BACTERIOPHAGE AND FOOD FERMENTATIONS. PHAGE ASSAY AND ENUMERATION

Michael Mullan
College of Agriculture, Food and Rural Enterprise

Aims

This presentation will provide an introduction to bacteriophages (phages) and their significance in food fermentations.

The plaque assay is extensively used to isolate, quantify phage numbers and in phage purification.

It can be difficult to get many phages to form plaques. Factors affecting plaque formation are discussed and recent research that may help solve the plaque count anomaly is reviewed.

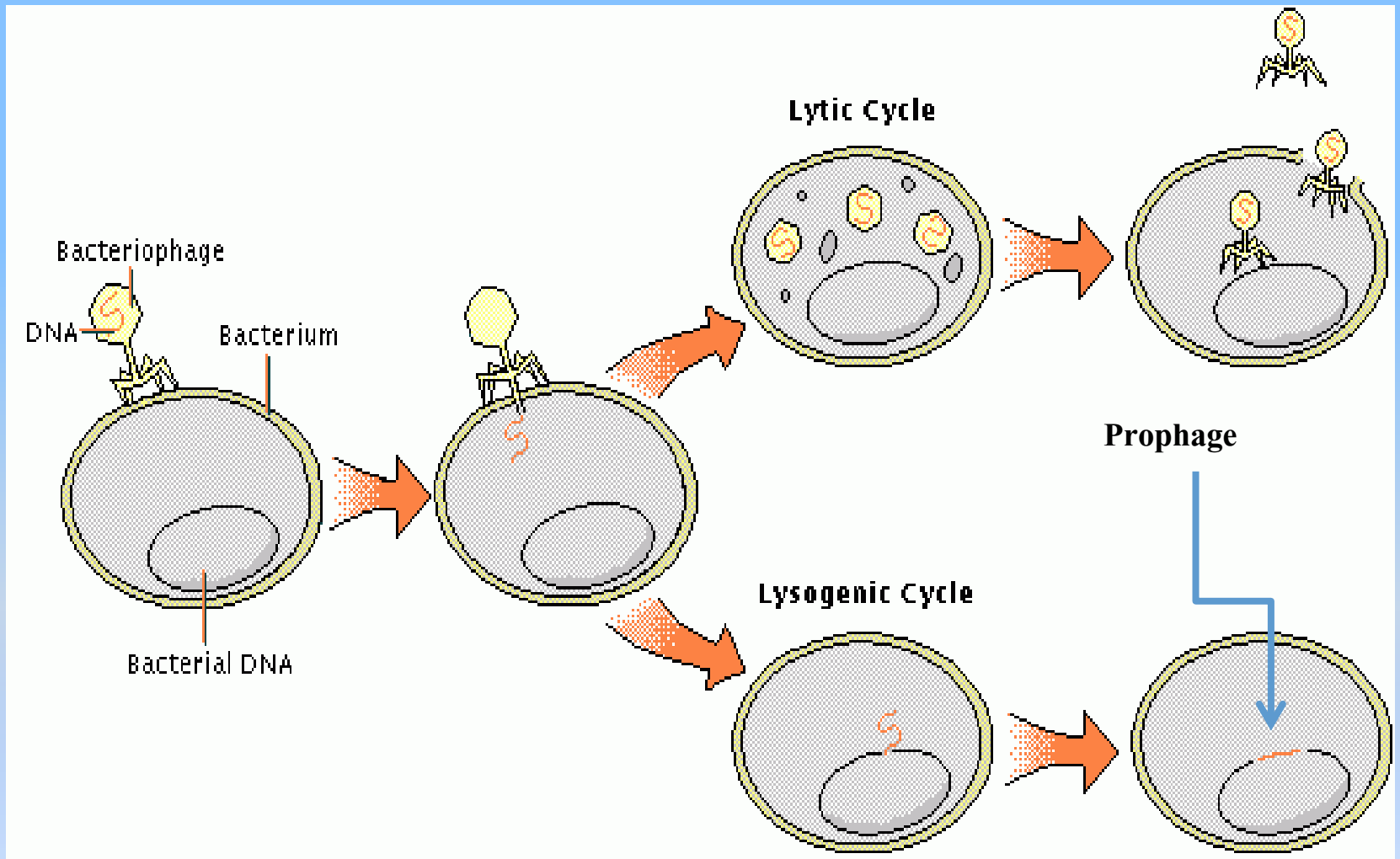
Topics

- Biology of bacteriophages
- Problems in food fermentations
- Assay and enumeration
- “Purification”
- Conclusions

Biology of bacteriophages

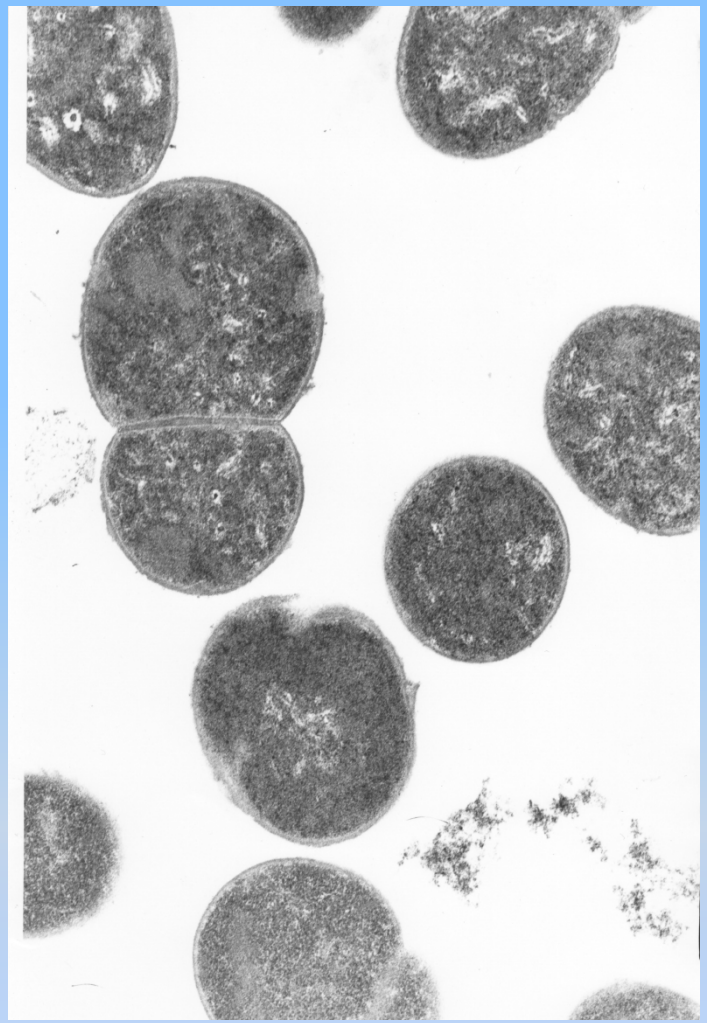
- Viruses obligately parasitic on bacteria
- Variable host range
- Classified on basis of ss vs. ds RNA or DNA **and** morphology
- > 96% reported phages are tailed- Order: Caudovirales
- Myo-, Sipho- and Podoviridae families significant in food fermentations, food quality/safety, bio-industries
- Role in regulating bacterial numbers in all habitats
- Major role in bacterial evolution and contribute to virulence - exotoxin production
- Their study has contributed to significant discoveries in biological science and they offer an enormous genetic resource

