Persisters show heritable phenotype and generate bacterial heterogeneity and noise in protein expression

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Abstract

Persisters are a small subpopulation of bacteria that survive lethal concentrations of antibiotic without antibiotic resistance genes. Isolation of persisters from normally dividing population is considered difficult due to their slow growth, low numbers and phenotypic shift i.e. when re-grown in antibiotic free medium, they revert to parent population. Inability to isolate persisters is a major hindrance in this field of research. 'Phenotypic shift' of persisters observed previously is questioned here. Persisters, on the other hand, may exhibit a heritable phenotype and hence can be easily isolated from a normally dividing population by allowing their selective growth. Rather than a single subset, they comprise many distinct subgroups each exhibiting different growth rates, colony sizes, antibiotic tolerance and protein expression levels. Clearly, they are one of the sources of bacterial heterogeneity and noise in protein expression. Existence of persisters in normally dividing population can explain some of the unsolved puzzles like antibiotic tolerance, post-antibiotic effect and viable but non-culturable bacterial state. It is hypothesized that persisters are aging bacteria.

Key words: Persisters, bacterial aging, post-antibiotic effect, small colony variants, antibiotic tolerance, viable but non-culturable bacteria

Introduction

Persisters were first described by Joseph Bigger in 1944 when he found that a culture of *Staphylococcus* spp. was not completely sterilized by a lethal concentration of ampicillin (5). Most of the bacteria were lysed by ampicillin, but a small subpopulation

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somehow survived. When the surviving bacteria were grown in antibiotic free medium, they grew just like parent population which was again susceptible to ampicillin. These surviving bacteria or persisters either grew slowly or did not grow at all in the presence of antibiotics but reverted to normal growth on removal of antibiotics (3, 21). Since the persisters reverted to original population on removal of antibiotics, they were considered as dormant bacteria that avoided killing by antibacterial agents (21). This phenotypic switch is widely considered as a survival strategy of bacteria against antibiotics (21). Due to this phenotypic shift, it was difficult to isolate persisters from a normally dividing population (21). Hence most of the studies on persisters were done with E.coli mutant hipA, which was reported to produce high frequency of persisters (6, 20, 23, 24).

Single persister cell was studied using microfluidic devices which identified at least two types of persisters (3). Type-1 persisters are formed in the stationary phase and thus constitute a pre-existing population of non-growing cells. However they revert to growing cells in antibiotic free medium with an extended time lag. On the other hand, type-II persisters do not originate from stationary phase but are continuously generated during the growth phase. Thus a wild type population consists of normal cells, type I and type II persisters (3).

Persisters are not mutants nor induced by the antibiotics, but are preformed in a culture (3, 23). During the lag and early exponential phase of growth, persister formation is very low but their number increases during mid to late exponential phase of growth (19). By growing bacteria repeatedly at early exponential phase of growth, persisters can be eliminated completely (19).

Persisters may be responsible for the resistance of biofilms to antibiotics (8, 33). A biofilm is formed when bacteria grow on a surface and is enclosed in an exopolymer matrix. Biofilms are nototrious for racalcitrant infections and resistance to antibiotics (1, 10) and is responsible for more than 65% of infections in the west (11). Isolation of persisters from a normally dividing population may help in understanding the pathogenesis of biofilm resistance to antibiotics.

Here it is reported that bacterial population consist of persisters that are tolerant to antibiotics. However, they show a heritable phenotype, rather than exhibiting a phenotypic shift. 'Phenotypic shift' of persisters reported earlier can be due to improper experimental settings. Persisters can be isolated from normally dividing population by utilizing the 'concentration dependent killing' property of aminoglycosides. Persisters thus isolated show many significant differences from those reported earlier apart from the lack of phenotypic shift. They comprise many subgroups which are formed during all phases of bacterial growth. Each subgroups exhibit their own characteristics properties including a difference in protein expression levels. The existence of persisters is suggested as one of the important source of bacterial heterogeneity and noise in protein expression. They can be aging bacteria and hence can be isolated from most bacterial cultures.

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Materials and methods

Bacterial strains, growth conditions, antibiotics and chemicals

The bacterial strains used were *E.Coli* DH5α and BL-21(DE-3) cells (Invitrogen). *Salmonella enterica* serovar Typhimurium was a generous gift from Dr. Suman Mukhopadhyay, VA-MD Regional college of Veterinary Medicine. *E.Coli* DH5α with pET 14-B plasmid carrying GFP was a gift from Dr. Iqbal Hamza, University of Maryland, College Park. All antibiotics (kanamycin, ampicillin, tetracycline and nalidixic acid) were obtained from Sigma. Concentrations of stock solution of the antibiotics were kanamycin-10mg/ml, ampicillin- 50mg/ml, tetracycline- 5 mg/ml and nalidixic acid-15 mg/ml.

Luria-Bertani (LB) broth and LB agar base were used to culture the above cells. Chemicals used were Glycine, Tris (Fischer), acrylamide, temed, SDS, Ammonium per sulphate, bromophenol blue (Biorad), BCA protein assay reagents (PIERCE), Thiamine, thymidine (sigma) mercaptoethanol (Invitrogen

Isolation of persisters using kanamycin

For isolating pure cultures of persisters, 50µl of overnight culture of *E.Coli* DH5α was added to 3 ml of fresh LB medium containing kanamycin at concentrations of 10, 20, 30, 40 and 50µg/ml and incubated at 37^0C for 48-60 hours at 240 r.p.m. When the culture reached an O.D. of approximately 0.5, 200 µl was withdrawn and centrifuged to remove the supernatant containing antibiotics. The pellet was washed with fresh LB medium twice. After the second wash, approximately equal concentration of bacteria was added to

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3ml of LB medium containing the same concentration of kanamycin and incubated as before. The whole procedure was repeated once again to ensure that persister culture was not contaminated by any normal cells. Growth rate of bacteria was determined from the O.D. measured at definite time periods using nanodrop ND-1000 spectrophotometer. The measurable range of O.D. by nanodrop spectrophotometer is 0.01- 0.2 which is almost equivalent to O.D. value of 0.1-2 in classical cuvette based systems, as per manufacturer's manual. Hence, the O.D. value obtained in nanodrop spectrophotometer will be multiplied by a factor of 10 to make the reading equivalent to cuvette based system.

For growth on agar, 50 µl of stationary phase culture was serially diluted 1 in 10 times up to 10^{-8} concentration. 30µl from each dilution was plated on LB agar and incubated at 37^0C for 48-60 hrs. Plates containing uniformly spread colonies were selected. Same method was followed for other antibiotics. Colonies on agar were then photographed using chemidoc system.

