Small colony variants and senescent bacteria

By

Jaison Jacob

Small colony variants (SCVs)

- SCVs are bacterial subpopulation that grow slowly and form smaller colonies
- SCVs have been described for a wide range of bacterial species. They have been most extensively studied for Staphylococci
- They are considered as mutants. Majority of them are auxotrophic to hemin, thiamine or thymidine. In the presence of auxotrophic agents, they revert to normal growth
- Can be selected using aminoglycoside antibiotics
- Are tolerant to many antibiotics
- Are implicated in chronic and persistent infections

Proctor, R. A. et al. 2006. Nature Reviews Microbiology 4: 295-305

Current Knowledge on SCVs

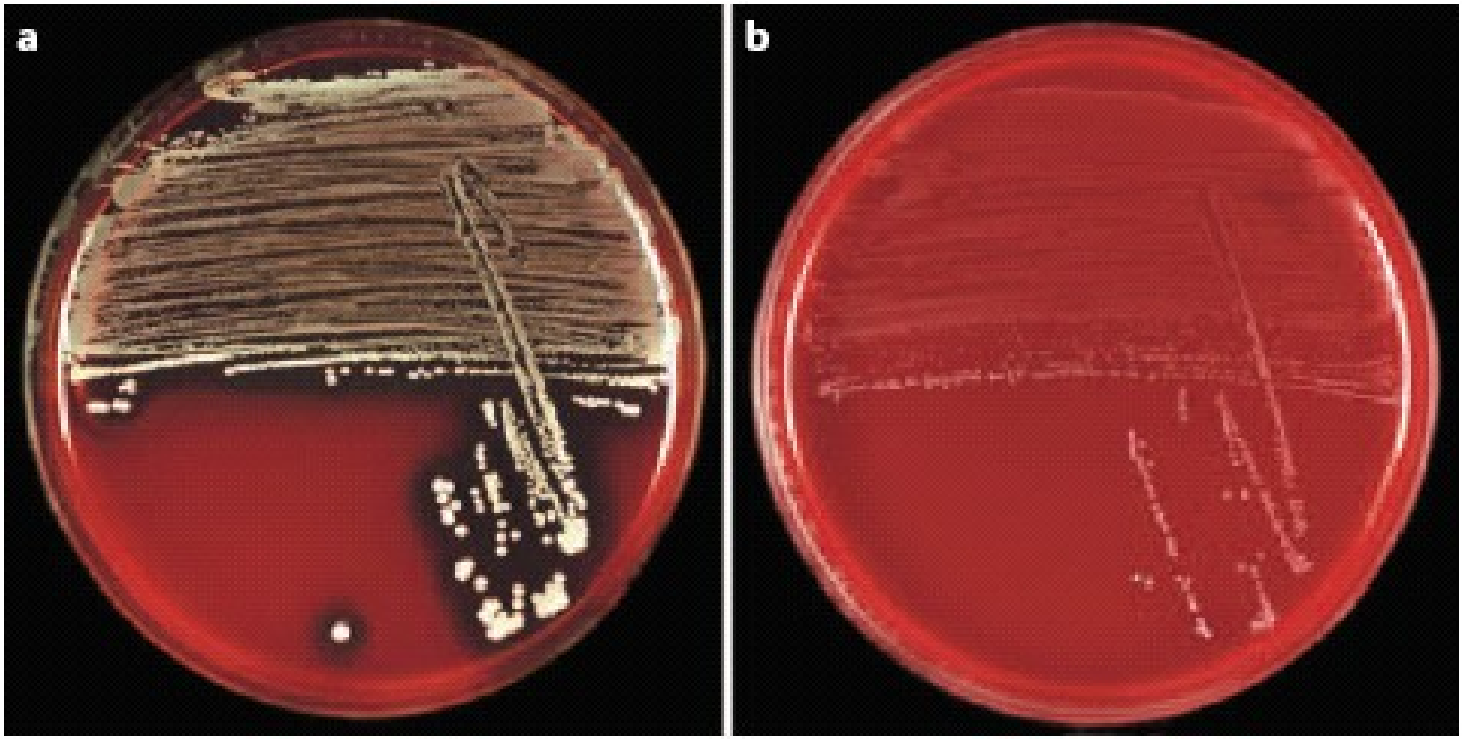
- Mutations in genes involved in hemin, thiamine or menadione production results in reduced TCA cycle metabolism and consequently reduced electron transport (ET), yielding SCVs

- Uptake of aminoglycoside antibiotics (kanamycin, gentamicin etc.) depend on bacterial membrane potential which in turn depend on ET. Hence reduced ET results in reduced uptake of aminoglycosides.

