Induction Signals and Functional Regulation of Antibiotic Tolerance in *Escherichia coli*

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Abstract

Bacteria respond swiftly to environmental perturbations, often becoming insensitive to bactericidal antibiotics. The underlying basis of this tolerance phenomenon, which presumably involves physiological adaptation mechanisms that counteract antibiotic-induced lethality in bacteria, remains poorly-defined. In this study, the fundamental issues of antibiotic tolerance development were addressed, with a focus on elucidating the environmental cues and genetic determinants that regulate this phenotypic switching process.

By examining the relationship between exogenous nutrition status and antibiotic susceptibility in bacteria, amino acids deprivation was identified as a prerequisite condition for tolerance development, during which a repertoire of drug-sepcific phenotypes evolved according to the relative abundances of other key essential nutrients. Sustainability of tolerance was highly dependent on a lack of carbon source and the duration of nutrition stress. Importantly, organisms which experienced prolonged starvation (over 24 h) were found to harbor subpopulations which remained drug-tolerant in nutrient-rich medium, suggesting that antibiotic persisters originated from starvation-induced precursor organisms.

Apart from the nutrition factor, a threshold cell density of 10^8 cells per ml was established as an independent mediator which could elicit phenotypic tolerance under nutrient-rich conditions, producing phenotypes which were markedly different from those observable under starvation in terms of drug specificity. Such cell density effects could be attributed to (i) impeded diffusion of drug and nutrient molecules, which simultaneously suppressed the deleterious effects of antibiotics and elicited cellular protection responses, and (ii) a hitherto undefined quorum sensing-like induction signal which was detectable in spent media of nutrient-supplemented but not starving populations. This finding indicates that bacteria can initiate active defense through cell density sensing even in the absence of starvation stress.

Comparative transcriptomic analysis showed that transient tolerance elicited by amino acids starvation was characterized by global metabolic down-regulation,

whereas emergence of sustainable phenotypes was tightly coupled to a metabolically active state. Gene knockout analysis on established tolerance determinants, such as *hipA*, *phoU* and *glpD*, revealed that their roles in tolerance development were condition and drug specific, suggesting that the cellular network governing starvation-mediated tolerance was highly complex. Studies on selected determinants further revealed the functional roles of multiple stress signaling and protection systems, including the stringent and SOS responses, heat shock proteins, oxidative defense enzymes, and several novel determinants. Among them, the SOS response was specifically required for development of tolerance to fluoroquinolones, whereas products of two novel genes, *yhfZ* and *yqgB*, were predominantly involved in protection against both fluoroquinolones and aminoglycosides. Taken together, results of gene expression and deletion studies depict the involvement of multiple protection systems in sustaining antibiotic stress for a prolonged period. This idea was supported by results of functional studies, which suggested that growth inhibition by bacteriostatic agents, impedance of antibiotic entry and neutralization of hydroxyl radicals were in each case not sufficient to produce significant phenotypic tolerance.

In conclusion, starvation and high cell density-mediated responses were identified as complementary tolerance induction factors in bacteria. Further elucidation of the core components of bacterial "multidrug tolerance regulon" should enable development of more effective strategies for combating resilient microbial infections.

摘要

細菌能因應外在因素而引發非遺傳性的抗藥狀態。這種統稱爲“耐藥性”現象的基本特性和機理，至今仍充滿謎團。這項研究主要探討各項生長參數對耐藥現象的誘發特性以及相應的防禦機制，從而了解這種複雜多變的適應系統。

我們發現，培養基的營養成份和耐藥性有着微妙的關係。其中胺基酸供缺是誘發耐藥反應的先決條件。從這基礎上配搭不同碳、氮、磷及核基的比例，能迅速引發不同程度和專性的耐藥現象，當中胺基酸和碳源的短缺能導致長效的多藥耐藥性。除此以外，較長期的營養短缺 (如多於 24 小時) 能引發持續耐藥細胞的產生，顯示此種不受養份供應約束的耐藥現象亦是源於饑餓反應。

此外，細胞密度是另一種有別於饑餓反應的耐藥性調控因素。於養份充足的狀態下，細胞密度如達臨界點每毫升 10^8 個細菌或以上亦能觸發一系列耐藥狀態。依目前的研究結論顯示，這種情況可歸類爲因細胞密度提升而相對造成的相對藥物稀釋和類饑餓作用，以及一種本質未明的聚量感應 (quorum sensing) 機制。

運用基因晶片轉錄子分析方法以及基因剔除實驗，我們發現如因胺基酸缺乏而導致之短暫性耐藥反應，應源自廣泛性新陳代謝遞減；反之，長效耐藥機制則主要涉及多項具調節內部壓力及防禦功能蛋白的表達如 Stringent 及 SOS 反應、熱休克反應蛋白、抗氧化酶等。其中 SOS 反應主要低禦氟化奎林酮類抗生素的殺菌藥效，而 *yhfZ* 及 *yqgB* 兩個新穎基因則與氨基配糖體及氟化奎林酮類抗生素耐藥性有關。另一方面，生長延緩，藥物滲透阻延及羥基自由基抵銷等推定機制皆不能有效達致長效耐藥性，這些數據進一步顯示細菌防禦功能對耐藥反應的重要性。

總括而言，抗生素耐藥性的誘發和表達涵蓋多種固有對應各種生理壓力的調節機制，並與環境因素及測試藥種息息相關。是項研究長遠有助了解細菌的耐藥機理，並對發展新一代抗菌素的研發有着重要啓示。

Acknowledgements

Four years have passed since I first stepped into the Department of Microbiology and this entirely new experience, in which I was given the great opportunity to design my own PhD project. With piles of Microbiology papers with no particular focus on hands, I began drafting notes on what I had read, without realizing that some of those early scripts, written even before I had a concrete research plan in mind, would become part of this thesis! Looking back, the feeling has been one of 'exciting yet unexpected' throughout the whole process in which I can watch this thesis mature and take shape; the same words can also be used to summarise my life as a PhD student. I would like to thank everyone who has contributed to my joy and tears over all these years, and I am especially grateful to:

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Chapter 1. Background of study

Bacteria are known to respond swiftly to environmental perturbations and undergo transient physiological changes that confer protection against the lethal effects of various bactericidal antibiotics (Greenwood, 1985; Levin and Rozen, 2006). This unique mode of survival strategy, collectively known as phenotypic tolerance or persistence, has been reported since the penicillin era, when it was first demonstrated that culture of drug-sensitive bacteria could not be completely sterilized using antimicrobial drugs as a result of the presence of "antibiotic persisters" (Bigger, 1944) and multiple exogenous factors which modulated bacterial physiological responses to the bactericidal effects of beta-lactam antibiotics (Tuomanen *et al.*, 1986). Although bacterial tolerance against penicillin was the first example of this phenomenon, it is now clear that treatment by other classes of antibiotics also fails to achieve complete microbial eradication (Levin and Rozen, 2006; Lewis, 2007). This chapter reviews our current knowledge on this fascinating yet often overlooked subject in microbiology.

1.1. Mechanisms of antibiotic-induced cell death

Understanding how antibiotics induce bacterial death is a vital step in unraveling the cellular basis of antibiotic tolerance. Despite the diversity of antimicrobial agents currently available for the treatment of bacterial infections, they can be classified into two general categories based on their ultimate effects on bacterial growth and survival: (i) bacteriostatic drugs (e.g. tetracyclines and rifampicin) which only inhibit cell growth, and (ii) bactericidal drugs (e.g. beta-lactams, fluoroquinolones and aminoglycosides) which can rapidly induce bacterial cell death and/or lysis.

Cell death elicited by cell wall inhibitors such as the beta-lactam antibiotics, which commonly cause rapid cell lysis, has been proposed to involve a two-step enzymatic process (Tomasz, 1979b). The first step involves binding and inhibition of its immediate drug target (e.g. penicillin binding proteins), suppressing peptidoglycan metabolism and producing a bacteriostatic effect; this is followed by both direct and indirect activation of endogenous murein hydrolases known as "autolysins", which

eventually lead to cell wall hydrolysis and osmotic burst. Recent studies on vancomycin-tolerant *Streptococcus pneumoniae* led to development of an advanced model of vancomycin-induced lysis, which involves secretion of the Pep27 "death peptide" through its associated transporter Vex, subsequently activating the vncRS two-component system and the corresponding lytic response (Novak *et al.*, 1999; Novak *et al.*, 2000). However the validity of this *pep27-vex-vncRS* pathway theory has been challenged by other workers (Robertson *et al.*, 2002). Although similar regulatory networks have not been reported for other bacterial species, an analogous two-step model (extensively reviewed in (Drlica *et al.*, 2008)) has been proposed for explaining the bactericidal action of DNA (Drlica *et al.*, 2008) synthesis inhibitors such as fluoroquinolones, which target DNA gyrase and topoisomerase IV, the enzymes which promote relaxation of positive DNA supercoils by introducing single-stranded chromosome breaks during the replication process. Binding of drug molecules to DNA-bound gyrase and topoisomerase IV results in the formation of "cleaved-complexes" and rapidly halts DNA replication; this process is reversible and by itself non-lethal in nature. However, the generation of cleaved complexes may trigger secondary events that ultimately lead to chromosome fragmentation and cell death in both protein synthesis-dependent and synthesis-independent manner (Malik *et al.*, 2006). On the other hand, prolonged inhibition of DNA replication may trigger other putative programmed cell death mechanisms such as activation of toxins and generation of hydroxyl radicals, initiating a "slow-killing" process in the absence of chromosome fragmentation. This hypothesis is supported by a recent finding which showed that fluoroquinolone-mediated, protein synthesis-dependent cell death also involved hydroxyl radical activities (Wang *et al.*, 2010).

Apart from antibiotics which target cell wall and DNA synthesis, translation inhibitors such as aminoglycosides are also capable of inducing rapid cell death; however, irreversible binding of this class of drug to 30S ribosomes and the subsequent inhibitory effect on protein synthesis cannot fully account for their unique bactericidal properties, since other types of protein synthesis inhibitors, such as tetracyclines, only produce bacteriostatic effects. Scattered findings (reviewed in (Davis, 1987)) suggested that cellular lethality induced by aminoglycosides likely stems from the production of mistranslated proteins which are incorporated into cell membranes, disrupting their integrity in a positive feedback manner that eventually

leads to excessive membrane leakage and free radical damages, and eventually cell death (Kohanski *et al.*, 2008).

In addition to the diverse yet drug-specific damages triggered by different classes of bactericidal antibiotics, recent studies also identified two common antibiotic-induced cell death mechanisms, namely, activation of chromosomal toxin-antitoxin (TA) modules and production of reactive oxygen species (ROS). TA module (e.g. *relBE* and *mazEF*) is a two-gene operon that encodes a stable proteinaceous toxin and a labile antitoxin, which were once regarded as a plasmid stabilization system until the identification of a large number of such loci in the chromosomes of different species of bacteria (Gerdes *et al.*, 2005). The unbound toxin, which functions as specific mRNA interferase, is a potent inhibitor of macromolecule synthesis *in vivo* (Yamaguchi and Inouye, 2009). Under normal physiological conditions, toxins are bound by their cognate antitoxins and had no detrimental effects on cellular activity. Exposure to environmental stresses or antibiotics may disrupt toxin-antitoxin coupling as a result of inadequate synthesis and/or degradation of antitoxin, the latter being mediated by the Lon protease (Gerdes *et al.*, 2005). These events in turn lead to rapid loss of cell viability (Amitai *et al.*, 2004; Sat, 2001), supporting the view that TA modules may serve as prokaryotic programmed cell death (PCD) regulators (Engelberg-Kulka *et al.*, 2004). Further characterization of the mazEF toxin-antitoxin systems led to identification of a novel extracellular death factor (EDF) which was shown to be essential for mazF-mediated lethality (Kolodkin-Gal *et al.*, 2007) through both ROS-dependent and ROS-independent pathways (Kolodkin-Gal *et al.*, 2008). Despite the recent advancement in the study of EDF/mazEF-induced PCD, the role of TA modules as regulators of antibiotic-mediated cell death has been challenged by a number of reports which suggest that these "toxins" are in fact involved in the development of antibiotic tolerance (Keren *et al.*, 2004b; Kim and Wood, 2009; Lewis, 2005). Resolution of such discrepancy awaits further experimental evidence.

Another popular model of antibiotic-induced cell death (Dwyer *et al.*, 2009) involves uncontrolled production of deleterious hydroxyl free radicals, which is elicited by the activities of bactericidal antibiotics including beta-lactams (Kohanski *et al.*, 2007), fluoroquinolones (Dwyer *et al.*, 2007) and aminoglycosides (Kohanski

et al., 2008). It was demonstrated that specific target inhibition by these bactericidal agents produced common downstream effects on metabolism such as stimulation of TCA cycle-associated NADH oxidative activities, release of intracellular ferrous iron, and increased production of hydroxyl radicals, eventually leading to viability loss. Consistent with this theory is the finding that supply of free-radical or ferrous-iron scavengers, such as thiourea and 2-2'-dipyrydyl respectively, could significantly reduce the rate of antibiotic-mediated bacterial cell death (Kohanski *et al.*, 2007). These studies therefore revealed an important mechanism of antibiotic-induced cellular damages; however, its relative contribution to drug-induced lethality with respect to those concerning the upstream drug target-specific molecular events remains elusive. Furthermore, a piece of recent evidence suggested a contradictory role of specific TCA cycle components, such as the SucB gene product, which seemed to promote survival rather than killing during antibiotic treatment (Ma *et al.*, 2009). Nevertheless, it is plausible that the process of antibiotic-induced cell death comprises multiple condition-specific pathways, and involves both drug specific and non-specific damages (Figure 1.1).

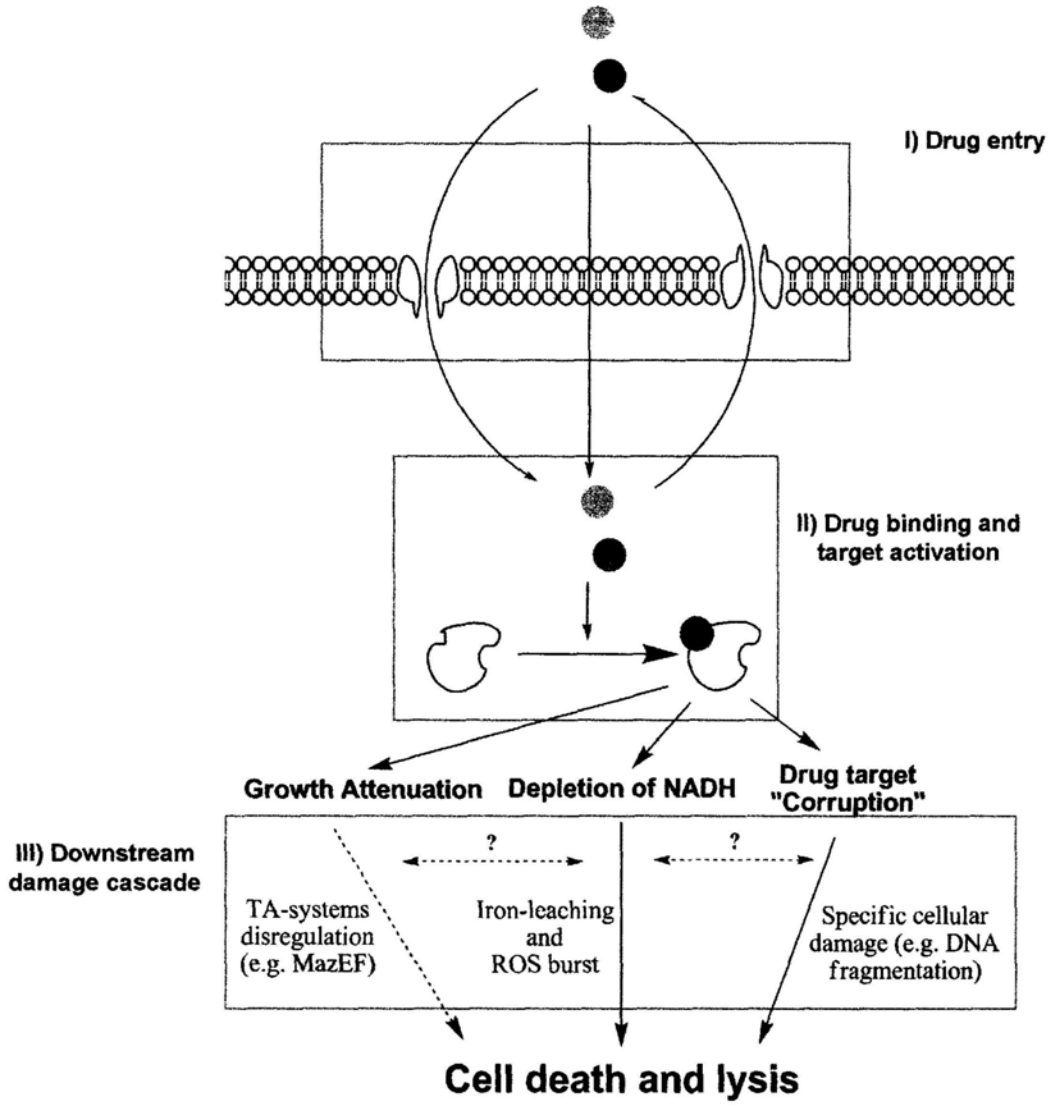


Figure 1.1. Antibiotic-mediated cell death. Bacteriostatic antibiotics inhibit cellular metabolism by binding to their respective drug targets, bringing bacterial cell growth to a halt. In addition to the bacteriostatic effects, bactericidal antibiotics such as beta-lactams, fluoroquinolones and aminoglycosides produce further cellular damages including those caused by accumulation of deleterious hydroxyl radicals, which eventually result in cell death.

1.2. Phenotypic tolerance and bacterial persisters

Phenotypic switching to antibiotic tolerance and genetical evolution of resistance can be regarded as two major survival strategies in bacteria which complement each other to counteract the lethal effects of antimicrobial agents: drug resistant organisms can grow at elevated antibiotic concentrations but rapidly lose viability once the drug concentration exceeds the MICs (i.e. increased MIC but MBC to MIC ratio remains unchanged). On the other hand, the drug tolerance state allows micro-organisms to withstand lethal concentrations of bactericidal antibiotics independent of their intrinsic drug susceptibility profile (i.e. MIC remains constant but MBC to MIC ratio is increased) (Figure 1.2). In contrast to genotypic tolerance (Tomasz *et al.*, 1970), which only occurs in a strain-specific manner and involves specific genetic defects in antibiotic-induced lytic pathways (Charpentier and Tuomanen, 2000; Handwerger and Tomasz, 1985; Novak *et al.*, 1999), all non-mutated and drug-susceptible organisms can transiently exhibit phenotypic tolerance to antibiotics in response to various exogenous stimuli (Handwerger and Tomasz, 1985). Environmental factors that mediate tolerance formation will be described in detail in the following sections.

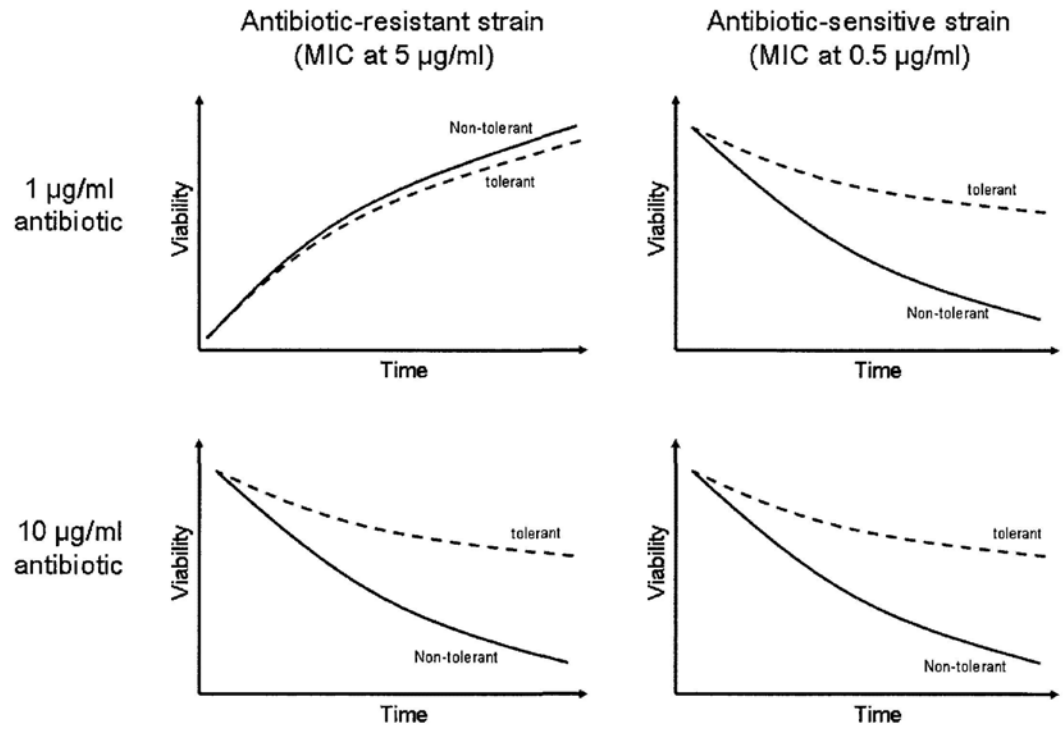


Figure 1.2. Schematic representation of the phenotypic characteristics of antibiotic tolerance and resistance. Antibiotic resistant bacteria can grow at antibiotic concentrations which can eradicate the majority of drug-sensitive organisms. Both antibiotic sensitive and resistant bacteria exhibit drug tolerance which enables prolonged survival under the respective lethal concentrations (above MIC) of antibiotics through development of inheritable genotypic tolerance or activation of non-inheritable phenotypic tolerance mechanisms.

Another phenomenon that phenotypically resembles non-inherited drug tolerance is that non-dividing "antibiotic persisters" are detectable in all bacterial populations regardless of their general drug susceptibility profiles (Figure 1.3). The discovery of heterogenic variants in bacterial populations can be dated back to 1944 when Dr. Joseph Bigger reported that penicillin failed to completely sterilize antibiotic sensitive staphylococcal populations, presumably because of the presence of persisters, which, as he described, are rare cells that existed at a ratio of 1 in 1,000,000 and possessed the ability to withstand the bactericidal effects of penicillin independent of growth conditions (Bigger, 1944). These penicillin-tolerant persisters are neither resistant mutants, since it can resume growth and give rise to antibiotic-sensitive offsprings in the absence of antibiotics (Bigger, 1944), nor penicillin-induced protoplasts (Lederberg, 1956), as they were found to be insensitive to osmotic changes and erythromycin (Greenwood and O'Grady, 1970). It is hypothesized that persisters may represent a small fraction of cells in the asynchronized bacterial population that reside in a physiological stage preceding cleavage of the existing murein network by peptidoglycan hydrolases, the enzymes responsible for the incorporation of newly synthesized murein during cell wall synthesis (Greenwood, 1972). Since peptidoglycan hydrolases are pencillin-binding proteins which can be inhibited by penicllin, the majority of organisms with cleaved cell wall would lyse as a result of continued cell elongation, whereas persisters survive with intact cell wall.