For heritability test, 50µl of persisters isolated were centrifuged to remove the supernatant containing antibiotics and the pellet was washed with fresh LB medium without antibiotics twice. Approximately equal concentration of bacteria, determined from O.D. of culture, from each group of persisters were added to 3 ml of fresh LB medium without antibiotics and incubated. Growth rate was determined as above. They were plated onto agar as described previously. The whole procedure was repeated twice to test the heritability.

Persister differences between antibiotics

50 µl of overnight culture of *E.Coli* DH5α was diluted to a concentration of $10^{-4}$to 10^{-5} and plated on LB agar. A hole was punch at the centre of the agar using a sterile pipette. Kanamycin (10 mg/ml) was added to fill the hole and the plate was then incubated at 37^{0}C for 48 hours. The same method was followed for ampicillin (50 mg/ml), tetracycline (5mg/ml) and nalidixic acid (10 mg/ml).

Initial inoculum size and time of exposure of antibiotic

50, 100, 250, 500 and 1,000 µl of overnight culture were added to fresh LB medium to make a final volume of 3 ml. 50µg of kanamycin was added to each and incubated as before. After 48 hours, approximately equal concentrations of bacteria (determined from O.D. values) were withdrawn, pelletted and washed with fresh LB twice and added to a final volume of 3 ml LB medium and incubated as before. Growth rate was determined as above.

To study the effect of time of exposure of antibiotics, 50 µl of overnight culture was added to 3 ml LB medium containing 50 µg/ml of kanamycin and incubated at 37^{0}as before. At designated time periods, samples containing approximately equal concentrations of bacteria (determined by O.D. values) were withdrawn and added to fresh LB medium without kanamycin to a final volume of 3 ml and incubated as before. Growth rate was determined as before.

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Persisters are not induced by antibiotics

50 µl of overnight stationary culture was added to 3 ml of LB medium and incubated as above. After reaching early exponential phase (O.D. approximately 0.5), 100µl of the culture was withdrawn and added to fresh medium and incubated again at same conditions. This cycle was repeated 4 times. 100µl of the mid-exponential phase culture from each cycle was added to 3 ml of fresh medium containing kanamycin at different concentrations as before and incubated for 48 hours and measured the growth rate. The mid exponential culture from the fourth cycle was allowed to reach a stationary phase by incubating overnight. This overnight culture with approximately equal concentration of bacteria as above was added to 3 ml of fresh medium containing same concentrations of kanamycin as above and incubated as before.

Auxotrophy of persisters

50 µl of persisters (K-30 and K-40) isolated were added to LB medium containing thiamine (Sigma) and thymidine (Sigma) at concentrations of 1, 3, 5, 10 and 20 µg/ml by adding these chemicals to LB medium and incubated as above. Growth rate was determined as before. They were also plated to agar containing thiamine and thymidine at these concentrations to test for reversion to large colonies.

Tolerance of persisters to different antibiotics

Approximately equal concentration of bacterial cultures of normal and persister cells were treated with the same and higher concentration of kanamycin at which they were

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isolated. Persisters were also treated with different concentrations of ampicillin and nalidixic acid. They were incubated for 48 hours as before.

SDS polyacrylamide gel electrophoresis and western blotting

pET-14B vector carrying GFP in *E.Coli* DH5α was transformed to BL21 (DE-3) cells by heat shock method. Persisters of BL21 (DE-3) cells were then isolated using kanamycin as before. Persisters thus isolated were grown in antibiotic free medium for 48 hours as described before. When persisters grown in antibiotic free medium reached stationary phase, they were pelletted, resuspended in PBS and heated at 95°C for 10 minutes. A small volume was used to determine total protein concentration by BCA protein assay (Pierce) with bovine serum albumin as standard and rest pelletted and stored at -20^0C . For immunoblotting, stored pellets were heated at 95°C for 2 minutes in the presence of 1xSDS loading buffer containing DTT. Samples were run on 12% SDS–PAGE and transferred onto nitrocellulose membrane (Biorad) using semi-dry apparatus (Biorad) at 9V for 45 minutes. Blots were blocked overnight using 5% non-fat dry milk and incubated with antibody against green fluorescent protein (Invitrogen) at a concentration of 1:5,000 for one and a half hours followed by incubation with secondary antibody(goat anti-rabbit conjugated with horse raddish peroxidase (biorad).Signal was detected using Amersham western blotting detection kit(GE healthcare)and blot quantified by chemidoc system. Protein bands were detected by Coomassie staining and molecular weight of proteins estimated using marker proteins (Kaleidoscope standards-Biorad).

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Immunofluorescence

Intensity of GFP fluorescence in normal wild type and persisters of BL21 (DE-3) cells were visualized from the fluorescent images captured on Leica DMIRE2 microscope fitted with a Q-imaging camera using Simple PCI software.

Statistical analysis

Data presented are given in mean ± standard error of mean (s.e.m.). The *n* for each data set is given below each table.

Results and discussion

Isolation of persisters

For the isolation of persisters, the 'concentration dependent killing 'property of kanamycin (12, 15, 36), an aminoglycoside that kills bacteria by inhibiting protein synthesis, is utilized here. Bacteria are killed by kanamycin in a growth rate dependent manner. At low concentration it kills fast dividing bacteria only, leaving the persisters which are slow growers. At high concentrations, slow growing cells also become succeptible, but again in a concentration dependent manner. This property of the antibiotic is utilized here to separate bacteria with different growth rates. The different groups of persisters will be designated as K-10, K-20, K-30 etc. depending on the concentration of kanamycin in μg/ml used for isolation of persisters. Among the persisters, K-10 had the highest growth rate and K-50, the least (Table.1). At kanamycin concentration of 60μg/ml, no growth could be detected. Since persisters divide slowly,

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they take more time to reach turbidity which is evident from the optical density (O.D.) readings of spectrophotometer. Extreme persisters like K-40 or K-50 never reach turbidity. They reached stationary phase before growing to turbidity**.** Above pattern of growth was observed with both *Escherichia coli* DH5-α and BL21 (DE-3) cells. With *Salmonella enteritica* serovar Typhimurium LT2, growth pattern was similar except that there was no growth of bacterium at kanamycin concentration above 30µg/ml (Table.1).

When plated on agar, they exhibited properties consistent with growth in liquid medium (Fig.1.A, B and C). The time taken for colonies to appear on agar increased with higher kanamycin concentrations. K-40 required 40-48 hours to form visible colonies on agar. There was clear difference in the size of colonies which decreased proportionally with higher concentration of antibiotic. Again in consistent with growth in liquid medium, the size of colonies of K-30 and K-40 did not increase much even after many days of incubation.

