

Nonmultiplying Bacteria are Profoundly Tolerant to Antibiotics

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Abstract Bacteria survive treatments with antimicrobial agents; they achieve this in two ways. Firstly, bacteria quickly become tolerant to these agents. This tolerance is temporary, reversible, and associated with slowing of the multiplication rate. Secondly, bacteria can undergo genetic mutations leading to permanent clonal resistance to antimicrobial agents. In patients with infections, nonmultiplying bacteria, some of which may be viable but nonculturable, exist side by side with multiplying bacteria. Current antibiotics capable of killing actively multiplying bacteria have very limited or no effect against nonmultiplying bacteria. Treatment

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of such infections requires a regimen of multiple antimicrobial agents in order to control nonmultiplying persistent bacteria. This is especially important in tuberculosis where there is co-existence of slowly multiplying tolerant bacteria with fast growing sensitive organisms. For this reason, a prolonged length of chemotherapy, lasting 6 months, is necessary to achieve cure. This long duration of treatment is due to the slow, inadequate effect of antibiotics on nonmultiplying persistent bacteria. Similar problems with eradication of persistent bacteria are evident in the treatment of biofilms. These bacteria serve as a pool for recurrent infections. Extended courses of antibiotics increase the likelihood of genetic resistance, raise the cost of treatments, and lead to more side effects.

Keywords Antibiotic tolerance • Nonmultiplying bacteria • Stationary phase • Persisters • Dormant bacteria

1 Nonmultiplying Bacteria and Persisters

In modern medicine, antibiotics are important in the fight against infections (Ball et al. 2004) such as bacteremia (Austrian and Gold 1964) and tuberculosis (Dineen et al. 1976) which used to have a poor survival rate. Today, bacterial resistance to antimicrobial agents is emerging at a rate which outpaces the discovery of new antibiotics (Vashishtha 2010) and multiple-drug-resistant (MDR) bacterial pathogens (Gootz 2010) are common. This resistance profile is due to genomic modifications of bacteria by point mutations and horizontal gene transfer which lead to permanent clonal resistance to antimicrobial agents. There is another mechanism of antibiotic resistance or tolerance which is primarily dependent on the bacterial physiological state. The efficacy of the majority of antibiotics depends on the target bacteria exhibiting a high level of metabolic activity. However, once growth rate of bacteria slows down, they become insensitive to antibiotic treatment. In infectious diseases, slow or nonmultiplying tolerant bacteria coexist with fast growing sensitive organisms (Seguin et al. 2003). Antibiotics are capable of killing actively multiplying bacteria, but are almost always only partially active against slowly multiplying, or are inactive against nonmultiplying persistent bacteria (Hu et al. 2010; Coates and Hu 2006; Hu and Coates 2005). Also more than 60% of all microbial infections are caused by nonmultiplying bacteria such as those present in biofilms (Lewis 2001). The persistent bacteria are responsible for recurrent infections as seen in tuberculosis. With the currently available antibiotics, most persistent infections cannot be eradicated and are therefore associated with poor clinical outcomes (Kyd et al. 2011; Wood and Douglas 2010). Antibiotic tolerance is an important problem in patients with infections because prolonged treatment with multiple doses of antimicrobial agents is required for most bacterial infections. Compared to shorter courses with a single agent, this prolonged treatment with multiple antibiotic regimens can increase the frequency of genetic resistance associated with poor patient compliance (Coates and Hu 2006; Pechere et al. 2007), increase the cost of the treatment, and cause more side effects.

1.1 Nonmultiplying Stationary-Phase Bacteria

Bacteria grow and divide exponentially in cell suspension when growth conditions are favorable. If a certain nutrient in a medium is reduced below a threshold level, it halts key metabolic processes, such as DNA replication, and growth is arrested (Navarro Llorens et al. 2010). In order to survive nutrient deprivation, the bacteria are able to make an orderly transition from exponential growth phase to stationary phase. Cells in stationary-phase culture can survive long periods of starvation (Hu and Coates 2005) in the nonmultiplying stage (Roostalu et al. 2008). The stationary-phase nonmultiplying bacteria are able to resume growth rapidly when nutrients again become available (Siegele and Kolter 1992, 1993). Kolter et al. (1993) reported that entry into stationary phase involves a process of transition which starts at a time point in the exponential phase when DNA, proteins, and total cell mass stop increasing and continues until no further increase in cell numbers is detected. Cells are then in the stationary phase. Many factors can be responsible for this phenomenon. The most clearly defined one is starvation for a single nutrient required for growth. Some bacteria form endospores and myxospores in response to starvation (Kaiser 1986; Losick et al. 1986), but most bacterial species do not generate such differentiated cells. Only nondifferentiating bacteria are discussed in this chapter.