Studies on antibiotic persisters achieved little progress until 1983 when Moyed and coworkers isolated the first "high persistence" mutant and subsequently identified *hipA*, the gene which presumably regulated the proportion of persisters in bacterial population (Black *et al.*, 1991; Moyed and Bertrand, 1983; Moyed and Broderick, 1986). Moyed's studies also showed that persisters were tolerant to multiple antibiotics (Black *et al.*, 1991), such property was consistent with those of recent findings regarding the drug tolerance characteristics of persisters (Wiuff, 2005; Wiuff and Andersson, 2007), as well as that of a report which showed that the locus *hipQ* could mediate variation in the abundance of both fluoroquinolone and beta lactam persisters (Wolfson *et al.*, 1990).

The origin and identity of persisters remained elusive until recent years, when

several independent studies suggested that they were metabolically dormant cells (Shah, 2006) formed either during stationary-phase passage, or randomly within a growing population (Balaban *et al.*, 2004). This process was presumably driven by intrinsic molecular "noises" (Avery, 2006; Keren *et al.*, 2004b; Losick and Desplan, 2008; Thattai and van Oudenaarden, 2004) on the expression of persister genes, such as *hipA*, which led to expression of heterogeneous properties pertinent to persister formation within an isogenic population. However, an alternative hypothesis suggested that persisters might be cells which were under extensive DNA repair (Debbia *et al.*, 2001) or undergoing an aging process (Klapper *et al.*, 2007). While it is now clear that microbial populations contain specialized cells which are phenotypically tolerant to multiple antibiotics, their formation as well as the underlying mechanisms involved remain largely unknown. Interestingly, a recent study reported that development of persisters involved a brief period of protein synthesis (Gefen *et al.*, 2008) prior to the entry into the antibiotic tolerant state. The variety of theories on persister formation appears to suggest that antibiotic persistence is governed by a diversity of molecular mechanisms.

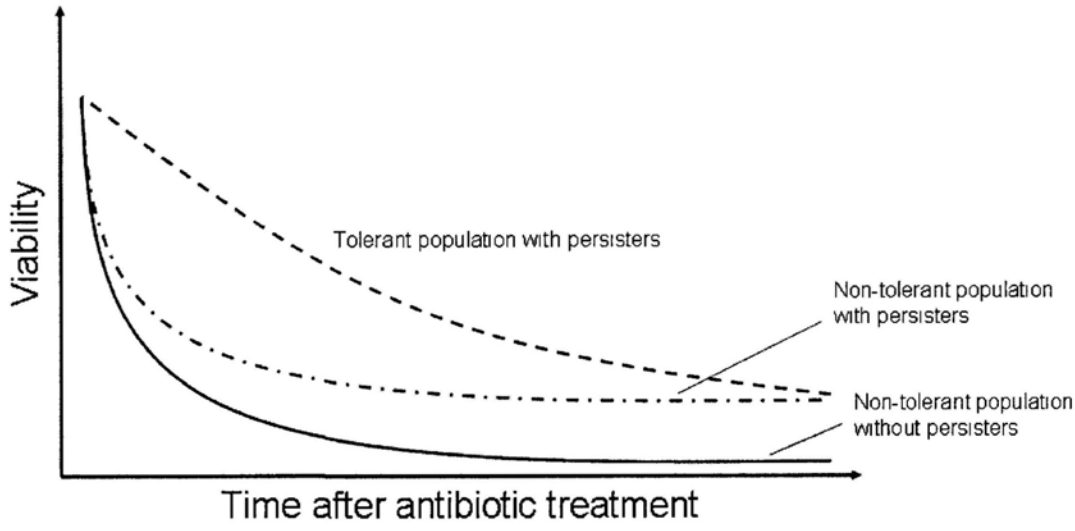


Figure 1.3. The antibiotic tolerance and antibiotic persister phenomena. Antibiotic persisters are rare specialized cells that are phenotypically tolerant to antibiotics and detectable in both drug tolerant and non-tolerant populations.

1.3. Protection against drug lethality

Development of antibiotic tolerance stems from reduced antibiotic-induced lethality (Figure 1.4). Early studies on phenotypic tolerance have been focused on the underlying mechanisms that protect bacteria against the effects of beta-lactam antibiotics, which have a unique property of rapidly lysing susceptible organisms (Tomasz, 1979a). Lytic responses to beta-lactams are believed to be mediated through autolysins which are cell wall biosynthesis enzymes (Holtje, 1995), since genotypically tolerant mutants were found to harbour loss of function mutation in the *lytA* gene, which encodes for the *N*-acetylmuramoyl-L-alanine amidase, an autolysin in *Strepococcus pneumoniae* (Tomasz *et al.*, 1970). Based on this pilot study, early reports on various factors or conditions which elicit development of phenotypic tolerance to beta-lactams, such as growth inhibition (Handwerger and Tomasz, 1985) and modulation (Tuomanen *et al.*, 1986), reduction in pH (Goodell *et al.*, 1976), protease treatment (Handwerger and Tomasz, 1985) and autolysin inhibitors (Handwerger and Tomasz, 1985) suggested that the tolerance inducing effects were in most cases mediated through direct and/or indirect inhibition of autolysin activities. In the case of fluoroquinolones, it was reported that anaerobiosis and/or translation inhibition could alleviate the lethal effects of this class of antibiotic (Malik *et al.*, 2007). Similar findings were also documented on the effect of translation block using chloramphenicol, which was shown to suppress the bactericidal effects of aminoglycosides and produce the phenomenon of antibiotic antagonism (Jewetz *et al.*, 1952).

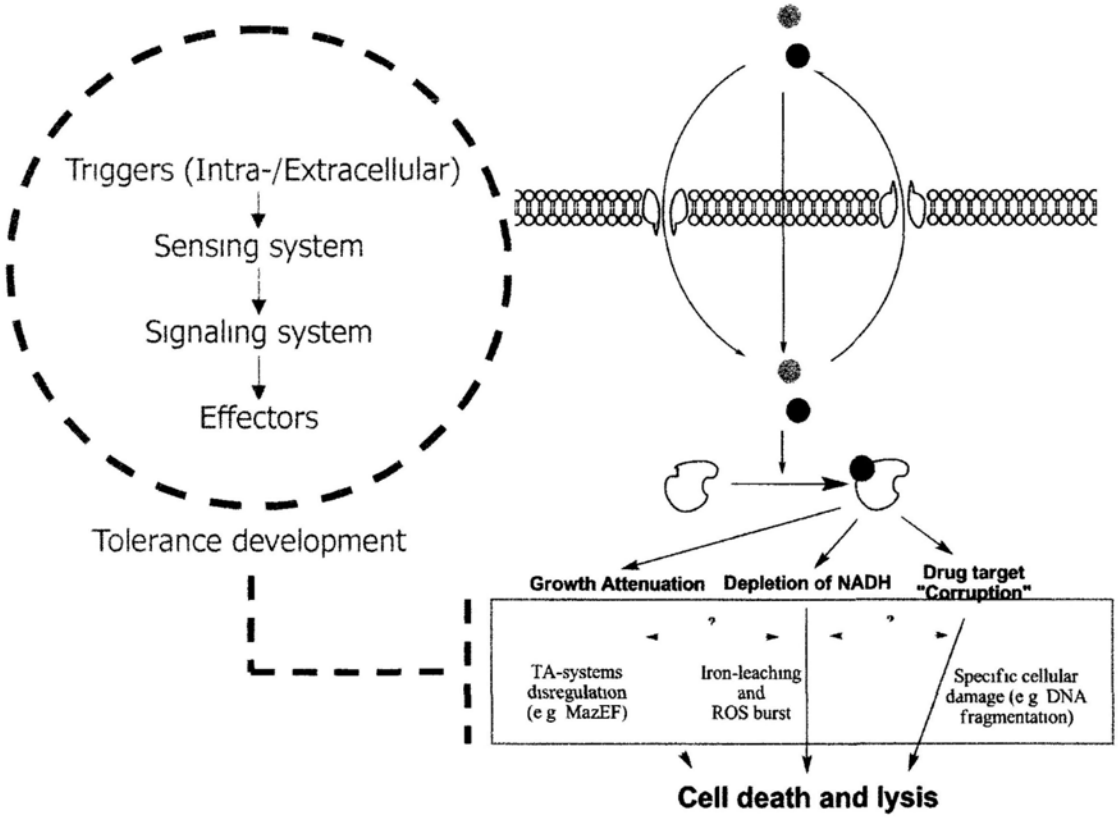


Figure 1.4. Principle of antibiotic tolerance development. Reduced lethality to antibiotics occur when the putative drug-induced killing processes are interrupted by direct inhibition of bactericidal mechanisms (e.g. mutation of *lyt*A gene) or induced responses (e.g. nutrient limitation) that activate pre-emptive protection mechanisms and mediate repair of cellular damages.

Among the plethora of extracellular factors which may lead to reduced susceptibility to killing by bactericidal antibiotics (Handwerger and Tomasz, 1985), the environmental state eliciting growth transition from log-phase to stationary-phase may be considered as one of the most important and concerted driving force that mediate development of multidrug tolerance (Keren *et al.*, 2004a; Kolter *et al.*, 1993; Levin and Rozen, 2006; Matin *et al.*, 1989; Novak and Tuomanen, 2002; Nystrom, 2004) and multi-stress resistance (Kolter *et al.*, 1993; Matin *et al.*, 1989). The physiological changes associated with entry into stationary-phase has largely been attributed to the activation of an adaptation program known as the Stringent Response, which is mediated by the bacterial alarmone (p)ppGpp (Potrykus and Cashel, 2008; Srivatsan and Wang, 2008). In response to different starvation signals, such as low levels of amino acids, carbon sources or phosphate, the (p)ppGpp synthetase RelA and/or the bifunctional (p)ppGpp synthetase and hydrolase SpoT are activated, resulting in rapid synthesis of the nucleotide derivatives pppGpp and subsequently ppGpp (Potrykus and Cashel, 2008). ppGpp binds to the RNA polymerase molecule and triggers global transcriptional re-programming of the bacterial cell by regulating the promoter binding and recognition activities of RNA polymerase through open complex destabilization and selective modulation of sigma factor binding, respectively. This cascade of events results in global down-regulation of growth and proliferation activities (e.g. reduced stable RNA and DNA synthesis) (Srivatsan and Wang, 2008) as well as up-regulation of amino acids biosynthesis and multiple stress defense mechanisms via mobilization of the stationary-phase sigma factor RpoS and expression of universal stress proteins (USPs) (Magnusson *et al.*, 2005).

In *Escherichia coli*, it has been demonstrated that development of penicillin tolerance during amino acid starvation is closely related to an elevation in ppGpp levels, which can be further linked to inhibition of phospholipid biosynthesis (Rodionov and Ishiguro, 1995). In another study, onset of amino acids starvation and development of penicillin tolerance in *Escherichia coli* is accompanied by the production of structurally modified murein, which is progressively less hydrolysable by autolysin; importantly, such physiological change is also partially dependent on the stringent response (Goodell and Tomasz, 1980). Further investigation of the observations in pneumococci suggested that phenotypic tolerance to pencillin

inducible by amino acids starvation is a two-stage and time-dependent process that involves disengagement or loss of autolysin from their target sites (phase 1), followed by introduction of non-hydrolysable cell wall material into the growth zone (phase 2) (Tuomanen and Tomasz, 1990). However, to date, it is still unclear how the onset of stringent response results in cell wall modification and whether this physiological response can fully account for the robust drug tolerance phenotypes typically observed in starved or stationary-phase organisms.

Apart from the stringent response regulated by RelA/SpoT, another global regulator which is inducible under various stress conditions as well as stationary-phase onset is the alternative sigma factor RpoS (Hengge-Aronis, 2002), which may directly or indirectly regulate about 10% of all *Escherichia coli* genes including those involved in multiple stress survival, protein processing and energy metabolism (Klauck *et al.*, 2007; Weber *et al.*, 2005). While RpoS activity is subjected to complex, multi-level regulation (Klauck *et al.*, 2007), it is clear that this important stress response regulator is responsive to different nutrition-limiting conditions (Peterson *et al.*, 2005) and is positively regulated by the stringent response (Magnusson *et al.*, 2005). The functional relationship between RpoS and antibiotic tolerance is not well defined but recent studies indicated that RpoS is at least partially responsible for stationary-phase antibiotic tolerance in a *mazEF*-dependent manner (Kolodkin-Gal and Engelberg-Kulka, 2009), as well as formation of ofloxacin tolerance in both *Escherichia coli* (Hansen *et al.*, 2008) and *Pseudomonas aeruginosa* (Murakami *et al.*, 2005). There is suggestion that this fluoroquinolone tolerance induction property is at least partially dependent on the DNA-binding and oxidative stress protection protein Dps (Almiron *et al.*, 1992); however, the role of RpoS-dependent protection against multiple antibiotic stresses has yet to be determined.

Despite the consensus on the role of stringent and RpoS mediated responses in development of starvation-induced drug tolerance, alternative mechanisms have also been implicated in alleviating the lethality of antibiotics under stationary-phase, these include the heat shock proteins DnaK, GroEL and HtpG, the expression of which are *rpoH* (the heat shock sigma factor) but not *rpoS*-dependent (McCann *et al.*, 1991). Indeed, it has been reported that expression of multiple heat shock proteins led to

inhibition of beta-lactam-induced lysis in the absence of RelA (Powell and Young, 1991); recent studies on antibiotic persisters undoubtedly revealed a growing number of candidate genes that may have diverse functions in mediating antibiotic tolerance. These putative tolerance genes will be discussed individually in the following sections:

Cell dormancy mediated by the hip loci and Toxin-Antitoxin (TA) modules

As discussed above, toxin-antitoxin (TA) pairs are commonly found in low-copy number plasmids and bacterial chromosomes which are believed to serve diverse cellular functions such as plasmid stabilization, growth regulation and programmed cell death (PCD) (Van Melderen and Saavedra De Bast, 2009). The functional relationship between antibiotic persistence and TA modules was abridged by the discovery of the *hipA7* high-persistence mutant, which in turn led to the identification of the *hipBA* operon, a putative TA module, in the 1980s by Moyed and coworkers (Black *et al.*, 1991; Moyed and Bertrand, 1983; Moyed and Broderick, 1986). The *hipA7* strain, selected by ethyl methanesulfonate (EMS) mutagenesis, has a 100-fold higher frequency of persisters during the log-phase as compared to its isogenic hipA+ parent when exposed to various lethal stresses including antibiotic exposure and thymine starvation (Moyed and Bertrand, 1983). The wild-type *hipA* gene encodes a 48-kDa protein which is lethal when expressed alone, presumably through inhibition of macromolecular synthesis (Moyed and Broderick, 1986). The *hipA7* toxin allele habours two point mutations and was found to be non-toxic as it can be expressed in the absence of the *hipB* antitoxin gene (Korch *et al.*, 2003). How this mutant allele gives rise to a higher proportion of persisters and how such gain-of-function mutation modulates the molecular functions of the wild-type HipA is still unclear. The fact that disruption of *relA* and *spoT* abolished the mutant phenotype of the *hipA7* strain (Korch *et al.*, 2003) further suggested the involvement of stringent response in drug tolerance; moreover, it was also speculated that the persister induction modes of HipA7 and HipA were not identical (Korch and Hill, 2006).

Evidence regarding the functional similarities between *hipBA* and the established *relBE* or *mazEF* TA systems, as well as the observation that the expression level of several TA modules was elevated in *hipA7* persisters, fuel the speculation that

antibiotic persistence is regulated by the stochastic expression and reversible bacteriostasis effects mediated by the environmental sensing and regulatory functions of products of this gene family (Keren *et al.*, 2004b; Pedersen *et al.*, 2002). Indeed, overexpression of *hipA* or *relE* resulted in a higher level of persisters (Korch and Hill, 2006), but this conclusion has been suggested to be erroneous since the effect of protein over-expression may elicit secondary stresses that in turn trigger the formation of persisters (Vazquez-Laslop *et al.*, 2006). On the other hand, deletion of *hipBA* but not *relBE* or *mazEF* had a significant impact on antibiotic persistence (Keren *et al.*, 2004b), suggesting the possibility that only specific TA modules may contribute to persister formation. In addition, HipA is the first toxin which was found to possess kinase activity (Correia *et al.*, 2006), with the translation elongation factor EF-Tu being its immediate target (Schumacher *et al.*, 2009). However, the proposed roles of *hipBA* or other TA modules have been challenged by recent reports which demonstrated that deletion of these genetic components had no detectable effect in persistence (Li and Zhang, 2007) or general stress survival (Tsilibaris *et al.*, 2007). Nevertheless, the number of TA modules postulated to play a role in persister formation has increased markedly in recent years; these include the recently identified MqsRA and the corresponding toxin CspD (Kim and Wood, 2009; Kim *et al.*, 2010; Shah, 2006), and the SOS response-dependent toxin TisAB (Dorr *et al.*, 2010). These findings indicate that further experimental proof is required to validate the "toxin and antitoxin" hypothesis on antibiotic persister development.

SOS response and DNA repair

Fluoroquinolones belong to a major class of bactericidal antibiotic which introduces lethal DNA damages in bacteria (Drlica *et al.*, 2008). However, bacteria are known to exhibit stress defense response against DNA lesions caused by these drugs. Such response, which is attributed to an endogenous DNA repair program known as the SOS response (Little and Mount, 1982), is mediated through a group of SOS genes which are normally repressed by the LexA repressor protein. The accumulated effects of DNA lesions induce binding of RecA to the single-stranded DNA regions, triggering self-cleavage of LexA. This process in turn enables de-repression of SOS genes in a time-dependent manner, leading to cell cycle arrest and DNA repair (Janion, 2008). RecA also facilitates homologous recombination at the damaged site during the repair process. In addition, expression of early SOS

genes such as *uvrABCD* is known to mediate nucleotide excision repair (NER), and late SOS genes such as *umuDC* have also been found to mediate mutagenic repair by the error-prone polymerase V (Janion, 2008).

Mutants defective in SOS response were found to result in reduced antibiotic tolerance, suggesting that antibiotic persisters may be organisms in which cell division is halted while undergoing SOS-mediated DNA repair (Debbia *et al.*, 2001). Moreover, induction of SOS response by the addition of mitomycin C or subinhibitory concentration of fluoroquinolones resulted in an increase in persister subpopulation (Debbia *et al.*, 2001; Dorr *et al.*, 2009). Beta-lactam-mediated inhibition of PBP3 has been found to activate the SOS response through the *dpiBA* two-component system, and disruption of this pathway resulted in an increased susceptibility to killing by ampicillin (Miller *et al.*, 2004). A recent study also reported that SOS-mediated tolerance to fluoroquinolones involved activation of the LexA-dependent toxin TisB (Dorr *et al.*, 2010), this finding suggested a concomitant role of cell dormancy and DNA repair in the formation of antibiotic persisters. Although a clear role of SOS response in antibiotic tolerance has been demonstrated, the upstream triggers as well as the downstream protective mechanisms conferring stationary-phase antibiotic tolerance in bacteria remain undefined.

Heat shock response and molecular chaperones

The heat shock response is a stress protection mechanism which assists in protein folding and repair when bacteria encounter heat and oxidative stresses, which may disrupt the normal functioning of cellular proteins (Yura and Nakahigashi, 1999). This response is mainly governed by RpoH, the heat-shock sigma factor, as well as RpoE, the envelope stress sigma factor, both of which are sensitive to the level of intracellular unfolded proteins (Gruber and Gross, 2003). RpoH is known to regulate the expression of a variety of heat shock proteins (HSPs) including the DnaK/DnaJ/GrpE and GroES/GroEL chaperone systems (Lund, 2001), as well as the ClpAP and Lon proteases; these proteins act in concert to regulate the level of unfolded proteins inside the cell (Yura and Nakahigashi, 1999). Chaperones such as DnaK, DnaJ and GrpE are inducible during stationary-phase entry in a RpoH-dependent manner and are responsible for survival against heat and oxidative stress under starvation (Matin *et al.*, 1989). Scattered evidence has suggested that

HSPs may also affect survival against antibiotics (Hansen *et al.*, 2008; Powell and Young, 1991; Wolska *et al.*, 2000). However, whether these "protein stabilizers" mediate stationary-phase antibiotic tolerance through direct protection of essential cellular proteins or by indirect maintenance or potentiation of other antibiotic tolerance components awaits further clarification.

Metabolic control and drug tolerance

A number of recent studies have shown that disruption of specific metabolic regulators, such as those involved in glycerol metabolism (*plsB* and *glpD*) (Spoering *et al.*, 2006), phosphate metabolism (*phoU*) (Li and Zhang, 2007) and energy production (*sucB* and *ubiF*) (Ma *et al.*, 2009), led to reduced tolerance to antibiotics. These studies unequivocally suggested that the effects are gene-specific, i.e. disruption of other components in the metabolic pathway had no effect on tolerance. While the mechanisms underlying the metabolite effects on tolerance formation is still poorly understood, it is likely that such metabolic regulators may have diverse regulatory functions during tolerance development. Indeed disruption of the phosphate regulator *phoU* alone resulted in global up-regulation of genes responsible for energy production and chemotaxis, enhancing cellular activities and susceptibility to antibiotics (Li and Zhang, 2007). Although this finding suggested that an active metabolic state is coupled to low tolerance level, it was shown that the absence of enzymes involved in energy production also resulted in enhanced antibiotic susceptibility (Ma *et al.*, 2009). The precise role of energy production proteins in tolerance development entails further investigation.

1.4. Clinical impact of antibiotic tolerance

Antibiotic tolerance, which may be regarded as a phenotypic switch controlled by intrinsic cellular mechanisms, enhances survival of bacteria against antibiotic stress and often complicates treatment of microbial infections (Bigger, 1944; Lam and Bayer, 1983; Novak and Tuomanen, 2002). This basic phenomenon offers a reasonable explanation to the *in vitro* and *in vivo* discrepancy in the therapeutic outcome of antimicrobial chemotherapy (Dhar and McKinney, 2007; Lam and Bayer, 1983). Such tolerance concept has been partially validated by studies involving various animal models, which suggested that the *in vivo* micro environment at the site of infection (e.g. amino acids limitations in CSF) could induce development of stationary-phase drug tolerance (Davey *et al.*, 1988; Entenza *et al.*, 1997; Lam and Bayer, 1983; Lam and Bayer, 1983; Novak and Tuomanen, 2002).

Another clinically relevant example of antibiotic tolerance is the extremely long treatment strategy (from 6 months to 2 years) commonly employed for the treatment of *Mycobacterium tuberculosis* (MTB) infections (Zhang, 2007), a major infectious disease which accounts for about 2 million deaths and more than 9 million new cases per year (World Health Organization, 2009). It has been suggested that ineffective treatment of MTB stems from the presence of antibiotic persisters which are phenotypically tolerant to antibiotics (Gomez and McKinney, 2004; Hu *et al.*, 2003; Mitchison *et al.*, 2007; Wallis *et al.*, 1999; Zhang, 2007). Although the mechanism of drug tolerance in MTB is largely undefined, a recent study hinted that the regulation mechanism may be similar to that in *Escherichia coli*, which involves the induction of stringent response (Stallings *et al.*, 2009).

