switching in *B. subtilis***
- Two point mutations are needed for L-form growth in *B. subtilis*

1. *rodA* -> induces L-form, unable to grow
   - Peptidoglycan glycosyltransferase RodA
   - **cell wall elongation** and the **maintenance** of the **rod** cell **shape**

2. *ispA* -> prevent lysis of L-form during growth
   - catalyzes the formation **farnesyl pyrophosphate**
   - required for synthesis of the precursors for **peptidoglycan** (lipid II) and **wall teichoic acid**

Cell
Volume 152, Issue 5, 28 February 2013, Pages 997-1007

Article

Excess Membrane Synthesis Drives a Primitive Mode of Cell Proliferation

Romain Mercier [1, 2], Yoshikazu Kawai [1, 2], Jeff Errington [1]

Show more

+ Add to Mendeley Share Cite

Current Biology
Volume 25, Issue 12, 15 June 2015, Pages 1613-1618

Report

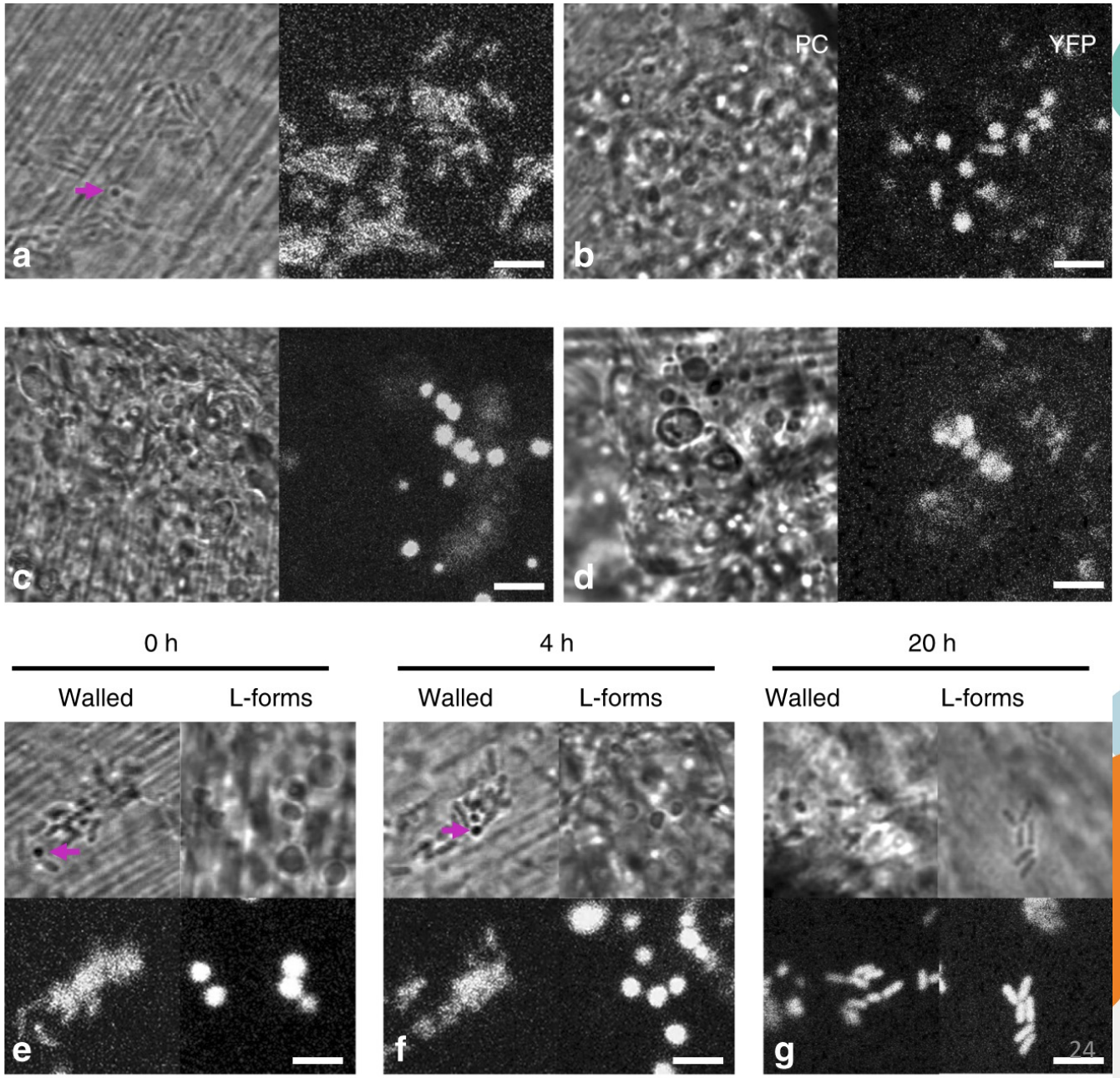
Cell Growth of Wall-Free L-Form Bacteria Is Limited by Oxidative Damage