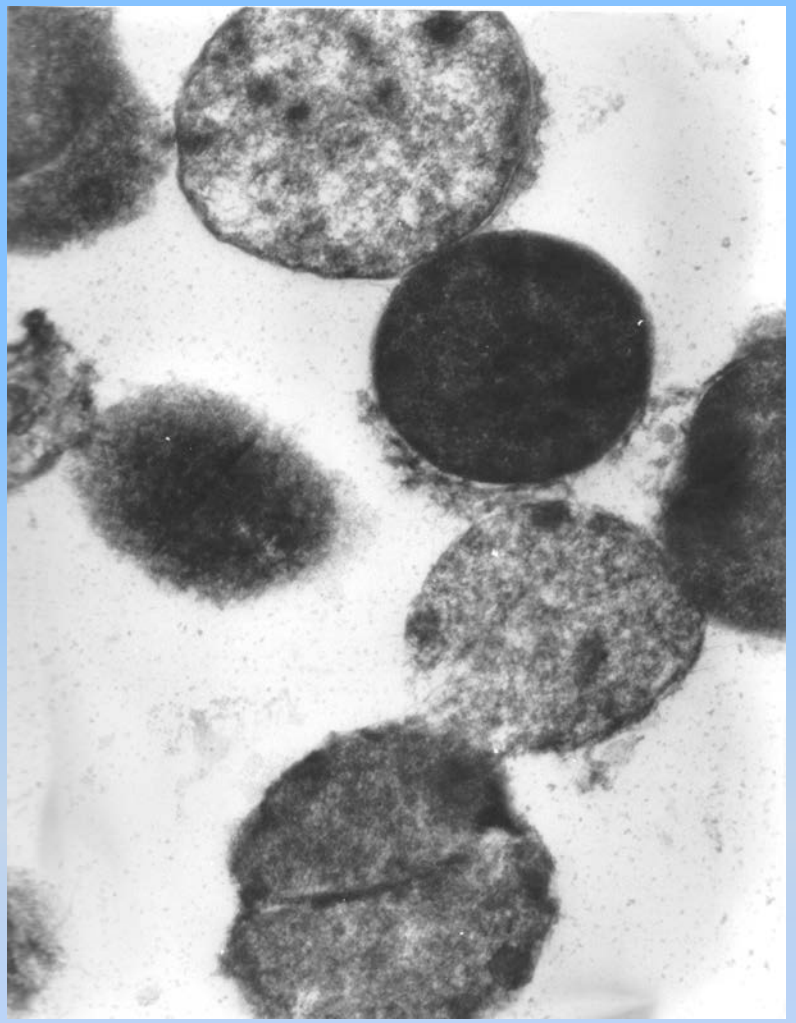
Biology of bacteriophages - life cycle



Source: http://mining.ubc.ca/cerm3/lytic%20vs%20lysogenic.gif

Biology of bacteriophages – phage lysin

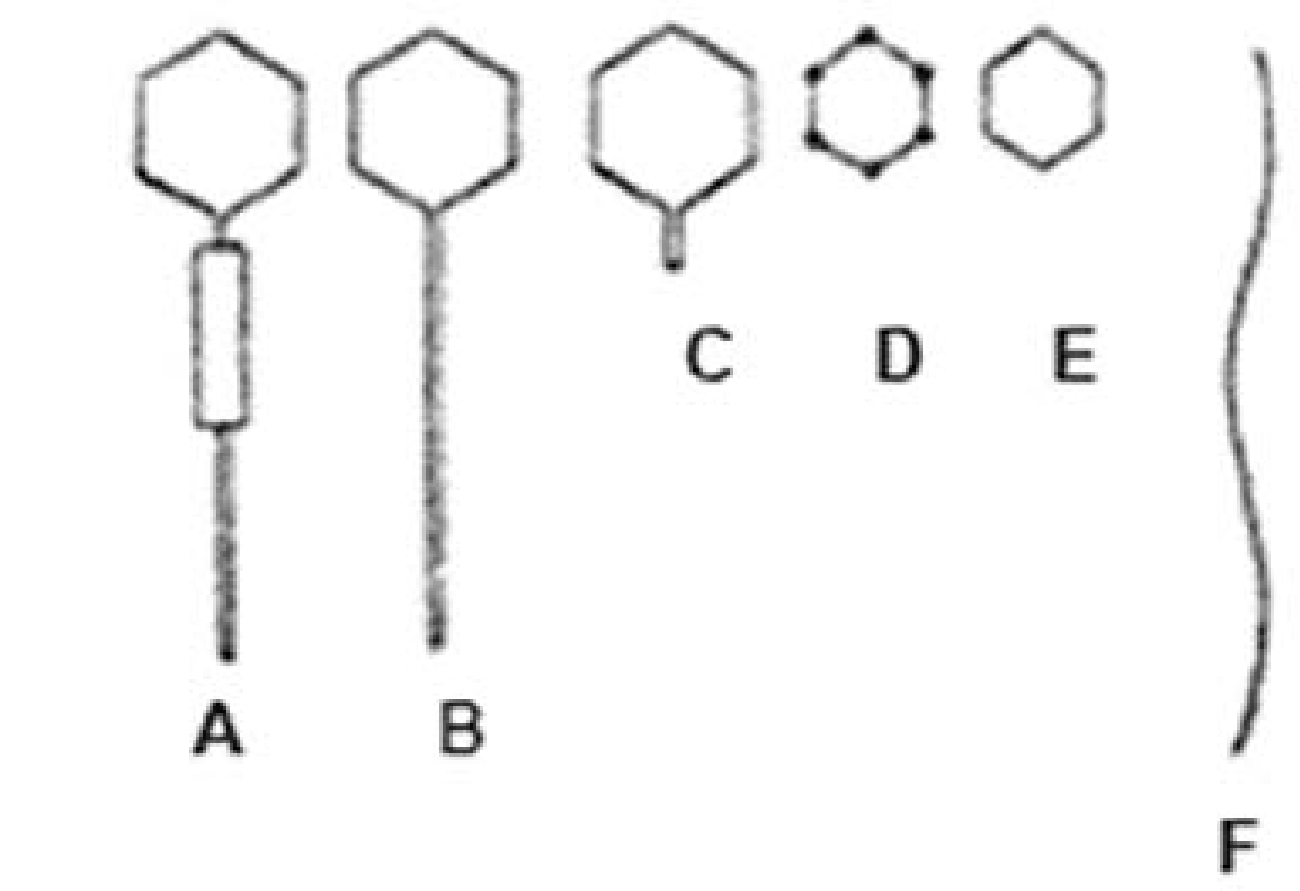




Control. *L. lactis* C10 x 40,000 C10 + 300 u/ml lysin. 150 s, 37°C x 54,000

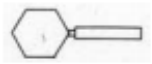
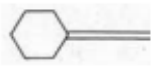
From: Mullan and Crawford (1981)