The stationary-phase response gives rise to dramatic changes in cell morphology, physiology, and gene expression (Smith 1995; Wortinger et al. 1998; Navarro Llorens et al. 2010). Lange and Hengge-Aronis examined *Escherichia coli* cells by light microscopy (Lange and Hengge-Aronis 1991). They found that *E. coli* cells became much smaller and almost spherical by reductive division when they entered into stationary phase. Some bacteria greatly reduce their size during starvation and form ultramicrocells which are less than 0.4 μm in diameter (Yourassowsky et al. 1979; Lee and Veeranagouda 2009). Changes in chromosomal topology accompanied with reduced cell division were also observed. After a few hours in stationary phase, changes in the negative superhelical density of plasmids can be detected in *E. coli* (Balke and Gralla 1987). The stationary-phase cultures of *S. aureus* produce small-colony variants (Fig. 1) which are very tolerant to antibiotics and are responsible for recurrent infections in humans (Proctor et al. 1998, 2006; Singh et al. 2009).

The changes in cell envelope and cell membrane of starved cells mirror those which insulate and protect bacteria from stress (Martinez-Rodriguez and Mackey 2005; Siegele and Kolter 1992; Tuomanen and Tomasz 1990). For example, many stationary-phase marine bacteria coat their surfaces with hydrophobic materials that lead to cell adhesion and aggregation. Alterations in membrane composition such as fatty acids result in less fluid and less permeable membranes (Siegele and Kolter 1992; Cronan 1968).

As bacteria enter into stationary phase, their overall metabolic rate decreases but a low level of endogenous metabolism is retained (Hu et al. 2000), which enables the bacteria to take substrates into the cell during starvation and resume growth when nutrients become available (Siegele and Kolter 1992). At the same time

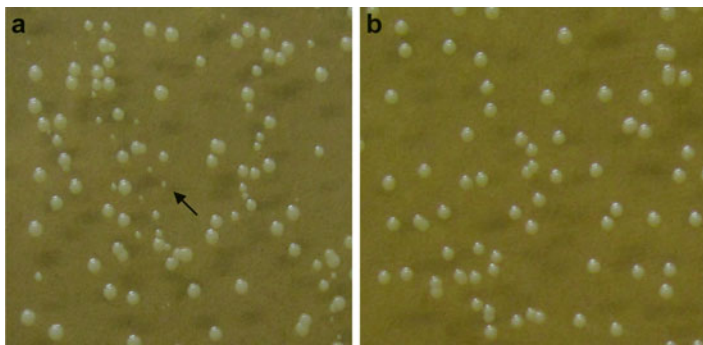


Fig. 1 Colony morphology of *S. aureus* in stationary-phase cultures. *S. aureus* was grown in broth culture for 6 days. The stationary-phase culture was diluted and plated onto a nutrient agar plate. There are many small colony variants formed in the stationary-phase culture indicated by the arrow (a) compared to the colonies formed by an exponential growth phase culture (b)

stationary-phase bacteria synthesize and accumulate storage compounds, such as glycogen and polyphosphates and protective substances, such as trehalose (Hengge-Aronis et al. 1991).

Stationary-phase bacteria are more resistant to stress conditions such as heat shock, osmotic challenge, acidic and oxidative stress than exponential-phase bacteria (Soares et al. 2010; Hengge-Aronis 1993; Jenkins et al. 1988, 1990; Cornet et al. 2010; Davis et al. 1996; Steels et al. 1994; Arnold and Kaspar 1995; Navarro Llorens et al. 2010; Zech et al. 2009). Particularly, stationary-phase bacteria are insensitive to conventional antibiotic treatment and can only be killed by multiple doses of antibiotics at high concentration (McLeod and Spector 1996; Tuomanen and Tomasz 1990; Hu et al. 2000; Dorr et al. 2009). This is called antibiotic indifference (Jayaraman 2008).

1.2 Biofilms: Another Form of Nonmultiplying Bacteria

In nature, apart from growing in a planktonic form which is suspended or growing in a fluid environment, bacteria will also grow on surfaces to form biofilms (Trautner and Darouiche 2004). A biofilm is highly organized. It is a compact multicellular community which is on a liquid–surface interface and is embedded in a self-produced exopolysaccharide matrix. Multiplying cells occur superficially and slow or nonmultipliers live in the deeper layers. Biofilm formation is an inevitable key step in the life cycle of most microorganisms and found on many biological and nonbiological surfaces. They are associated with many infectious diseases, such as infective endocarditis, chronic skin wounds, osteomyelitis, dental plaques, infective cystic fibrosis, and infections due to indwelling medical devices such as catheters, prosthetic heart valves, and shunts (Fux et al. 2005; Mittal et al. 2009). Antibiotic therapy of infections which are associated with biofilms often lead to a poor clinical