Two factors are important in the isolation of persisters without 'contamination' by normal fast dividing wild type bacteria. 1. low initial inoculum size and 2. prolonged time of exposure to antibiotics (Table.3). As initial inoculum size increases, the chances for bacteria to escape killing is more. The presence of a few normal cells that escape killing can completely change the picture as they have a definite growth advantage over persisters; hence it is important that all susceptible bacteria must be killed, leaving only the persisters. This can be ensured by repeatedly treating persisters at exponential phase of growth with the same concentration of kanamycin used to isolate them. 50µl of stationary phase bacterial culture was used for isolation of persisters which was the ideal

inocculum size for *E.coli* and *S.enteritica*. Similarly, an extended time period is also necessary to avoid ‘contamination’ by normal cells and to allow sufficient time for growth of persisters. A short exposure time below 9h did not kill all susceptible bacteria.

It is important to know whether the growth characteristics exhibited by different persisters are induced by the antibiotic itself. Earlier, it was reported that persisters are not induced by antibiotics (19). However it was also assumed that persisters comprise a single subpopulation which starts to appear in the early exponential phase, followed by a sharp increase in mid-exponential phase and reaches maximum in stationary phase forming approximately 1% of the total population. The experiment was repeated since a number of subgroups of persisters were isolated rather than a single subset. For this purpose,the procedure by Keren *et.al* (19) was followed with some modifications as described in methods section. By repeatedly growing bacteria at early-exponential phase of the cycle, bacteria with high growth rates were selected while slow growing ones were gradually diluted out. As the number of cycles increased, there was a gradual reduction in the number of persisters (Table.5). By the fourth cycle K-30, K-40 and K-50 were almost completely eliminated, but K-10 and K-20 still remained. However, all persisters reappeared in cultures from the fourth cycle incubated overnight. This experiment demonstrates that persisters are normally present in a bacterial population and they are not induced by antibiotics. Similarly, they comprise a number of subgroups and are formed during all phases of bacterial growth. In fact, stage of bacterial growth does not influence the formation of persisters. It depends only on the rate of growth of individual persister groups. Though all persister groups are present in a normal population, all of them cannot be detected in agar unless they are selected by eliminating fast dividing

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bacteria. They have definite growth disadvantage in the presence of normal wild type bacteria due to their slow growth and low numbers. Thus, extreme persisters like K-40 or K-50 can be included under viable but non-culturable bacteria (VNBC), bacteria that remain viable but cannot be cultured under normal circumstances (7, 25, 26).

Heritability of persisters

As the persisters showed visible differences of growth pattern in liquid and solid medium, the heritability of the persisters was tested by growing them in antibiotic free medium. Persisters grown in LB medium without antibiotics showed the same pattern of growth rate as the parent persister population even though the growth of persisters were faster when compared to their growth in presence of antibiotic (Table.1). This faster growth rate is due to the higher initial inoculum size of persisters. In solid medium, there were differences in the total time taken to form visible colonies, colony sizes and the growth rate of colonies (Table.1 and Fig.1.D and E). Similarly when largest colony from each group were picked and grown in antibiotic free medium for 48 hours followed by plating on agar, the same pattern was again observed. This was true at least for three generations. A small colony was never seen to revert back to a larger one.

Phenotypic shift by bacteria

Since persisters were reported to exhibit a phenotypic shift, the phenomenon need to be studied more precisely. For this purpose, bacteria were grown in a medium containing lethal concentrations of antibiotics as well as other stressors like low pH and high pH. It was observed that at lethal concentrations of any of these stressors, most of the bacteria

were killed and there was no visible growth (Table.4 and Table.2) However, when the bacteria were transferred to medium without the stressors, they showed luxuriant growth just as normal cells. These bacteria were not mutants as they were again susceptible to the same concentration of stressors previously used. This experiment showed that 'phenotypic shift' reported is not a property seen with antibiotics alone, but rather with any stressors. It can be assumed that, due to the lysis of majority of the bacteria by lethal concentrations of antibiotics, growth medium might be accumulating materials (probably some quorum sensing molecules) released from those lysed bacteria which inhibit the growth of others and transiently protect them from the action of antibiotics. To test this hypothesis, bacteria were grown in the presence of lethal concentrations of ampicillin (15µg/ml). Once most of the bacteria were killed and there was no visible growth, they were pelletted, washed twice and then grown in fresh medium and also in medium containing the same concentration of ampicillin (15µg/ml) previously used to kill them and incubated for another round. While turbidity was noticed in ampicillin-free medium after overnight incubation, no growth was detected in medium containing ampicillin. It was evident that all bacteria were killed by the second round of ampicillin treatment since no growth was detected after re-incubation following removal of ampicillin (data not shown). If bacteria were exhibiting a 'true phenotypic shift', they would have escaped the bactericidal action of ampicillin for the second round also, since they were growing under the same conditions of growth as the first round. This experiment may also explain why more number of bacteria survives when a high initial inoculum size is used. With high inoculum size, the amount of materials released by lysed bacteria is also higher which may inhibit the growth of more number of bacteria resulting in more survivors.

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Most of the experiments demonstrating phenotypic shift were done after incubating bacteria for a short period of time (19). For ampicillin and other antibiotics which exhibit a 'time dependent killing property' it is highly important to incubate bacteria for longer time. It is well documented that for such antibiotics, the time of incubation is more critical than concentration of antibiotics (12, 40, 41). This is very important since a short incubation cannot kill all susceptible bacteria and hence re-inoculation may show a 'false phenotypic shift'. (Even for kanamycin, a 'concentration dependent killing' antibiotic, all susceptible bacteria are not killed by incubating for a short period (Table.3.). Moreover, earlier experiments with persisters were using high initial inoculum size by growing bacteria to exponential phase (19). As noted earlier, a high inoculum size could also give false results since the survival of some normal cells is sufficient to give false phenotypic shift (Table.3.). Similarly, a very high concentration of antibiotics above MIC can result in a 'paradoxical effect' wherein significant number of bacteria escapes killing by antibiotics (42, 43) due to mechanisms that are not clear. Previously it was demonstrated that maximal killing of bacteria occurs over a narrow range of antibiotic concentrations above MIC and the number of survivors increase with higher concentrations above this value (42, 43). Since some of the experiments with persisters might have used high concentrations of ampicillin above MIC (3, 19), the chance of giving a 'false phenotypic shift' is higher.