Biology of bacteriophages - morphology



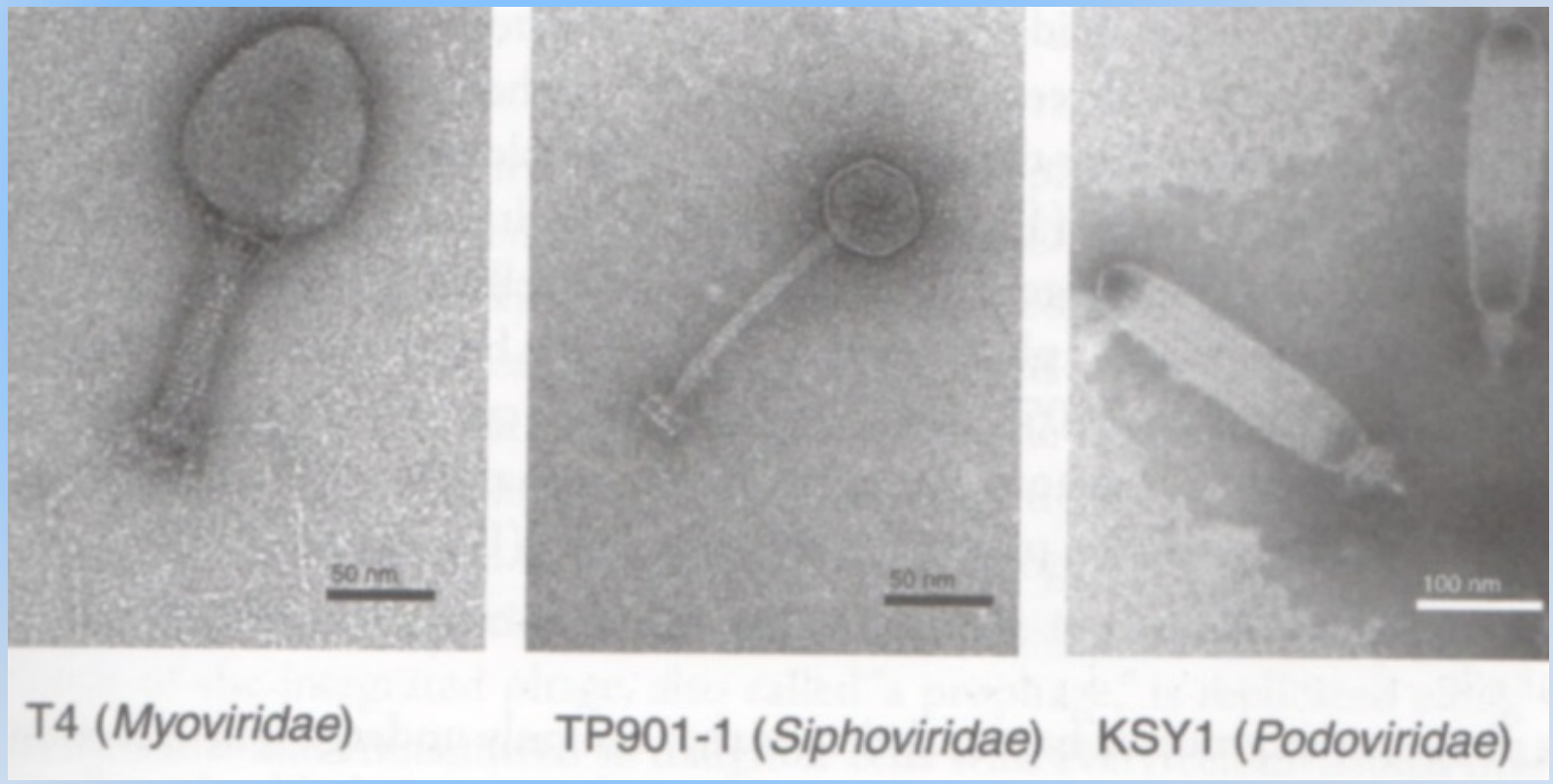
Basic morphological phage types
Bradley (1967)

Biology of bacteriophages- current phage classification

Shape	Order or family	Nucleic acid, particulars, size	Member	Number[a]
	Caudovirales	dsDNA (L), no envelope		
	*Myoviridae*	Tail contractile	T4	1312
	*Siphoviridae*	Tail long, noncontractile	λ	3262
	*Podoviridae*	Tail short	T7	771
	*Microviridae*	ssDNA (C), 27 nm, 12 knoblike capsomers	ϕX174	38
	*Corticoviridae*	dsDNA (C), complex capsid, lipids, 63 nm	PM2	3?
	*Tectiviridae*	dsDNA (L), inner lipid vesicle, pseudo-tail, 60 nm	PRD1	19
	*Leviviridae*	ssRNA (L), 23 nm, like poliovirus	MS2	38
	*Cystoviridae*	dsRNA (L), segmented, lipidic envelope, 70–80 nm	ϕ6	3
	*Inoviridae*	ssDNA (C), filaments or rods, 85–1950 x 7 nm	fd	66
	*Plasmaviridae*	dsDNA (C), lipidic envelope, no capsid, 80 nm	MVL2	5

From: Ackermann, H.-W. (2007) 5500 Phages examined in the electron microscope. *Arch. Virol.* 152, 277–243.

Biology of bacteriophages - Caudovirales examples



From: Emond and Moineau (2007)

Problems in food fermentations

- **Any bioconversion process whereby substrates are fermented to give value-added products e.g. proteases, antibiotics, solvents, is threatened by phage infection**

- Phage problems have been particularly well described in the dairy industry e.g. in cheese manufacture