Bacterial or fungal pathogens may also lead to development of implant-related infections or dental plaque through the formation of biofilm (Monds and O'Toole, 2009), which is commonly regarded as structured microbial communities bound by an extracellular matrix. Biofilms are particularly difficult to eradicate compared to planktonic populations presumably due to impeded entry of antibiotic molecules and decreased growth rate of biofilm cells (Costerton *et al.*, 1999). However this model is far from being sufficient in explaining biofilm resistance, since some antibiotics such

as fluoroquinolones (Brooun *et al.*, 2000) or toxic metals (Harrison *et al.*, 2005a) can penetrate the matrix effectively. Various recent findings also suggested that the presence of antibiotic tolerant persister cells in biofilm constitutes the basis of its recalcitrant nature (Arciola, 2008; Haagensen *et al.*, 2007; Harrison *et al.*, 2005b; Lewis, 2001; Lewis, 2007). Further understanding of persister physiology and the underlying drug tolerance mechanisms involved will undoubtedly contribute to development of better treatment strategies against biofilm-related infections.

Non-inheritable protection against drug lethality associated with phenotypic tolerance may have long-term benefits to microbial populations since development of antibiotic resistant variants may be promoted through a process known as adaptive mutation, which becomes active during the tolerant state (Foster, 2007; Sandegren and Andersson, 2009). Antibiotics which elicit DNA damages are also known to be mutagenic (Riesenfeld *et al.*, 1997), presumably due to activation of endogenous error-prone DNA repair mechanisms controlled by the SOS response (Sutton *et al.*, 2000). As suggested by *in silico* studies of antibiotic treatment on bacterial populations containing antibiotic tolerant cells (Levin and Rozen, 2006), it is also possible that enhanced survival against antibiotic stress may increase the likelihood of resistance development. Although the mutation induction potential in tolerant organisms has yet to be elucidated by *in vivo* studies; interference of antibiotic tolerance development may potentially serve as an important strategy in delaying emergence of antibiotic resistant variants in future drug development.

1.5. Aim of study

The objective of this study is to elucidate the environmental induction factors and the corresponding cellular sensing and response network governing the development of phenotypic antibiotic tolerance in bacteria. Despite the renewed interest in this area, efforts to better understand this complex physiological change have been hampered by the lack of reproducible findings among studies performed by different research groups (Hansen *et al.*, 2008; Li and Zhang, 2007; Ma *et al.*, 2009). We believe that such discrepancy may stem from different choices of bacterial model and induction / selection criteria. Another factor which may have been overlooked in the current study approaches concerns the concept of "stationary-phase", which is primarily a term used to describe a specific phase of balanced microbial growth and cell death dynamics elicited and maintained by limitation of one or more growth parameters (Kolter *et al.*, 1993), with concomitant activation of different stress response pathways. It should be emphasized that conventional antibiotic tolerance studies commonly utilize stationary-phase populations in which the organisms have been subjected to nutrient deprivation and other stresses (Eng *et al.*, 1991; Hansen *et al.*, 2008). A recent study by Hansen *et al.* suggested that antibiotic tolerance was mediated by multiple global regulators which were redundant in nature (Hansen *et al.*, 2008). Hence, identification of individual genetic component of such redundant antibiotic tolerance mechanisms is not readily achievable if the population is subjected to multiple stresses simultaneously.

To delineate the inductive and functional factors involved in tolerance formation, drug susceptible log-phase bacteria were exposed to a panel of strictly defined conditions, followed by analysis of the subtle inductive effects of specific environmental parameter on the development of phenotypic tolerance. Based on the condition-specific antibiotic tolerance profiles, we were able to validate the role of individual response and protection component in mediating antibiotic stress survival by means of functional and genetic approaches. Findings in this work demonstrate that bacteria are highly sensitive to fluctuation in exogenous nutrient composition and population cell density in terms of phenotypic variation in antibiotic susceptibility and persister population size. The following chapters describe the

process of phenotypic characterization and identification of genetic components responsible for the overlapping condition of sensing and protection functions in *Escherichia coli*.

Chapter 2. Starvation mediated antibiotic tolerance

2.1. Introduction

Starvation of essential nutrients has long been regarded as the pivotal physiological factor in bacteria that promotes entry into the stationary-phase (Kolter *et al.*, 1993). This dormant state is characterized by the development of multiple stress resistance through starvation-inducible defense pathways such as the stringent response and major reprogramming of cellular functions, in which multiple alternative sigma factors also play important roles (Potrykus and Cashel, 2008; Srivatsan and Wang, 2008). While the underlying basis of starvation-mediated antibiotic tolerance has been investigated by multiple workers (Eng *et al.*, 1991; Handwerger and Tomasz, 1985; Tuomanen *et al.*, 1986), evidence suggesting that limitation of different nutrition factors can elicit production of diverse tolerance regulatory signals in bacteria remains scattered. This chapter describes the use of phenotypic approaches to elucidate the relative inductive properties of major nutrients, and findings which allow establishment of a framework for further delineation of the role of the nutrition sensing and response network that regulates antibiotic tolerance in bacteria.

2.2. Methods

Bacterial strains and culture medium.

The *Escherichia coli.* K-12 strain BW25113 was used in all experiments (kindly provided by H. Mori, Nara Institute of Science and Technology). All cultures utilized the rich defined medium (RDM) (Teknova, Holister, CA, USA) unless stated otherwise. This medium, developed by Neidhardt *et al* (Neidhardt *et al.*, 1974) and later modified by Blattner and coworkers (medium recipe available at http://www.genome.wisc.edu/resources/protocols/ezmedium.htm), comprised six major components: (i) MOPS (3-(N-morpholino)propanesulfonic acid) buffer, (ii) glucose, (iii) ammonium salts, (iv) inorganic phosphate, (v) nucleobases and (vi) amino acids mix with trace vitamins. The composition of this growth medium could be precisely manipulated according to instructions of the manufacturer. Based on the

RDM recipe, a panel of defined media consisting of different compositions as denoted in text was constructed for the tolerance induction assays. Standard LB agar (Difco, Leeuwarden, The Netherlands) was used for assessing the proportion of bacterial population that survived in the assay.

Drugs and chemicals.

All antibiotics were purchased from Sigma (St. Louis, MO). Sodium azide and potassium cyanide were obtained from Merck and BDH Chemicals, respectively.

Growth and assay media.

Fresh bacterial colonies were inoculated into RDM and grown overnight at 37 °C under constant agitation (200 rpm). The overnight culture was diluted 10,000-fold in RDM and cultivated for about 4 h until the exponential growth phase was reached (~10^7 cells/ml). Aliquots of this exponential phase culture were washed and resuspended in the assay medium, followed by the tolerance induction assays:

(i) For analysis of the effect of different nutrient compositions on induction and maintenance of tolerance to specific bactericidal antibiotics, various recipes ranging from complete RDM to one which was deprived of all test nutrients (MOPS) were prepared (Fig. 1). Specific growth rate (μ) prior to antibiotic treatments were determined by measuring the changes in cell density in the untreated controls using the formula $\mu = ((\log_{10} N_3 - \log_{10} N_0)\ 2.303) / (t_3 - t_0)$, where N_0 and N_3 represent the initial and final cell density within the first 3 h of incubation upon switching to the test medium ($t_3 - t_0$).

(ii) For comparative assessment of the tolerance induction effect of bacteriostatic conditions in a nutrient rich environment, complete RDM was supplemented with individual bacteriostatic agents (tetracycline, 2 μg/ml, rifampicin, 16 μg/ml, 5 mM sodium azide and 5 mM potassium cyanide) and used to resuspend the test population prior to the assays.

Tolerance induction assay.

Bacterial populations were pre-incubated with the test medium at 37°C for 2 h and subjected to antibiotic challenge using ampicillin (100 μg/ml), ofloxacin (0.75 μg/ml)

and gentamicin (6.25 μg/ml) at a working concentration of 25 times the respective MIC of the test strain (BW25113). Cells were incubated for 48 h at 37 °C under constant shaking (200 rpm). Standard serial dilution and plating on LB-agar was performed at 0, 3 and 48 h. The plates were incubated at 37 °C and colonies counts were recorded for two consecutive days to account for possible discrepancies due to residual drug effects. Changes in the size of surviving cell populations over time were recorded and compared to that prior to antibiotic challenge for assessment of short-term (3 h) and long-term (48 h) drug tolerance. At least three independent experiments were performed in each assay to assess reproducibility of the induction effect. Surviving cells were routinely tested for antibiotic susceptibility by using the agar dilution method to determine the MICs of isolates randomly selected from the tolerant population.

2.3. Results

In this part of the study, the effect of different nutrient limitations on the development of drug tolerance phenotypes was assessed by resuspension (at a cell density of 10^7 cells/ml) and brief incubation of actively growing cells in a panel of defined media with known nutrient compositions (Figure 2.1), followed by antibiotic treatment for up to 48 h. Relative population survival was assessed by comparing the viable population size before and after antibiotic challenge for 3 h and 48 h, for which we termed short and long-term tolerance, respectively. In addition to antibiotic survival, bacterial growth rate under different test media was also recorded in the untreated samples for assessment of its relationship with the antibiotic tolerance phenotype characteristic of each nutrition background.

2.3.1. Four major classes of drug tolerance responses

The diverse spectrum of nutrient compositions tested was found to produce complex and condition-dependent antibiotic susceptibility phenotypes which could be divided into four major categories according to their drug specificity and sustainability (Figure 2.1). Firstly, a total of five conditions, including the full RDM recipe, failed to support survival against the bactericidal effects of the three test drugs (Figure 2.1, Category A). These conditions mainly involved depletion of a single

nutrient except amino acids, but also included one in which three nutrient classes, glucose, ammonium salts and nucleobases, were simultaneously depleted from the growth medium. Secondly, two conditions could specifically produce transient tolerance to ofloxacin but not the other two drugs, in both cases, phosphate was depleted from the medium (Figure 2.1, Category B). Thirdly, a total of eleven recipes produced mixed tolerance responses to ampicillin and ofloxacin (Figure 2.1, Category C). The recipes that produced Category C responses ranged from a lack of amino acids alone (Figure 2.1, C_1iii), to simultaneous depletion of up to four nutrients (Figure 2.1, C_3). Apart from specificity and sustainability, the strength of drug tolerance, recorded as the size of tolerant population produced and maintained during the assay, also varied extensively between different nutrient compositions. Finally, eight conditions, all involving media which lacked glucose and amino acids, were found to elicit tolerance to all the three test drugs. The phenotypes that belonged to this category typically included sustainable tolerance to both ampicillin and ofloxacin, and transient tolerance to gentamicin (Figure 2.1, Category D).

2.3.2. Inductive and regulatory effects of amino acids and nucleobases

Detailed analysis of the relationship between nutrient compositions and the corresponding drug susceptibility phenotypes revealed several important tolerance induction characteristics which could only be made apparent by comparing the induction effects of differential nutrient compositions. Firstly, depletion of amino acids from the growth medium was found to be a prerequisite for onset of the vast majority of tolerance phenotypes; exception included several phosphate-limiting recipes which could induce short term tolerance to ofloxacin, or ofloxacin and ampicillin even in the presence of amino acids (Figure 2.1, Bi and ii, C_1ii,vi and vii). Hence depletion of amino acids produced a much more diverse and far-reaching effect than the lack of any other compounds in eliciting antibiotic tolerance, although the sustainability and drug specificity of the phenotypes produced were highly dependent on the nature of nutrients which were concomitantly depleted.

The second important feature observable during nutrient depletion-mediated tolerance induction is that several induction processes appeared to be highly sensitive to the relative abundance of nucleobases. In the case of ampicillin but not ofloxacin, simultaneous depletion of amino acids and nucleobases consistently produced a

much weaker induction effect than a medium which was deprived of amino acids only (Figure 2.1, compare C_1i and C_1iii). Although this finding appeared to indicate that the presence of nucleobases could enhance the development of ampicillin tolerance mediated by depletion of amino acids, a contradictory role of these molecules in tolerance regulation was observable in assays with other nutrient compositions. Notably, a prolonged ampicillin tolerance phenotype conferred by a lack of amino acids and ammonium salts could not be supported if nucleobases were present (Figure 2.1, compare C_1iv to C_2ii), suggesting a suppressive effect of nucleobases on specific tolerance induction pathways. In contrast, prolonged ampicillin tolerance triggered by glucose limitation was not affected, as long as amino acids were absent (Figure 2.1, Di and iv). Apart from ampicillin tolerance, the specific inhibitory effects of nucleobases were also observable in ofloxacin tolerance induction; in this case, simultaneous depletion of amino acids and phosphate produced sustainable ofloxacin tolerance only in the absence of nucleobases (Figure 2.1, compare C_1v to C_4i).

2.3.3. Induction of prolonged tolerance by depletion of multiple nutrients

The third important feature of nutrient-sensitive tolerance regulation is that bacteria were able to exhibit prolonged tolerance to antibiotics under specific conditions in which key nutrients were further removed from an amino acids limitation background. This finding was mainly based on our observation that highly sustainable tolerance to ampicillin and/or ofloxacin could only be produced by simultaneous depletion of amino acids and glucose (Figure 2.1, Category D), or amino acids and ammonium salts, plus either phosphate or nucleobases (Figure 2.1, $C_{2,3,4}$). Absence of amino acids and glucose was also a prerequisite for induction of short term tolerance to gentamicin, a phenotype which consistently emerged along with sustainable tolerance to ampicillin and ofloxacin (Figure 2.1, Category D).

Data from this work indicated that prolonged antibiotic tolerance was likely mediated by complex adaptive and stress responses inducible when specific nutrients other than amino acids became limiting. Such responses, apart from being subjected to negative regulation by amino acids, appeared to produce synergistic induction effects with those of amino acids depletion. Firstly, we noted that simultaneous limitation of glucose, ammonium salts, and phosphate did not support even short

term ampicillin tolerance when amino acids were present (Figure 2.1, Bii and C_1ii), yet the presence of these three nutrients could reverse most of the MOPS-induced phenotypes even in the absence of amino acids (Figure 2.1, compare C_1i with Category D conditions). We therefore hypothesized that a lack of one or more of these compounds could induce a tolerance response which was negatively regulated by amino acids and distinguishable from the one triggered by amino acids depletion alone, which was nucleobases-dependent (Figure 2.1, compare C_1i to C_1iii). We noticed that, although each of these components could not fully reverse MOPS-induced ampicillin tolerance (Figure 2.1, C_3i, Dvi and vii), further depletion of ammonium salts or glucose (Figure 2.1, C_2ii and Div), but not phosphate (Figure 2.1, C_4i), from a medium background which failed to induce the putative amino acids depletion-mediated ampicillin tolerance response (lack of amino acids and nucleobases simultaneously, Figure 2.1, C_1i), could induce sustainable ampicillin tolerance. Corroborating the idea that prolonged tolerance was regulated by de-repressible mechanisms was the observation that ofloxacin tolerance was inducible by two independent pathways. Firstly, a lack of either amino acids or phosphate induced primary tolerance to ofloxacin at three h treatment (Figure 2.1, Bi and C_1iii); the phosphate depletion-mediated response being insensitive to suppression by amino acids. Secondly, a stronger and more persistent phenotype resulted from a combined depletion of amino acids, phosphate and nucleobases, or amino acids and carbon sources (Figure 2.1, C_3i, C_4i and all Category D conditions). It should also be noted that, unlike amino acids and nucleobases, glucose, phosphate and ammonium could not individually confer negative regulation on antibiotic tolerance formation. In this study, we confirmed that long term tolerance to ampicillin and ofloxacin was not due to selection and proliferation of resistant mutants, since all tolerant populations were found to contain isolates which remained susceptible to the test drugs in MIC determination.

2.3.4. Growth rate and antibiotic tolerance

In addition to the relationship between nutrition recipes and tolerance formation, we further analyzed the correlation between growth rate and phenotypic tolerance under the test conditions. We found that a high specific growth rate (an average of 1 hr^{-1} or above between 0 – 3 hr, Figure 2.1, Category A) was not compatible with tolerance development, whereas conditions that suppressed bacterial growth (with

specific growth rates of 0 hr^{-1} or below) generally conferred sustainable tolerance to both ampicillin and ofloxacin as well as short term tolerance to gentamicin (Figure 2.1, Di-iv,viii). However, the relationship between growth rate and drug susceptibility became much less apparent under conditions in which bacteria grew at a relatively slow pace, when induction of antibiotic tolerance in a growth rate-independent manner was often observed. For example, a medium which was depleted of amino acids and ammonium salts induced significant short term tolerance to ampicillin (Figure 2.1, C_1iv), yet another medium which lacked glucose, ammonium salts and nucleobases (Figure 2.1, Av) did not confer detectable ampicillin tolerance despite the fact that the test populations exhibited similar growth rate in these media (exhibiting specific growth rates of 0.69 hr^{-1} and 0.62 hr^{-1} respectively). Likewise, a number of other conditions that supported identical growth rate (ranging from 0.32 to 0.36 hr^{-1}) were found to induce a spectrum of tolerance phenotypes with significant variation in drug specificity and the degree of sustainability (Figure 2.1, C_1v, C_4i and Dv). In addition, we do not observe significant correlation between the capacity to exhibit prolonged or re-growth in the test medium (exhibited by increase in population size between 3 and 48 hr, Figure 2.1), which is a hallmark of adaptive biosynthesis, and development of tolerance.

2.3.5. Tolerance induction potential of bacteriostatic agents

To determine whether growth inhibition alone could contribute to tolerance formation, we examined the effects of bacteriostatic agents in eliciting tolerance in an actively growing population. Potassium cyanide and sodium azide, both inhibitor of aerobic respiration, were the only agents which continued to exert bacteriostatic effects throughout 48 h treatment (Figure 2.2). In general, the tolerance profiles elicited by bacteriostatic agents were highly dissimilar to those conferred by MOPS or limitation of major nutrients, which consistently supported prolonged tolerance to ampicillin and ofloxacin, and short term tolerance to gentamicin (Figure 2.1, Category D). Potassium cyanide was the only agent which produced prolonged tolerance to both ampicillin and ofloxacin and elicited short term gentamicin tolerance in a situation where starvation signals and detectable bacterial growth were both absent (Figure 2.2), although the size of the tolerant population was smaller than that observed during nutrient depletion conditions. Interestingly, the bacteriostatic

effects triggered by sodium azide were not associated with any detectable tolerance phenotypes (Figure 2.2).

2.3.6. Phenotypic difference between starvation and stationary-phase-induced tolerance

To assess the relative contribution of nutrient deprivation in producing multidrug tolerance in stationary-phase populations, a spent medium assay was performed to determine if the cell-free supernatant obtained from stationary-phase culture, which had been grown overnight in RDM, could induce formation of a phenotype which matched those produced by the tested recipes. Our results showed that this spent medium supported tolerance development in a manner similar to that of MOPS base (Figure 2.3). This induction effect was found to be due to nutrient limitation but not accumulation of toxic metabolites produced during prolonged culture, as it could be abolished by replenishment of nutrients to the spent medium (Figure 4.2). These findings confirmed that nutrients in an overnight culture have been depleted to a level that would support development of tolerance phenotypes comparable but not entirely similar to those observed in stationary-phase populations, which exhibited prolonged gentamicin tolerance.

2.4. Summary

This study clearly demonstrated that drug-sensitive bacteria could rapidly switch to a multidrug tolerant state in which the relative abundances of exogenous nutrients were tightly coupled to drug specificity and sustainability of tolerance. Deprivation of amino acids from the growth medium was a prerequisite for tolerance formation, consistently conferring condition-specific phenotypes against inhibitors of cell wall synthesis and DNA replication (ampicillin and ofloxacin respectively) depending on the availability of ammonium salts, phosphate, and nucleobases. Upon further depletion of glucose, this variable phase consistently evolved into a sustainable mode, along with enhanced capacity to withstand the effect of the protein synthesis inhibitor gentamicin. While a general correlation on the reduction of growth rate and drug tolerance was observed, our results also provided indirect evidence that bacteriostasis is not the sole determining factor for antibiotic tolerance development, as multiple

growth retardation conditions were found to be insufficient to produce a significant tolerance phenotype. Furthermore, utilization of complex defined media in our tolerance induction assay enabled us to identify novel, yet subtle interaction between different nutrient components, such as those between amino acids and nucleobases, on mediating antibiotic tolerance. On the other hand, although transient exposure to starvation alone was capable of transforming cells into multidrug tolerant states, their long term survival capacity were significantly lower than those exhibited by overnight stationary-phase populations. Such discrepancy prompted us to explore the possibility that alternative environmental parameters might be involved in promoting the production of tolerance phenotypes comparable in strength and drug spectrum to those observable in overnight bacterial culture.

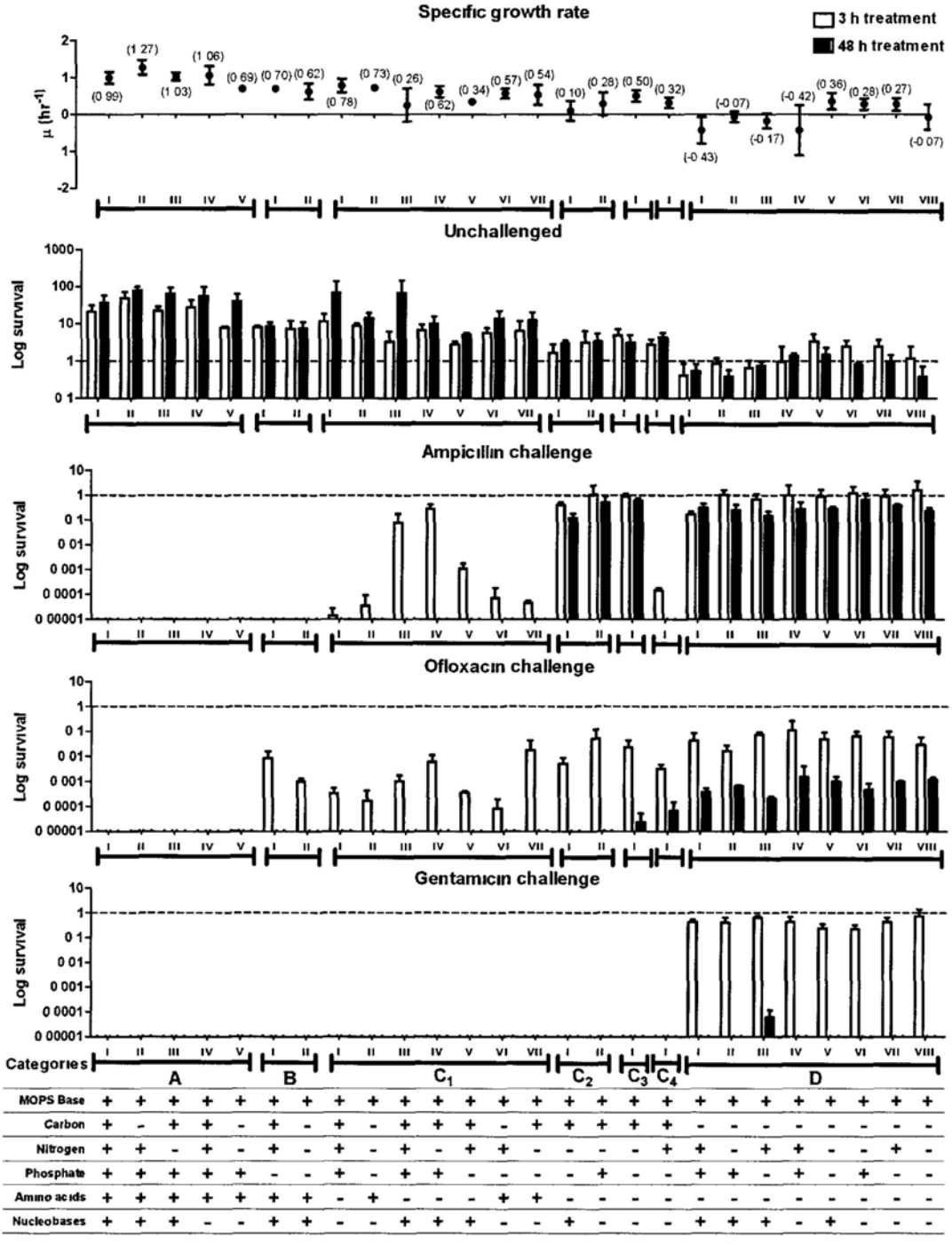

Specific growth rate
3 h treatment
48 h treatment
μ (hr-1)
(0 99) (1 27) (1 03) (1 06) (0 69) (0 70) (0 62) (0 78) (0 73) (0 26) (0 62) (0 34) (0 57) (0 54) (0 10) (0 28) (0 50) (0 32) (-0 43) (-0 07) (-0 17) (-0 42) (0 36) (0 28) (0 27) (-0 07)
Unchallenged
Log survival
Ampicillin challenge
Ofloxacin challenge
Gentamicin challenge
Categories
A
B
C1
C2
C3
C4
D
MOPS Base +
Carbon + - + + - + - + - + + + - + + + + + - - - - - - - -
Nitrogen + + - + - + - + - + - + + - - - - + + - + + - - + -
Phosphate + + + + + - - + - + + - - - - + - - + + - + - + - -
Amino acids + + + + + + + - + - - - + + - - - - - - - - - - - -
Nucleobases + + + - - + + - - + + + - - + - - - + + + - + - - -

Figure 2.1. Effects of differential nutrient compositions on the induction and sustainability of antibiotic tolerance. Exponentially growing *Escherichia coli* BW25113 were washed, resuspended and incubated at 37°C for 2 h in a panel of defined media composed of various combinations of five major nutrient classes (glucose, ammonium salts, inorganic phosphate, amino acids mix and nucleobases) starting from full RDM, which contained all the five components, to the MOPS base which was deprived of all nutrients. The pre-incubated cells were either untreated or subjected to antibiotic treatments using 100 μg/ml ampicillin, 0.75 μg/ml ofloxacin or 6.25 μg/ml gentamicin for up to 48 h. The degree of population survival was assessed by comparing the log-based population size prior to drug treatment to those determined upon 3 and 48 h treatment, which reflected short-term and long-term tolerance respectively. The nutrition recipes are categorized according to the drug specificity and level of sustainability of tolerance phenotypes inducible under each test condition. Category A: undetectable tolerance to all the three antibiotics; Category B: tolerance to ofloxacin only; Category C: tolerance to both ampicillin and ofloxacin, this category is sub-divided on the basis of sustainability to these two drugs; Category D: tolerance to all the three test drugs. Population growth / viability in the untreated control was depicted by the specific growth rate (μ), which was determined on the basis of changes in cell density within the first 3 h of incubation upon switching to the test medium.