- Aminoglycosides, thus, can be used to select SCVs

Proctor, R. A. et al. 2006. Nature Reviews Microbiology 4: 295-305

Small colony variants



Normal *Staphylococcus aureus* (a) and their SCVs (b) grown on Columbia blood agar plates

Proctor, R. A. *et al.* 2006. *Nature Reviews Microbiology* **4**: 295-305.

Current Knowledge on SCVs (cont.)

- Switch between SCV and normal (revertant) phenotype may occur when bacteria is exposed to cycles of gentamicin followed by antibiotic free medium (1)
- SCVs have the ability to enter into and persist within epithelial and endothelial cells (2)
- They have low levels of toxin production, are more resistant to intracellular host defence and show decreased activation of host immune system (3)
- Frequency of SVCs vary and most of them are reported to be isolated from clinical cases exposed to long antibiotic therapy (4).
- In one study (4), no SCVs of *S.aureus* were recovered from 10 patients who did not receive gentamicin beads indicating that SCVs can be isolated only in certain conditions

Isolation of *E.coli* and *Salmonella enteriticus* SCVs

- 50 µl of *E.coli* DH5α , BL21 DE-3 and *Salmonella enteriticus* grown overnight, was added to 3 ml LB medium containing 0, 10, 20, 30, 40, 50, 60 and 70 µg kanamycin and incubated at 37 degrees for 48h

- Cells were plated on LB agar

Isolation of SCVs- critical steps

- Stage of bacterial growth: stationary phase bacterial cultures have more SCVs and hence should be used
- Initial size of inoculum: If high, some normal bacteria may escape killing, which will repopulate on antibiotic-free medium giving false negative results
- To ensure complete killing of normal bacteria, 50 µl of sample was withdrawn when the cells incubated with kanamicin reached an O.D. of 0.2 to 0.3, and was added to fresh LB medium containing the same concentrations of kanamicin. This was followed by incubation for 48 h

Results

- SCVs could be isolated from any normal colonies of *E.coli* DH5α , BL21 DE-3 and *Salmonella enteriticus*

- SCVs were not a single subpopulation, but rather comprise many subpopulations. As concentration of kanamycin increased, the size of colonies decreased sequentially

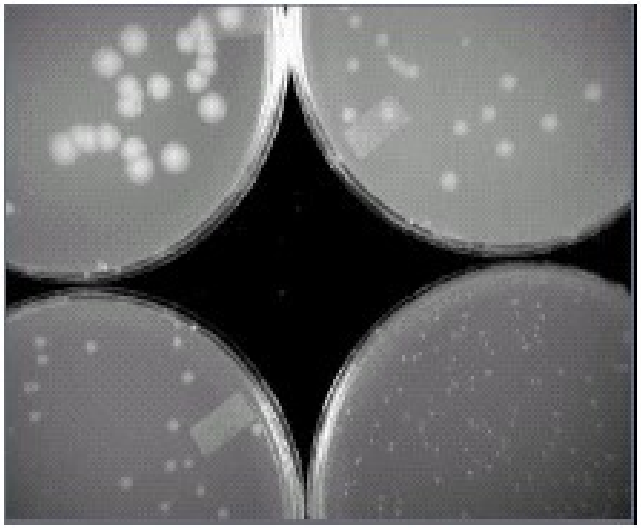
- No switching between SCVs and normal phenotype noticed