outcome, because the bacteria within the biofilms are extremely tolerant to antimicrobial agents (Lewis 2008). Successful treatment of biofilm-associated diseases requires multiple doses of high concentration antibiotic regimens or the removal of foreign body devices (Trautner and Darouiche 2004). Bacteria within biofilms are not genetically resistant strains per say. When the same cells are spread out and re-cultured in a planktonic culture, they retain sensitivity to the antibiotics (Spoering and Lewis 2001). The physiological states of the bacteria in the depth of a biofilm are very similar to those in stationary-phase planktonic culture. They are induced by nutrient starvation, high density of cell population, and accumulation of metabolic waste (Spoering and Lewis 2001; Fux et al. 2005). Biofilms are resistant to environmental stresses such as altered pH, osmolarity, and nutrient limitation. Furthermore, biofilm formation enables the bacteria to become resistant to host immune defenses (Vuong et al. 2004; Fux et al. 2005).

It has been suggested that biofilm tolerance to antibiotics and host immunity is due to the following (Lewis 2001; Fux et al. 2005): Firstly, slime matrix limits penetration of the antibiotic (Renslow et al. 2010). The exopolysaccharide matrix in which bacteria are embedded plays a key role in preventing large or small antimicrobial agents from binding to and penetrating into a biofilm. The slime matrix also protects bacteria from being engulfed during phagocytosis (Rohde et al. 2005; Vuong et al. 2004) and enhances bacterial virulence (Begun et al. 2007). Antibodies bind to the matrix and are not able to penetrate into biofilms (de Beer et al. 1997). Bacterial proteins in the biofilm matrix render them more resistant to attacks by complement (Simmons and Dybvig 2007). The exopolysaccharide matrix is likely to prevent smaller antibiotics, such as glycopeptides, crossing the diffusion barrier (Lewis 2001; Singh et al. 2010). In addition, the negatively charged exopolysaccharide binds to the positively charged antibiotics such as aminoglycosides, which effectively blocks antibiotic activity (Walters et al. 2003). Secondly, different physiological states of bacteria determine antibiotic tolerance. Biofilm formation undergoes highly regulated processes including surface attachment, cellular proliferation by cell–cell interactions, maturation by producing matrix, antibiotic tolerance development and detachment which results in bacteria regaining planktonic growth mode (O’Toole et al. 2000). In the early stage of biofilm formation, when bacteria are actively replicating, the cells are sensitive to eradication by antimicrobial agents (Gunther et al. 2009). In a mature biofilm, bacteria slow down or terminate replication and become tolerant to antibiotics. A small proportion of nonmultiplying bacteria in a biofilm cannot be eradicated by any antimicrobial agents and human immune clearance (Lewis 2008). Once the antimicrobial agents become unavailable, these persistent bacteria reform the biofilm and this leads to the relapse of infection.

1.3 Persisters

In the 1940s, Bigger (Bigger 1944) noticed that a culture of *S. aureus* could not be completely killed by penicillin, even at high concentration. He called these surviving bacterial cells “persisters.” Persistent cells are also present in the

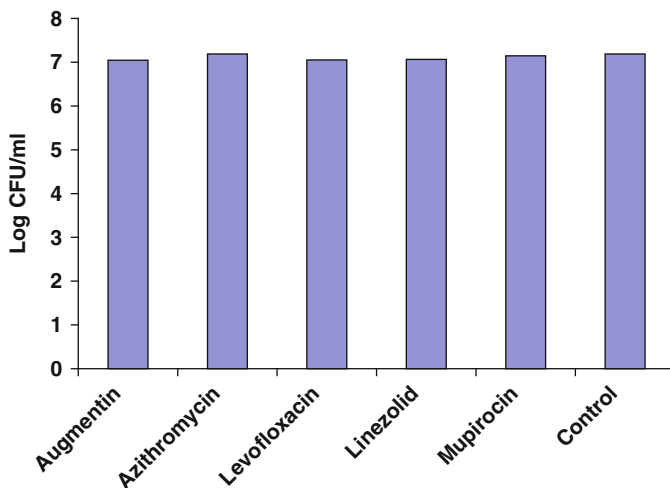


Fig. 2 Activities of currently marketed antibiotics against stationary-phase nonmultiplying *S. aureus*. The 6-day stationary-phase culture was incubated with augmentin (amoxicillin/clavulanate), azithromycin, levofloxacin, linezolid, and mupirocin at 100 $\mu\text{g/ml}$ for 24 h. Viability of the bacteria was determined by colony forming unit counts. There were no antimicrobial activities observed against the stationary-phase nonmultiplying bacteria for any the tested antibiotics