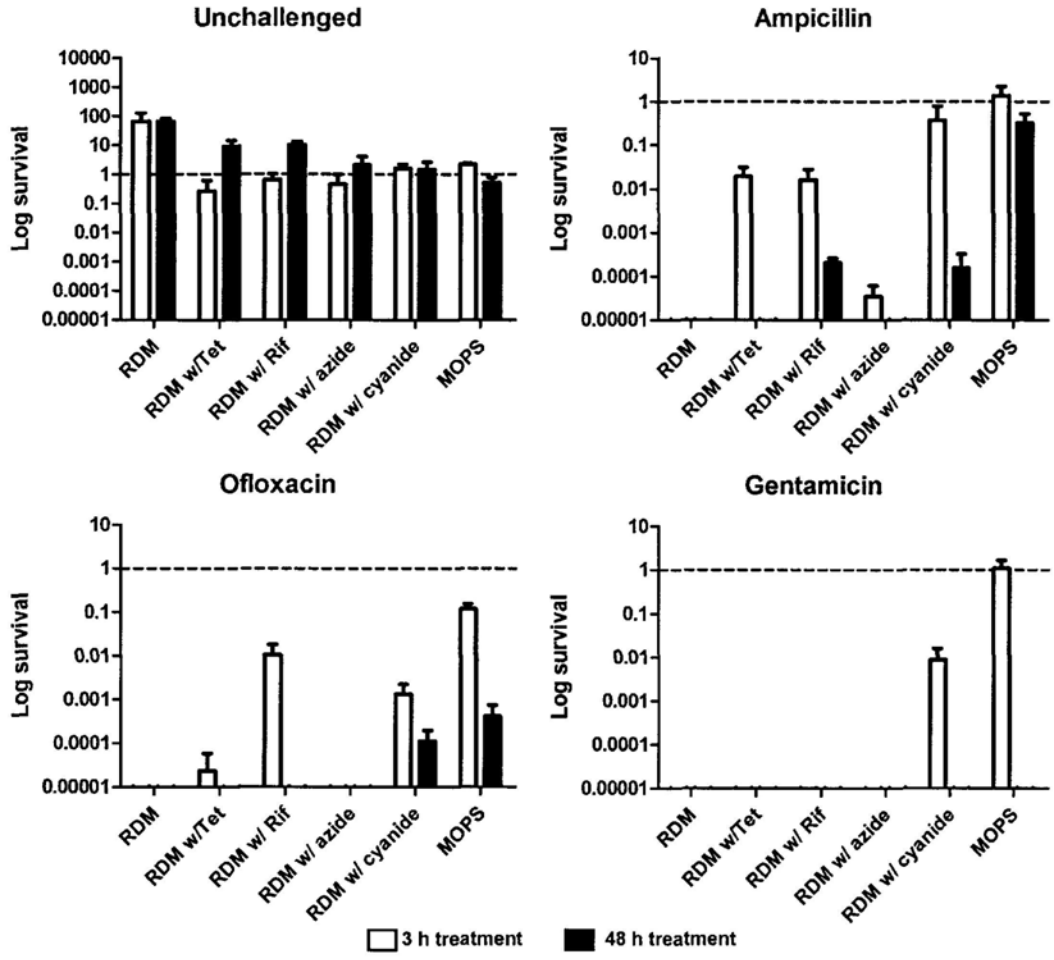


Figure 2.2. Induction of tolerance by bacteriostatic agents. Exponentially growing cells were washed and pre-incubated in full RDM containing different classes of bacteriostatic agents (Tet: tetracycline (2 μg/ml), Rif: rifampicin (16 μg/ml), Azide: sodium azide (5 mM), Cyanide: potassium cyanide (5 mM)) for 2 h before drug treatment and assessment for survival. Exponentially-growing cells resuspended in RDM (RDM) and MOPS base (MOPS) were included as control.

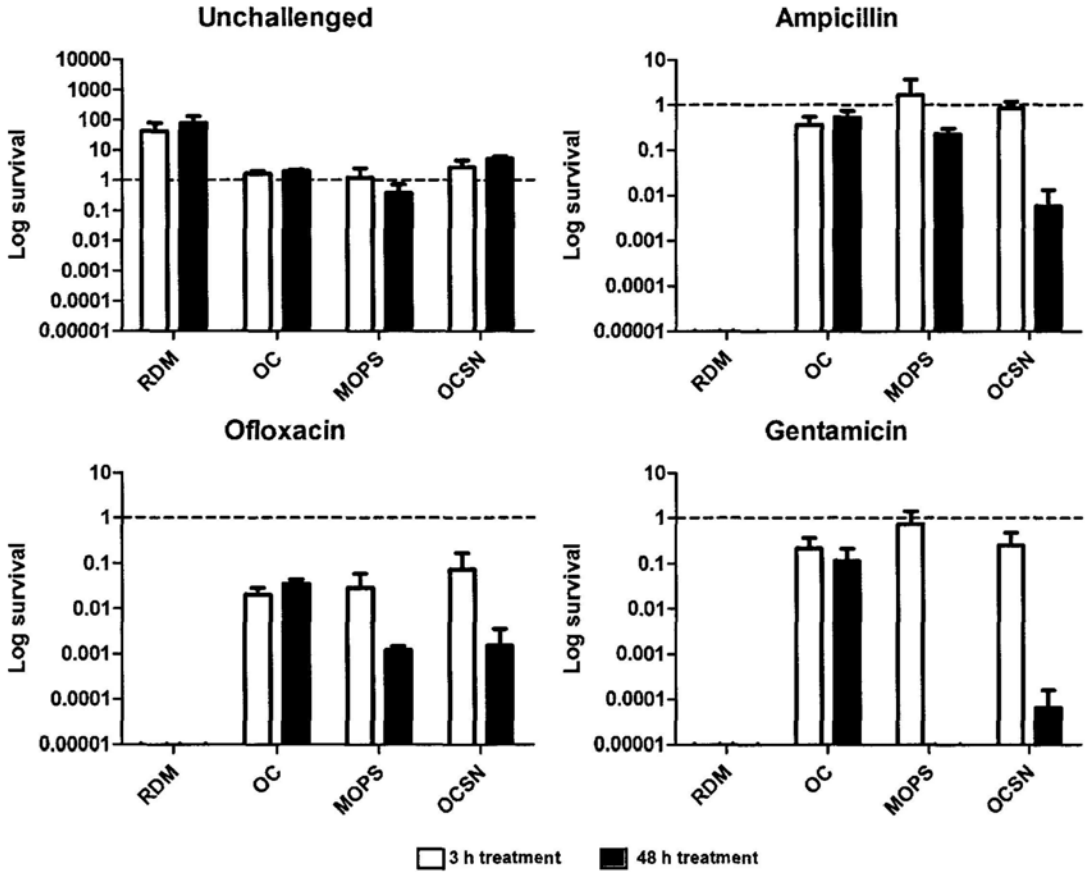


Figure 2.3. Analysis of tolerance induction effects of cell-free supernatant of multidrug tolerant stationary-phase culture on log-phase, drug-sensitive population. Exponentially growing cells were pre-incubated in filter-sterilized supernatant of the stationary-phase culture for 2 h (OCSN), followed by treatment with 100 μg/ml ampicillin, 0.75 μg/ml ofloxacin or 6.25 μg/ml gentamicin for 3 and 48 h and assessment of population survival. The following controls were included in the assay: stationary-phase culture (OC), log-phase populations reconstituted in RDM (RDM) and MOPS base (MOPS).

Chapter 3. Tolerance induction by prolonged starvation

3.1. Introduction

As described in Chapter 2, starvation of essential nutrients was identified as a major regulatory factor that predisposed development of phenotypes that are tolerant to bactericidal antibiotics. However, the rapid physiological response to abrupt alteration in exogenous nutrient composition is inadequate to account for the robust and sustainable tolerance phenotypes observable in the stationary-phase population (Figure 2.3). Despite the fact that bacterial population size remains constant during the stationary-phase, the organisms concerned have been suggested to undergo dynamic and time-dependent physiological changes which enhance survival fitness under unfavorable environmental conditions (Kolter *et al.*, 1993). On the other hand, various previous reports have suggested that induction of beta-lactam tolerance by starvation is also time-dependent (Goodell and Tomasz, 1980; Novak and Tuomanen, 2002), and that passage of stationary-phase organisms results in emergence of subpopulation persisters which remain drug-tolerant in nutrient-rich medium (Balaban *et al.*, 2004; Lewis, 2007). Based on such background evidence, we sought to further explore the tolerance induction potential of prolonged starvation stress, and examined whether the time factor was essential in producing phenotypes that resembled those elicited by prolonged nutritional stress encountered by organisms of stationary-phase populations.

3.2. Methods

Bacterial strains and culture conditions

Escherichia coli K-12 strains and their isogenic derivatives used in this study were listed in Table 3.1. All broth cultures were grown in RDM as described previously unless otherwise specified. LB agar was used as solid medium for antibiotic survival assays. In the case of stringent response mutants, bacteria were also plated on M9 glucose agar to check for compensatory mutants.

Table 3.1. *Escherichia coli* strains used in phenotypic and genetic characterization of prolonged starvation-induced tolerance.

Strain	Genotype	Source (reference)
BW25113	*lacI*^{q} *rrnB*$_{T14}$ Δ*lacZ*$_{WJ16}$ *hsdR514* Δ*araBAD*$_{AH533}$ Δ*rhaBAD*$_{LD78}$	Baba *et al* (Baba *et al*, 2006)
JW3389	BW25113 *glpD* Km^R	As above
JW1500	BW25113 *hipA* Km^R	As above
JW3702	BW25113 *phoU* Km^R	As above
JW2669	BW25113 *recA* Km^R	As above
JW0176	BW25113 *sucB* Km^R	As above
JW0659	BW25113 *ubiF* Km^R	As above
CF1943	Wild-type W3110	Xiao *et al* (Xiao *et al*, 1991)
CF1946	CF1943 Δ*relA251 kan* Δ*spoT207 cat*	As above

Tolerance induction and persister assay

Escherichia coli BW25113 was grown in RDM to exponential phase with a cell density of about 10^7 cells/ml. Cells were washed once and incubated in MOPS base, which was deprived of all essential nutrients compared to the RDM. Induction time was set at 10, 30 and 90 min, and 24 h, followed by treatment with three antibiotics (ampicillin, ofloxacin and gentamicin at 25 XMIC) for 3 and 48 h, and assessment of the relative size of surviving population as described previously (Fung *et al.*, 2010). A parallel persister assay was performed in which organisms recovered from the starvation experiments were resuspended in Rich Defined Medium or RDM (Teknova, Holister, CA, USA) and incubated at 37°C for 15 min, followed by drug treatment and determination of the size of persister population. The stationary-phase population of the test strain was included as a control to depict the relative tolerance and persistence characteristics of this physiological stage.

3.3. Results

3.3.1. Progressive increase in sustainable tolerance rate during prolonged starvation

The time-dependent induction effects of nutrient limitation on phenotypic antibiotic tolerance were studied by exposing RDM-grown log-phase cells to MOPS base for a range of test periods (10 min to 24 h), followed by assessment of the relationship between the exposure time and the drug tolerance phenotypes including the proportion of persisters recoverable from the test population. As shown in Figure

3.1, the tolerance induction effects appeared to be accumulative during a prolonged starvation process. For each of the three test drugs, the size of the emerging tolerant population increased proportionately with the length of starvation period. Induction for as short as 10 min was nevertheless sufficient to produce detectable and often sustainable tolerance; however, a significantly higher level (>10 fold) of sustainable tolerance was consistently produced if the organisms were starved for 24 h. Such prolonged starvation-mediated effects therefore vaguely resembled those of overnight culture (Figure 3.1), although the level of sustainable gentamicin tolerance was still substantially lower than that of the stationary-phase population. This phenotypic difference in the relative degree of sustainable tolerance shows that divergent population structures exist between organisms subjected to starvation stress alone and those recoverable in stationary-phase conditions.

3.3.2. Persisters as slow-recovering tolerant organisms

Upon reconstitution in RDM, each of the test populations which had been subjected to starvation for 30 min or more was found to contain subpopulation persisters that exhibited at least 3-h tolerance to at least two of the three test drugs (Figure 3.1). The population size of gentamicin persisters was consistently smaller than those of ampicillin and ofloxacin in all cases (Figure 3.1), indicating that persisters were not necessarily multidrug tolerant. Bacterial populations that experienced starvation for 10 min or less prior to antibiotic challenge did not harbor any persisters, despite the fact that they exhibited sustainable tolerance to ampicillin and ofloxacin in MOPS base. Resembling the tolerance induction characteristics, development of persistence potential was apparently time-dependent, with the level of persisters inducible by brief starvation (30 min), which is considerably smaller than those which had been subjected to an extended starvation period (90 min and 24 h). Surprisingly, the persistence strength elicited by prolonged starvation was even higher than that detectable in a stationary-phase culture, despite the fact that the latter was more tolerant to the test drugs under nutrient-limiting conditions (Figure 3.1).

3.3.3. Gene knockout studies

A gene knockout study was performed to investigate whether genetic determinants which have been postulated to coordinate antibiotic tolerance development were also involved in regulation of the sustainable tolerance and persistence phenotypes

observable during prolonged starvation and the subsequent resuscitation experiments, respectively. As shown in Figure 3.2, which depicts the relative gene knockout effects as compared to the wild-type, the genetic determinants tested were found to play common, differential or drug-specific roles in the starvation responses. Of particular interest is that the *ubiF* and *sucB* loci, both energy production genes implicated in persister formation (Ma *et al.*, 2009), were indeed important for such process. However, we noted that expression of sustainable tolerance to the test drugs was not affected in the Δ*ubiF* mutant, indicating that the product of this gene is highly specific in mediating persister formation. On the other hand, deletion of *sucB* resulted in defect in producing all phenotypes, except sustainable tolerance to ofloxacin. Taken together, it appears that energy production is required for maintaining the tolerance mode upon nutrient replenishment but not absolutely necessary for sustaining such phenotype during starvation. However, the inconsistency between the *ubiF* and *sucB* knockout results also highlights the possibility that these gene products may regulate the tolerance mechanisms in an energy-independent manner. The Δ *recA* mutant, which is defective in SOS response-mediated DNA repair, was able to develop sustainable tolerance and the corresponding persistence to ampicillin and gentamicin, but not ofloxacin. These findings indicate that common or compensatory mechanisms which confer tolerance to multiple antibiotics are not inducible during prolonged starvation, at least in a background incapable of eliciting SOS response. The fact that prolonged starvation stress enabled this mutant to develop sustainable tolerance to gentamicin, a phenotype not inducible during brief starvation (Figure 2.1), further confirmed that progressive physiological changes occurred during prolonged starvation. The Δ*relA* Δ *spoT* or stringent response mutant was another strain which exhibited compromised ability to produce sustainable tolerance and persisters for all the three antibiotic classes. This finding complements our previous observation that a lack of stringent response abrogated the capability of the bacteria to produce sustainable ofloxacin tolerance phenotype during brief starvation (Fung *et al.*, 2010). The relatively mild deletion effect of the putative tolerance genes encoding the HipA toxin (Keren *et al.*, 2004b) and metabolic regulators GlpD (Spoering *et al.*, 2006) and PhoU (Li and Zhang, 2007) suggests that they play a rather indirect role in responses mediated by prolonged starvation.

3.4. Summary

Results in this part of the study depict a sequential developmental pattern of antibiotic tolerance within a population of drug-sensitive organisms, in which phenotypic responses to nutritional stress are highly dependent on the duration of stress exposure (Figure 3.3). Extended (e.g. 24 h) starvation was found to elicit robust ampicillin and ofloxacin tolerance comparable in strength and sustainability to that of the stationary-phase populations. Such tolerant populations were also found to harbor persister organisms whose population size varies according to the length of the preceding starvation period. Preliminary results of the gene knockout experiments confirmed the presence of independent yet overlapping genetic pathways regulating the drug specificity, sustainability and reversibility potential of antibiotic tolerance. Since emergence of tolerance must precede that of persisters, we expect that specific cellular mechanisms inducible during prolonged starvation must play a role in the concomitant development of sustainable tolerance and persisters, even though the latter is not necessarily a subpopulation of sustainably tolerant organisms. However, the specific knockout effect of certain determinants, such as *ubiF*, which affected formation of persisters but not the sustainable tolerance phenotypes, suggests that tolerance induction mechanisms are highly redundant, and that some of these mechanisms do not necessarily drive persister formation. All in all, this stepwise induction model may serve as a valuable platform that allows differential analysis of phenotypic tolerance and persistence, which are often regarded as the same phenomenon due to their non-inheritable nature and drug tolerance characteristics. Furthermore, the finding that prolonged starvation only produced a low level of gentamicin tolerance suggests the presence of nutrient-independent mechanisms which may contribute to development of robust tolerance against this antibiotic during stationary-phase conditions.

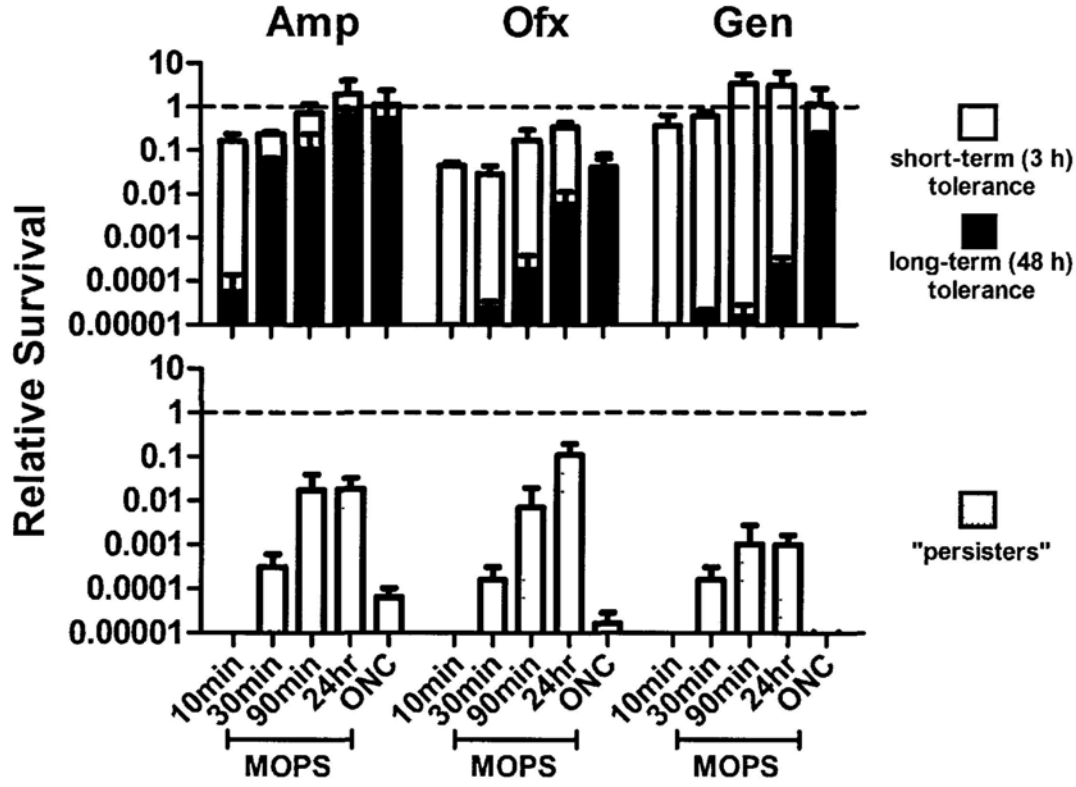


Figure 3.1. Progressive development of antibiotic persisters within starvation-induced antibiotic-tolerant populations. Exponentially growing *Escherichia coli* BW25113 populations (at ~10^7/ml) in RDM were washed and incubated in MOPS base. At indicated times, aliquots were assayed for tolerance to three antibiotics (top panel) as described previously, and the relative abundance of persisters (bottom panel) by reconstituting the MOPS-starved cells in RDM for 15 min, followed by 3-h antibiotic challenge and assessment of the survival rate. Persisters abundance in overnight culture (ONC) was determined by 100-fold dilution in RDM followed by 15-min incubation prior to antibiotic challenge and was included as control in both assays.