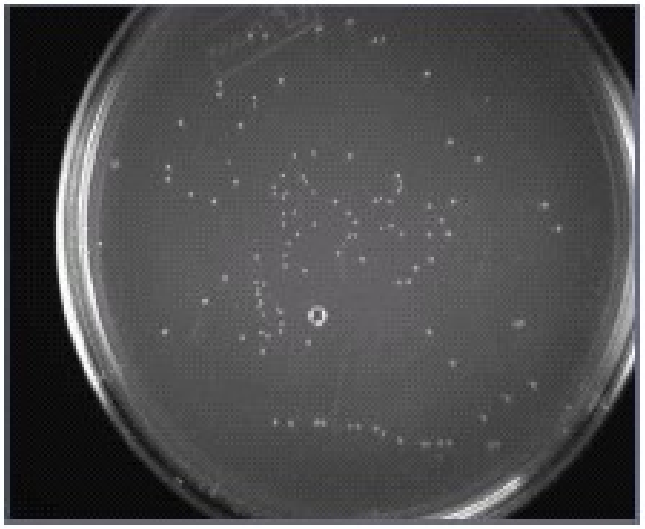
- Slow growing bacteria can be removed after repeatedly growing in early exponential phase. However, they reappeared following overnight incubation

- SCVs have reduced GFP expression

Results

A





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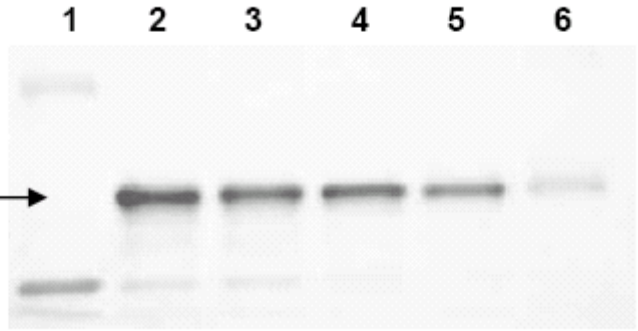

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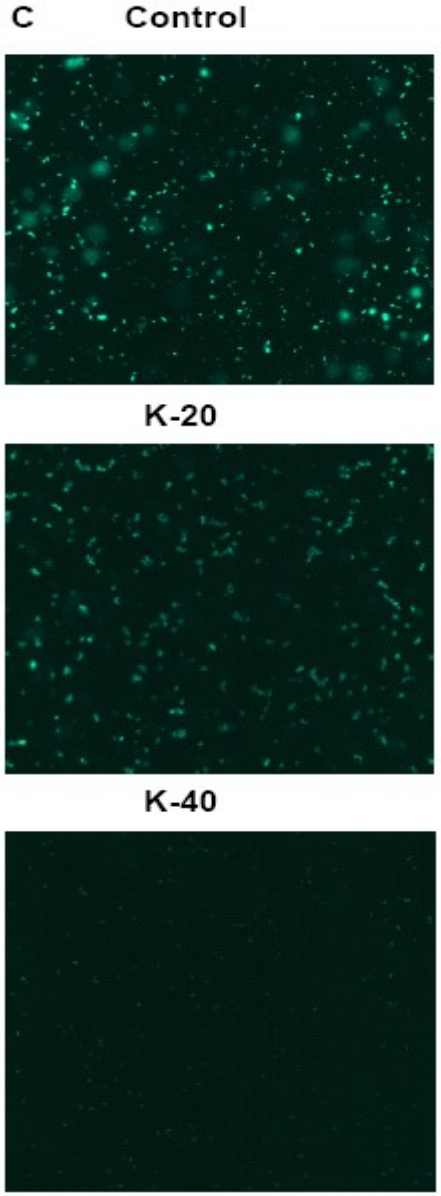


A. Subpopulations of SCVs isolated using different kanamycin concentrations

B. GFP expression levels of different subpopulations by western blot

C. GFP expression using immunoflouroscence

Conclusion

Majority of SCVs are senescent bacteria that do not revert to normal phenotype

Senescent bacteria

- The uptake of aminoglycosides into bacteria depends on their membrane potential (MP)
- In many species, MP reduces with age due to reduced expression of electron transport (ET) pathway and seems to be a common signature of aging (5)
- Even though lifespan varies greatly between species, the level of age regulation of ET pathway is nearly the same (5)
- It can be assumed that this is true in bacteria also
- Young, active bacteria may have high MP resulting in a higher uptake of aminoglycoside leading to their rapid elimination. Aging bacteria may have low MP and escape killing due to reduced uptake and are thus selectively grown.