populations of “old” stationary-phase bacteria in batch cultures (Kaprelyants and Kell 1993; Kaprelyants et al. 1993). Old persisters can survive for a long time—it has been demonstrated that a 38-year-old stationary-phase *E. coli* culture contained more than 10^5 viable bacteria/ml in the nonmultiplying persistent stage (Eisenstark et al. 1992). Generally, persistent bacteria can be isolated by antibiotic treatment which eliminates the multiplying bacterial population (Hu et al. 2000). Persisters can be found in exponential growth culture of *E. coli* (Balaban et al. 2004), and this has been observed in single cells using a microfluidic device. So, persisters preexist the addition of an antibiotic to a culture and are not actually produced by such treatment. The proportion of the persistent bacteria increases upon entry into stationary phase and during biofilm formation. Persisters are nonmultiplying (Roostalu et al. 2008) and constitute 1% of the bacterial population in stationary-phase cultures and biofilms (Shah et al. 2006; Balaban et al. 2004; Spoering and Lewis 2001). These persisters are not genetically modified mutants, but are transient phenotypic variants of their parental population (Wiuff et al. 2005). They are profoundly tolerant to most marketed antibiotics (Wiuff et al. 2005; Hu et al. 2010; Keren et al. 2004a, b). If *E. coli* or *S. aureus* cells in a late stationary-phase culture are resuspended in phosphate buffered saline, the nonmultiplying persistent bacteria are very tolerant to high doses of the current antibiotics (Hu et al. 2010) as seen in Fig 2. After removal of antibiotic pressure, the small fraction of the persistent cells restore growth and become sensitive to antibiotics again (Bigger 1944).

1.4 Dormant Bacteria

A number of studies have indicated that there is a subgroup of dormant microbes among nonsporulating bacterial species which are unable to divide or form colonies on agar plates but are able to produce daughter cells under appropriate conditions (Kaprelyants et al. 1993). They are formed as a result of adaptation to starvation or other unfavorable conditions. The term “viable but nonculturable” (VBNC) was introduced by Colwell et al. (1985) and includes the dormant bacteria which are present in populations of some Gram-negative bacteria such as *E. coli* (Darcan et al. 2009), *Salmonella* (Turner et al. 2000), and *Vibrio* spp. (Chaiyanan et al. 2007; Baffone et al. 2003) starved in an aquatic environment. VBNC cells are also evident in many human pathogens such as *S. enteritidis*, *V. cholerae*, *Shigella sonnei*, *S. flexneri* (Colwell et al. 1985; Roszak et al. 1984), *Listeria monocytogenes* (Lindback et al. 2010; Cappelier et al. 2005, 2007), *Enterococcus* spp. (Leo et al. 2001), *Campylobacter jejuni* (Jackson et al. 2009; Klancnik et al. 2009; Baffone et al. 2006), *Helicobacter pylori* (Saito et al. 2003), *Staphylococcus aureus* (Masmoudi et al. 2010), *Mycobacterium tuberculosis* (Hu et al. 2000), and *Pseudomonas aeruginosa* (Moore et al. 2007).

VBNC cells are usually smaller than the normal cells (Roszak and Colwell 1987). Torrellar and Morita (1981) observed very small cells of marine bacteria (ultramicrocells) which had reduced growth rates. Nilsson and colleagues (1991) also found that nonculturable *V. vulnificus* formed small cocci of 1.0 μm in diameter. In contrast, their rod-shaped (3.0 μm in length) vegetative cells are much larger. In general, most ultramicrobacteria of marine bacteria are unable to form colonies on agar plates (Morita 1988) but can exist as VBNCs. These cells still exhibit low metabolic activity (Barcina et al. 1989). Also, it has been found that Gram-negative and Gram-positive VBNC cells alter their cell wall components which results in robust resistance to environmental stress (Signoretto et al. 2000, 2002; Costa et al. 1999). VBNC cells can be resuscitated. Successful in vitro resuscitation and growth of *S. enteritidis* has been reported by Roszak et al. (1984). After addition of nutrients to microcosms (sterile river water cultures) for 25 h, the nonculturable cells produce colonies on solid agar plates.