Yoshikazu Kawai [1, 4], Romain Mercier [1, 4], Ling Juan Wu [1], Patricia Domínguez-Cuevas [2], Taku Oshima [3], Jeff Errington [1]

Show more

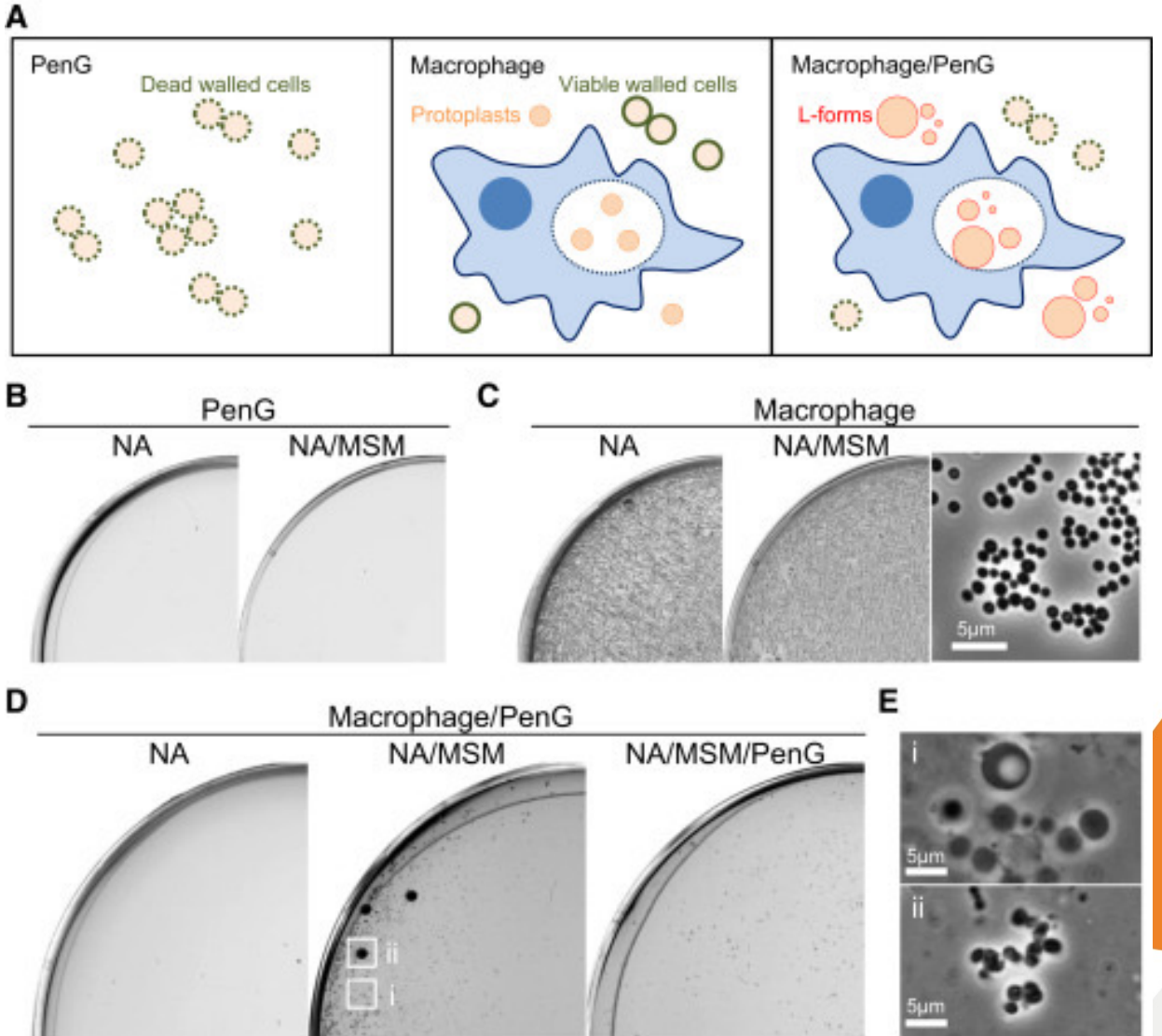
In vivo model

- Transparent zebrafish larvae
- A-D: L-form *E. coli* injected *in vivo* with/without antb
  - L in antb
  - L->N without antb
- E-G: Injected with walled/L-form *E. coli* without antb
  - L->N switch *in vivo*
- Homogenise embryo ☹ and plated then counted colonies



Other ways in which L-forms can be generated naturally

- Bacterial Cells Evade Antibiotic Action of PenG by Interacting with Macrophages
- Interaction with **lysozymes** can induce L-form switching



(Kawai et al., 2018, *Cell*)

Other ways in which L-forms can be generated naturally

"L-form switching is a **common response** of pathogenic E. coli strains to **cell wall targeting antibiotics** and that the most commonly used **lytic bacteriophages** are ineffective against them in this state"

L-form switching confers antibiotic, phage and stress tolerance in pathogenic *Escherichia coli*

Aleksandra Petrovic Fabijan[1], Muhammad Kamruzzaman[1], David Martinez-Martin[2,3], Carola Venturini[1,4], Katarzyna Mickiewicz[5], Neftali Flores-Rodriguez[6], Jeff Errington[5], Jonathan R. Iredell[1,4,7]