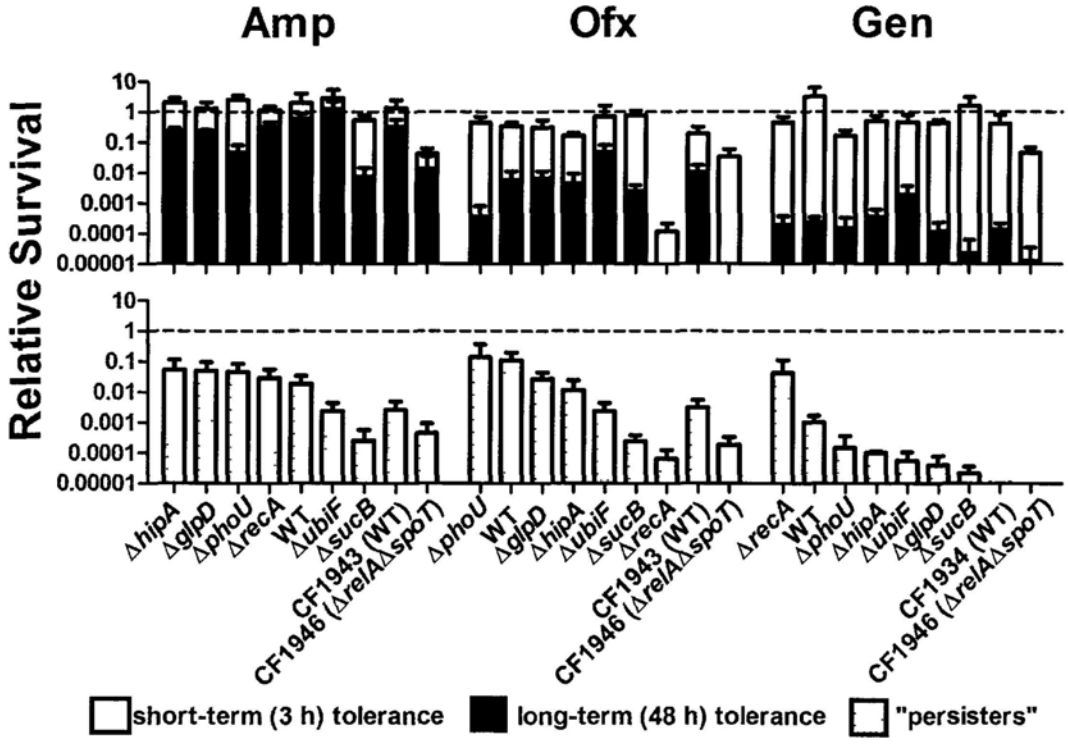


Figure 3.2. Gene knockout analysis of the relative role of putative persister genes on starvation-induced persister formation. RDM grown, log-phase populations with indicated isogenic gene deletions (arranged in descending order by their relative persister abundance except for the ppGpp+ strain and its corresponding ppGpp0 mutant) were subjected to starvation in MOPS for 24 h and subsequently analysed for tolerance (top panel) and persister development (bottom panel).

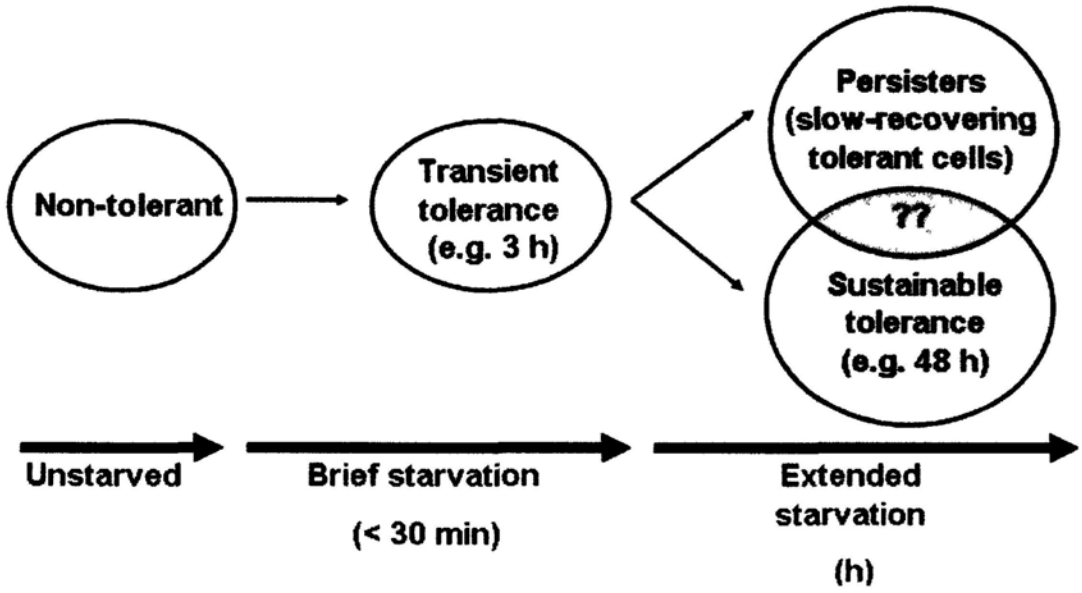


Figure 3.3. Progressive changes in tolerant population structure upon nutrient depletion. Non-tolerant cells (light blue) rapidly switch to the transient but multidrug tolerance state (light orange) in response to nutritional stress; sustained nutrition starvation leads to further enhancement of tolerance strength characterized by emergence of multiple subpopulations of differential tolerance features. These include organisms which can survive prolonged antibiotic treatment (orange) and persister cells (purple) which can transiently retain their drug-tolerant status upon nutrient replenishment. Organisms with combined phenotypes (marked by "??") may also exist and are expected to account for an increasing proportion of the total tolerant population during the course of starvation.

Chapter 4. Cell density-mediated antibiotic tolerance

4.1. Introduction

The phenotypic discrepancy between stationary-phase and starvation-based antibiotic tolerance prompted us to search for other environmental induction factors that may elicit alteration in susceptibility to bactericidal drugs. Apart from nutrition depletion, another potentially negative impact on bacterial cell growth during the transition between the log and stationary-phases can be attributed to the effects of greatly increased cell density (typically 100-fold), which is an inevitable result of population expansion itself. During such transition period, cell density-dependent accumulation of extracellular quorum sensing signals (Lazazzera, 2000) and / or activation of contact-dependent mechanisms (Blango and Mulvey, 2009) may elicit stress responses which in turn confer protection against antibiotic-mediated killing. Quorum sensing-dependent regulation of antibiotic tolerance has been reported in *Pseudomonas aeruginosa* (Bjarnsholt *et al.*, 2005; Kayama *et al.*, 2009; Moker *et al.*, 2010) but not in *Escherichia coli*. Moreover, a recent study on contact-dependent growth inhibitory functions of the *Escherichia coli* cdiAB systems (Aoki *et al.*, 2005; Aoki *et al.*, 2009) opened up the possibility that growth attenuation can be mediated through cell-to-cell contact, with a resultant drug tolerance induction effect. Currently, cell density mediated antibiotic tolerance in *Escherichia coli* is largely an unexplored area. In view of the finding that starvation stress alone cannot fully account for the sustainable multidrug tolerance phenotypes observable in stationary-phase populations, we proceeded to investigate the role of cell density upshift on antibiotic tolerance development.

4.2. Methods

Bacterial strains and growth conditions.

Bacterial strains used in this study include *Escherichia coli* K-12 BW25113 and its Δ*luxS* derivative (Baba *et al.*, 2006). Broth cultures were grown in Rich Defined Medium (RDM) or nitrogen-free MOPS-base (Teknova) unless otherwise specified. LB-agar (Difco) was used as solid medium in all cases.

Tolerance induction under high cell density conditions.

Overnight population of BW25113 *Escherichia coli* and/or its isogenic derivatives were inoculated into fresh RDM at 1:10000 dilution and grew to exponential phase at about 10^7/ml under constant agitation (200 rpm) at 37℃. Cultures were then pelleted by centrifugation and resuspended in 1/10 or 1/100 of its original culture volume achieving 10-fold or 100-fold concentration of cell density in fresh RDM or MOPS base as mentioned in the text. The concentrated cultures were incubated at 37℃ under shaking (200 rpm) for 30 min and subjected to either direct antibiotic challenge or spent-medium extraction by membrane filtration using 0.2-μm filter membranes. The spent-media were either untreated or re-conditioned by addition of concentrated stocks of glucose, ammonium, phosphate, nucleotides and amino acids equivalent to the RDM. Batches of spent-media were used to resuspend and pre-incubate RDM-grown, exponentially growing BW25113 (10^7/ml) for subsequent antibiotic tolerance assay. For comparative studies on stationary-phase culture, adjustment of cell density to create various combinations of cell density and medium compositions was performed according to specification in the text, followed by assessment of drug susceptibility under the test conditions, using protocols as described herein.

Antibiotic tolerance assay.

Bacterial populations were assayed for antibiotic tolerance as described (Fung *et al.*, 2010). In brief, pre-incubated exponentially growing BW25113 (at 10^7/ml) were subjected to antibiotic treatment at roughly 25X MIC with ampicillin (100 μg/ml), ofloxacin (0.75 μg/ml) or gentamicin (6.25 μg/ml) for up to 48 h at 37℃ under constant shaking (200 rpm). Population viability was determined at 0, 3 and 48 h after treatment using by standard serial dilution and plating on LB-agar. Survival for each test condition was presented in the form of "survival ratio", which depicts the comparative log-based population size before and after drug treatment for 3 h and 48 h.

Assessment of tolerance induction potential of late log-phase spent medium

The test strain (BW25113) was grown in RDM to late log-phase with a cell density of approximately 10^9 per ml. Upon centrifugation, cell-free supernatant was collected

from this high density culture and reconstituted to the composition of RDM by addition of a concentrated stock of glucose, ammonium, phosphate, nucleotides and amino acids. This conditioned supernatant was tested for its ability to induce tolerance in a fresh log-phase population as described in antibiotic tolerance assay.

Determination of tolerance induction potential of acetate

Exponentially growing organisms of BW25113 (10^7 cells per ml) were pelleted and resuspended in RDM alone and RDM containing 10, 100 or 1000 μg/ml sodium acetate (pH 7), followed by incubation at 37℃ for 15 min and assessment of antibiotic susceptibility as described in antibiotic tolerance assay.

4.3. Results

4.3.1. Cell density-dependent drug tolerance

Experiments described in this section were designed to elucidate the molecular events underlying the progressive reduction in antibiotic susceptibility of an actively growing bacterial population, especially when exogenous nutrients were not limiting. As multidrug tolerance emerges predominantly at stationary-phase, in which bacterial density is 100-fold higher than that of a log-phase culture, we hypothesize that such phenomenon could be due to both starvation responses and increasing physical constraint as a result of increasing cell density, which may to some extent impede permeation and reduce availability of both nutrients and drug molecules per unit cell, producing a characteristic cell density-dependent tolerance phenotype independent of starvation responses during the later stages of exponential growth. To test this possibility, we reconstituted a log-phase culture in smaller volumes of fresh and nutrient-rich medium (RDM) to prevent starvation signal induction, followed by assessment of antibiotic susceptibility as previously described through determining the proportion of the starting population that would become tolerant to the three test drugs after treatment for 3 and 48 h respectively. Surprisingly, a 10-fold concentration of a drug-susceptible log-phase population, with a resulting cell density of approximately 10^8 cells per ml, was sufficient to support development of sustainable (48 h) tolerance to ofloxacin and gentamicin; however, this condensed population remained highly susceptible to ampicillin even during short term

treatment (3 h) (Figure 4.1). It should be noted that such tolerance profile was not observable under all nutrition recipes which we tested previously (Figure 2.1), indicating that this phenomenon was unlikely due to starvation responses. When the cell density was increased 100-fold to 10^9 cells per ml, which was equivalent to that of an overnight culture, prolonged tolerance to ampicillin also became apparent in a significant proportion of the test population. Nevertheless, the proportion of high density bacterial suspension which exhibited prolonged or 48 h tolerance to the test drugs was substantially smaller than that observed in overnight culture, primarily due to the fact that the number of organisms which could survive 48 h of ofloxacin treatment was relatively small (Figure 4.1). To delineate the complex factors underlying the development of high cell density-mediated tolerance, we next performed a series of nutrient and drug supplementation, as well as density adjustment experiments, so as to investigate whether the high cell density-associated tolerance phenomenon was solely due to decreased nutrient and/or drug availability, which would simultaneously trigger onset of starvation-mediated tolerance responses as we previously demonstrated and decrease the effectiveness of bactericidal action of the test drugs.

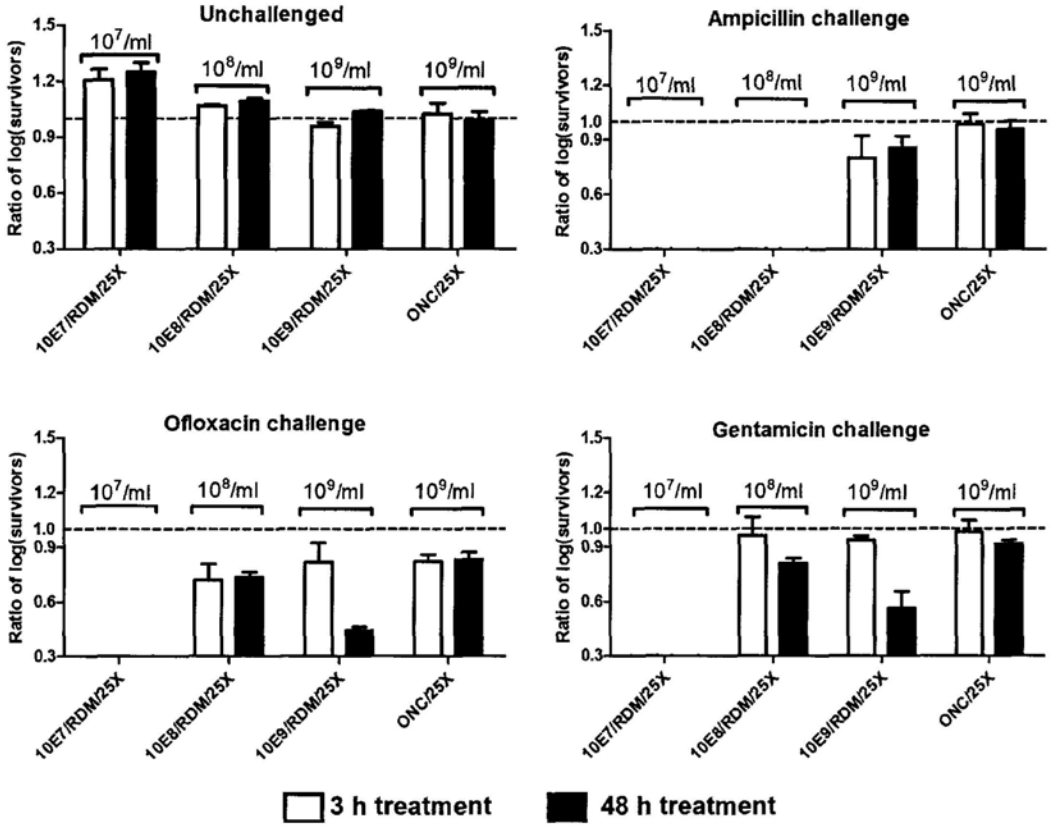


Figure 4.1. Effect of increasing population densities on antibiotic tolerance development. RDM-grown exponentially growing cells (at 10^7 cells per ml) (10E7) were concentrated by 10- or 100-fold in smaller volumes of RDM to 10^8 (10E8) or 10^9 (10E9) cells per ml respectively and assayed for their tolerance phenotypes. An RDM-grown overnight culture (at 10^9 cells per ml) (ONC) was included as control. A survival ratio of 1 represents complete tolerance, whereas values below or above 1 describe partial tolerance or net population growth, respectively.

4.3.2. Effect of nutrient supplementation on high cell density-mediated tolerance

We envisaged that if drug tolerance of the artificially constructed high density population was caused by reduced nutrient penetration, this phenomenon should at least be partially offset by supplementing an arbitrarily higher amount of nutrients to the cell suspension. We verified this view by preparing a high density log-phase cell suspension in an enriched RDM comprised of higher concentrations of glucose (4X), ammonium (2X), phosphate (4X) and amino acids (2X). Surprisingly, enhanced nutrient supplementation to all test populations (at 10^8 and 10^9 cells per ml) resulted in a markedly lower survival rate at 48 h even in the absence of antibiotics (Figure 4.2). However, the relative tolerance levels of such high density cell suspension were not significantly altered in the presence of excessive nutrients, indicating that the cell density-dependent phenotypes were not elicited by deficiency of nutrients within the microenvironment of the test organisms. In order to further determine whether tolerance formation in such condensed bacterial population was attributed to rapid consumption of nutrients, which could have triggered tolerance formation via starvation-induced stress responses, cell-free supernatant of the 10^8 and 10^9 cells per ml populations was prepared and respectively tested for their potency to induce tolerance development in a fresh, low density (10^7 cells per ml) and non-tolerant log-phase population. The cell-free supernatant of the 10^8 cells per ml population failed to induce tolerance to the three test drugs, indicating that sufficient nutrients were present in this high density cell suspension; in contrast, those of the 10^9 cells per ml suspension was able to elicit development of significant short term (3 h) tolerance to the three antibiotics as well as sustainable tolerance to both ofloxacin and gentamicin but not ampicillin (Figure 4.2). Such tolerance profiles were similar to those exhibited by the 10^8 cells per ml population but could not be generated using all available nutrient depletion recipes (Figure 2.1). In addition, expansion in population size was observed during the course of drug treatment, indicating that nutrients were still available. To confirm that the tolerance induction effect of the cell-free supernatant of the 10^9 population was not elicited through starvation response, we replenished the test supernatant with a concentrated nutrient stock to a composition equivalent to that of RDM, which should abolish the ability of a nutrient-deficient medium to induce tolerance in a log-phase population via starvation responses. Our results showed that nutrient supplementation could abrogate the sustainability of the tolerance phenotypes inducible by spent medium of

high cell density population (Figure 4.2). This nutrient-replenished medium, however, continued to exhibit the ability to induce significant short term (3 h) tolerance to all the three test drugs. Importantly, such tolerance profile was also not inducible under all possible nutrient depletion conditions (Figure 2.1). In this case, bacterial growth was also found to be inhibited by excessive nutrients. These data showed that spent medium recovered from high cell density, nutrient-rich culture contained some unidentified factors which could specifically mediate changes in physiology and hence antibiotic susceptibility in the absence of starvation signals.

These preliminary findings prompted us to examine whether late log-phase culture of a cell density of 10^9 cells per ml also contained tolerance induction signals. By testing the spent medium of such culture against log-phase cells, we could detect short term tolerance to the three test drugs, yet the phenotypes could not be maintained through 48 h (Figure 4.2). This tolerance profile is not compatible with those generated under starvation conditions. Nevertheless, nutrient replenishment to the late log-phase spent medium could abolish all phenotypes (Figure 4.2). In contrast, spent medium of stationary-phase culture could induce prolonged tolerance to the test drugs except gentamicin, a phenotype similar to those triggered by starvation (Figure 4.2). These data confirmed that starvation is not the only factor governing tolerance formation during the advanced stage of exponential growth.

4.3.3. Effect of drug supplementation on high cell density-mediated tolerance

Apart from nutrition factor, we also examined the possible drug limitation effect in the log-phase-derived high density population by testing the effect of increasing drug concentration in tolerance induction assays. A 10 and 100 fold increase in drug concentration during treatment, without a concomitant increase in nutrient level, was respectively found to abolish all detectable prolonged tolerance phenotypes for all the three test drugs in the 10^8 and 10^9 cells per ml populations. The level of short term tolerance was also markedly reduced but not totally abolished (Figure 4.2). These findings indicated either that high cell density might result in reduced efficacy of the bactericidal action of antibiotics, or that high cell density mediated tolerance could not withstand higher drug concentration during treatment. At this stage, we were not able to distinguish these two possibilities by phenotypic approaches.

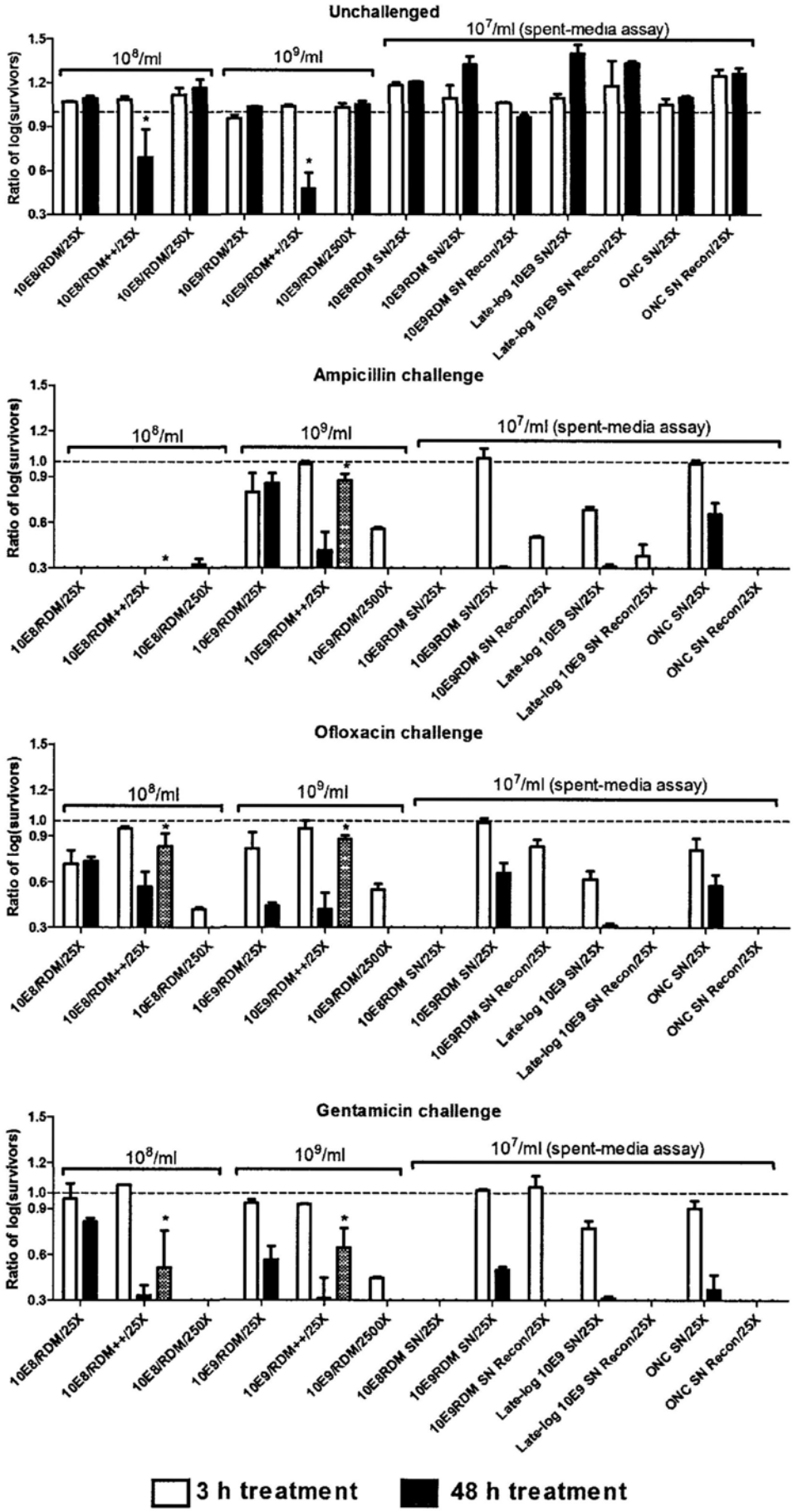

Unchallenged
Ratio of log(survivors)
10^8/ml
10^9/ml
10^7/ml (spent-media assay)
10E8/RDM/25X
10E8/RDM++/25X
10E8/RDM/250X
10E9/RDM/25X
10E9/RDM++/25X
10E9/RDM/2500X
10E8RDM SN/25X
10E9RDM SN/25X
10E9RDM SN Recon/25X
Late-log 10E9 SN/25X
Late-log 10E9 SN Recon/25X
ONC SN/25X
ONC SN Recon/25X
Ampicillin challenge
Ofloxacin challenge
Gentamicin challenge
3 h treatment
48 h treatment

Figure 4.2. Analysis of cell density-dependent tolerance by nutrient or drug supplementation. Exponentially growing cells collected at 10^7 cells/ml were concentrated to 10^8 (labeled with 10E8) or 10^9 (labeled with 10E9) cells per ml in RDM with enhanced nutrient concentrations (labeled with RDM++) and subjected to standard antibiotic treatment at 25X MIC (labeled with 25X), or resuspended in normal RDM and exposed to proportionately higher concentrations of antibiotics (labeled with 250X or 2500X). In the cases where additional supply of nutrients resulted in significant antibiotic-independent viability loss (denoted by asterisks), antibiotic survival relative to the diminished population size of the unchallenged control was also determined (shaded bars). For the spent-medium assays, filter-sterilized spent media from the high cell density populations (log cells concentrated to 10^8 cells per ml ("10E8 RDM SN"), log cells concentrated to 10^9 cells per ml ("10E9 RDM SN"), late-log-phase populations at 10^9 cells per ml ("late-log 10E9 SN") or overnight stationary-phase populations at 10^9 cells per ml ("ONC SN")) were either reconditioned (labeled with Recon) back to RDM or directly used to resuspend RDM-grown log-phase cells at 10^7 cells per ml for the sequential assessment of their tolerance-inducing effects. Survival ratios below or above 1 describe partial tolerance or net population growth, respectively.