2 Clinical Importance of Persistent Bacteria

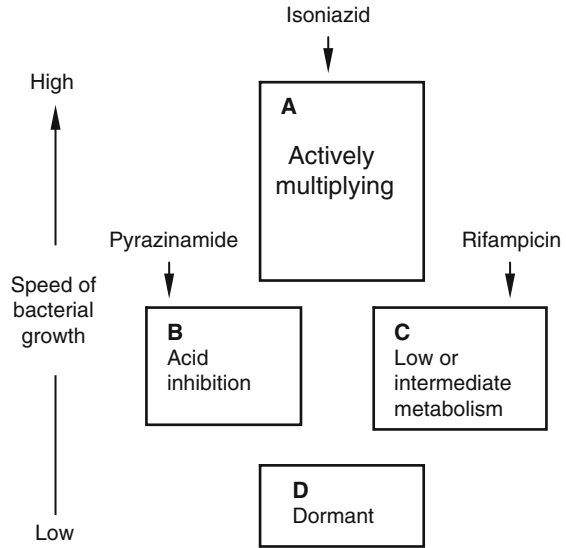
Persistent bacteria including the VBNC forms are present in many chronic human infectious diseases (Rihl et al. 2006; Coates et al. 2008). Although persisters such as VBNC bacteria cannot cause overt disease, they form an important reservoir of latent survivors and are responsible for persistent and recurrent infections (Velazquez and Feirtag 1999). Persistent infections almost always need multiple antibiotic regimens and long periods of treatment (El Solh et al. 2008). As these persistent bacteria are nonmultiplying and cannot be cultured by traditional

microbiological methods, it has proved to be difficult to detect the presence of these populations in an infection (Leo et al. 2007; Signoretto et al. 2004). Persisters retain their pathogenicity as evidenced by maintaining their ability to adhere to human tissues and to produce toxins (Oliver 1995; Fischer-Le Saux et al. 2002; Pruzzo et al. 2002, 2003; Pommepuy et al. 1996). In chronic urinary tract infections (UTIs), a nonculturable form of *E. coli* with a round shape has been detected in bladder epithelial cells, and this may serve as a pool for recurrent UTIs (Anderson et al. 2003, 2004; Rivers and Steck 2001; Mulvey et al. 2001). Persistent bacteria which are present in UTIs are extremely tolerant to the highest achievable serum concentrations of all antibiotics commonly prescribed for this disease (Seguin et al. 2003). This means that eradication of persisters needs long-term antibiotic treatment, and under certain circumstances, such as catheter-associated infections, persisters cannot be eradicated by antibiotics at all, so the infected catheter has to be physically removed from the patient. This situation also applies to many other implant-associated infections. It has also been found that VBNC *H. pylori* is present in the gastric mucosa. These bacteria change shape from vegetative rods to an unusual round form (Kusters et al. 2006). Reactive arthritis is associated with bacterial infections which are caused by pathogens such as *Chlamydia* spp., *Salmonella* spp., *Yersinia* spp., *Shigella* spp., *Campylobacter* spp., and *Clostridium* spp. It is believed that there may be small numbers of VBNC persisters present in the joint. These bacteria cannot be cultured from synovial specimens, but their existence can be confirmed by the presence of bacterial antigens which are synthesized by metabolically active bacteria (Colmegna et al. 2004; Rihl et al. 2006). Prolonged antibiotic therapy of 12–50 weeks duration is required to cure the disease (Rihl et al. 2006) due to these persistent nonculturable bacteria.

3 Persistent *M. tuberculosis*

One of the most important characteristics of *M. tuberculosis* in the pathogenesis of the disease is its ability to persist in human tissue for the life span of the human host. During *M. tuberculosis* infection, the acquired immune response inhibits the replication of tubercle bacilli, but not all bacilli are destroyed. A small proportion of the bacilli remain in a dormant or persistent form (Dannebery and Rook 1994) causing latent infections. Dormant *M. tuberculosis* is important not only because it can survive attacks by the immune response but also because, when compared to actively growing bacteria, it is more tolerant to antibacterial agents. This means prolonged chemotherapy for 6 months is required to produce a cure (Mitchison 2004). These persistent bacteria are usually drug sensitive at relapse, so their resistance to chemotherapy is phenotypic tolerance (Hu et al. 2000) rather than genetic mutation. It is suggested that an altered physiological state of persistent *M. tuberculosis* accounts for its tolerance to drugs as well as the ability to survive in the host for many years. Persistence is likely to be a combined effect of both the immune system and bacterial physiology, resulting in what is generally referred to as a dormant or a latent state (Bloom and McKinney 1999).

Fig. 3 Hypothesis of special populations of tubercle bacilli within lesions killed by the bactericidal and sterilizing drugs (Mitchison 1979)



3.1 Different Populations of *M. tuberculosis* in Human Lesions

It has been suggested (Mitchison 1979) that there are four different components of the bacterial population in tuberculosis lesions. As shown in Fig. 3, the majority of bacilli in the lesions of an untreated patient with tuberculosis are actively multiplying, probably at a rate similar to that in a log phase culture (population A). These bacilli may be present in the lining of open cavities with an abundant of oxygen supply, which favors the bacilli to grow rapidly. There is also a small proportion of dormant bacilli (population D) whose metabolism and growth is almost completely inhibited by an unfavorable environment, such as in closed lesions where oxygen becomes unavailable. In addition there are semidormant bacilli: Population B consists of those organisms which are inhibited by an acid environment, such as in early acute inflammatory lesions or within the phagolysosomes of macrophages. Population C is assumed to be those bacilli which contain brief or intermittent metabolic activity.