- Look at the significance of phage infection in cheese manufacture

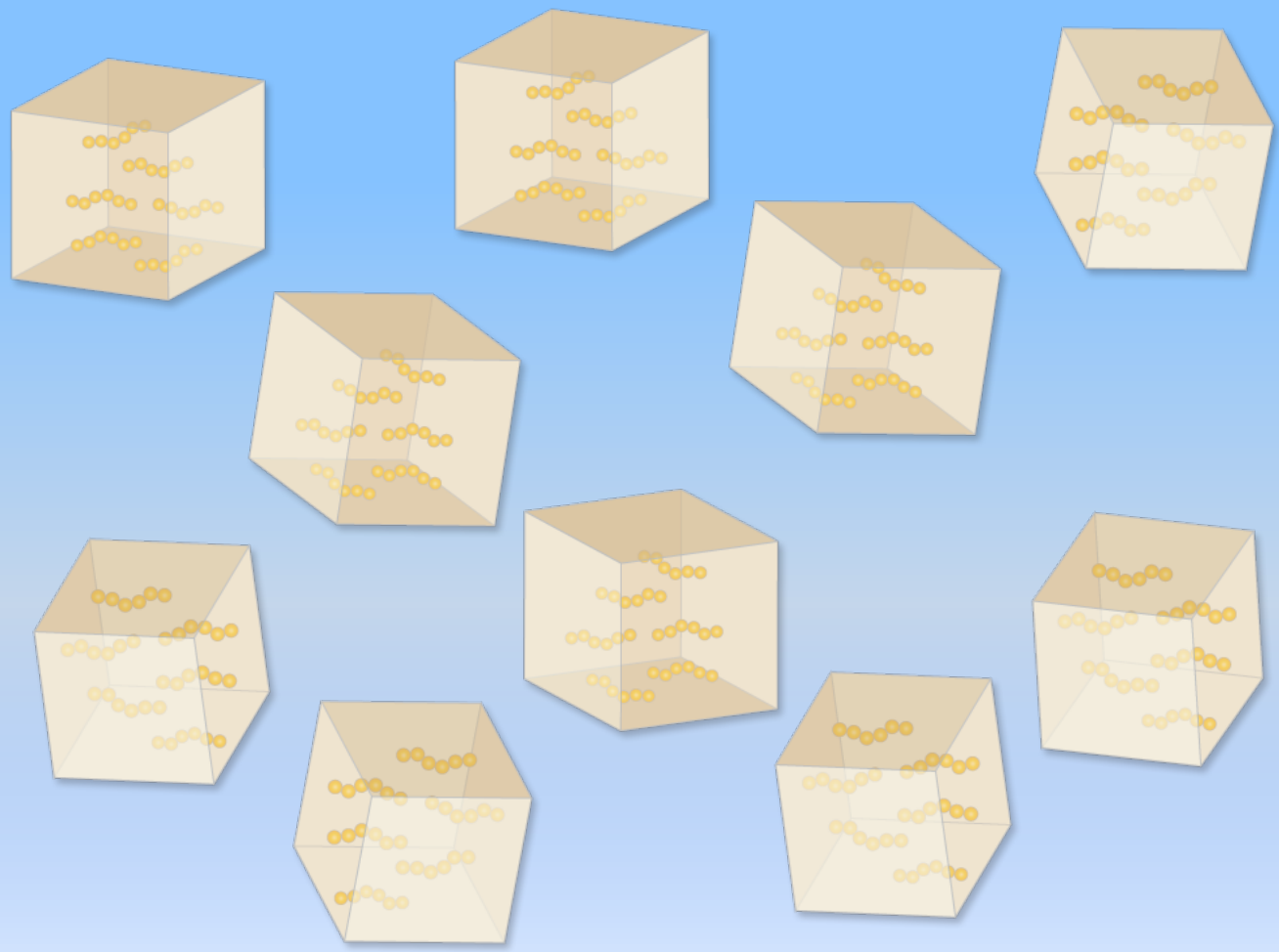
Dairy fermentations - role of lactic starter cultures

Function	Result / Mechanism
Acid production	Gel formation Whey expulsion (syneresis) Preservation Flavour development
Flavour compound production	Formation of diacetyl and acetaldehyde
Preservation	Lowering of pH and redox potential Production of bacteriocins e.g. nisin Production of hydrogen peroxide Formation of D-leucine Production of lactate / lactic acid Acetate formation
Gas formation	Eyehole formation Production of openness to facilitate 'blue veining'
Stabiliser formation	Body and viscosity improvement Increase cheese yield? Reduced use of milk powder in yoghurt

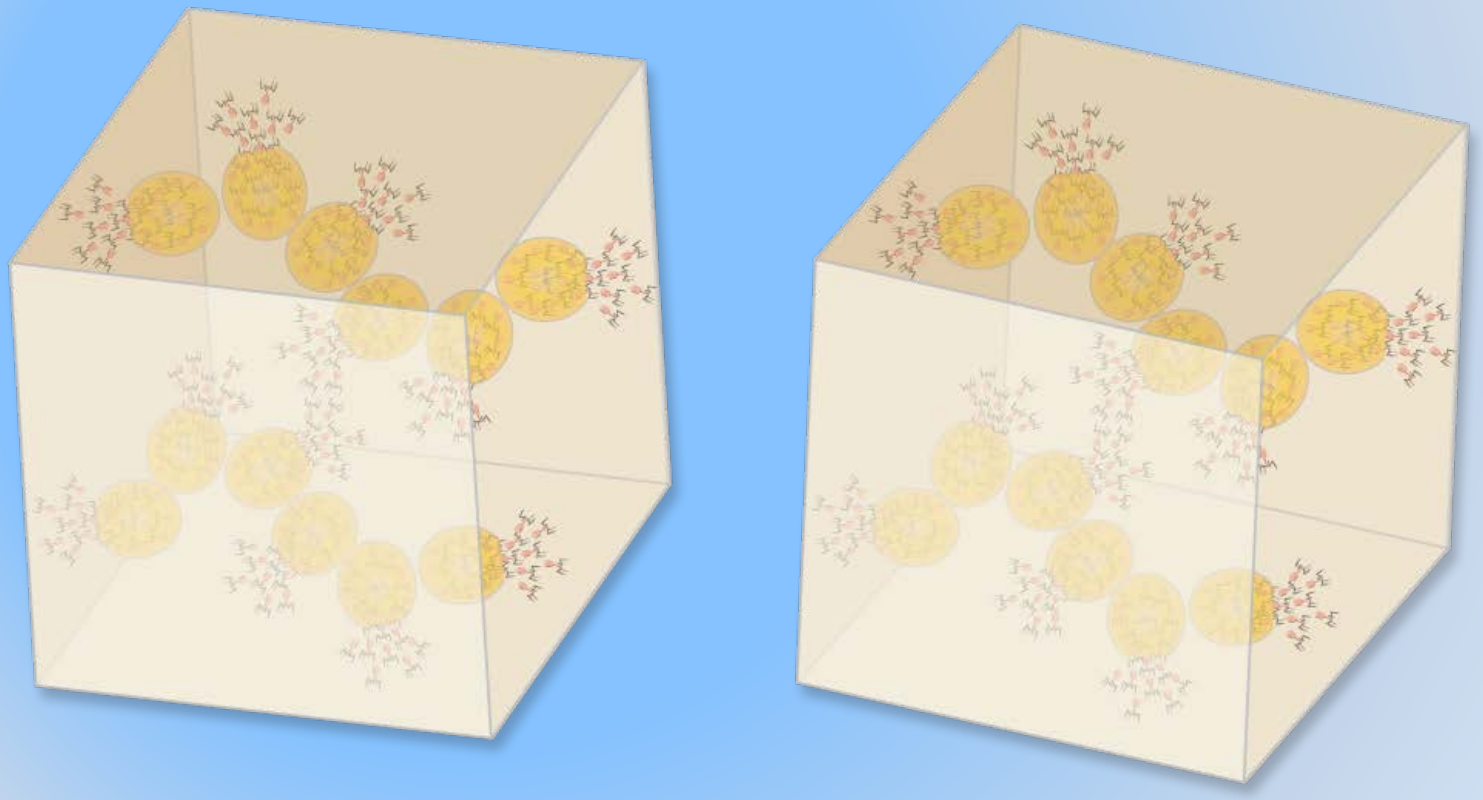
Cheesemaking - consequences of phage infection

Consequences of cell lysis include:	
“Dead vats”	Economic loss
Disruption of timed schedules	Economic loss
Low acid production and high pH cheese	Growth of pathogens Toxin production Food poisoning Economic loss
Reduced synerysis	Cheese of variable moisture concentration Inconsistent quality Economic loss
“Sweet cheese”	Little or no utilisation of residual lactose
“Gassy cheese”	Gas production from lactose by non starter lactic acid bacteria

Cheesemaking - consequences of phage infection



Cheesemaking - consequences of phage infection



Modern methods for controlling phage problems in cheesemaking

- Major imbalance between reproductive rates of phage and bacteria. One “non-infected” bacterium produces four new bacteria in two generations of fission. A typical lytic phage will produce >22,000 phage particles in this time.