Types of SCVs and their growth characteristics

- Most SCVs are considered as mutants
- However, my findings indicate that majority of them can be senescent bacteria
- Senescent bacteria and mutants can be differentiated by growing them in medium containing known auxotrophic agents
- Apart from these two, bacteria under repair or those which have a transient growth inhibition may also form SCVs. They are normal bacteria that do not grow during repair phase and hence form small colonies. After penicillin treatment, many SCVs can be isolated that revert to large colonies on subculture

Types of SCVs and their growth characteristics

Type of bacteria forming SCVs	**Colony sizes on subculture**	
	Minimal media	Rich media
Respiratory deficient mutants	small	large
Senescent bacteria	small	small
Bacteria under repair	large	large

Aminoglycoside resistance by SCVs

- At 60 µg/ml of kanamycin, no growth of E.coli was noticed
- At 50 µg/ml, SCVs were isolated, that, when grown were resistant to even 100 µg/ml of kanamycin
- If SCVs were mutants, they could have been isolated from parent culture itself treated with 100 µg/ml of kanamycin
- ‘ Mutant theory’ thus cannot explain the increased antibiotic resistance by SCVs

Aminoglycoside resistance by senescent bacteria

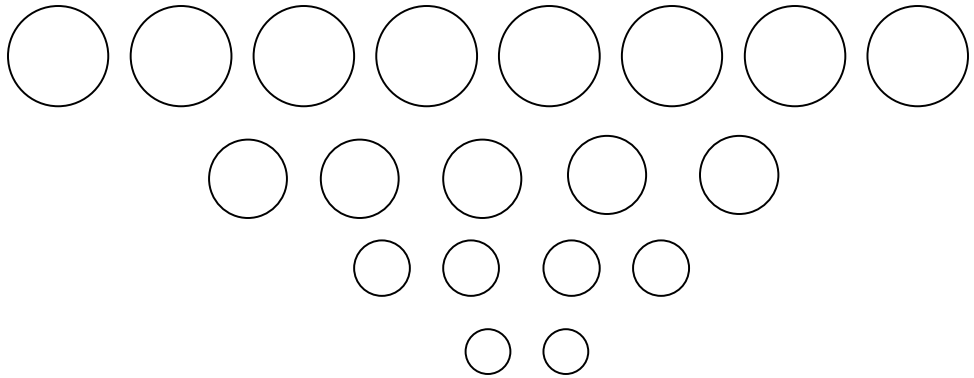
- In the parent culture, senescent bacteria can not divide more as they have the growth disadvantage. By the time the culture reaches stationary phase, it mostly consists of fast growing young population

- However, on removal of fast growing population by kanamycin, senescent bacteria are selected which then can further divide and give rise to more senescent ones which are more tolerant to aminoglycosides as they have still lower membrane potential

- Thus, the production of still more aged bacterial population can explain the increased resistance of SCVs to kanamycin

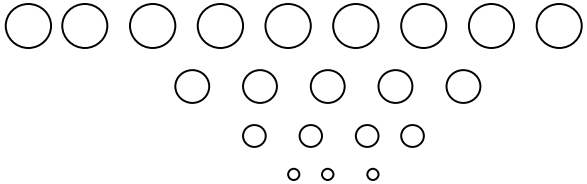
Aminoglycoside resistance by senescent bacteria

At stationary phase



At stationary phase, most of the bacteria are normal fast dividing ones. Senescent bacteria is unable to divide further due to their growth disadvantage.