Mechanism of persister formation is largely unknown. It was shown that cells over-expressing HipA, RelE and other toxin-antitoxin (TA) modules resulted in formation of high frequency of persisters and hence these proteins were implicated in the persister formation (21). These proteins may increase the persister population by slowing

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down or stopping the cell division and thus evade the action of antibiotics (21). However, Vazquez-Laslop et.al (2006) questioned the specific roles of HipA and RelE in the formation of persisters. They found that proteins such as DnaJ and PmrC that are unrelated to TA modules can also result in high frequency of persisters when they are over-expressed in cells. They concluded that when cells are expressing proteins to the toxic levels, frequency of persisters increase regardless of the kind of proteins.

Based on the above findings, it is assumed that phenotypic shift of persisters observed earlier may be due to faulty experimental settings.

Persister formation by other antibiotics

The procedure used to isolate persisters using kanamycin was repeated with tetracycline, ampicillin and nalidixic acid. A growth pattern similar to kanamycin could not be seen with any of these antibiotics (Table.2). With high concentration of tetracycline, O.D. did not change much after a period of time indicating that there was no bacterial growth. With lower concentrations, medium showed turbidity. When bacteria were plated on agar, they grew like normal cells and the size of the colonies were similar to that of control (data not shown). This was not surprising as tetracycline is bacteriostatic and hence bacteria that remained after tetracycline treatment could revert to normal population once the antibiotic was removed. With ampicillin and nalidixic acid, a lot of bacterial lysis was seen in liquid medium within 3 hours of incubation. At low antibiotic concentrations, the surviving bacteria resumed growth and reached turbidity. However at high concentrations, there were no signs of growth. When surviving cells were incubated in fresh medium without antibiotics, the effect was all or none

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phenomenon i.e. either they grew to turbidity or they did not grow at all. On agar also, either there were no colonies or normal large colonies with occasional smaller ones were detected (data not shown). This indicates that the bacteria that survived ampicillin or nalidixic acid treatment cannot be distinguished from the normal cells which are consistent with the findings of Keren et.al (19). The difference between kanamycin and persisters of other antibiotics was also evident from the zone of inhibition by antibiotics (Fig.2. A and B). The area immediately outside the zone of inhibition by kanamycin consisted of only small colonies and the size of colonies gradually increased as antibiotic concentration reduced. With ampicillin, tetracycline and nalidixic acid, the zone of inhibition consisted of both large and small colonies (Fig.1.B shows persisters of ampicillin. Same pattern was seen with tetracycline and nalidixic acid). Our studies indicate that among the four antibiotics, only kanamycin can produce a pure culture of persisters .Hence persisters isolated by kanamycin exhibit a heritable phenotype which was not observed with persisters isolated with other antibiotics. Since other antibiotics cannot select a pure culture of persisters, 'phenotypic shift' exhibited by these persisters was only due to the presence of some normal fast dividing cells. The selection of persisters and their slow growth can partly explain the long post-antibiotic effect (period of time after removal of antibiotics when bacterial growth is not observed) exhibited by aminoglycosides (16, 18). *In vitro* post-antibiotic effect of kanamycin against *E.coli* DH5-α can be as long as 40-45 hours, provided a pure culture of extreme persisters only remain.

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Tolerance of persisters to antibiotics

Persisters are reported to be responsible for recalcitrant infections and can tolerate high antibiotic concentrations (21).The ability of different persister groups to tolerate higher kanamycin concentration was tested by treating each group of the persisters. Each group of the persisters were treated with the same concentration of kanamycin at which they were isolated and with higher concentrations. Persisters showed increased tolerance to kanamycin which is evident from the high MIC value (Table.6). All persisters were completely tolerant to the concentration at which they were isolated i.e. there was no significant difference between the growth rate of K-40 grown in antibiotic free medium and those grown in kanamycin concentration of 40µg/ml (data not shown). However, with increasing concentrations, they became more and more sensitive. Depending on the type of persisters, an increased MIC up to 2-7 folds was noticed.

Antibiotic tolerance exhibited by persisters may be clinically significant because a sub lethal concentration of an aminoglycoside can result in the selection of persisters which may cause a latent infection later and will be difficult to eradicate since a high concentration of antibiotic need to be used. In this aspect, persisters are similar to small colony variants (scv), variants of bacteria that grow slowly, form small colonies and are tolerant to antibiotics especially aminoglycosides (28, 29). Most of the scvs reported are mutants of *Staphylococcus aureus* isolated from clinical infections and are auxotrophic to hemin, thiamine or thymidine (28, 37, 38). *E.coli* lack the ability to take up hemin (31, 32) and the persisters of E.coli did not revert at any of the five different concentrations of thiamine or thymidine (data not shown). Moreover, persisters comprise a number of

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distinct subgroups with different growth characteristics which is against the possibility of them being mutants. Aminoglycoside tolerance exhibited by majority of scvs is due to defects in electron transport resulting in reduced transmembrane potential that donot favor the uptake of aminoglycosides (28). Aminoglycoside uptake and its killing rate are directly dependent on transmembrane potential (13). Whereas scvs may arise from genetic mutations (28), persisters may have defects in electron transport without carrying mutations in electron transport chain pathway. SCVs that are auxotrophic for a number of agents other than hemin, thiamine or thymidine as well as stable scvs not auxotrophic to any of these agents are also reported (28). Since persisters form colonies of all sizes and were noticed with all bacterial cultures tested (Table.1., Fig.1, Fig.2.A.) and showed no reversion on addition of thiamine or thymidine, it can be assumed that they are naturally occurring forms. Moreover, after eliminating extreme persisters by repeatedly growing them in early exponential phase, all of them reappeared after overnight incubation (Table.5). This raises the possibility that they arise from natural process rather than from mutations and that these stable scvs may be different from the mutants reported earlier. It is not documented whether electron transport defective scvs also form colonies of various sizes. In such cases, the term ‘small colony variants’ itself become a misnomer as these variants can produce larger colonies also (the colony size of K-10 was indistinguishable from control after 36-40 hours).

Aging can reduce the transmembrane potential since the expression of electron transport chain pathway decreases with age and is common to humans, mouse and fly (39). Whether this is true for bacteria is not known. However, bacteria which were considered to be functionally immortal may also undergo aging and death (2, 34, 44).

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Stewart *et.al* (34) studied the replicative senescence of E.coli and found that bacteria divide asymmetrically. They observed a rate of decline of growth by 1% in cells that inherited old poles. The cells growing slower were the ones that have more often inherited old poles. Since persisters are not mutants and comprise many subgroups that can be isolated from all cultures used and show a gradual reduction in growth rate and colony sizes which cannot be reverted back, it is hypothesized that the persisters are bacteria at different stages of aging which become tolerant to aminoglycosides due to reduced uptake of the antibiotic resulting from a lower expression of electron transport chain pathway (Fig.3.B. show some differences in the protein expression profile of control and persisters).