Keep phage concentrations low!

- Phage infected cells may also overproduce phage lysin. Phage lysin typically has a broader host range that the phage.

Lysin sensitivity is also important when developing starters!

Modern methods for controlling phage problems in cheesemaking

- Use of starters containing phage-unrelated or phage-insensitive strains
- Production of 'phage-free' bulk starter
- Minimising the concentration of phage in processing plants
- Other measures

For a review see "Bacteriophage control in cheese manufacture" at http://www.dairyscience.info/index.php/bacteriophage-control.html

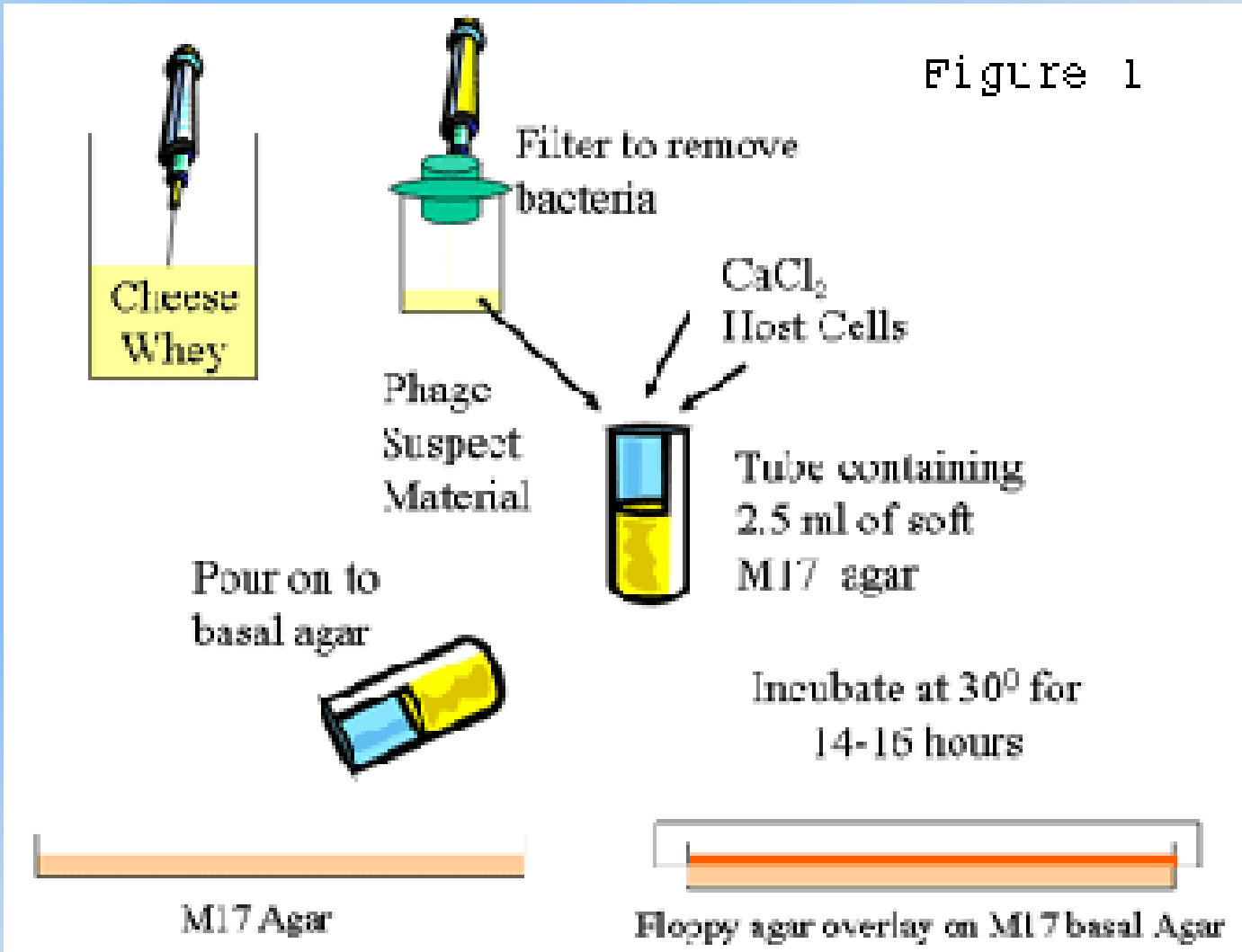
Assay and enumeration. Methods

- Microbiological methods e.g. plaque assay
- Biochemical methods that measure phage components or cell components released following cell lysis
- Direct visualisation of virus particles - generally negatively stained using electron microscopy

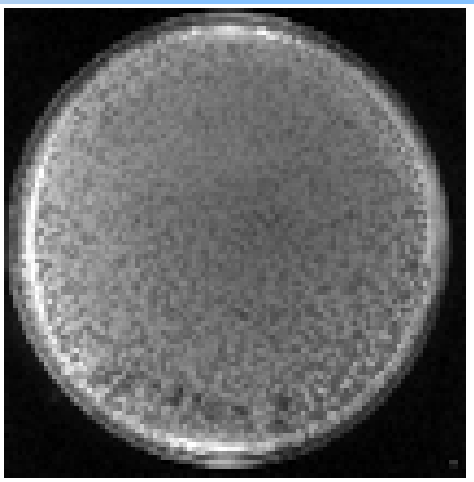
Assay and enumeration. Plaque assay

- Phage suspension and host cells, are mixed in molten, 'soft' agar. The suspension is poured on to a basal agar medium which hardens and immobilises the bacteria.
- During incubation uninfected bacteria multiply to form a confluent film of growth. Each infected bacterium, bursts and liberates progeny phages that infect adjacent bacteria. This 'chain' reaction spreads in a circular motion until bacterial growth ceases. The visible, circular area of clearing in the confluent bacterial growth is known as a plaque.
- The number of plaques multiplied by the dilution factor t gives the number of plaque forming units (PFU) / ml

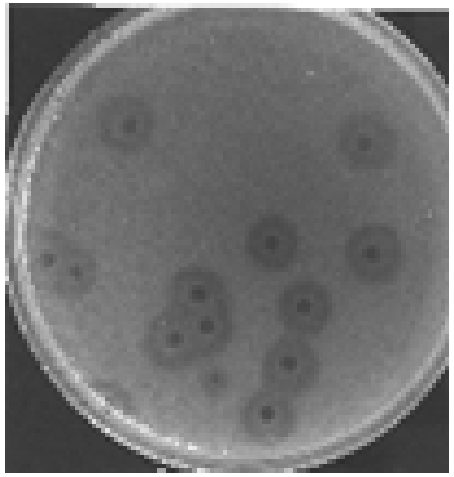
Assay and enumeration. Plaque assay



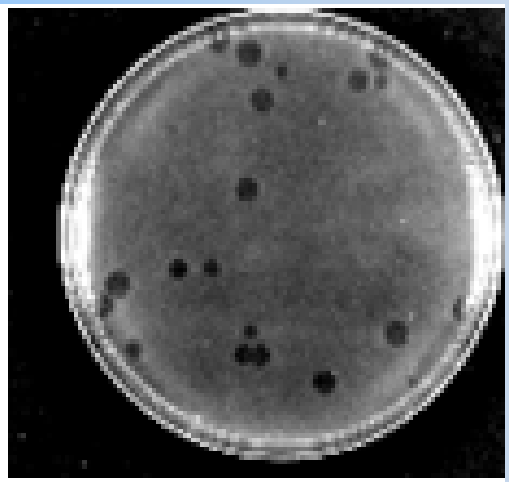
Assay and enumeration. Plaque assay



ø712



øc2(w)