4.3.4. Effect of supplementation of excessive nutrients or drugs on stationary-phase tolerance

In view of the negligible effects of supplementation of amino acids and glucose on the multidrug tolerance phenotypes of high density log-phase populations, we tested whether supplementing an overnight culture with a similarly higher dosage of nutrients indeed had no effect on reversing the tolerance phenotypes exhibited by such population. Surprisingly, our results showed that the effect of nutrient supplementation on stationary-phase population was dosage-dependent, and that at the same nutrient to cell density ratio as log-phase culture, there was a drastic reduction in ampicillin-tolerant population in both 3 and 48 h treatment; in addition, the ability of this resuscitated overnight culture to exhibit prolonged tolerance to gentamicin was also markedly inhibited under this condition (Figure 4.3). However, sustainable tolerance to ofloxacin was not significantly affected by nutrient supplementation (Figure 4.3). These findings are contradictory to that observed in the log-phase-derived 10^9 cells per ml population, which continued to exhibit a significant degree of sustainable tolerance to all the three antibiotics in the presence of essential nutrients (Figure 4.2).

Inconsistent findings were also obtained in experiments designed to test the effects of high drug dosage on log-phase population-derived high density culture and stationary-phase organisms. Our data showed that an elevated drug concentration could abolish most tolerance phenotypes observable in high density log-phase populations (Figure 4.2), whereas a 100-fold increase in concentration of antibiotics had no effect even on prolonged tolerance of stationary-phase cells to both ampicillin and ofloxacin (Figure 4.3). Under such treatment condition, however, the gentamicin tolerance phenotype of stationary-phase population was significantly diminished in the short term treatment and completely abolished during long term treatment (Figure 4.3).

Since the results of individual nutrient and drug supplementation experiments respectively indicated that the multidrug tolerance phenotypes in both stationary-phase and log-phase derived high density population could at least be partially reversed by increasing per unit cell availability of nutrients or drugs, we further sought to determine if such phenotypes could be totally abolished by a

simultaneous increase in the availability of both agents. Another purpose of this experiment was to confirm whether multidrug tolerance observable in stationary-phase population was due to a putative bacterial stress response which could be specifically activated under high cell density condition even in the absence of all starvation and physical limitation factors. This experiment therefore effectively constituted a tolerance induction assay on stationary-phase organisms in which the levels of both nutrients and drug were not limiting; therefore, any signs of emergence of tolerance in this assay could not have been due to starvation-induced stress responses or physical constraints that reduced nutrient and drug entry. Our results indicated that a simultaneous increase in nutrient and drug concentration in the assay medium led to eradication of both short and long term tolerance of a stationary-phase population to ampicillin and gentamicin (Figure 4.3). However, ofloxacin tolerance was found to persist in such high nutrient and drug concentration environment (Figure 4.3). The fact that nutrient-replenished spent medium of stationary-phase culture could not induce tolerance development in actively growing organisms indicated that the irreversible ofloxacin tolerance phenotype of stationary-phase population was not attributed to extracellular induction signals (Figure 4.2).

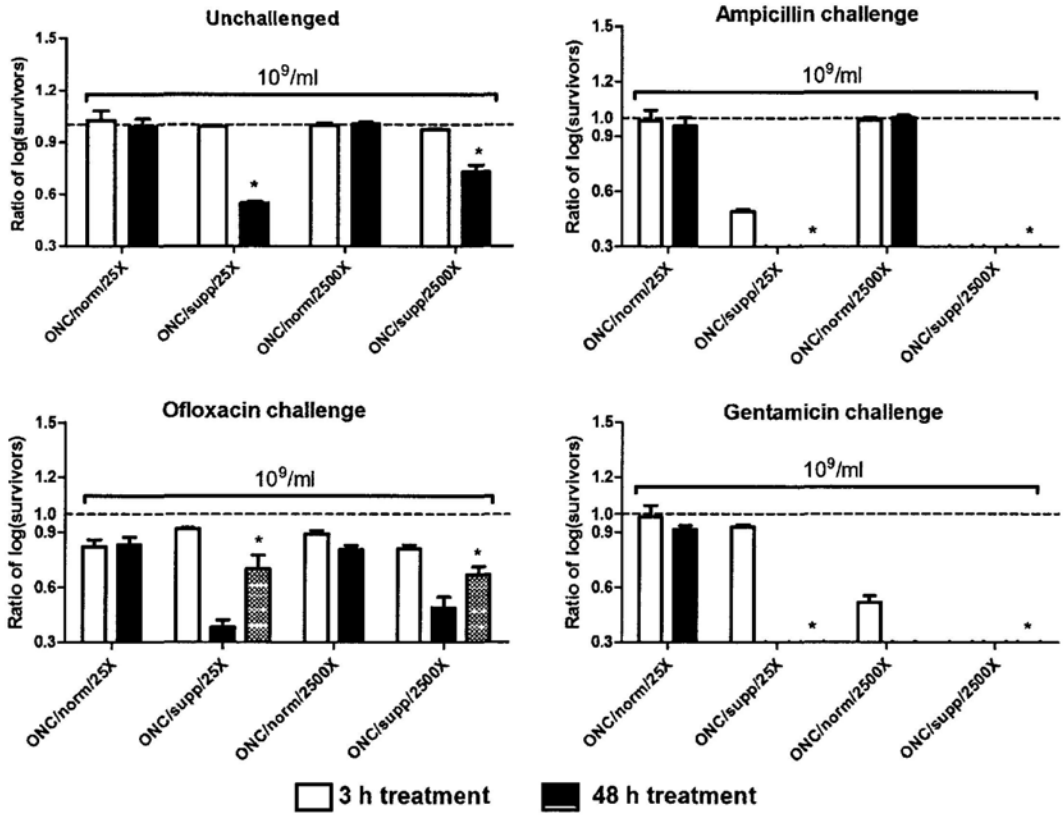


Figure 4.3. Effect of nutrient or drug supplementation on stationary-phase drug tolerant populations. RDM-grown overnight populations were either supplemented with excess nutrients ("ONC/supp/25X"), exposed to proportionately higher antibiotic dosage ("ONC/norm/2500X"), or both ("ONC/supp/2500X"). In the cases where additional supply of nutrients resulted in significant antibiotic-independent viability loss (denoted by asterisks), antibiotic survival relative to the diminished population size of the unchallenged control was also determined (shaded bars).

4.3.5. Effect of reduced cell density on stationary-phase tolerance

On the basis of the cell density effects on development of antibiotic tolerance in the absence of starvation signals, we further attempted to compare the relative features of development of cell density-mediated tolerance in different physiological backgrounds. We envisaged that, if high cell density in the vicinity of 10^8 cells per ml could result in tolerance formation by either physical limitations of solute diffusion or quorum sensing responses, the same level of antibiotic tolerance should be detectable if a stationary-phase population was diluted in RDM, as long as the cell density remained above 10^8 cells per ml. However, we found that upon 10-fold dilution in RDM, the tolerance level exhibited by this reduced-density (10^8 cells per ml) stationary-phase population was substantially lower than that of a log-phase population adjusted to the same cell density in terms of both tolerant population size and sustainability of tolerance phenotypes, i.e. despite being at the same cell density (10^8 cells per ml), a diluted stationary-phase population exhibited only low level and short term tolerance to the three test drugs whereas a condensed log-phase population exhibited significant and sustainable tolerance to both ofloxacin and gentamicin, but not ampicillin (Figure 4.4). Consistent with this finding is the observation that the level of antibiotic tolerance exhibited by an overnight culture could be drastically reduced if the cell density was lowered to that of a log-phase culture, using its own cell-free spent medium which was shown to be able to induce tolerance in a log-phase population (Figure 4.4). Interestingly, the tolerance profile of this reduced-density stationary-phase population differed significantly from that of a log-phase population which had been subjected to induction by cell-free supernatant of the same stationary-phase population (Figure 4.4); the former exhibited a barely detectable level of sustainable tolerance to ampicillin but a significant level of prolonged tolerance to gentamicin, whereas in the latter situation, prolonged tolerance to ampicillin but not gentamicin was observable.

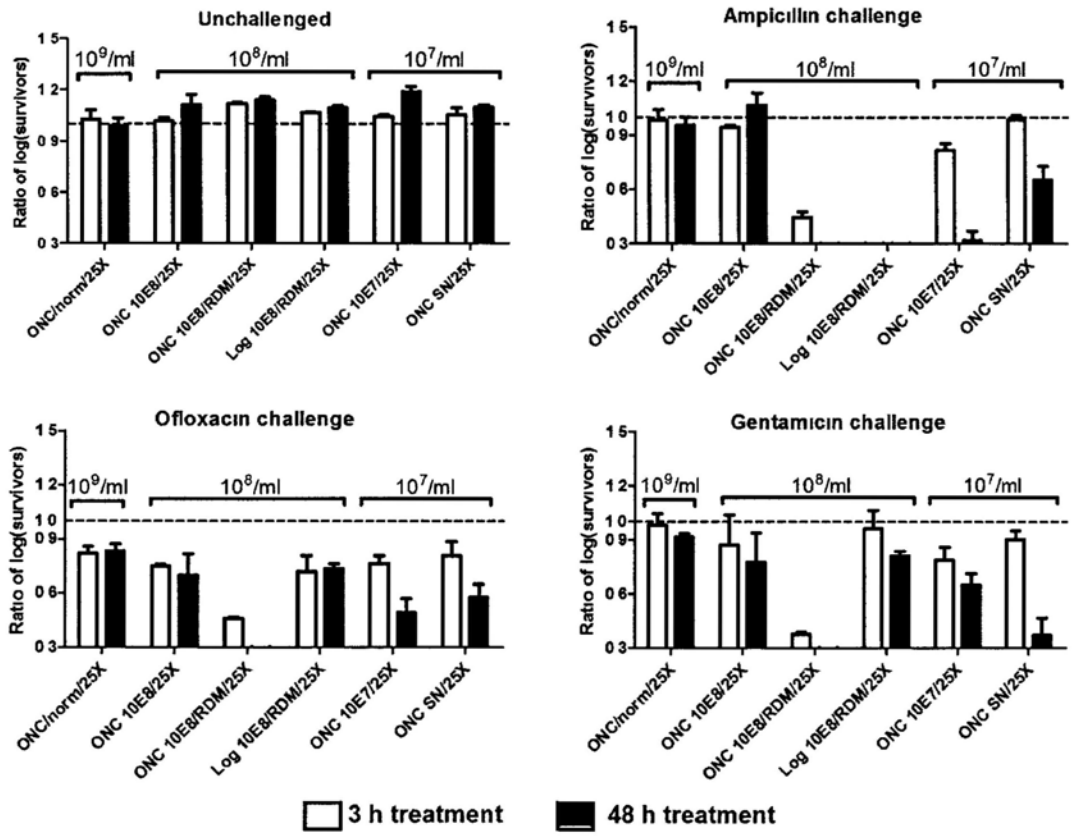


Figure 4.4. Effect of cell density reduction on antibiotic tolerance in stationary-phase populations. Overnight culture grown to 10^9 cells per ml were diluted either in its native supernatant to 10^8 ("ONC 10E8") or 10^7 ("ONC 10E7") cells per ml, or in RDM to a cell density of 10^8 cells per ml ("ONC 10E8/RDM"). Log-phase cells concentrated to 10^8 cells per ml in RDM ("Log 10E8/RDM") was included for comparison. Tolerance assays were performed at standard 25X MIC concentrations.

4.3.6. Effect of nutrient depletion on high cell density-mediated tolerance

The observation of a putative starvation-independent tolerance induction pathway in log-phase population and the failure of cell-free supernatant of stationary-phase population to induce tolerance in the same manner as that of an artificially adjusted high density population prompted us to test whether quorum sensing signals were produced only in exponentially growing organisms. We showed that bacteria which had been subjected to starvation were nevertheless tolerant to the test drugs when their cell density was adjusted to 10^9 cells per ml with MOPS base. However, cell-free supernatant of this cell suspension had a substantially weaker tolerance induction effect upon supplementation of nutrients (Figure 4.5), suggesting that the induction signals observable in a nutrient-rich culture were less abundant. Corroborating with this finding is the observation that adjustment of a MOPS-based population to 10^8 cells per ml resulted in significantly lower tolerance to prolonged ofloxacin and gentamicin treatment as compared to the RDM-based culture (Figure 4.5). These results suggested that development of specific drug tolerance under high cell density conditions may be subjected to nutrient availability.

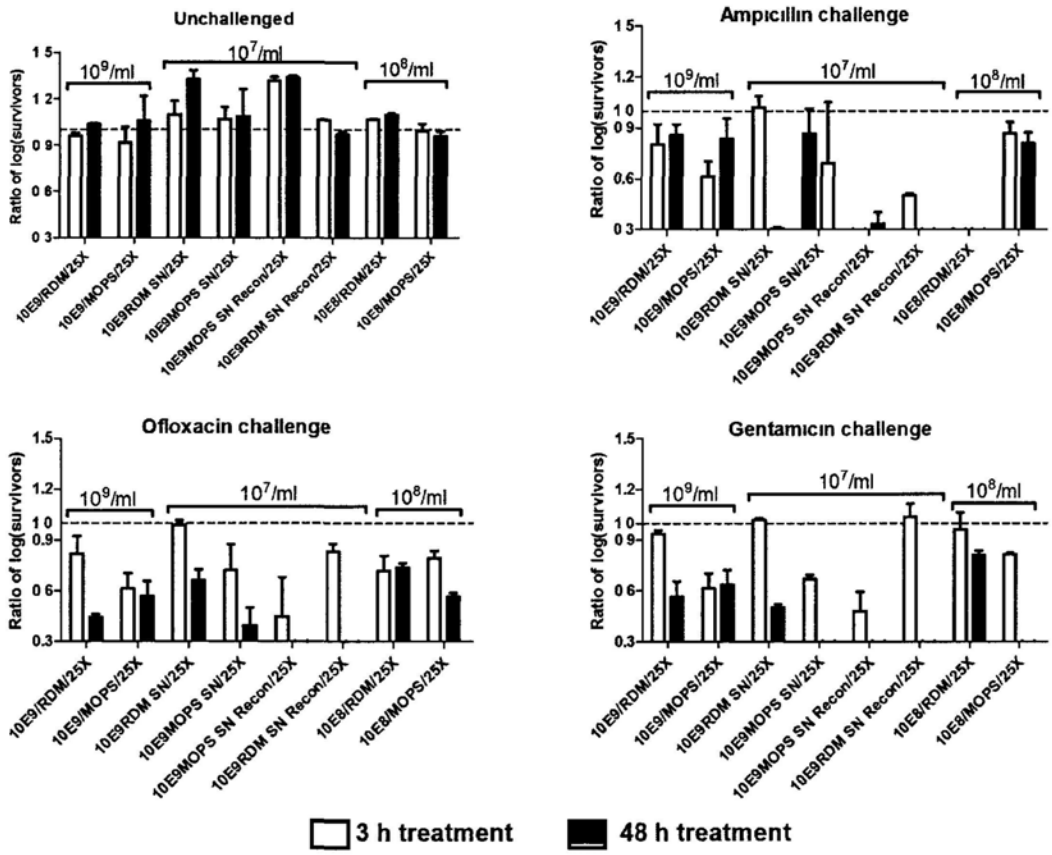


Figure 4.5. Effect of high population densities under nutrient-limiting conditions. Exponentially growing cells (10^7 cells/ml) were concentrated in MOPS base to 10^8 ("10E8/MOPS") or 10^9 ("10E9/MOPS") cells per ml; cell-free supernatant of these suspensions was isolated for tolerance induction assays using RDM-grown log-phase cells at 10^7 cell per ml ("10E9MOPS SN" and "10E9MOPS SN Recon"). Identical experiments using RDM, as described previously, were included as controls.

4.3.7. Tolerance profiles of high density tolerant population-derived persisters

As stationary-phase population is known to contain antibiotic persisters observable upon dilution in nutrient broth, we sought to investigate if a high density log-phase cell suspension also contained persisters. As shown in Figure 4.6, 10^8 cells per ml suspension in RDM did not produce detectable persisters upon dilution to 10^7 cells per ml in RDM, a condition in which both starvation and cell density-associated drug tolerance should fail to develop. However, at 10^9 cells per ml, a population density similar to that found in RDM-grown overnight cultures, was found to consistently contain a detectable amount of antibiotic persisters comparable to that observed in stationary-phase cells (Figure 4.6), indicating that persister development can be attributed to both starvation and high density induction.

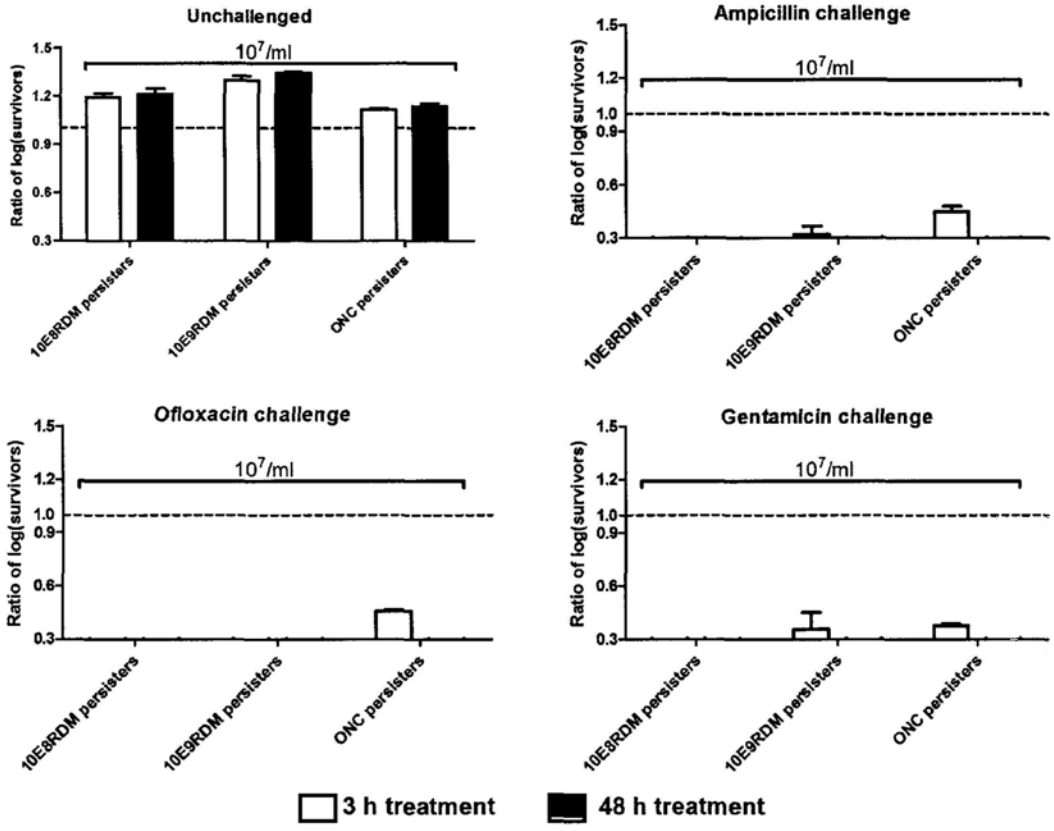


Figure 4.6. Induction of persister formation by high population density. Log-phase cells-derived high cell density populations (at 10^8 or 10^9 cells per ml) were diluted into fresh RDM to produce a final cell density of 10^7 cells/ml and assayed for antibiotic tolerant persister cells ("10E8RDM persisters" and "10E9RDM persisters"). Diluted overnight stationary-phase populations (10^7 cells/ml), which consistently contain drug-tolerant persister cells ("ONC persisters"), were included as positive control.

4.3.8. Putative high cell density mediated tolerance mechanisms

One of the most intriguing findings in this study is the demonstration of tolerance inducing properties of the nutrient-replenished supernatant of a log-phase-derived high density culture but not stationary-phase population (Figure 4.2). In a preliminary attempt to elucidate potential signals that might elicit tolerance formation in log-phase cells, we tested whether this ability was abolished in a strain in which the *luxS* gene, which encoded for the only autoinducer (AI-2) synthase known to exist in *Escherichia coli*, was deleted. As shown in Figure 4.7, the Δ*luxS* mutant also possessed the ability to produce a tolerance-inducible supernatant at high concentration, indicating that this property is not associated with the AI-2 based quorum sensing system. Apart from the AI-2 system, we also tested whether the high cell density-dependent phenomenon was caused by excretion of acetate, which is known to accumulate at a high cell density condition as a result of a metabolic overflow mechanism, potentially exerting transient growth inhibitory or tolerance effects (Arnold *et al.*, 2001; Wolfe, 2005). Information regarding whether acetate can induce antibiotic tolerance development, however, is not available in the literature. In a preliminary attempt to examine whether this metabolite could induce antibiotic tolerance, we added sodium acetate to a log-phase culture and assayed for its effect on drug susceptibility. None of the concentrations (10-1000 μg/ml) tested, including the level known to induce tolerance to both heat shock and peroxide treatment (100μg/ml) (Arnold *et al.*, 2001), could elicit tolerance to the three antibiotics.