As per the model for bacterial aging proposed by Ackermann *et.al* (2) and Stewart *et.al* (34), when a bacterium divides, the mother cell becomes older whereas the daughter cell is a rejuvenated young offspring which has a growth rate similar to normal cells (Fig.4, model 2). However, the model proposed here is different (Fig.4, model 3) because, on bacterial reproduction, the mother cell becomes older whereas the daughter cell do not become a rejuvenated offspring but rather has the same age as the mother cell, a concept earlier proposed by Liu (44). The reason why the rejuvenated offspring hypothesis is not supported is that persisters never attain the growth rate of normal cells even after they were passaged for 3-4 times. Rather they maintained their own growth rate which was slower than the normal cells.

To test whether kanamycin tolerant persisters also exhibit tolerance to other antibiotics, persisters isolated using kanamycin`were treated with different concentrations

of ampicillin and nalidixic acid. While there was no significant difference between control, K-10 and K-20 group as far as MIC was considered, K-30 and K-40 did show increased MIC up to 2-3 folds (Table.6). The result demonstrates that persisters isolated by kanamycin may offer some tolerance to other antibiotics also, but not to the extent reported earlier (22).

Persisters generate noise in protein expression

Individual cells of a genetically identical homogenous population of bacteria may show different protein expression levels referred to as noise in protein expression (4, 14, 27, 30). Noise can be extrinsic when protein expression levels differ between individual cells of a genetically identical homogenous population or intrinsic when the differences arise due to inherent stochasticity of individual cells with proteins produced in random bursts (9, 35). Sources of noise can be multiple such as variation in cell cycle stage, aging, epigenetic regulation, unequal segregation of mitochondria during cell division, fluctuations in upstream signalling, subtle differences in surrounding environments etc (17, 35). To determine whether persisters are responsible for generation of noise, the expression levels of green fluorescent protein (GFP), an unnecessary protein for the bacteria, in normal cells and in persisters was measured. BL21 (DE-3) cells with plasmid containing gene for GFP and which have high basal level of GFP expression was used for this purpose. Since the presence of stressors like antibiotics, especially kanamycin that inhibits protein synthesis may affect protein expression, GFP expression was measured in persisters grown in antibiotic free medium. For this purpose, persisters of BL21 (DE-3) cells were isolated first, followed by washing to remove the antibiotics and further

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incubation in fresh medium without antibiotic. This was repeated once again to eliminate any effects of antibiotic in protein expression. GFP expression by persisters was then determined by western blot which showed a decreasing gradient with persisters isolated using higher kanamycin concentrations (Fig.3.A). It is predictable that there will be innumerable number of subgroups with subtle differences in protein expression levels. The difference in the protein expression is not due to stochastic variation but results from the existence of distinct persister populations. The noise was also evident from immunofluorescence (Fig.3.C). The intensity of fluorescence by GFP decreased as its expression was reduced which was consistent with western blot results. However, an entire range of intensity was not obtained as in western blot. No difference in intensity was noticed between K-10 and K-20 and also between K-30 and K-40. The difference in protein expression was also visible from the intensity of fluorescence of colonies in solid medium (data not shown). While control gave the strongest intensity, K-40 showed only very low intensity. Again, a decreasing gradient of fluorescence was noticed as in western blot.

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Significance of persisters

Persisters are slow growing bacteria present in normally dividing bacterial population which are neither mutant nor induced by antibiotics. ‘Phenotypic shift’of persisters reported earlier can be due to faulty experimental set up and hence is rejected. Persisters can be aging bacteria and can be selected by aminoglycosides due to the peculiar property of aminoglycoside uptake by bacteria which depends on the transmembrane potential. They comprise a number of subgroups and are one of the

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sources of bacterial heterogeneity and noise in protein expression. Even though persisters generate phenotypic heterogeneity, they may not offer an appreciable survival advantage to the population as they do not revert to parent population. However, they can tolerate high concentrations of antibiotics and hence can be clinically significant. Persisters may be aging bacteria with reduced levels of protein expression and thus may provide an excellent model for bacterial aging. The hypothesis that a 'true VBNC' state results from bacterial senescence is also supported here. Existence of many subgroups of persisters with different growth rates, colony sizes, antibiotic tolerance and protein expression levels warrant revision on some of the fundamental concepts in microbiology including colony forming units, stationary phase physiology, VBNC, post-antibiotic effect and aging.

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Table.1. Growth characteristics of persisters isolated by by kanamycin

Bacterial strain	KAN* (ug/ml)	O.D. of bacterial culture						Colony growth (h)$^{\Delta}$
		In presence of antibiotics			On removal of antibiotics[§]			
		9h	24h	48h	9h	24h	48h	
E.coli DH-5α	0	t	t	t	t	t	t	>15
	10	0.23±0.02	t	t	1.04±0.09	t	t	>15
	20	>0.1	0.82±0.08	t	0.43±0.04	t	t	17±1.2
	30	>0.1	0.14±0.02	0.53±0.05	0.16±0.02	0.74±0.05	0.82±0.06	24±1.3
	40	>0.1	>0.1	0.18±0.02	>0.1	0.1±0.02	0.25±0.03	48±1.7
	50	>0.1	>0.1	0.16±0.01	>0.1	0.1±0.01	0.2±0.02	53±5.6
	60	>0.1	>0.1	>0.1	>0.1	>0.1	>0.1	-
S.enteritica	0	t	t	t	t	t	t	>15
	10	>0.1	t	t	t	t	t	>15
	20	>0.1	0.36±0.03	0.77±0.07	0.21±0.04	1.03±0.08	1.31±0.14	25±2.4
	30	>0.1	>0.1	0.12±0.01	>0.1	>0.1	0.15±0.01	32±3.6
	40	>0.1	>0.1	>0.1	>0.1	>0.1	>0.1	-

Results are shown as mean ± s.e.m.; *n*=3 for *E.coli* DH-5α; *n*=2 for *S.enteritica*

t – turbid

*Concentration of kanamycin (ug/ml) used for isolation of persisters

[§] persisters isolated were washed twice and incubated in fresh LB medium for 48 hours. This procedure was repeated again

$^{\Delta}$Time taken to form visible colonies after antibiotic-free persister cultures were plated on agar.