øsk3

Figure3. Lactococcal phage plaques on M17

Assay and enumeration. Difficulties in enumerating phage

- Current data indicate that some 10^{31} bacteriophages exist globally, including about 10^{8} genotypes and possibly most of **the earth's gene diversity** e.g. Williamson *et al.* (2005).

- Less than 1% of the observed bacteriophages have ever been grown in culture (this is sometimes called "the great plaque count anomaly" e.g. Serwer *et al.(2007).*

- It can be challenging to culture bacteriophages. Without plaques, and ultimately, pure phage it can be challenging to do ‘cutting edge’ phage research.

- Can this plaque count anomaly be resolved?

Assay and enumeration. Difficulties in enumerating phage. Insights from models

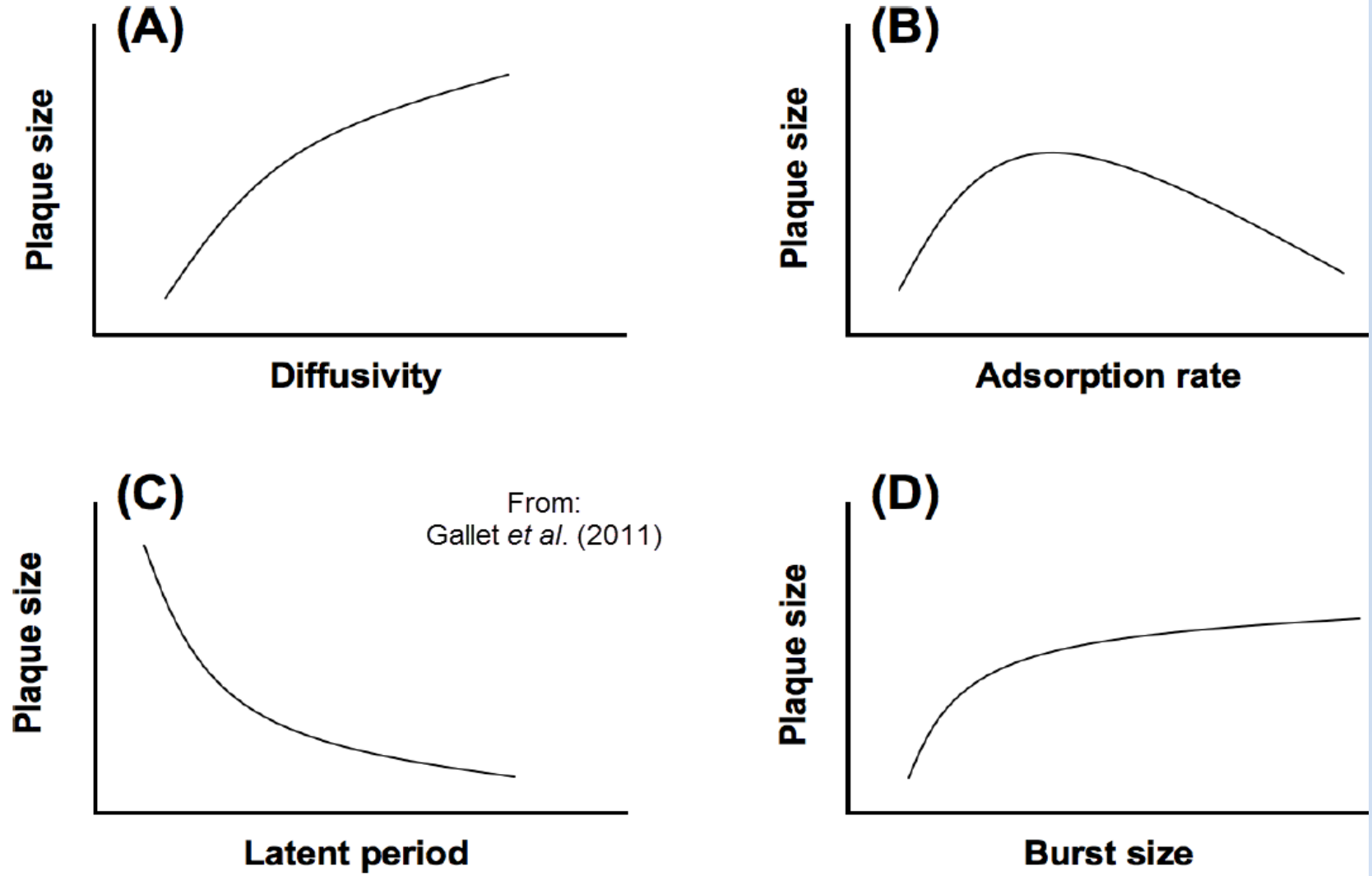


Figure 1 The expected relationships between plaque size and various phage traits as summarized by Abedon and Yin [12].

Assay and enumeration. Maximising Efficiency of Plating (EOP)

- Not every phage particle will produce a plaque. Nor will every plaque be produced by one phage. The plaque count does not give the absolute number of phage particles present in a PSM.

- Careful choice of assay media and conditions can give accurate and reproducible results.

- Such observations have led to the concept of efficiency of plating (EOP); EOP may be defined as the plaque count obtained under a certain set of conditions relative to the plaque count obtained under standard conditions.

Assay and enumeration. Maximising EOP

- Electrolyte or co-factor requirements
- Use of indicator strains
- Growth phase of host
- Hydrogen-ion concentration
- Influence of growth medium
- Incubation temperature
- Temperature at which phage adsorption occurs
- Incorporation of glycine in growth media
- Enhancing plaque definition by using dyes (TTC) or ferric ammonium citrate and sodium thiosulfate (FACST)

Assay and enumeration. Maximising EOP

- Electrolyte or co-factor requirements
- Use of indicator strains
- Growth phase of host
- Hydrogen-ion concentration
- Influence of growth medium
- Incubation temperature
- Temperature at which phage adsorption occurs
- Incorporation of glycine in growth media
- Enhancing plaque definition by using dyes (TTC) or ferric ammonium citrate and sodium thiosulfate (FACST)

Assay and enumeration. Effect of host and growth phase

PFU/ml *

	HOMOLOGOUS HOST		HETEROLOGOUS HOST**	
Phage	**Logarithmic phase (a)**	**Stationary phase (b)**	**Logarithmic phase (a)**	**Stationary phase (b)**
am1	13.0×10^8	8.0×10^8	19.0×10^8	24.0×10^8
am2	10.5×10^8	2.0×10^8	15.0×10^8	22.0×10^8