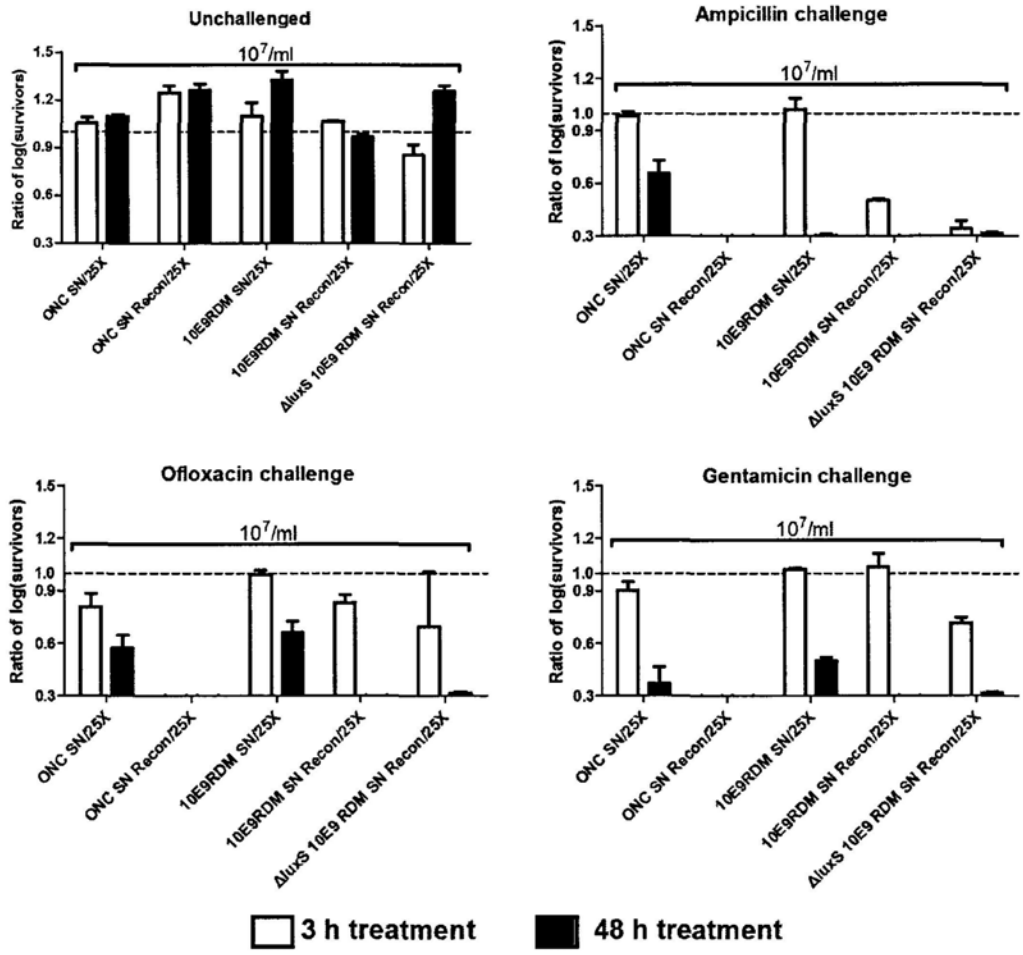


Figure 4.7. Role of extracellular signals mediating high cell density induced tolerance. Cell-free supernatant isolated from both overnight-grown and log-phase cells derived high density (10^9 cells/ml) populations were re-conditioned to RDM and subsequently used to incubate RDM-grown log-phase cells (10^7 cells/ml) and assayed for the development of drug tolerance ("ONC SN Recon" and "10E9RDM SN Recon"). A parallel study using spent-medium from the isogenic *luxS*-null mutant ("Δ*luxS* 10E9 RDM SN Recon"), which encodes the only known autoinducer synthase in *Escherichia coli,* was included.

4.4. Summary

Exponentially growing bacteria are known to exhibit progressively reduced susceptibility to antibiotics, becoming multidrug-tolerant at stationary-phase. To elucidate the cellular basis of this phenomenon, we analyzed the physical and physiological parameters that governed cellular responses to bactericidal antibiotics at different growth stages and cell densities. Phenotypic tolerance to fluoroquinolones and aminoglycosides became detectable in logarithmic-phase organisms when the population was artificially reconstituted to 10^8 cells per ml; such tolerance profile was extended to cover beta-lactams upon a further 10-fold concentration of the cell suspension. The differences in tolerance phenotypes were partially explainable by physical limitation of drug molecules and/or indirect starvation responses. By means of supplementation and density adjustment approaches, it was further demonstrated that logarithmic and stationary-phase organisms exhibited drastically different tolerance profiles with a varied degree of nutrient sensitivity even at identical population density, indicating that bacterial physiological status was also responsible for determining drug susceptibility prior to stationary-phase or exhaustion of nutrients. Corroborating with this finding was the observation that spent medium of condensed logarithmic phase bacterial suspension exhibited tolerance induction potential even after nutrient replenishment of the medium, confirming that the high cell density-dependent phenotypes were not solely elicited by starvation. Such tolerance signals were, however, absent in stationary-phase population or cell suspension in which nutrients were depleted from the reconstitution medium, indicating that their production was nutrient-dependent. These findings suggest that bacteria possess preparatory defense mechanisms which become functional beyond a threshold density, thereby mediating tolerance development when growth conditions are still favorable. Preliminary analysis showed that autoinducer-II (synthesized by the *luxS* gene product (Walters and Sperandio, 2006)), or extracellular metabolites such as acetate were not involved in regulating such mechanisms.

Chapter 5. Investigation of starvation-induced antibiotic tolerance mechanisms

5.1. Introduction

Phenotypic studies as described in the previous chapters show that bacteria can spontaneously switch to an antibiotic-tolerant state with condition-sensitive characteristics of target drug specificity and sustainability of the tolerance mode. Unlike the conventional study approach which employs a single induction parameter, namely, the stationary-phase condition in which bacteria are subjected to induction by multiple starvation signals, our condition-specific tolerance assays allowed us to identify the unique induction profile supported by each condition. Such "phenotypic tolerance maps" provided a unique platform that facilitated further analysis of the role of specific stress protection system in development of antibiotic tolerance during nutrition starvation.

Since development of antibiotic tolerance is expected to correlate with an induced ability to disrupt one or more steps of antibiotic-mediated cell death pathways, it is envisaged that cellular mechanisms which can confer a protective effect against drug-induced damages must produce at least one of the following effects: (i) altered membrane permeability and/or activation of efflux pumps which impede drug entry or extrude imported antibiotic molecules, (ii) activation of antioxidant defense to neutralize antibiotic-induced accumulation of deleterious free radicals, (iii) enhanced production of molecular chaperones or other protection molecules which minimize drug-elicited damages, (iv) up-regulation of cellular repair system such as the SOS response which restores normal functioning of essential cellular apparatus, and (v) global down-regulation of biosynthetic pathways leading to reduced sensitively to drug action. In this part of the project we chose to test whether starvation-mediated tolerance resulted in active interference of drug killing processes, using both functional and molecular approaches. Data produced in this work will help to validate the hypothesis that starvation-activated mechanisms that confer several lines of protection against drug-induced lethality constitute the molecular basis of phenotypic antibiotic tolerance.

5.2. Methods

Bacterial strains and culture media

Escherichia coli K-12 strains and their isogenic derivatives used in this study were listed in Table 5.1. All broth cultures were grown in RDM as described previously (Fung *et al.*, 2010) unless otherwise specified. LB agar was used as solid medium for antibiotic survival assays. In the case of stringent response mutants, bacteria were also plated on M9 glucose agar to check for emergence of compensatory mutants.

Table 5.1. *Escherichia coli* strains used in studies on starvation-induced antibiotic tolerance mechanisms.

Strain	Genotype	Source (reference)
BW25113	*lacI*^{q} *rrnB*$_{T14}$ Δ*lacZ*$_{WJ16}$ *hsdR514* Δ*araBAD*$_{AH533}$ Δ*rhaBAD*$_{LD78}$	Baba *et al* (Baba *et al*, 2006)
JW3974	BW25113 *aceB* Km^{R}	As above
JW0452	BW25113 *acrA* Km^{R}	As above
JW0451	BW25113 *acrB* Km^{R}	As above
JW0598	BW25113 *ahpC* Km^{R}	As above
JW0599	BW25113 *ahpF* Km^{R}	As above
JW0048	BW25113 *apaH* Km^{R}	As above
JW4364	BW25113 *arcA* Km^{R}	As above
JW5536	BW25113 *arcB* Km^{R}	As above
JW3711	BW25113 *atpG* Km^{R}	As above
JW5060	BW25113 *bolA* Km^{R}	As above
JW3882	BW25113 *cpxA* Km^{R}	As above
JW5558	BW25113 *cpxP* Km^{R}	As above
JW3883	BW25113 *cpxR* Km^{R}	As above
JW0590	BW25113 *cstA* Km^{R}	As above
JW3779	BW25113 *cyaY* Km^{R}	As above
JW0157	BW25113 *degP* Km^{R}	As above
JW0141	BW25113 *dksA* Km^{R}	As above
JW0014	BW25113 *dnaJ* Km^{R}	As above
JW0013	BW25113 *dnaK* Km^{R}	As above
JW0797	BW25113 *dps* Km^{R}	As above
JW3865	BW25113 *fdoG* Km^{R}	As above
JW3863	BW25113 *fdoI* Km^{R}	As above
JW4253	BW25113 *fecI* Km^{R}	As above
JW3229	BW25113 *fis* Km^{R}	As above
JW5603	BW25113 *gppA* Km^{R}	As above
JW4103	BW25113 *groL* Km^{R}	As above

Table 5.1. *Escherichia coli* strains used in studies on starvation-induced antibiotic tolerance mechanisms. (cont'd)

Strain	Genotype	Source (reference)
JW0833	BW25113 *grxA* Km^R	As above
JW2663	BW25113 *gshA* Km^R	As above
JW1225	BW25113 *hns* Km^R	As above
JW5692	BW25113 *hslO* Km^R	As above
JW0462	BW25113 *htpG* Km^R	As above
JW3664	BW25113 *ibpA* Km^R	As above
JW5782	BW25113 *iraD* Km^R	As above
JW2515	BW25113 *iscR* Km^R	As above
JW1721	BW25113 *katE* Km^R	As above
JW3914	BW25113 *katG* Km^R	As above
JW3423	BW25113 *livK* Km^R	As above
JW1505	BW25113 *lsrR* Km^R	As above
JW2662	BW25113 *luxS* Km^R	As above
JW5249	BW25113 *marA* Km^R	As above
JW5248	BW25113 *marR* Km^R	As above
JW2753	BW25113 *mazF* Km^R	As above
JW1100	BW25113 *mfd* Km^R	As above
JW5129	BW25113 *mgsA* Km^R	As above
JW2990	BW25113 *mqsR* Km^R	As above
JW3252	BW25113 *mscL* Km^R	As above
JW2891	BW25113 *mscS* Km^R	As above
JW1275	BW25113 *osmB* Km^R	As above
JW3933	BW25113 *oxyR* Km^R	As above
JW4072	BW25113 *proP* Km^R	As above
JW3990	BW25113 *psiE* Km^R	As above
JW3706	BW25113 *pstS* Km^R	As above
JW2205	BW25113 *rcsB* Km^R	As above
JW5917	BW25113 *rcsC* Km^R	As above
JW2669	BW25113 *recA* Km^R	As above
JW1555	BW25113 *relE* Km^R	As above
JW0184	BW25113 *rof* Km^R	As above
JW3039	BW25113 *rpoD* Km^R	As above
JW1907	BW25113 *rpoF* Km^R	As above
JW3169	BW25113 *rpoN* Km^R	As above
JW5437	BW25113 *rpoS* Km^R	As above
JW3624	BW25113 *rpoZ* Km^R	As above
JW1901	BW25113 *sdiA* Km^R	As above
JW3879	BW25113 *sodA* Km^R	As above
JW1648	BW25113 *sodB* Km^R	As above
JW1638	BW25113 *sodC* Km^R	As above

Table 5.1. *Escherichia coli* strains used in studies on starvation-induced antibiotic tolerance mechanisms. (cont'd)

Strain	Genotype	Source (reference)
JW1223	BW25113 *sprE* Km^R	As above
JW0716	BW25113 *sucB* Km^R	As above
JW0941	BW25113 *sulA* Km^R	As above
JW0052	BW25113 *surA* Km^R	As above
JW5503	BW25113 *tolC* Km^R	As above
JW5856	BW25113 *trxA* Km^R	As above
JW0659	BW25113 *ubiF* Km^R	As above
JW3462	BW25113 *uspA* Km^R	As above
JW3461	BW25113 *uspB* Km^R	As above
JW1884	BW25113 *uspC* Km^R	As above
JW3894	BW25113 *uspD* Km^R	As above
JW1327	BW25113 *uspE* Km^R	As above
JW1370	BW25113 *uspF* Km^R	As above
JW0600	BW25113 *uspG* Km^R	As above
JW5446	BW25113 *xni* Km^R	As above
JW0004	BW25113 *yaaX* Km^R	As above
JW0774	BW25113 *ybhQ* Km^R	As above
JW5200	BW25113 *yciW* Km^R	As above
JW1995	BW25113 *yeeE* Km^R	As above
JW5395	BW25113 *yfeW* Km^R	As above
JW2879	BW25113 *ygfA* Km^R	As above
JW3325	BW25113 *yhfG* Km^R	As above
JW5948	BW25113 *yhfZ* Km^R	As above
JW3785	BW25113 *yigB* Km^R	As above
JW5588	BW25113 *yigI* Km^R	As above
JW3844	BW25113 *yihM* Km^R	As above
JW5559	BW25113 *yiiM* Km^R	As above
JW5742	BW25113 *yjfN* Km^R	As above
JW5786	BW25113 *yjiM* Km^R	As above
JW5791	BW25113 *yjiY* Km^R	As above
JW4342	BW25113 *yjjW* Km^R	As above
JW1714	BW25113 *yniA* Km^R	As above
JW2906	BW25113 *yqgB* Km^R	As above
CF1943	Wild-type W3110	Xiao *et al* (Xiao *et al* , 1991)
CF1946	CF1943 Δ*relA251 kan* Δ*spoT207 cat*	As above
RW118	*thr-1 araD139* Δ *(gpt-proA)62 lacY1 tsx-33 supE44 galK2 hisG4 rpsL31 xyl-5 mtl-1 argE3 thi-1 sulA211*	Woodgate *et al* (Fernandez De Henestrosa *et al* , 2000)
RW434	RW118 *lexA3*(Ind-)	As above
RW542	RW118 *lexA51*(Def)	As above

Hydroxyl radical scavenger protection assay

To determine the protective effects of free radical scavengers against antibiotic-induced killing, RDM-incubated log-phase cells were subjected to antibiotic treatment (at 5X or 25X MIC) with or without 150 mM thiourea. Survivors at 3-h and 48-h were enumerated by standard viability counts and relative population survival was calculated with respect to the population size prior to drug treatment.

Antibiotic uptake and two-step killing experiment

Antibiotic entry in antibiotic-tolerant cells was assayed using tritium-labeled antibiotics purchased from American Radiolabeled Chemicals (St. Louis, MO). RDM or MOPS incubated cells prepared as described previously were incubated in sub-inhibitory concentrations of [3H] penicillin G (0.5 μCi/ml), [3H] norfloxacin (0.3 μCi/ml) or [3H] gentamicin (0.5 μCi/ml) for 30 min along with untreated controls. Samples were taken at 0, 2, 15 and 30 min post treatment, washed in their respective growth media, followed by measurement of radioactivity using the PerkinElmer scintillation counter. Parallel measurement of population viability was performed by serial dilution and plating on LB agar. Antibiotic uptake over time was calculated and presented as CPM (Counts per Minute) per 10^7 cells so that growth difference between RDM and MOPS incubated cells was normalized. A control drug-tolerance assay was performed using bactericidal concentrations of non-labeled pencillin-G, norfloxacin and gentamicin in both RDM-primed and MOPS-primed populations to assess variation in population size during the course of uptake assay. A two-step killing experiment was performed to determine the immediate effects of drug uptake. In this experiment, RDM-grown log-phase cells were pelleted and incubated in either RDM or MOPS for 2 h and aliquots were briefly exposed to 25X MIC of either ampicillin (15 min), ofloxacin (10 min) or gentamicin (5 min). A portion of the drug-treated cells were switched to drug-free RDM and population survival was monitored for up to 1 h and compared to the original population which were subjected to continuous drug exposure.

Tolerance inhibition by translation inhibitors

To test the effect of transcription and protein synthesis inhibitors on starvation-induced tolerance development, tetracycline (4 μg/ml) was added to a RDM-grown log-phase culture and incubated for 2 h at 37°C, the treated population

was subjected to centrifugation, washed once, resuspended in MOPS base which also contained tetracycline at 4 μg/ml, and incubated at 37°C for 2 h. The cell suspension was then subjected to antibiotic challenge as described in tolerance induction assay.

Microarray experiments

Microarray analysis using the Affymetrix *Escherichia coli* 2.0 Genechip was performed according to the supplier's specification. In brief, total RNA was extracted from RDM-grown log-phase populations which had been resuspended in (i) RDM, (ii) RDM minus glucose, (iii) RDM minus amino acids, or (iv) RDM minus both glucose and amino acids, using the Qiagen RNeasy extraction kit. Total RNAs were reverse-transcribed, fragmented and labeled with biotin according to the manufacturer's protocol. Hybridization and scanning service of Affymetrix Genechip was provided by the Li Ka Shing Institute of Health Sciences (LiHS) core facilities. The array data was analysed using ArrayStar 3.0 (DNASTAR, Inc.).

Phenotypic screening of putative tolerance determinants

Shortlisted genes which were implicated in antibiotic tolerance development were subjected to starvation-induced tolerance assay to assess the gene deletion effect. For testing the relative role of putative tolerance genes in tolerance development, RDM-grown log-phase cells were exposed to a specific test conditions followed by drug treatment and assessment of population survival as described (Fung *et al.*, 2010). Information on the identity of the test genes and conditions is described in detail in the Results Section. Those selected on the basis of microarray data as well as their biological functions were subjected to a quick screening test. Briefly, log-phase organisms were subjected to amino acids and carbon starvation, followed by antibiotic treatment. Cell viability at 3 h and 48 h post treatment was assessed by plating 10 μl of diluted culture on LB agar. Mutants which demonstrated reduction in tolerance were subjected to a confirmation test using the standard plate-count approach.

5.3. Results

Putative mechanisms underlying the development of antibiotic tolerance upon nutrient starvation were investigated from the perspective of cellular functions, gene expression features and outcome of specific gene knockout. Consistency between global and specific analyses of tolerance determinants was checked to identify the key functional components responsible for the initiation and maintenance of starvation-mediated tolerance phenotypes. In experiments designed to elucidate the complex relationship between nutritional composition and bacterial susceptibility to specific antibiotic classes, we identified several key conditions which could respectively elicit tolerance of different strength and drug combinations (Fung *et al.*, 2010). On the basis of these tolerance induction criteria and the corresponding phenotypic features, we performed a microarray analysis of the gene expression profile under several key tolerance inducing conditions. These conditions included one in which amino acids were depleted from a full nutrient recipe (RDM), which triggered development of short tolerance to ampicillin and ofloxacin. The second condition chosen for expression analysis involved depletion of both amino acids and glucose from the growth medium, which was shown to induce prolonged tolerance to ampicillin and ofloxacin, as well as short term tolerance to gentamicin. The phenotypic difference between the tolerance inducing abilities of these two conditions suggested that the physiological changes that occurred under these conditions were not identical, and some of those discrepancies, which might be reflected in the gene expression patterns, were expected to be responsible for the differential tolerance phenotypes observable under the two conditions. Interestingly, we found that carbon starvation alone could not elicit any phenotypes. This condition was also included to depict the genes whose expression was altered during nutrition starvation only. Together with RDM, which does not support tolerance, a total of four nutrient recipes were included in microarray analyses. Table 5.2 summarizes the composition of these recipes and the respective antibiotic susceptibility profiles detectable in an exponentially growing bacterial population. According to the nutrient and tolerance relationship depicted in the table, genetic components responsible for mediating short term tolerance to ampicillin and ofloxacin should comprise at least part of the gene pool which exhibits differential expression level

when the expression profile of cells grown in RDM is compared to that of cells grown in a medium in which amino acids are depleted from RDM (RDM-AA). On the basis of this principle, we postulate that the molecular basis of long-term tolerance can be revealed by comparing the expression profiles of cells grown in RDM or RDM-AA to that of RDM-AAC, a medium in which both amino acids and glucose were depleted from RDM. Since differentially expressed genes selected by comparing these two inducing conditions might not necessarily be responsible for maintenance of tolerance, we performed comparative analysis of genes which were differentially expressed when cells grown in a medium lacking glucose (RDM-C) was compared to cells grown in RDM itself or RDM-AAC. The former condition was expected to yield genes which were responsive to starvation but did not contribute to tolerance induction, whereas the latter might allow the identification of genes which might play predominant roles in the maintenance of tolerance, complementing those selected by comparing cells grown in RDM and RDM-AA to those grown in RDM-AAC. Two basic selection criteria were adopted for short-listing differentially expressed genes in pairwise comparison of the effects of test conditions: genes depicting an average of at least two fold differences in expression level calculated on the basis of three independent experiments were chosen and categorized according to their biological functions. For these selected genes, the degree of reproducibility in relative gene expression status (i.e. up-regulation or down-regulation) among three experiments was assessed.

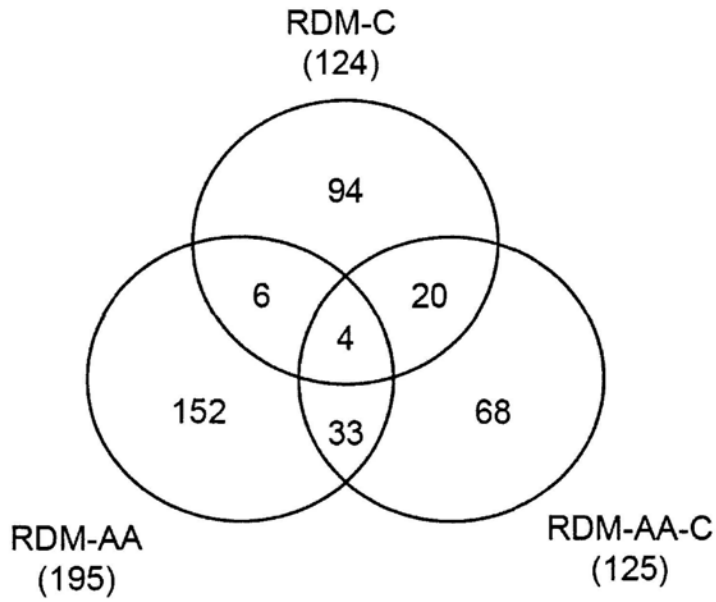
5.3.1. Overview of starvation-mediated gene expression profile

Using the four-fold difference criterion, a distinct feature of starvation response was observable when the gene expression profiles recorded under the three tolerance inducing conditions were compared to that of RDM. For each condition, significant differential expression was detectable in over 100 genes; however, the degree of overlap among these selected gene-sets was found to be small. For example, a total of 195 genes were differentially expressed under the condition of RDM-AA (Figure 5.1), yet out of these 195 genes, as many as 152 or 78% were uniquely detected under this condition. This rate was 94 out of 124 or 76% for the condition of RDM-C. For RDM-AAC, 68 out of the 125 differentially expressed genes (54%) were recorded only under such condition. Nevertheless, the expression level of 6 genes was found to be altered in both RDM-AA and RDM-C. The number of overlapping

genes among the RDM-C and RDM-AAC groups was 20. On the other hand, a total of 33 genes were found to span both RDM-AA and RDM-AAC gene sets. Furthermore, four genes were found to be differentially expressed in all the three test conditions. From the perspective of relevance to tolerance, a total of 222 genes whose expression was exclusively altered under the conditions of RDM-AA and / or RDM-AAC were regarded as potential candidates for tolerance regulation. The 124 genes whose expression were altered in RDM-C, either uniquely or in combination with the other two conditions, were regarded as being responsible for starvation response with little contributive effect on tolerance development or maintenance. In order to reveal the nature of functional changes under specific conditions, we categorize all selected genes according to their known biological functions. As shown in Figure 5.1, the proportion of up-regulated genes was markedly lower than that of down-regulated genes in all functional categories under the condition of RDM-AA. As many as 79 genes which were known to be involved in biosynthesis and metabolism were significantly down-regulated, whereas only 21 genes in this category were over-expressed. However, the opposite situation was observable in the case of RDM-AAC; under this condition, up-regulated gene expression was predominant despite the fact that carbon source was further depleted from the growth medium. In this case, 43 out of the 62 biosynthesis or metabolism genes which exhibited four-fold difference in gene expression were in fact up-regulated. This trend was consistent with that of the differential gene expression pattern of the RDM-C background, in which the vast majority of the short-listed genes (109 out of 124 or 88%) were significantly up-regulated when compared to RDM.