After kanamycin treatment



After kanamycin treatment, fast growing bacteria are selectively killed, leaving senescent ones, which further divides to give rise to more senescent ones, that are more resistant to kanamycin due to its reduced uptake resulting from their still lower membrane potential

Concentration dependent killing of aminoglycosides

- Aminoglycosides exhibit concentration dependent killing (unlike penicillin group which exhibit time dependent killing)
- Senescent bacteria can explain this phenomenon
- At low aminoglycoside concentrations, only the young, fast dividing bacteria are killed, leaving senescent, slow dividing, small colony forming bacteria
- With increasing concentrations, more and more of the senescent bacteria are killed till it reaches MBC, wherein all bacteria are killed
- Thus, killing of bacteria by aminoglycosides depends on the antibiotic concentration

Long post-antibiotic effect (PAE) of aminoglycosides

PAE is the period of time after removal of an antibiotic during which there is no growth of the bacteria (6)

Aminoglycosides exhibit long post-antibiotic effect

Senescent bacteria can explain this phenomenon

At concentrations below MBC, fast growing bacteria are selectively killed, leaving mainly slow growing bacteria which takes longer time for re-growth

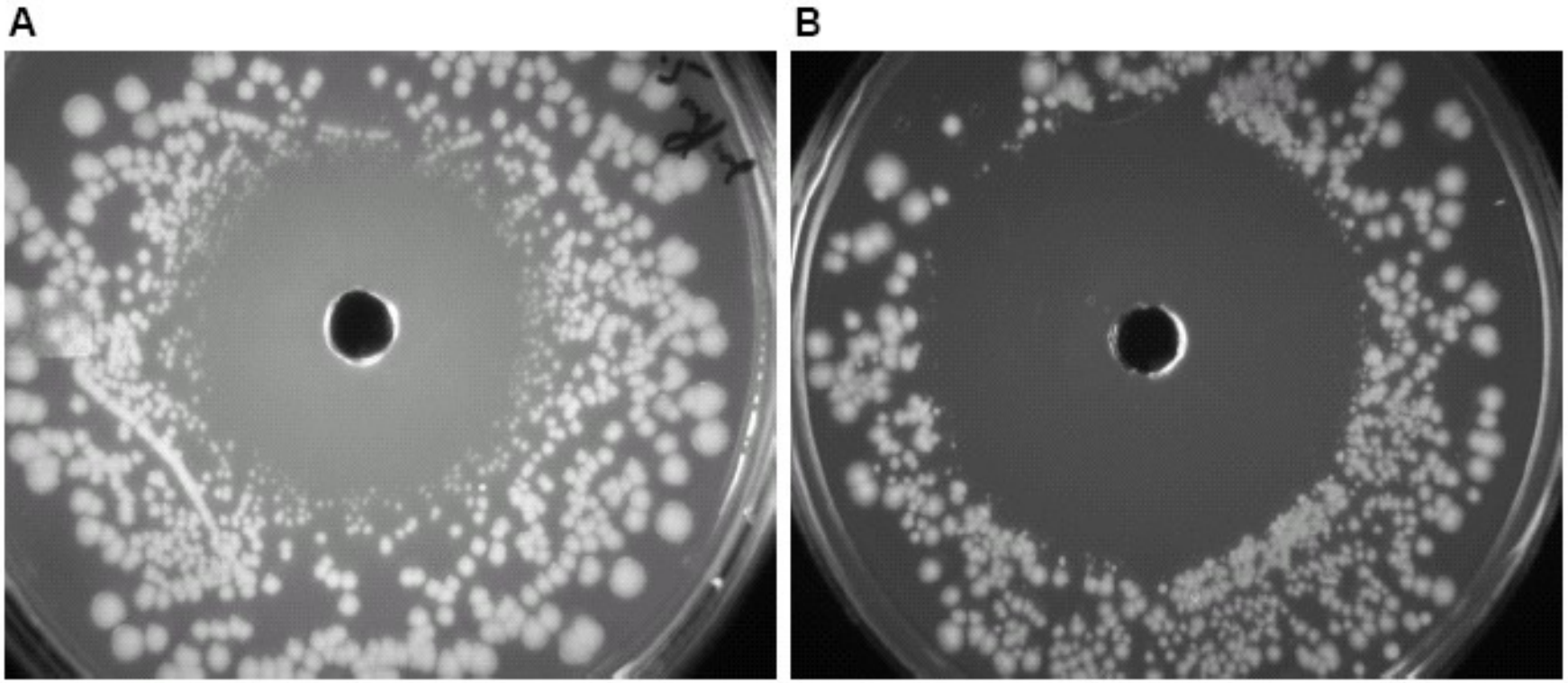
Long PAE of aminoglycosides

PAE of aminoglycosides can be increased by

- Increasing the concentration of aminoglycosides (more slow growing bacteria are selected; hence take longer time for growth)
- Increasing the time of incubation (more ‘ perfect’ killing of fast dividing normal bacteria)
- Using optimal initial inoculum size (a high initial inoculum size may not kill all normal, fast dividing bacteria)