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Table.2. Growth characteristics of persisters of *E.coli* DH-5α isolated by different antibiotics

Antibiotic*	Concn (ug/ml)	O.D. of bacterial culture						Colony growth (h)$^{\Delta}$
		In presence of antibiotics			On removal of antibiotics§			
		9h	12h	24h	9h	12h	24h	
TET	0	t	t	t	t	t	-	>15
	5	0.43±0.08	0.4±0.08	0.38±0.05	t	t	-	>15
	10	0.23±0.02	0.2±0.01	0.21±0.02	0.83±0.1	t	-	>15
	15	0.13±0.02	0.11±0.01	0.12±0.02	0.57±0.05	t	-	>15
	20	0.1±0.01	0.1±0.01	0.1±0.01	0.17±0.01	t	-	>15
AMP	0	t	t	t	t	t	t	>15
	2	0.33±0.03(L)	t(L)	t(L)	t	t	t	>15
	4	0.26±0.02(L)	0.74±0.12	t(L)	t	t	t	>15
	6	L	0.5±0.06	t(L)	t	t	t	>15
	8	L	L	t(L)	t	t	t	>15
	10	L	L	t(L)	t	t	t	>15
	12	L	L	L	1.08±0.15	t	t	>15
	16	L	L	L	0.34±0.04	t	t	>15
	20	L	L	L	>0.1	>0.1	>0.1	-
	25	L	L	L	>0.1	>0.1	>0.1	-
NAL	0	t	t	t	t	t	-	>15
	5	0.28±0.05(L)	t(L)	t(L)	t	t	-	>15
	10	0.16±0.03(L)	t(L)	t(L)	t	t	-	>15
	15	L	L	0.22±0.03(L)	t	t	-	>15
	20	L	L	L	0.37±0.05	t	-	>15
	25	L	L	L	0.44±0.08	t	-	>15

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Results are shown as mean ± s.e.m.; *n*=3 for all antibiotics.

t – turbid ; L – lysis ; t(L) – turbid with lysis

* TET-tetracycline; AMP-ampicillin; NAL-nalidixic acid

§ persisters isolated were washed twice and incubated in fresh LB medium for 48 hours.

Δ Time taken to form visible colonies after antibiotic-free persister cultures were plated on agar.

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Table.3. Effect of inoculum size and exposure time on persister isolation

Initial inoculum size					Total time of exposure to antibiotics		
Initial inoculum size(μl)[§]	Spectrophotometer readings (O.D.)				exposure time to kanamycin (50 μg/ml)	O.D. after removal of kanamycin	
	With kanamycin (50 μg/ml)		After removal of kanamycin				
	24h	48h	24 h	48h		24 h	48 h
50	>0.1	0.18±0.01	>0.1	0.25±0.02	1.5h	t	t
100	>0.1	0.22±0.01	>0.1	0.23±0.02	3h	t	t
250	0.74±0.12(L)	t(L)	t	t	6h	t	t
500	t(L)	t(L)	t	t	9h	t	t
1000	t(L)	t(L)	t	t	24h	>0.1	0.22±0.02
					48h	>0.1	0.24±0.01

Results are shown as mean ± s.e.m.; *n* =3

t – turbid

L – lysis

t(L) – turbid with lysis

[§] overnight culture of E.coli DH-5α

Table.4. Effect of pH on growth characteritics of E.coli persisters

Conditions of growth[§]	pH	O.D. of bacterial culture after 6 h of incubation	
		In the presence of stressors	On removal of stressors
Optimal pH	7.2	t	t
Acidic pH	5.1	>0.1	t
	4.6	>0.1	t
	3.5	>0.1	>0.1
Alkaline pH	8.2	>0.1	t
	8.8	>0.1	t
	10.6	>0.1	>0.1

Results are shown as mean ± s.e.m.; *n* =2

[§]Overnight culture of 50 μl was used for incubation

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Table.5. Elemination of persisters and their re-growth.[§]

Kanamycin (µg/ml)	control	cycle 1	cycle 2	cycle 3	cycle 4	stationary phase of cycle 4
0	t	t	t	t	t	t
10	t	t	t	t	t	t
20	t	1.8±0.3	1.4±0.2	1.18±0.2	0.83±0.07	t
30	0.66±0.06	0.54±0.08	0.33±0.07	0.1±0.02	>0.1	1.1±0.27
40	0.22±0.03	0.22±0.03	0.11±0.03	>0.1	>0.1	0.44±0.08
50	0.12±0.01	0.13±0.01	>0.1	>0.1	>0.1	0.19±0.04
60	>0.1	>0.1	>0.1	>0.1	>0.1	0.12±0.05

Results are shown as mean ± s.e.m.; *n* =3

t – turbid

[§] Early exponential phase culture of E.coli was incubated, diluted in fresh medium and reinocculated for four cycles. Bacterial culture from each cycle was treated with kanamycin at varying concentrations and incubated for 48 hours. The exponential phase culture from the fourth cycle was allowed to reach stationary phase by incubating overnight which was then treated with same concentrations of kanamycin.

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Table.6. Tolerance of persisters to antibiotics

Type of cell	Minimum inhibitory concentration[§]		
	Kanamycin (μg/ml)	Ampicillin (μg/ml)	Nalidixic acid(μg/ml)
con	53.3 ± 3.3	10.67± 0.7	14.3 ± 0.3
K-10	110 ± 5.8	10.4±0.5	14.7 ± 0.7
K-20	170 ± 14.6	11.3 ± 1.3	14.6 ± 0.3
K-30	283.3 ± 8.8	23.3 ± 1.7	24.7 ± 2
K-40	346.7 ± 26.1	28.7 ± 1.7	32.7 ± 5.5

Results are shown as mean ± s.e.m.; *n* =3

[§] Minimum concentration of antibiotic that inhibited the visible growth of test organism after 48 hours of incubation. MIC is usually determined after overnight incubation. Since persisters require more time for growth, MIC of all antibiotics was determined after 48 hours of incubation.

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Figure legends

Fig.1. Persisters comprise many subpopulations having different growth rates. A. Colonies of DH5-α normal cells and persisters isolated by kanamycin after 18 hours following agar plating. No colonies were formed by K-30 and K-40 at 18 hours. Upper left- control, upper right- K-10 and down- K-20. B. colonies of control, K-10, K-20 and K-30 after 24 hours. Upper left- control, upper right- K-10, lower left- K-20 and lower right- K-30. C. Colonies of K-40 after 50 hours. D. Colonies of control, K-10, K-20 and K-30 grown in antibiotic-free medium after 26 hours following agar plating. Upper left-control, upper right- K-10, lower left- K-20 and lower right- K-30. E. colonies of K-40 grown in antibiotic-free medium after 2 days. F. colonies of K-40 after incubation for 21 days followed by plating on agar.

