Ability of Urografin Density Gradients to Separate Nonculturable Subpopulations of *Escherichia coli*

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Definition of Viable but nonculturable (VBNC) cells
Intact and metabolically active but nonculturable bacterial cells

Significance
- Cellular deterioration, conducting to bacterial death? (1)
- Response to adverse conditions, similar to spore formation? (2)

Question
Can VBNC cells recover culturability? Resuscitation? (3)

Problem (1)
- Recovery of growth capacity in true VBNC cells?
- Growth of a few remaining culturable cells?

Methods
- Elimination of remaining culturable cells by dilution techniques (4)
- Inhibition of growth of remaining culturable cells (5)
- Separation of nonculturable subpopulations from heterogeneous suspensions (6)

Aim of the work

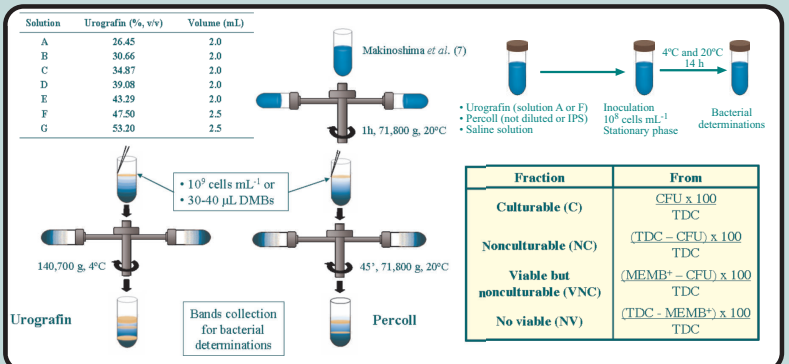
To assess the ability of Urografin to separate nonculturable subpopulations from heterogeneous bacterial suspensions

METHODOLOGY

Strain: *E. coli* STCC 416
Origin of cells: growth curves (MB, TSB, LB), mixed cultures (stationary phase and aged cultures) or stress exposure (starvation or acid pH)
External standard of density: Density Marker Beads (DMBs, Amersham Biosciences)

Bacterial determinations
Total number of bacteria (**TDC**) (8), bacteria with intact cytoplasmic membranes (**MEMB$^+$**) (9), colony-forming units (**CFU**) on Tryptone Soya Agar plates. Flow cytometry measurements with a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA).

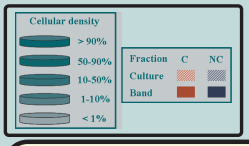
Solution	Urografin (%, v/v)	Volume (mL)
A	26.45	2.0
B	30.66	2.0
C	34.87	2.0
D	39.08	2.0
E	43.29	2.0
F	47.50	2.5
G	53.20	2.5



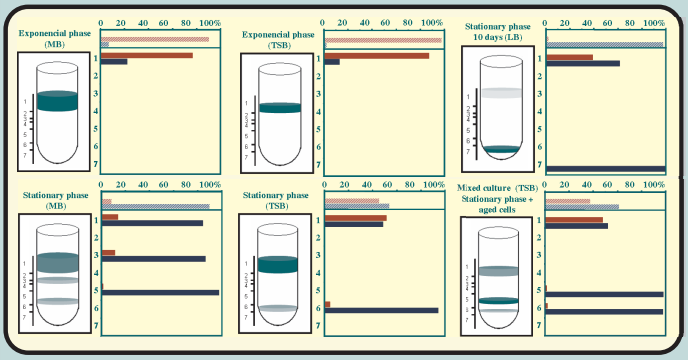
Fraction	From
Culturable (C)	CFU x 100 / TDC
Nonculturable (NC)	(TDC – CFU) x 100 / TDC
Viable but nonculturable (VNC)	(MEMB$^+$ – CFU) x 100 / TDC
No viable (NV)	(TDC - MEMB$^+$) x 100 / TDC