*Plaque forming units
**Determined on *Str. cremoris* US3
(a) 1% inoculum, 6 hours, 30°C
(b) 1% inoculum, 18 hours, 30°C
From: Mullan (1979)

Assay and enumeration. Effect of growth media

	PFU/ml*		
Phage	**PLGYG**	**PLGYG + 1.18 % glycerophosphate***	**M17**
am1	8.0×10^8	6×10^8	$<10^7$
am2	1.5×10^9	$<10^7$	$<10^7$
c10(1)	5.1×10^9	5.1×10^9	5.2×10^9
c13	3.2×10^9	3.0×10^9	3.1×10^9

*Plaque forming units
Contains the same glycerophosphate content as M17

Assay and enumeration. Effect of temperature of phage adsorption on

	PFU / ml* 10^8	
Bacteriophage	**Adsorption at 46°C (agar)**	**Adsorption at 20°C**
am1	3.3	8.0
am2	2.2	6.2
c8	32.0	38.0

*Plaque forming units

Contains the same glycerophosphate content as M17

From: Mullan, W.M.A. (1979)

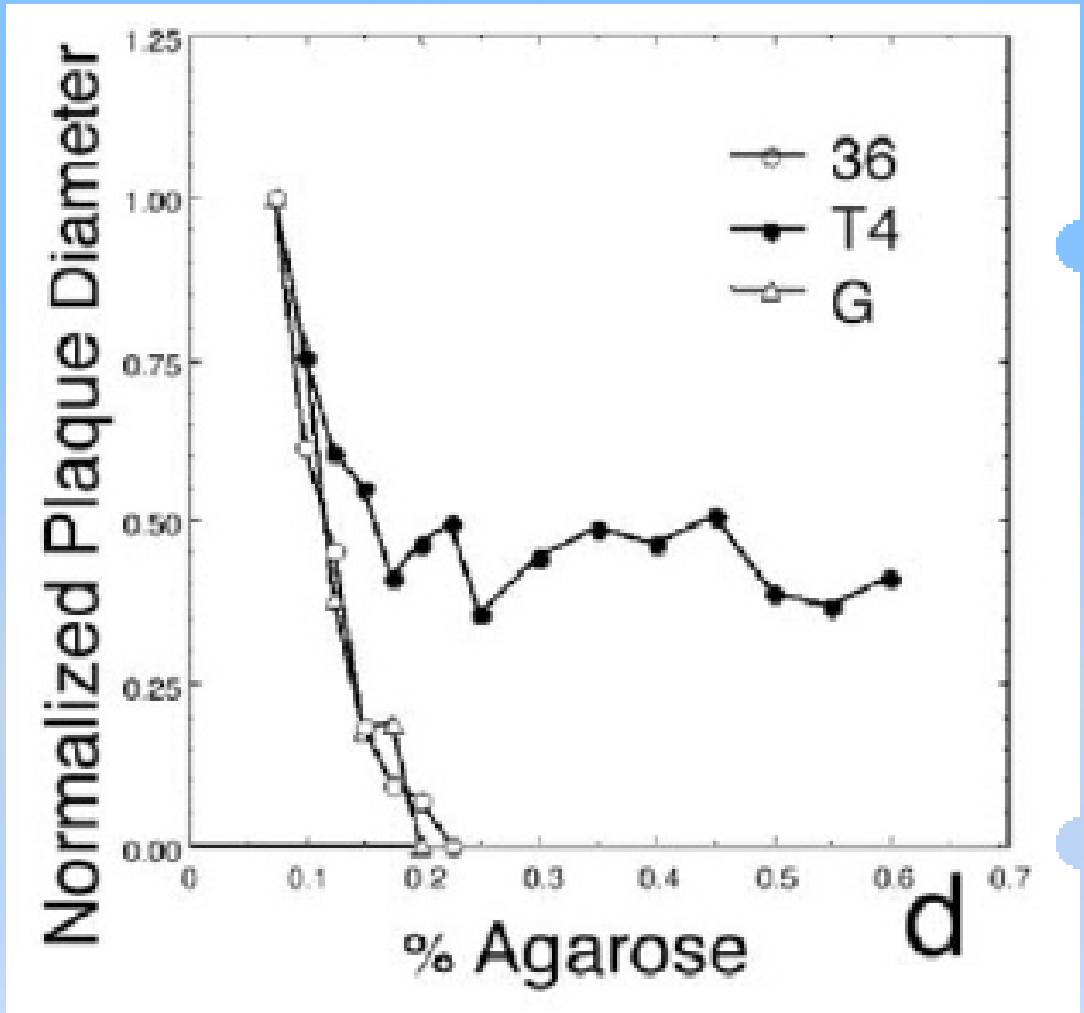
Assay and enumeration. Solving the great plaque count anomaly?

- Agrose is preferred to agar because of its reduced concentration of agaropectin, the component containing most of the sulphate and carboxyl groups which may inhibit some viruses

- Serwer *et al*. (2007) found that a *Bacillus thuringiensis* phage* would plaque if the agrose concentration was < 0.2%. **Good results with 0.15% agrose. > 4% no plaques.**

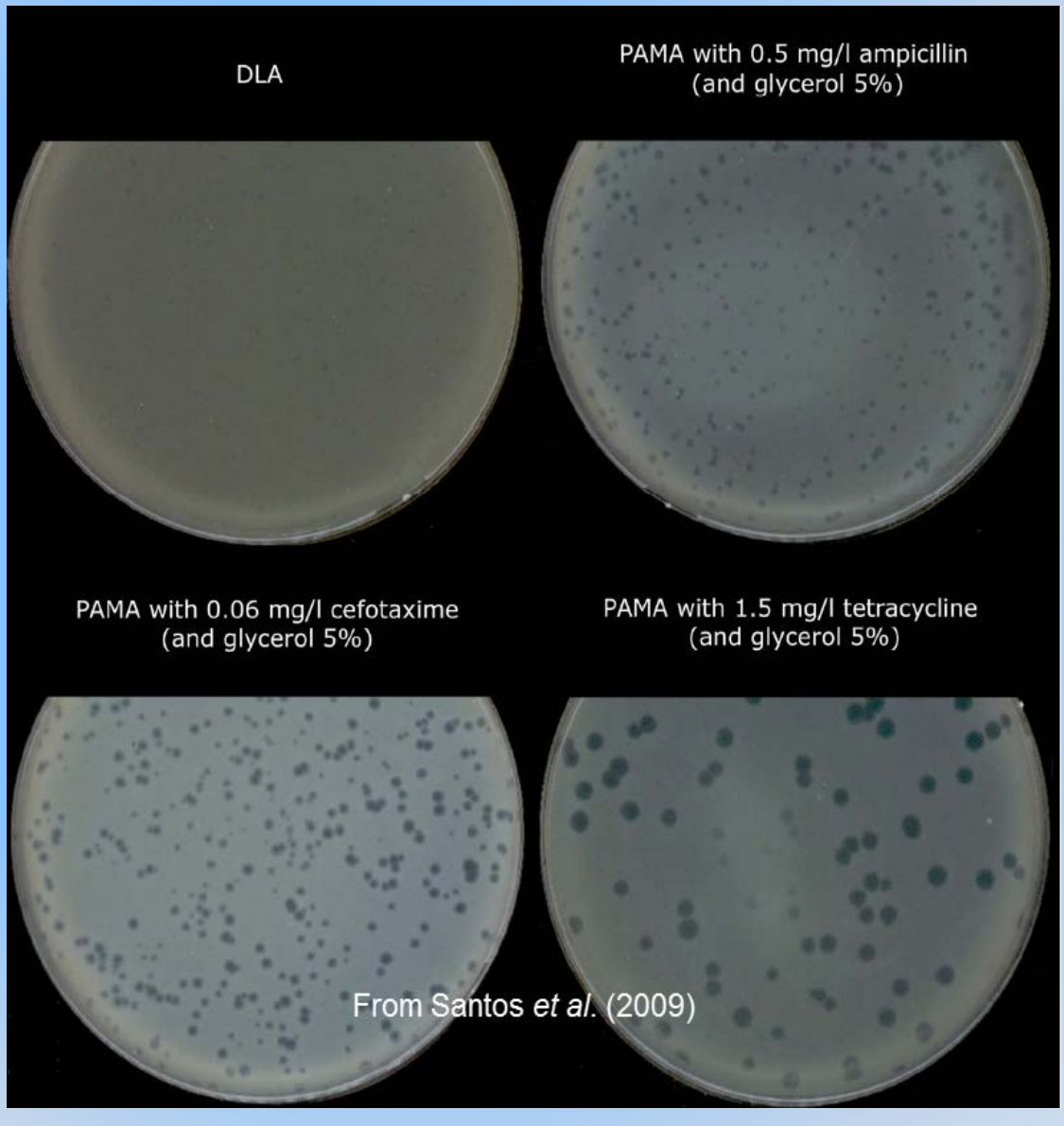
* 0305ϕ8-36 . Source soil. Aggregates. Slow growing. Apparent burst size = 22–30 particles after 60 min.