Fig.2. Aminoglycosides select persisters with different growth rates. 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.

Figure.3. Persisters generate noise in protein expression. A. Western blot showing GFP expression by normal and persisters of BL21 (DE-3) cells. Lane 1 protein marker, lane 2-control, lane 3- K-10, lane 4-K-20, Lane 5- K-30 and lane 6-K-40. B. Protein fractions of BL21 (DE-3) normal and persisters resolved on 12% SDS-PAGE and visualized by Coomassie staining. Lane1- protein marker,

lane 2- control, lane 3- K-10, lane 4- K-20, Lane 5- K-30 and lane 6- K-40. C. Intensity of GFP fluorescence in normal, K-20 and K-40 persisters of BL21 (DE-3) by fluorescence microscopy.

Figure.4. Models of bacterial aging. Model.1 assumes that bacteria are functionally immortal and there is no aging in bacteria. Here ‘X’ is considered as the starting age of bacterium. Model.2. As per this model, bacteria undergoes aging. When bacterium reproduces, the mother cell becomes older and shows slower growth rate but the daughter cell is a rejuvenated offspring which has the same growth rate as normal cell. In this case, bacterial lineage is never lost. Due to the production of rejuvenated offspring, a culture of aging bacteria will eventually have the fast growth rate as normal cells. Model.3. proposes that as a bacterium reproduces, mother cell becomes older whereas daughter cell maintains the same age as the mother. Here also there is no loss of bacterial lineage. However, a culture of aging bacteria can never attain the same growth rate as normal since the daughter cell is not a rejuvenated fast growing offspring. This model is supported by our findings on persisters and by Liu (44). Model.4. As a bacterium divides, both mother and daughter cells becomes older. Here there will be a complete loss of bacterial lineage after a number of divisions. Even though we do not support this model, it can not rejected based on our results on persisters.

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Figure.1.

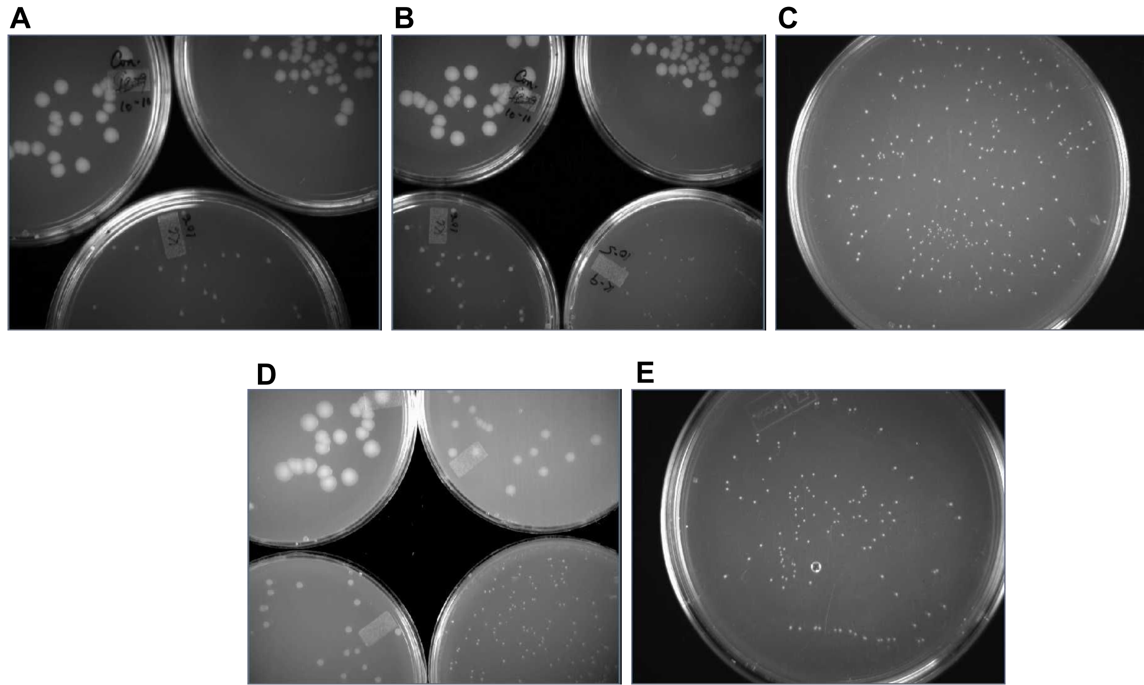

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Figure.2.

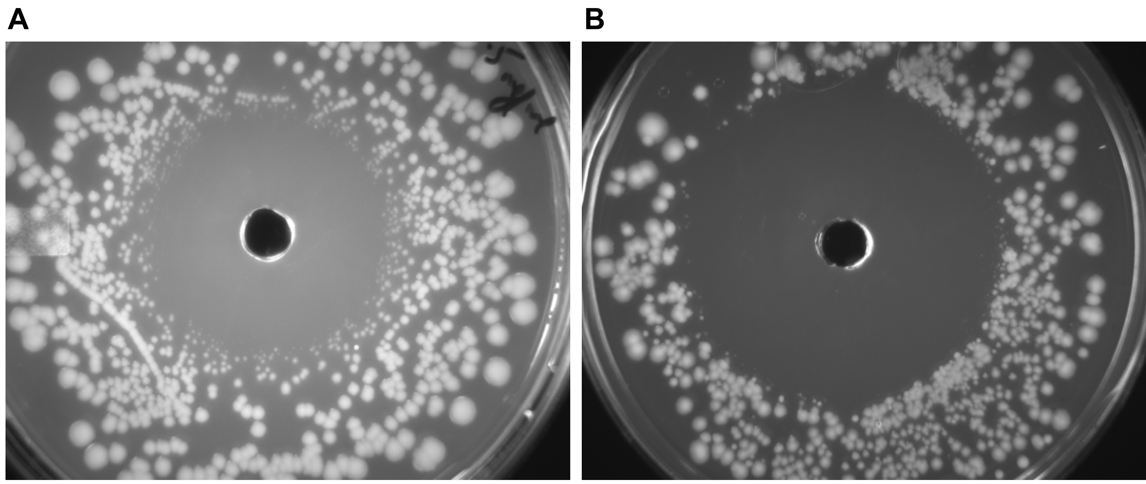

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Figure.3.