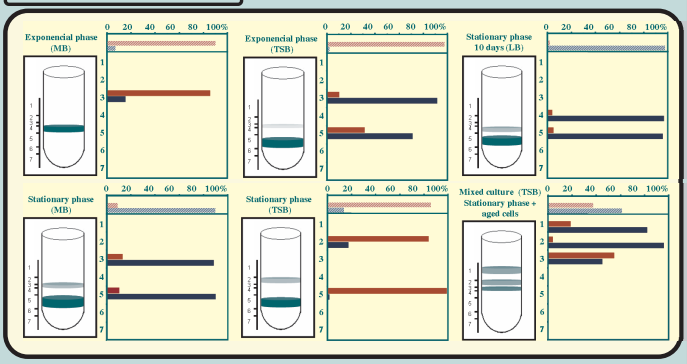
REMARKABLE FACTS

Fractionation in Urografin gradients: very diverse results were obtained when physiologically heterogeneous populations from different culture media, in different phases of growth, and even populations subjected to stress were examined. Formation of low- and high-density bands was observed. The high-density bands (horizon 5 and below) showed a homogeneous composition, mainly of nonculturable cells. In contrast, low-density bands had a heterogeneous composition, being mixtures of culturable and nonculturable cells.

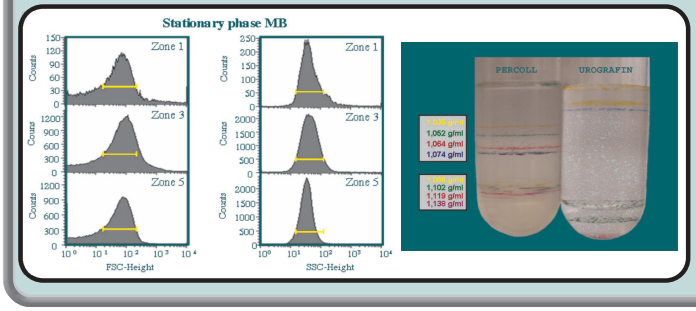


Fractionation in Percoll gradients: greta differenceds with respect to the centrifugation in Urografin gradien were detected. A great heterogeneity was observed in number and distribution of bands regardless of the origin of layered cells. Moreover, there was no correlation between band location and the kind of cells





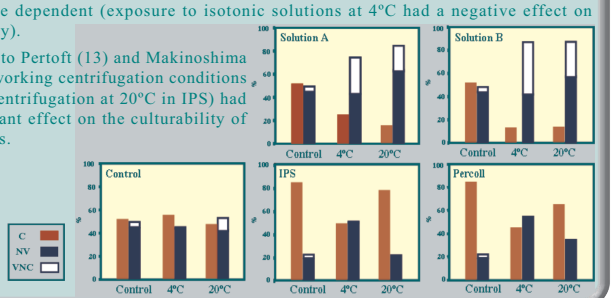
Bases for separation of VBNC cells: The separation of culturable and nonculturable cells by means of Urografin gradients has been reported (6, 10) to be based on slight difference in cell densities. A greater density is assigned to nonvulturable cells, which are thus understood to concentrate in high-density band.
From our results: 1) the increase in cells density would not be to all nonculturable cells (part of these cells banded in the same zone as culturable cells), 2) significant differences in cells size (FSC-Height) or complexity (SSC-Height) were no found between bands, 3) in Urografin gradients, DMBs were distributed in each band according to density, but vertical distribution was not observed.



Toxicity of fractionation methods based on density gradient: Urografin has been described as toxic to bacterial cells (11, 12). Centrifugation in density gradients of Urografin may have a harmful effect upon cellular state. This effect depends on time and temperature of centrifugation, as well as on concentration. Our results indicate that Urografin has a toxic effect on cell culturability. The decrease in percentage of culturable cell, and the concomitant increase in the nonculturable fraction, was mainly due to the formation of viable but nonculturable cells rather than the loss of viability.

Percoll is regarded as non-toxic to cells (13). Undiluted Percoll had a more negative effect upon culturability than isotonic Percoll solution (IPS) and the effect of Percoll was temperature dependent (exposure to isotonic solutions at 4ºC had a negative effect on culturability).

According to Pertoft (13) and Makinoshima et al. (7), working centrifugation conditions (< 1 h of centrifugation at 20ºC in IPS) had no significant effect on the culturability of *E. coli* cells.



CONCLUSION

We can thus conclude that Urografin density gradients are a good tool to obtain homogeneous nonculturable subpopulations from a heterogeneous *E. coli* populations. However, it is necessary to pay special attention to working conditions to avoid Urografin toxicity.

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