Solving the great plaque count anomaly? Importance of concentration of gelling agent in the soft agar



From: Serwer *et al*. (2007)

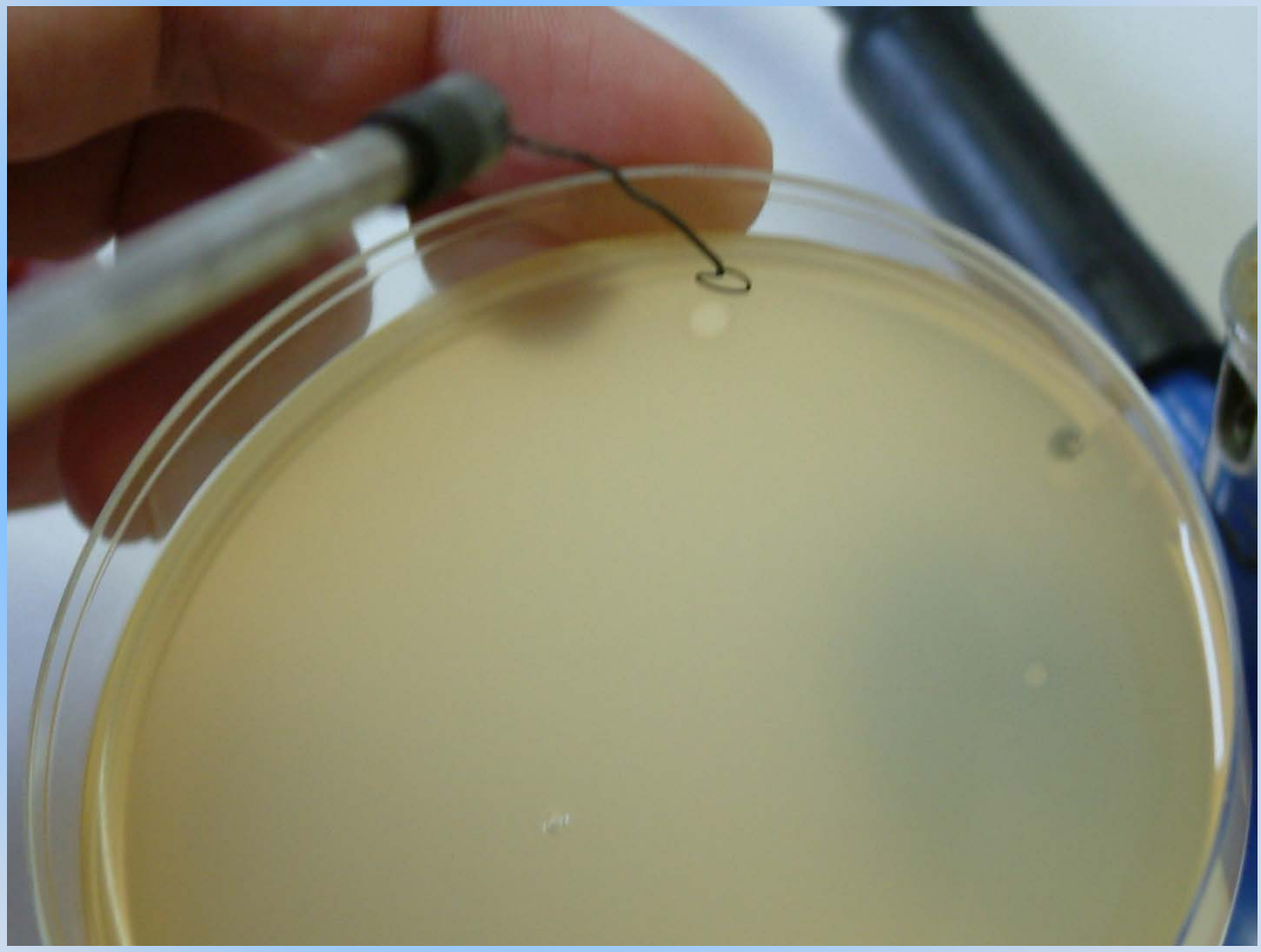
Solving the great plaque count anomaly? Use of antibiotics and glycerol



Solving the great plaque count anomaly? Development of phage sensitive indicator strains

- Over 50 known natural phage resistance mechanisms have been found in lactococci (Coffey and Ross, 2002).
- Adsorption blocking, DNA injection blocking, Restriction Modification and Abortive infection
- Exopolysaccharide or capsule production may also be a potential defence mechanism
- Most natural defensive mechanisms are not integrated into the bacterial chromosome e.g. on plasmids
- Superinfection immunity can be conferred by resident prophages and can prevent initiation of the lytic cycle.
- Bacteria can easily be cured of plasmids and many prophage suggesting the possibility of engineering indicator strains with broad phage sensitivity.

Isolating and purifying phage



Isolating and purifying phage 1

Isolating and purifying phage 2

Conclusions

- Phage enumeration is important in phage control programmes and the plaque assay remains an important analytical method.
- There is now a significant body of research suggesting how “non plaquing phages” can be made to plaque.
- While I have discussed many of the negative factors associated with phages they can be used to control pathogens: on field crops, foods, and food environments. Their potential role in eliminating biofilms in food plants is one of the many applications being actively pursued.

Thank you!