1. *Centre for Infectious Diseases and Microbiology, Westmead Institute for Medical Research, Sydney, New South Wales, Australia*
2. *School of Biomedical Engineering, The University of Sydney, Sydney, New South Wales, Australia*
3. *The University of Sydney Nano Institute, The University of Sydney, Sydney, New South Wales 2006, Australia*
4. *School of Medicine, Sydney Medical School, The University of Sydney, Sydney, New South Wales, Australia*
5. *Centre for Bacterial Cell Biology, Institute for Cell and Molecular Biosciences, Newcastle University, Newcastle upon Tyne, United Kingdom*
6. *Sydney Microscopy and Microanalysis, Charles Perkins, The University of Sydney, Sydney, New South Wales, Australia*
7. *Westmead Hospital, Western Sydney Local Health District, Sydney, New South Wales, Australia*

(Fabijan et al., 2021, *bioRxiv* preprint)

Other ways in which L-forms can be generated naturally

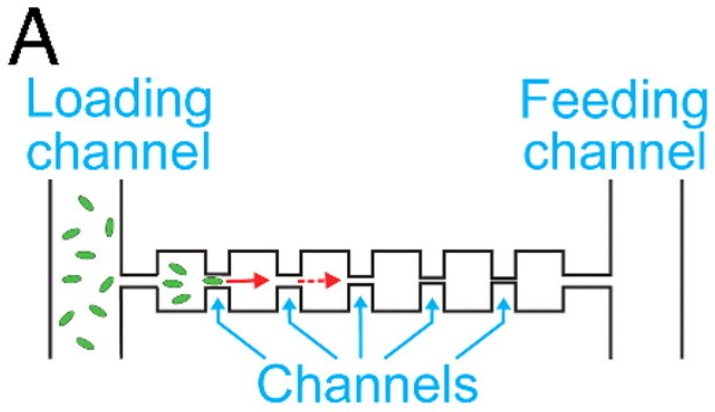
- Microfluidic channels (constrictions)
- *E. coli* L-forms are derived after penetrating channels with a width that is smaller than their diameter
- L-form switching appears to be a stress response