3.2 Dormant *M. tuberculosis* in Animal Models

The existence of dormant or persistent *M. tuberculosis* after chemotherapy was shown for the first time in an animal model by McCune and colleagues more than half century ago (McCune and Tompsett 1956; McCune et al. 1966b). The “Cornell model” which was named after Cornell University where the work was performed is considered to be the most useful model for demonstrating and studying mycobacterial dormancy. In this model, a large number of mice are infected

intravenously with 10^5 – 10^6 CFU of *M. tuberculosis* H37Rv. Therapy with isoniazid and pyrazinamide for 12 weeks is started immediately after infection. After termination of treatment, a “sterile” state is achieved. At this stage, no tubercle bacilli are detected from homogenates of lungs or spleens of the mice by bacteriological culture and microscopy. However, the disappearance of the bacilli does not mean that the organisms have been eliminated from the tissues. About 3 months after the end of drug treatment, culturable bacilli are detected from about one-third of the mice. The reappearance of the tubercle bacilli in another two-thirds of the animals occurs within 9 months after termination of the chemotherapy. Administration of large doses of cortisone, which suppresses the immune system of the mice, accelerates the reactivation of the tubercle bacilli. The persistence of the tubercle bacilli after 12 weeks of chemotherapy is not due to the emergence of drug-resistant mutants since the bacilli which are recovered from the mice remain essentially drug sensitive (McCune et al. 1956). Some similar studies were performed later with a combination of isoniazid and rifampicin. Isoniazid and rifampicin in combination were given to *M. tuberculosis* infected mice for 12 months. A high relapse rate of 60% was observed by giving the mice 2 months of high dose of cortisone (Grosset 1978). The original Cornell model and the later studies show that human tuberculosis can be converted from active disease to a latent infection with chemotherapy. The dormant bacilli which survive the drug treatment can be reactivated and can cause active disease later in life. So, latent infection in animal models is characterized by the presence of the tubercle bacilli which cannot be detected by microscopy or by culture and can only be demonstrated by the emergence of active disease (McCune et al. 1966a).

3.3 Subpopulations of Nonmultiplying *M. tuberculosis* In Vitro

Subpopulations of nonmultiplying bacteria have been modeled in experimental cultures. *M. tuberculosis* is allowed to grow in a culture without agitation. The initial growth of bacilli occurs at an exponential rate with a doubling time of 16–18 h (population A) until the cell density reaches 4×10^8 CFU/ml. At this stage, dissolved oxygen becomes limiting, and the growth rate decreases while the bacilli settle to the bottom of the container (Wayne 1976, 1994). The organisms in the sediment adapt to microaerophilic conditions and enter a homogenous physiological state of dormancy. The bacilli in the deposit of the settling culture can persist for long periods after transfer to anaerobic conditions (Wayne 1977). When an old, micro-aerophilically adapted culture is treated with a high dose of rifampicin, the antibiotic kills most of the bacilli which actively replicate (A) or shows intermittent metabolic activity (population B and C), but fails to remove a small proportion of a bacterial population (D) (Hu et al. 2000) which are rifampicin tolerant persisters. These persisters rapidly lose the ability to grow on solid medium

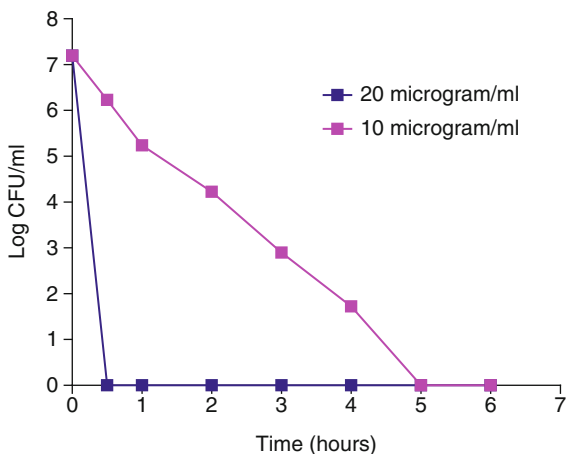
plates, but are alive and able to grow in liquid medium which contains no rifampicin (Hu et al. 2000). In such liquid subcultures, they regain the ability to grow on plates after 7 days of incubation and are then fully susceptible to rifampicin. During chemotherapy with rifampicin, tolerant bacilli that persist during treatment with rifampicin are likely to be the most difficult to kill, so that the *in vitro* models should reflect the likely sterilizing activity during human treatment of the drug under test, in combination with rifampicin.