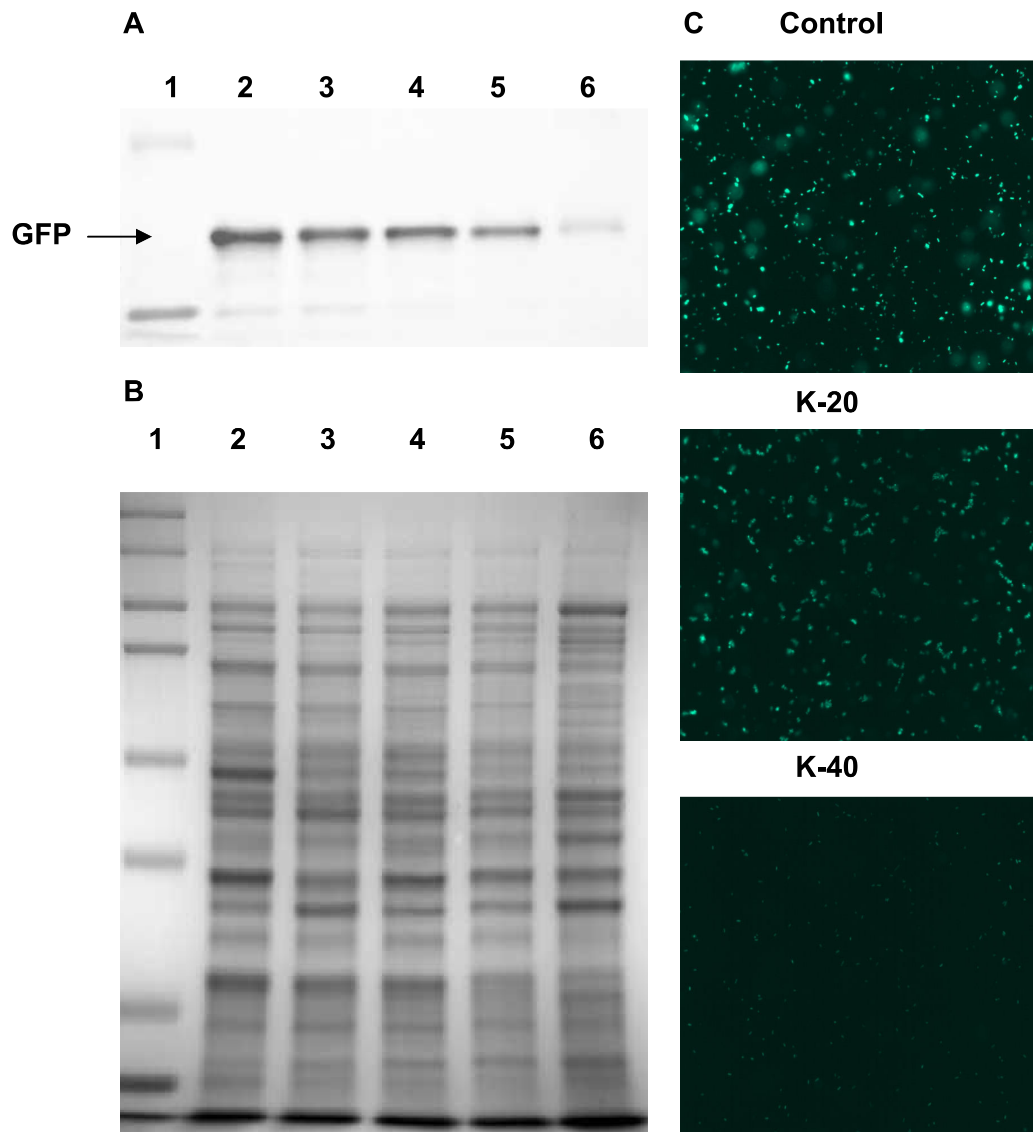

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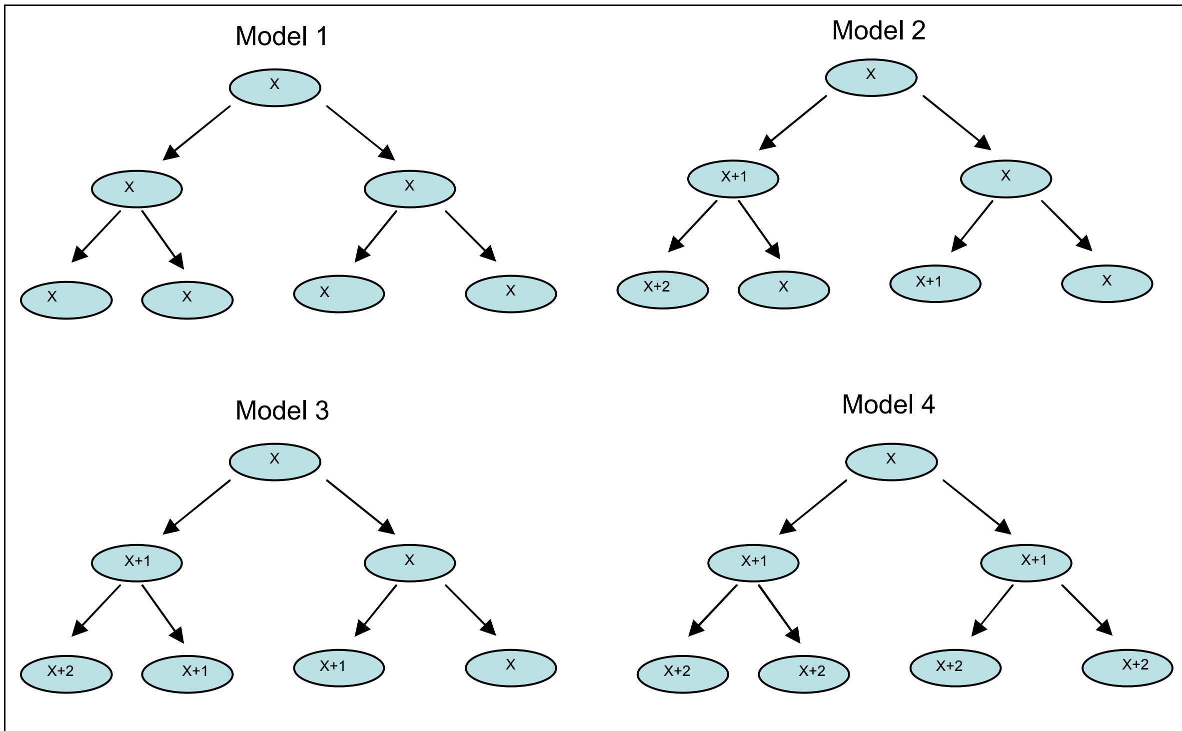
Figure 4



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