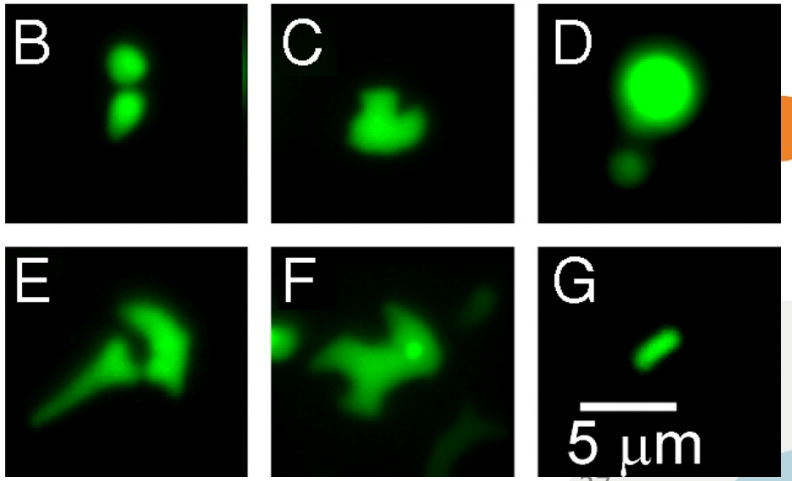
Bacterial growth and motility in sub-micron constrictions

Jaan Männik, Rosalie Driessen, Peter Galajda, Juan E. Keymer, and Cees Dekker

+ See all authors and affiliations

PNAS September 1, 2009 106 (35) 14861-14866; https://doi.org/10.1073/pnas.0907542106





(Männik et al., 2009, PNAS)

Other strains of pathogenic E. coli isolated from other specimen types display L-form switching due to stress from meropenem and lytic phages

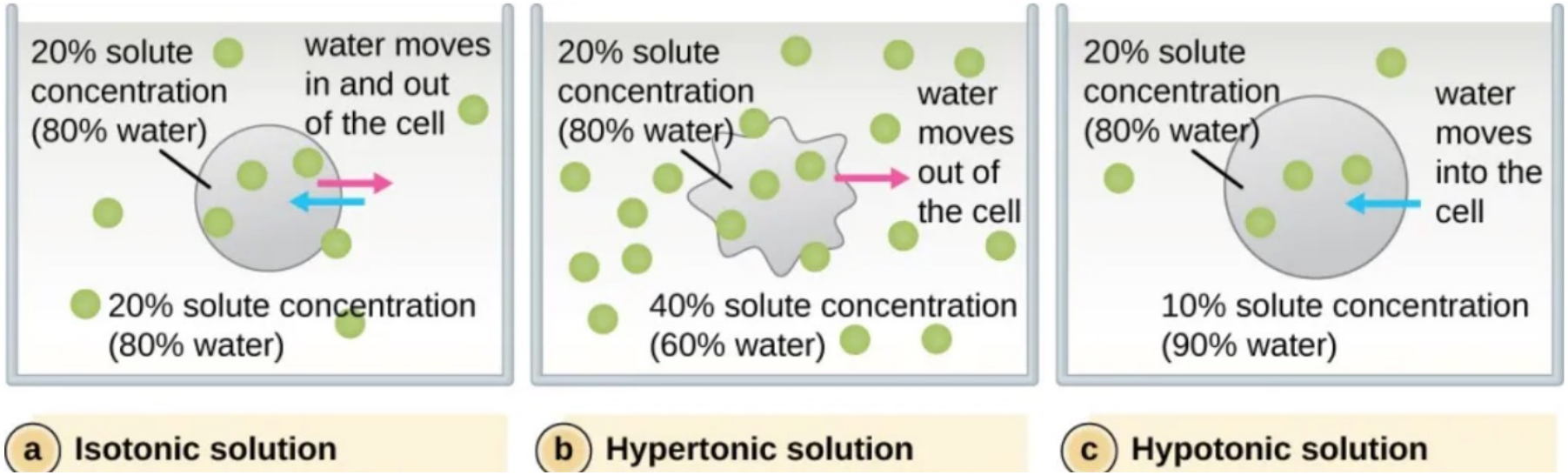
E. coli strain ID	Specimen	ST (clade)	Antimicrobial resistance genes	Meropenem MIC (µg/ml)		Specific phages			
				WT	Revertant	Eco2	Eco6	Eco11	Eco12
SYD045	Urine	ST1193	*bla*$_{CTX-M-14a}$, *bla*$_{TEM-1b}$, *aac(3)-IId*	<0.25	ND				
SYD252	Urine	ST131 (B)	*bla*$_{CTX-M-15}$, *bla*$_{TEM-1b}$, *aac(3)-IId*, *dfrA17-aadA5*, *sul1*, *mph*(A), *sul2*, *strAB*, *tet*(A)	0.031	0.031				
SYD449	Blood culture	ST131 (A)	None	0.031	0.031				
SYD214	Urine	ST648	*bla*$_{TEM-1b}$, *aac(3)-IIe*	<0.25	ND				
SYD402	Urine	ST73	*bla*$_{TEM-1b}$, *sul2*	0.031	0.015				
SYD009	Blood culture	ST95	*bla*$_{DHA-1}$, *qnrB4*, *dfrA17*, *sul1*, *mph*(A), *sul2*, *strAB*, *tet*(B),	0.015	0.015				
SYD074	Blood culture	ST58	*bla*$_{TEM-1b}$, *dfrA5*, *strAB*, *sul2*, *tet*(A),	0.015	0.015				
SYD066	Urine	ST405	*bla*$_{CTX-M-15}$, *aac(3)-IIe*, *dfrA17-aadA5*, *sul1*, *mph*(A), *tet*(B)	<0.25	ND				
SYD259	Urine	ST998	*bla*$_{CTX-M-15}$, *bla*$_{TEM-1b}$, *bla*$_{OXA-1}$, *aac(6')-Ib-cr*, *aac(3)-IIe*, *aphA1*, *dfrA5*, *dfrA17-aadA5*, *sul1*, *mph*(A), *sul2*, *floR*	0.015	0.015				
SYD001	Urine	ST38	*bla*$_{CTX-M-27}$, *dfrA17-aadA5*, *sul1*, *mph(A)*, *sul2*, *strAB*, *tet*(A),	0.031	0.031				
SYD421	Urine	ST349	*bla*$_{TEM-1b}$, *dfrA14*, *sul2*, *strAB*	0.031	0.031				
JIE4039	Urine	ST963	*bla*$_{CMY-2}$	0.031	0.063				
B36	Blood culture	ST131 (C)	*bla*$_{CTX-M-15}$, *bla*$_{TEM-1b}$, *bla*$_{OXA-1}$, *dfrA17-aadA5*, *sul1*, *mph*(A), *sul2*, *strAB*, *tet*(A),	0.031	0.031				
J53	Stool	ST10	None	<0.25	ND				
WH62	Stool	ST127	None	0.063	0.063				