4 Antibiotics Kill Nonmultiplying Bacteria

4.1 Bactericidal and Sterilizing Antibiotics

Currently, although certain antibiotics are capable of killing nonmultiplying bacteria, a very limited number of antibiotics can kill persistent or dormant bacteria. It is well established that antibiotics are ineffective against bacteria whose growth or metabolic activity has been almost completely inhibited. For example, antibiotics in the penicillin family such as penicillin, ampicillin, and amoxicillin only kill rapidly growing cells, but are ineffective against nonmultiplying cells. Although some of the antibiotics such as aminoglycosides and fluoroquinolone have activities against nongrowing bacteria such as those in biofilms, they are more effective in killing growing bacteria. In tuberculosis, antituberculous drugs have been shown to be both bactericidal and sterilizing (Fig. 3). Bactericidal activity of a drug is defined as the ability to kill rapidly replicating bacilli (population A) and is mostly effective at the beginning of treatment, such as isoniazid. The sterilizing activity of drugs such as rifampicin and pyrazinamide depends on their ability to kill semidormant bacilli (population B and C) which can persist for long periods during chemotherapy and give rise to relapses. Sterilizing activity may start early in treatment but is more evident in the later stages of treatment after bactericidal activity has declined (Mitchison and Fourie 2010). The sterilizing activity of drugs is of great clinical importance, since it determines the length of the chemotherapy. Neither bactericidal nor sterilizing drugs are currently available to kill the population D of dormant bacilli. The success of a combination of isoniazid, rifampicin, streptomycin, and pyrazinamide or some other drugs in the short-course chemotherapy lies in their capacity to attack the semidormant as well as the actively replicating bacilli. In a nonmultiplying culture of *M. tuberculosis*, addition of fluoroquinolones such as moxifloxacin and gatifloxacin to the drug regimen of isoniazid, rifampicin, and pyrazinamide reduces the numbers of persisters (Hu et al. 2003, 2006a). This indicates a need for new antibiotics which target nonmultiplying bacteria (Coates and Hu 2006, 2008) in order to shorten the length of chemotherapy.

Fig. 4 Fast speed of kill by HT61 against nonmultiplying *S. aureus*. HT61 was incubated with nonmultiplying *S. aureus* at different concentrations for 6 h. At different time points, CFU counts were performed



4.2 Antimicrobials Targeting Cell Membrane and Cell Wall

There is growing evidence indicating that antimicrobial drugs against nonmultiplying bacteria are likely to target the bacterial cell membrane (Hurdle et al. 2011). The recently approved lipopeptide antibiotic, daptomycin, exhibits activity against stationary-phase nonmultiplying bacteria (Mascio et al. 2007). The mode of action of this drug is associated with modification of cell membrane potential by depolarization of the bacterial membrane (Silverman et al. 2003). Telavancin is a lipoglycopeptide derivative of vancomycin. Its mechanism of bactericidal action against nonmultiplying bacteria (Gander et al. 2005) is dependent on its ability to depolarize the bacterial cell membrane and to increase membrane permeability as well as inhibiting cell wall synthesis (Lunde et al. 2009; Nannini et al. 2010; Higgins et al. 2005). There are some antimicrobial drugs either in preclinical or clinical development targeting bacterial cell membrane or cell walls. For example, other lipoglycopeptide antibiotics such as oritavancin and dalbavancin are able to kill nonmultiplying *S. aureus* in stationary-phase cultures and biofilms (Belley et al. 2009; Darouiche and Mansouri 2005). The mechanism of action is to disrupt membrane potentials and to enhance the permeability of cell membranes (Belley et al. 2009). A small quinoline-derived compound HT61 was developed in an antibiotic discovery program which targets nonmultiplying bacteria from the onset of antibiotic development process (Hu et al. 2010). This is the first time that nonmultiplying cells have been targeted during the initial phases of antibiotic discovery. HT61 is very potent against nonmultiplying Gram-positive bacteria, including those that are methicillin sensitive and resistant, as well as Panton-Valentine leukocidin-carrying *S. aureus*. It also kills mupirocin-resistant MRSA. The action of the drug is fast, showing a complete kill within 0.5–1 h (Fig. 4). The mechanism of action of the drug is depolarization of the cell membrane and destruction of the cell wall (Hu et al. 2010). HT61 is in clinical trials

with aim of decolonizing the nose of *S. aureus* including MRSA. Other examples of these membrane acting drugs are XF-70 and XF-73, which are porphyrin-derived compounds (Ooi et al. 2009). XF-70 and XF-73 show significant potency against nonmultiplying or slowly multiplying bacteria (Ooi et al. 2010). Ceragenins such as CSA-13 are derived from bile acid which kills bacteria by depolarization of bacterial cell membrane (Epand et al. 2010).