Penicillin exhibit low PAE (there is no selection of slow growing bacteria; hence bacteria that survives are normal population which grow immediately)

E.Coli killing by penicillin and aminoglycosides



A. Area at the zone of inhibition after kanamycin treatment consists of only small colonies. Larger colonies are seen radiating out as the antibiotic concentration decreases.
B. Persisters remaining after ampicillin treatment. The zone of inhibition is abrupt and has both large and small colonies.

Jacob, Jaison. 2009. Available from Nature Precedings
http://hdl.handle.net/10101/npre.2009.1411.2

New findings and hypothesis on SCVs

Current Knowledge	New findings and hypothesis
SCVs are reported as single subpopulation	SCVs consists of a number of subpopulations with different growth rates
Are mutants	Majority are normal senescent bacteria; occasionally mutants may also be present
Can be isolated from a few colonies only	Can be isolated from any colonies sensitive to aminoglycosides
Revert in the presence of auxotrophic agents	Senescent bacteria do not revert; mutants revert in presence of auxotrophic agents
Are less virulent	Less virulent
Responsible for chronic infections	Few may cause chronic infections. Higly senescent bacteria may not cause chronic infection
Tolerant to many antibiotics	Tolerant to antibiotics; but can be killed by increasing the duration of treatment (except by aminoglycosides)
Pose public health hazards	Most of them do not pose public health hazards as they are less virulent and do not revert. Mutants may pose threats.

Future directions

Senescent bacteria- a gold mine for immunologists?

- As bacteria undergoes senescence, they exhibit less growth rate, form small colonies and are less virulent
- More knowledge on the regulation of virulence can be obtained by studying bacterial subpopulations of increasing senescence
- Senescent bacteria are potential live vaccines as they do not revert and are less virulent
- They are good model for aging and epigenetics research

Senescent bacteria as potential live vaccines

- **‘ Senescent bacterial vaccines’ have the potential to become an effective and ideal group of vaccines because**
- They may elicit good immune responses as they are live and are capable of multiplication.
- Their virulence is, predictably, very low. They are slow dividing and exhibit reduced virulence, electron transport chain pathway, GFP levels etc.
- Reversion frequency may be low or absent as they are attenuated naturally by aging.
- The use of naturally attenuated bacteria stimulates immune responses to antigen in their natural conformation itself and can be superior to the traditional methods of attenuation.

Other predictable advantages of ‘ senescent bacterial vaccines’ include

- Easy to isolate senescent bacteria required for vaccine development
- Vaccines can possibly be developed against most vaccine preventable diseases of bacterial origin as aging can be a universal phenomenon.
- Prior knowledge on virulent proteins/genes not required.

Conclusions

- SCVs comprises many subpopulations
- SCVs can be isolated from any bacterial colonies sensitive to aminoglycosides
- Isolation of SCVs alone may not indicate chronic infections
- Concentration dependent killing by aminoglycosides can be explained by senescent bacteria
- Long PAE of aminoglycosides can be explained by senescent bacteria
- Aminoglycoside resistance by SCVs can be explained by bacterial senescence, but not by ‘ mutant theory’
- Senescent bacteria is an important source of bacterial heterogeneity and noise in protein expression

Proposal

- Senescent bacteria can be potential live vaccines
- They can be excellent models for future studies in aging, epigenetics and immunology

Prediction

- SCVs can still be isolated from hemB mutants (auxotrophic to hemin) and menD mutants (auxotrophic to menadione) grown in the presence of hemin and menadione respectively
- Logic: Mutants will revert to normal bacteria in the presence of auxotrophic agents which on further mutiplication will give rise to senescent bacteria that will form SCVs which will not revert in the presence of these agents

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