**MIC=minimal inhibitory concentration; ND=not determine; Coloured cells represent phage activity on CWB lawn: dark green=clear plaques, light green=slightly turbid plaques, yellow=turbid plaques and grey=no activity.*

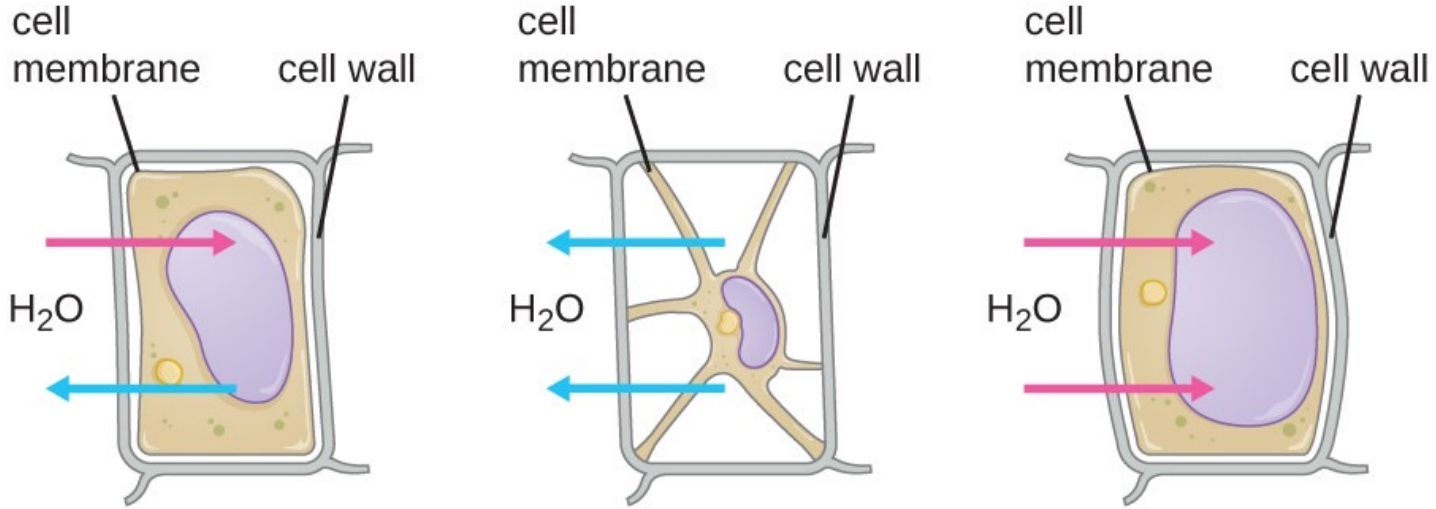
(Fabijan et al., 2021, *bioRxiv* preprint)