Potential advantages of these membrane targeting antibiotics are firstly, they are not only active against actively multiplying bacterial but most importantly they are active against nonmultiplying bacteria. Treatment using single-drug regimens may remove all populations of bacteria in different physiological states which may lead to shortened or efficient antibiotic therapy. Secondly, experimental data demonstrates that there is a very low frequency of resistance development (Van Bambeke et al. 2008; Hu et al. 2010) against this class of antibiotics. After 50 passages of *S. aureus* with sub-MIC concentrations of HT61, no HT61 resistant strains were selected (Hu et al. 2010). The cell membrane is essential. In order for bacteria to develop resistance to membrane active drugs, they need to modify the charges on their membrane lipids or cell membrane components. This almost always leads to death (Van Bambeke et al. 2008). Thirdly, membrane active agents normally possess rapid bactericidal activities (Hu et al. 2010) which remove the persistent bacteria prior to the drug serum level dropping below their therapeutic concentrations. In other words, these agents destroy the bacteria before resistance has a chance to occur. Mutations which are capable of conferring drug resistance cannot occur in dead bacteria.

4.3 Antipersister Formation and Waking up Dormancy

The presence of persisters in human infections is the main reason for prolonged antibiotic treatment and recurrent infection. The mechanisms in which a bacterial community forms persisters are largely unknown. Obviously, a global metabolic shut down and a program of differential gene expression play very important roles. Protein and RNA synthesis associated with stationary phase has been intensively studied in bacteria such as *E. coli*, *S. typhimurium*, and *M. tuberculosis* (Hu et al. 1998; Ward et al. 2010; Soares et al. 2010; Chaussee et al. 2008; Spector and Cubitt 1992; Spector et al. 1988). The stationary-phase response involves the synthesis of a characteristic set of proteins (Dong and Schellhorn 2009) which is accompanied by a gradual decrease in total protein synthesis (Groat et al. 1986; Hu et al. 1998). Most of these proteins are unique to stationary phase (De Groote et al. 2009). This essential de novo protein synthesis, accompanied by the metabolic shutdown, affords the bacteria a remarkable degree of resistance to many stress conditions. Furthermore, these processes enable the bacteria to withstand prolonged periods of

stationary phase. Using transposon mutagenesis to search for persistent genes, it has been demonstrated that an intergenic region of *E. coli* genome is responsible for the persisters' tolerance to high dose antibiotics (Hu and Coates 2005). In *M. tuberculosis*, genes which control bacterial growth have been found (Hu et al. 2006b; Parish et al. 2003). *hspX* gene which encodes an alpha-crystallin-like protein acts as a growth suppresser (Hu et al. 2006b; Stewart et al. 2006). Deletion of the gene results in a hyper-virulent mutant which grows faster than its parental strain (Hu et al. 2006b). This indicates that if the *hspX* gene product was inhibited by targeting with novel drugs, it might be possible to prevent the transition of the bacilli from log-phase growth to the nonmultiplying stage or reduce the speed of growth shifting. If persisters no longer existed or are formed at a significantly reduced speed, conventional antibiotics could be more effective.

VBNC cells can be resuscitated to normal vegetative cells when growth conditions become favorable again. Also bacterial cells are capable of producing growth promoting factors or resuscitation promoting factors such as proteins which stimulate the VBNC cells to regain growth (Mukamolova et al. 1998). If dormant bacteria in human infection can be woken up on a regular basis followed by treatments with the current antibiotic arsenal, it might be possible to effectively achieve complete sterilization and shorten the duration of chemotherapy.

5 Conclusion

Nonmultiplying persistent bacteria were discovered more than 67 years ago. It is commonly accepted that after initial exponential growth in nutrient-rich conditions, bacteria slow their metabolic activities and gradually reach a nonmultiplying stage. When bacteria grow on a solid surface, the growth mimics the profiles of planktonic culture except that biofilms are formed with a self-generated matrix. Nonmultiplying persisters, for example, VBNC cells are present in both planktonic cultures and biofilms and are also present in almost all bacterial infections. The clinical significance of these persisters is that they are profoundly tolerant to antibiotics, which leads to the need to prolong the duration of antibiotic therapy, to use high doses and to employ drug combinations. It is therefore critically important to search for antimicrobials which target nonmultiplying persisters. The most promising antibiotics discovered to date are those acting on structures such as the cell membrane or cell wall. Also, using novel agents to slow down persister formation or waking up dormant cells will be beneficial to potentiate the activities of current antibiotics. In addition, combination therapy with bactericidal and sterilizing antibiotics such as tuberculosis treatment may be able to achieve shorter and more effective antibiotic therapy with improved clinical outcomes and better patient compliance.

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