Routine microbiological media is hypotonic and does not support L-form growth

L-form

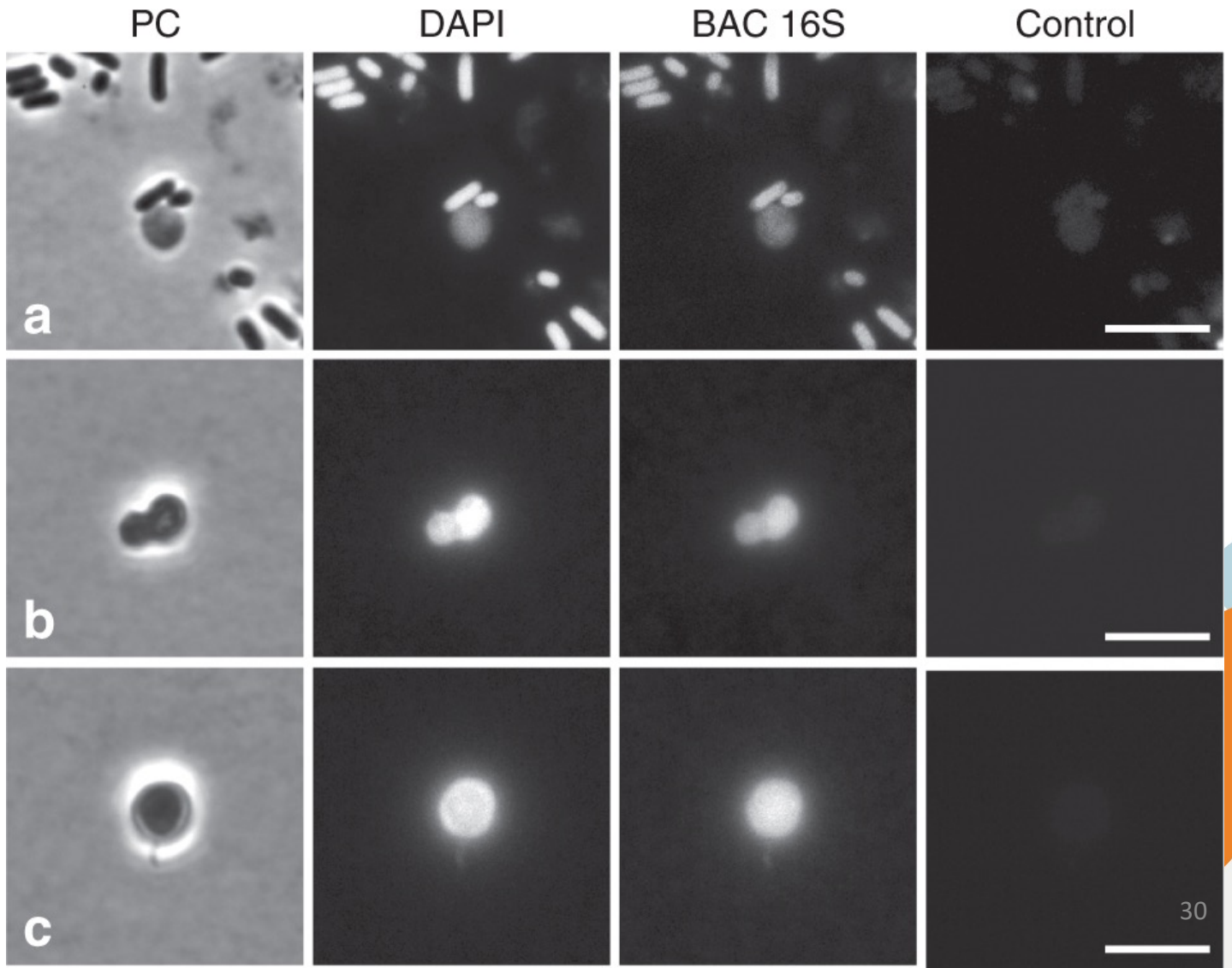


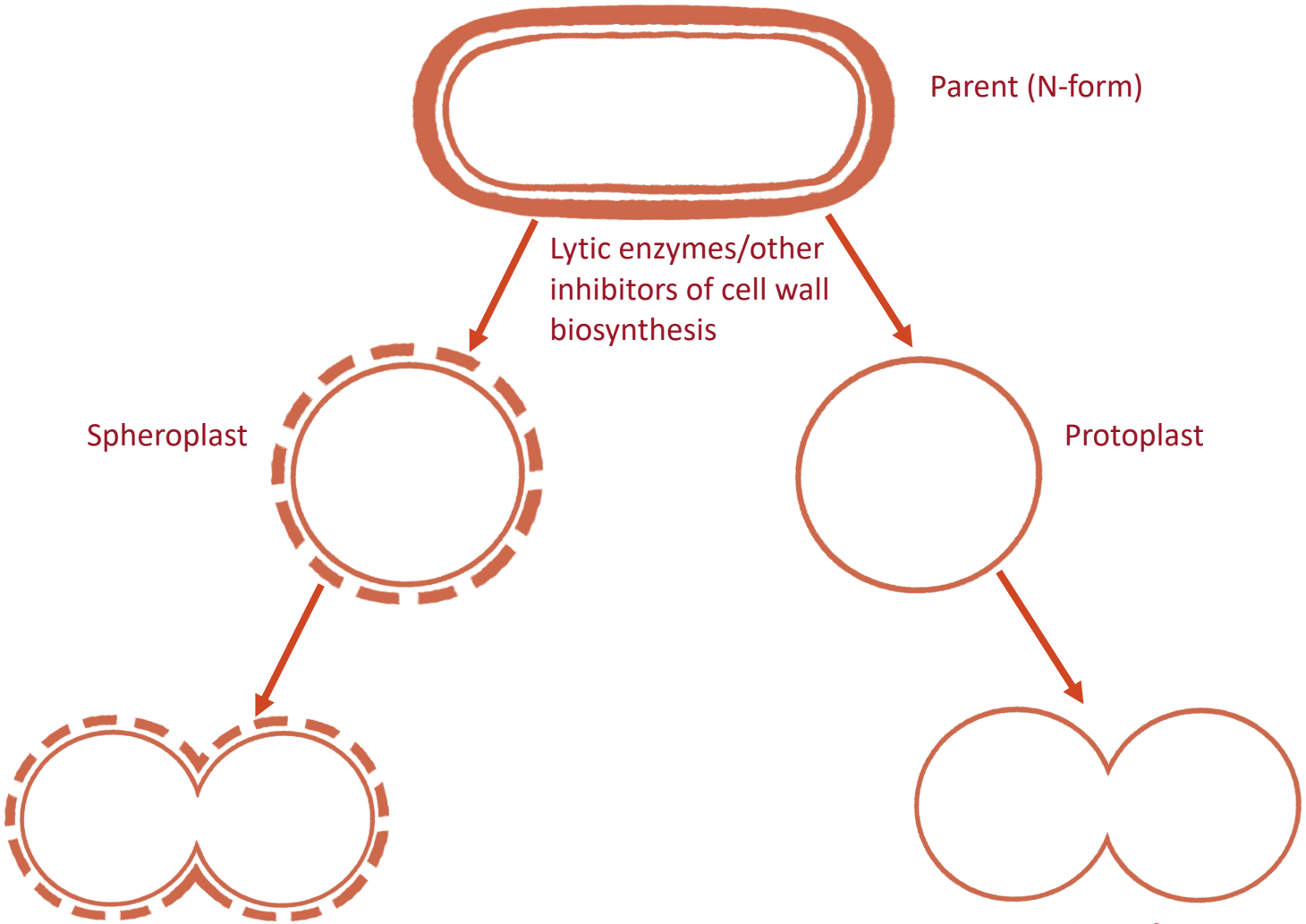
N-form

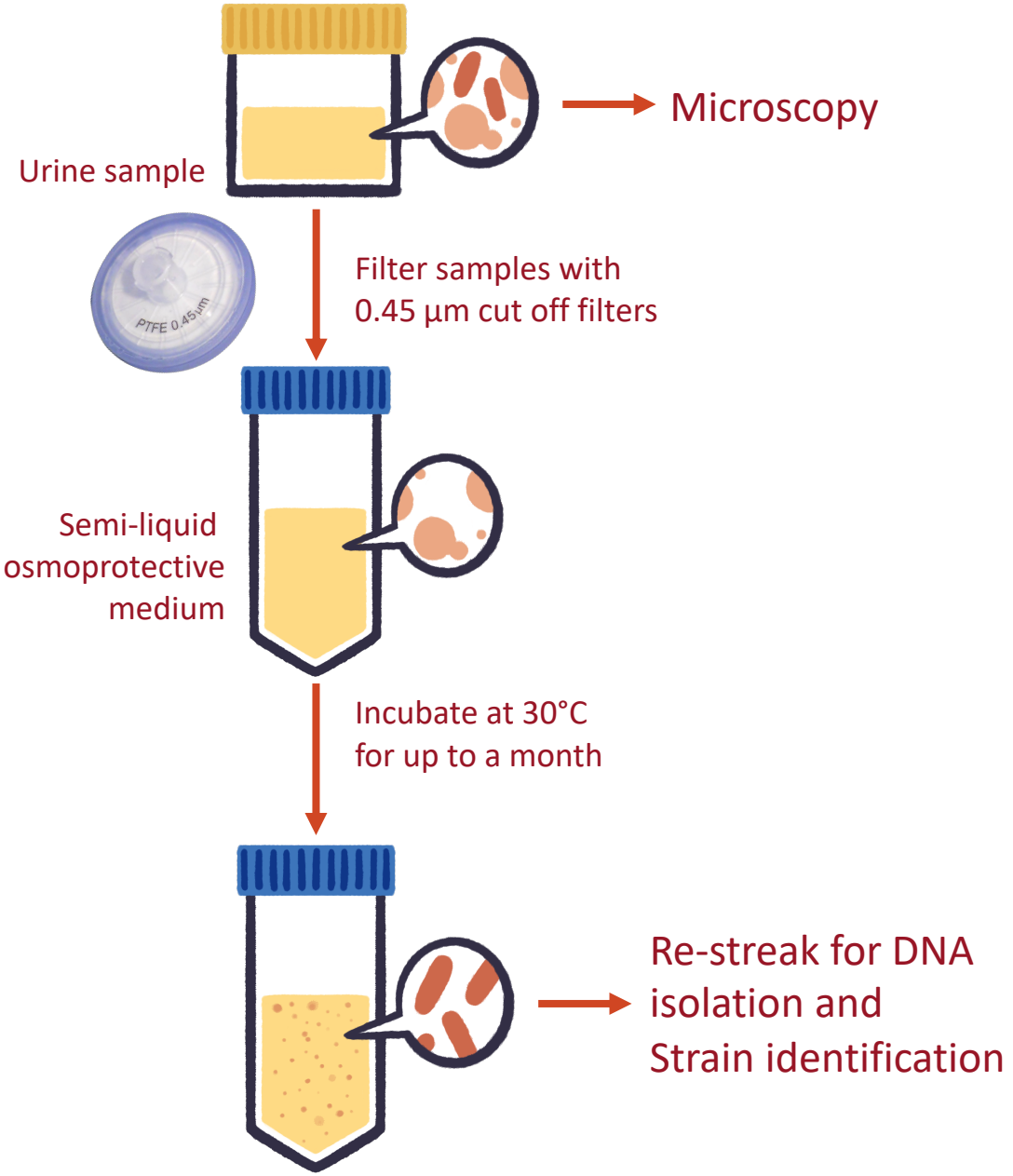


L-forms provide a route for antibiotic evasion

- patient UTI343
- treated with **phosphomycin** (donation 6)
- Significant viable bacterial >1 × 10^5
- **UPEC strain ST144**




Parent (N-form)
Lytic enzymes/other inhibitors of cell wall biosynthesis
Spheroplast
Protoplast
Spheroplast L-forms:
1. Stable
2. Unstable
Protoplast L-forms:
1. Stable
2. Unstable


Urine sample
Microscopy
PTFE 0.45µm
Filter samples with
0.45 µm cut off filters
Semi-liquid
osmoprotective
medium
Incubate at 30°C
for up to a month
Re-streak for DNA
isolation and
Strain identification