Table 5.2. Summary of nutrient recipes selected for microarray analysis. Four nutrition conditions (RDM: full RDM, RDM-C: full RDM minus carbon source (glucose), RDM-AA: full RDM minus all amino acids, RDM-AAC: full RDM minus all amino acids and carbon source) leading to differential starvation-mediated tolerance phenotypes (short: transient (3 h) tolerance, long: prolonged (48 h) tolerance) were selected from phenotypic studies (Figure 2.1) for further transcriptomic analysis

Condition	Ampicillin tolerance		Ofloxacin tolerance		Gentamicin tolerance	
	Short	Long	Short	Long	Short	Long
RDM	-	-	-	-	-	-
RDM-C	-	-	-	-	-	-
RDM-AA	+	-	+	-	-	-
RDM-AAC	+	+	+	+	+	-



Biological Process	Conditions								
	RDM-C/RDM			RDM-AA/RDM			RDM-AA-C/RDM		
	Total	Up	Down	Total	Up	Down	Total	Up	Down
a) Biosynthesis and metabolism	70	65	5	100	21	79	62	43	19
b) Transport	22	21	1	46	22	24	20	17	3
c) Protein processing	2	2	0	3	0	3	0	0	0
d) Energy production	1	1	0	5	1	4	1	0	1
e) Stress adaptation	7	5	2	13	5	7	9	6	3
f) Adhesion and motility	2	1	1	0	0	0	0	0	0
g) Signal transduction	1	1	0	0	0	0	1	1	0
h) Undefined functions	19	14	5	28	10	18	32	22	10
Total	124	109	14	195	59	136	125	89	36

Figure 5.1. Overview of starvation-induced transcriptome. RDM-grown log-phase cells were subjected to selected starvation conditions (RDM-C: RDM without carbon source (glucose), RDM-AA: RDM without amino acids and RDM-AA-C: RDM without carbon source and amino acids) followed by microarray analysis. Entities which are differentially expressed as compared to unstarved, RDM treated cells (4-fold or above) are shortlisted and represented in the form of Venn diagram, which shows the degree of overlap across conditions (top), or general functional clustering (bottom).

5.3.2. Selection of tolerance genes

In order to narrow down the list of putative tolerance genes from the differential gene expression data, we tightened the screening criteria and performed consistency check among datasets generated under the test conditions. Firstly, 152 genes for which gene expression was exclusively altered in RDM-AA were regarded as potential candidates for mediating short term tolerance, whereas a certain proportion of the 68 genes whose expression was significantly altered in RDM-AAC only were believed to play a role in long term tolerance. Comparison between these two sets of candidate genes showed that there were 37 genes in common. As described below, these genes were analyzed for determination of common regulators of tolerance formation and maintenance. On the other hand, our preliminary functional categorization data suggested that short term tolerance was likely to be caused by the effect of reduced metabolism, whereas the condition which elicited prolonged tolerance to at least ampicillin and ofloxacin appeared to produce active stress response despite a more severe degree of starvation; we therefore further sought to identify the genes whose expression level differed by four-fold when data obtained under the conditions of RDM-AA and RDM-AAC, which were known to induce short and long term tolerance respectively, were compared to each other. As shown in Table 5.3, a total of 117 genes fall into this category. Importantly, 97 of these 117 genes (83%) were in fact up-regulated under the condition of RDM-AAC, when the expression level recorded in RDM-AA was used as the baseline, thereby confirming the relatively active metabolic and biosynthetic status under a situation where amino acids and carbon source were simultaneously depleted from the growth medium. Reproducibility check showed that 87 of the 117 genes (74%) exhibited identical relative expression status among three different experiments (Table 5.3); in other words, each of these 87 genes was found to be either up-regulated or down-regulated in three independent experiments, indicating that the results were reproducible.

Table 5.3. Relative change in transcript expression level between the conditions of concomitant depletion of amino acids and carbon and amino acids limitation alone. Entities which were differentially expressed (4-fold or above) in RDM-AAC with respect to RDM-AA were shortlisted (117 entities). Relative expression of each entity in each of the three independent experiments was included to depict the reproducibility of these experiments. Underlined entities were selected for further deletion studies.

Gene Symbol	RDM-AAC vs RDM-AA	Gene Ontology Biological Process	AAC1vsAA1	AAC2vsAA2	AAC3vsAA3
adhE	6.174 up	oxidation reduction, alcohol metabolic process	3.125 up	1.488 up	4.639 up
aldA	8.778 up	oxidation reduction, metabolic process	25.917 up	1.431 up	10.821 up
aspA	5.585 up	tricarboxylic acid cycle, aspartate metabolic process	5.674 up	1.204 up	5.713 up
atoS	6.969 up	two-component signal transduction system (phosphorelay), regulation of transcription	41.945 up	1.013 down	2.697 up
atpG	6.190 up	ATP biosynthetic process, ion transport	7.934 up	1.450 up	4.344 up
cbl	6.729 down	regulation of transcription, DNA-dependent	53.020 down	1.529 down	17.966 down
cstA	5.326 up	cellular response to starvation	4.776 up	1.220 up	9.238 up
cysA	6.543 down	sulfate transport	27.608 down	1.322 down	12.549 down
cysH	5.171 down	cysteine biosynthetic process, oxidation reduction	44.098 down	1.485 down	1.950 down
cysJ	4.564 down	sulfate assimilation, cysteine biosynthetic process	13.579 down	1.598 down	9.321 down
dctA	6.353 up	transport, carbohydrate transport	23.795 up	1.133 down	3.658 up
dhaL	4.334 up	glycerol metabolic process	3.458 up	1.812 up	4.475 up
dhaM	6.593 up	transport, phosphorylation	5.850 up	1.516 up	6.895 up
evgA	5.059 down	two-component signal transduction system (phosphorelay), regulation of transcription, DNA-dependent	16.504 down	1.508 down	8.384 down
fadA	5.069 up	fatty acid metabolic process	8.227 up	1.074 down	6.458 up
fadB	36.728 up	oxidation reduction, fatty acid metabolic process	97.125 up	1.089 up	46.881 up
fadE	15.147 up	oxidation reduction, fatty acid metabolic process	40.220 up	1.304 up	26.958 up
fadH	19.519 up	oxidation reduction, metabolic process	134.451 up	1.091 down	29.851 up
fadI	5.015 up	fatty acid metabolic process	18.966 up	1.007 up	4.388 up
fadJ	6.787 up	fatty acid metabolic process, metabolic process	15.928 up	1.181 up	7.433 up
fpr	4.653 up	oxidation reduction	5.266 up	1.312 up	3.621 up
frdB	7.654 up	electron transport chain, oxidation reduction, tricarboxylic acid cycle	7.793 up	1.233 up	7.094 up

Table 5.3. Relative change in transcript expression level between the conditions of concomitant depletion of amino acids and carbon and amino acids limitation alone. (cont'd)

Gene Symbol	RDM-AAC vs RDM-AA	Gene Ontology Biological Process	AAC1vsAA1	AAC2vsAA2	AAC3vsAA3
fumA	4.285 up	generation of precursor metabolites and energy, tricarboxylic acid cycle	4.741 up	1.995 up	3.228 up
fusA	4.176 up	translational elongation	1.542 up	1.105 up	2.949 up
galS	8.976 up	regulation of transcription, DNA-dependent	57.345 up	1.185 up	2.717 up
garP	4.011 up	Transmembrane transport	7.494 up	1.013 down	1.888 up
gatY	5.356 up	Glycolysis	3.757 up	2.296 up	2.455 up
gatZ	5.818 up	galactitol metabolic process	3.034 up	1.858 up	5.689 up
glnP	4.261 down	amino acid transport	35.456 down	1.723 down	3.192 down
groL	4.144 up	protein folding	4.663 up	1.115 down	3.720 up
hybA	5.048 up	oxidation reduction	19.560 up	1.217 down	2.273 up
iraD	4.363 down	cellular response to stress	92.842 down	1.365 down	3.072 down
lpp	4.009 up	lipid modification	1.048 up	1.092 up	1.087 up
lsrK	4.073 up	carbohydrate metabolic process	8.247 up	1.186 up	2.115 up
malE	4.734 up	maltose transport	8.441 up	1.075 down	2.493 up
malK	4.598 up	maltose transport	18.066 up	1.218 down	1.426 up
malT	5.206 up	two-component signal transduction system (phosphorelay); carbohydrate metabolic process	15.673 up	1.158 down	4.332 up
melR	5.655 up	regulation of transcription	29.648 up	1.068 down	2.417 up
menC	4.315 up	menaquinone biosynthetic process	7.808 up	1.292 up	1.578 up
metB	5.158 down	methionine biosynthetic process	12.728 down	2.160 down	8.233 down
mfd	4.272 up	DNA repair, regulation of transcription, DNA-dependent; response to DNA damage stimulus	9.294 up	1.016 up	2.757 up
mglB	4.820 up	chemotaxis, carbohydrate transport	8.913 up	1.072 up	2.895 up
murQ	5.107 up	carbohydrate metabolic process, amino sugar metabolic process	18.309 up	1.233 up	1.827 up
mutL	4.191 down	DNA repair, mismatch repair, response to DNA damage stimulus	11.686 down	5.197 down	1.592 down
nadR	4.749 up	pyridine nucleotide biosynthetic process, regulation of transcription	4.816 up	1.120 up	2.695 up
napA	5.711 up	nitrate assimilation, oxidation reduction, Mo-molybdopterin cofactor biosynthetic process	5.490 up	1.881 up	5.004 up
narG	4.152 up	electron transport chain, nitrate metabolic process	9.911 up	1.079 down	3.485 up
nlpA	4.522 down	inner membrane lipoprotein	12.422 down	1.941 down	24.017 down
nmpC	9.490 up	silent gene, outer membrane porin	31.731 up	1.303 up	4.520 up
nrfA	4.066 up	nitrogen compound metabolic process, electron transport chain, oxidation reduction	9.429 up	1.512 down	1.908 up
nrfB	5.967 up	electron transport chain	23.005 up	1.290 down	2.402 up
nrfC	5.318 up	electron transport chain	23.676 up	1.148 down	2.664 up

Table 5.3. Relative change in transcript expression level between the conditions of concomitant depletion of amino acids and carbon and amino acids limitation alone. (cont'd)

Gene Symbol	RDM-AAC vs RDM-AA	Gene Ontology Biological Process	AAC1vsAA1	AAC2vsAA2	AAC3vsAA3
nrfD	4.341 up	nitrite reductase complex	12.402 up	1.107 down	1.587 up
nuoE	5.125 up	oxidation reduction	8.716 up	1.298 up	7.388 up
ompA	6.363 up	transport	1.828 up	1.687 up	4.513 up
ompF	5.288 up	Transport	1.939 up	2.814 up	2.314 up
pgi	5.374 up	gluconeogenesis, glycolysis	5.441 up	1.044 up	4.900 up
priB	7.254 up	DNA replication, synthesis of RNA primer	5.861 up	1.531 up	11.443 up
psiE	7.062 down	cellular response to phosphate starvation	78.183 down	1.314 down	6.247 down
pta	4.056 up	metabolic process	4.817 up	1.334 up	3.488 up
ptsG	4.960 up	phosphoenolpyruvate-dependent sugar phosphotransferase system, glucose transport	7.255 up	1.570 up	2.733 up
pykF	6.378 up	glycolysis	9.746 up	1.759 up	5.673 up
rbsA	8.592 up	transport, carbohydrate transport	37.441 up	1.016 down	3.161 up
rbsB	8.736 up	transport, chemotaxis, carbohydrate transport	16.949 up	1.082 down	7.854 up
rbsC	8.178 up	transport, carbohydrate transport	27.183 up	1.021 up	5.606 up
rbsD	5.545 up	carbohydrate metabolic process, carbohydrate transport	8.431 up	1.016 up	3.149 up
rnpB	4.134 up	RNAse P component	1.143 up	1.329 down	1.946 up
rplA	8.749 up	regulation of translation	6.532 up	1.700 up	7.102 up
rplI	4.913 up	Translation	5.139 up	1.290 up	3.817 up
rplK	6.514 up	Translation	4.251 up	1.358 up	6.981 up
rplO	4.380 up	Translation	1.816 up	1.535 up	3.639 up
rplR	4.770 up	Translation	1.764 up	2.466 up	2.972 up
rpmD	4.015 up	Translation	1.956 up	1.565 up	2.953 up
rpmE	5.669 up	Translation	1.900 up	1.307 up	7.422 up
rpoB	4.612 up	transcription, DNA-dependent	2.835 up	1.030 up	3.964 up
rpsA	5.972 up	translation	2.478 up	2.444 up	4.330 up
rpsB	5.421 up	Translation	2.745 up	1.693 up	5.614 up
rpsF	4.169 up	Translation	4.421 up	1.512 up	4.420 up
rpsR	7.347 up	Translation	7.074 up	1.602 up	7.501 up
rrsA, rrsB, rrsC, rrsD, rrsE, rrsG, rrsH, rrsH	5.599 up	rRNA operons	1.315 up	1.219 up	1.088 up
sbp	11.093 down	sulfate transport	83.505 down	2.718 down	34.044 down
soxS	6.817 up	regulation of transcription	7.305 up	1.030 down	8.479 up
ssrS	4.678 up	regulatory RNA, stationary-phase survival	1.555 up	1.378 down	1.107 up
tauA	7.203 down	transport	64.771 down	1.210 down	7.928 down
tauB	4.130 down	taurine transport, transport	38.329 down	1.033 up	5.329 down
tktA	4.642 up	metabolic process	5.168 up	1.187 up	3.172 up
trpE	6.765 down	tryptophan biosynthetic process	42.917 down	1.885 down	13.904 down
trpL	5.329 down	tryptophan biosynthetic process	6.913 down	4.156 down	17.296 down

Table 5.3. Relative change in transcript expression level between the conditions of concomitant depletion of amino acids and carbon and amino acids limitation alone. (cont'd)

Gene Symbol	RDM-AAC vs RDM-AA	Gene Ontology Biological Process	AAC1vsAA1	AAC2vsAA2	AAC3vsAA3
tsf	5.229 up	translational elongation	2.190 up	1.682 up	4.443 up
tuf	4.856 up	response to antibiotic, translational elongation	1.146 up	1.111 down	1.863 up
yagE	5.983 up	lysine biosynthetic process	18.321 up	1.035 up	3.349 up
yahN	4.659 up	amino acid transport	21.850 up	1.243 up	1.800 up
yaiY	4.578 up	inner membrane protein	15.833 up	1.220 down	2.502 up
ybaE	5.993 up	transport	30.218 up	1.105 up	2.442 up
ybeL	4.943 up	unknown function	2.874 up	2.261 up	2.685 up
ybhG	4.147 up	protein secretion	7.710 up	1.121 up	2.267 up
ycfF	4.345 down	purine nucleoside phosphoramidase	5.807 down	1.057 down	17.501 down
ychH	7.113 up	peroxide and cadmium stress response	4.297 up	4.043 up	4.056 up
yciW	7.230 down	predicted oxidoreductase	68.461 down	1.286 down	9.936 down
yeeE	5.188 down	putative transport protein	14.798 down	1.335 down	7.997 down
yeiQ	4.908 up	metabolic process, oxidation reduction	8.341 up	1.179 up	5.615 up
yfeW	4.804 up	unknown function	20.432 up	1.035 up	3.474 up
yfiQ	7.703 up	metabolic process	19.312 up	1.084 down	8.323 up
yghZ	4.644 up	potassium ion transport, oxidation reduction	12.977 up	1.456 down	2.837 up
ygiS	4.228 up	transport	8.854 up	1.017 down	2.003 up
ygjR	4.335 up	metabolic process, oxidation reduction	9.541 up	1.457 up	2.049 up
yhfZ	4.971 up	unknown function	20.136 up	1.039 down	1.608 up
yhjE	4.905 down	transmembrane transport	54.399 down	1.465 down	7.940 down
yihM	4.482 up	solvent stress response	11.984 up	1.361 down	2.068 up
yjfN	5.954 up	unknown function	10.988 up	1.140 up	3.086 up
yjiM	4.165 up	unknown function	7.775 up	1.296 up	1.973 up
yjiY	4.283 up	cellular response to starvation	2.957 up	1.216 up	3.428 up
yjiW	5.057 up	glucose metabolic process, oxygen and reactive oxygen species metabolic process, oxidation reduction	10.412 up	1.001 up	2.003 up
ykgM	4.260 up	translation	7.220 up	1.097 up	2.213 up
ynfF	4.793 up	oxidation reduction	8.332 up	1.194 up	4.990 up
ynfM	4.393 down	transmembrane transport	77.997 down	1.216 up	7.135 down
yniA	5.493 up	predicted kinase	4.313 up	1.580 up	4.324 up

We next performed a series of comparative analysis for selected datasets to identify specific genes whose expression was drastically altered under different tolerance inducing conditions. We found that out of the 117 differentially expressed genes selected by comparing the RDM-AA and RDM-AAC conditions, only 8 (*aldA*, *cstA*, *dctA*, *fadB*, *mglB*, *ptsG*, *yghZ* and *yjiY*) were on the list of the 124 genes selected by comparing RDM-C with RDM itself. This finding confirmed that the expression profile of organisms which experienced carbon starvation alone was significantly different from those subjected to starvation of both amino acids and carbon source. We then compared the RDM-AA / RDM-AAC dataset (117 genes) with the 125 genes in which four fold differences in the expression level was detected by comparing the RDM-AAC and RDM profiles (Table 5.4). Results of this comparative analysis showed that only 28 genes were simultaneously identified in both datasets. Importantly, 26 of these 28 genes were up-regulated in both cases, i.e. under the condition of RDM-AAC, these genes were expressed at a much higher level than that recorded in RDM-AA or RDM. The two down-regulated genes, *sbp* and *trpL*, were known to play a role in sulphate transport and tryptophan biosynthesis respectively. In addition, 7 of the 28 selected genes were found to be on the RDM-C list; hence these 7 genes (*cstA*, *dctA*, *fadB*, *fumA*, *mglB*, *yfiQ* and *yjiY*) were regarded as regulators of starvation responses but not tolerance development.

Table 5.4. Genes which were commonly found to be significantly up-regulated or down-regulated in comparison between amino acids and carbon starvation and amino acids starvation alone, or amino acids and carbon starvation and the unstarved control. A total of 28 common entities, each with 4-fold of above difference, were found in transcriptomic comparison between RDM-AAC and RDM-AA or RDM-AAC and RDM. Underlined entities were selected for further deletion studies.

Gene Symbol	AAC/AA	AAC/R	Gene Ontology Biological Process
atoS	6.969 up	5.323 up	two-component signal transduction system (phosphorelay), regulation of transcription
cstA	5.326 up	13.391 up	cellular response to starvation
dctA	6.353 up	5.928 up	transport, carbohydrate transport
fadA	5.069 up	4.747 up	fatty acid metabolic process
fadB	36.728 up	52.513 up	oxidation reduction, fatty acid metabolic process
fadE	15.147 up	19.395 up	oxidation reduction, fatty acid metabolic process
fadH	19.519 up	13.974 up	oxidation reduction, metabolic process
fadI	5.015 up	6.868 up	fatty acid metabolic process
fadJ	6.787 up	5.310 up	fatty acid metabolic process, metabolic process
fumA	4.285 up	7.189 up	generation of precursor metabolites and energy, tricarboxylic acid cycle
galS	8.976 up	8.227 up	regulation of transcription, DNA-dependent
melR	5.655 up	5.900 up	regulation of transcription
mglB	4.820 up	9.759 up	chemotaxis, carbohydrate transport
rbsD	5.545 up	6.264 up	carbohydrate metabolic process, carbohydrate transport
sbp	11.093 down	4.147 up	sulfate transport
soxS	6.817 up	13.945 up	regulation of transcription
trpL	5.329 down	8.241 up	tryptophan biosynthetic process
yagE	5.983 up	4.152 up	lysine biosynthetic process
yahN	4.659 up	4.999 up	amino acid transport
yaiY	4.578 up	10.644 up	inner membrane protein
ybaE	5.993 up	4.853 up	transport
ychH	7.113 up	11.658 up	peroxide and cadmium stress response
yeiQ	4.908 up	6.558 up	metabolic process, oxidation reduction
yfeW	4.804 up	4.286 up	unknown function
yfiQ	7.703 up	4.611 up	metabolic process
yjfN	5.954 up	9.966 up	unknown function
yjiY	4.283 up	5.357 up	cellular response to starvation
yniA	5.493 up	5.675 up	predicted kinase

We also performed comparative analysis of the datasets representative of short term and prolonged tolerance to determine if there were common determinants that regulated both formation and maintenance of tolerance. When the 195 genes of the RDM-AA / RDM list was checked against those (117 genes) selected by the RDM-AAC / RDM-AA comparison test, 44 common genes were identified (Table 5.5). However, only one of these 44 genes, *trpL*, overlapped with those (37 genes) which could be commonly found on the two gene sets selected under the RDM-AAC/RDM and RDM-AA/RDM background, suggesting that different genetic components were involved at different stages of physiological adaptation of progressive starvation. Under the background of RDM-AAC, the relative expression level in 29 of these 44 genes was much higher and comparable to that recorded in RDM. For the other 15 genes, the expression level was lower than that of RDM-AA; however, the expression level recorded in the RDM-AAC background was in each case higher than that of RDM. This finding further confirmed that gene expression was more active under RDM-AAC, with a profile markedly different from that of RDM-AA.

Table 5.5. Representative determinants selected under both long term and short term tolerance inducing conditions. Overlapping genes with 4-fold or more differences (44 in total) were identified on both RDM-AAC vs RDM and RDM-AA vs RDM comparison tests. Underlined entities were selected for further deletion studies.

Gene Symbol	AAC/AA	AA/R	Gene Ontology Biological Process
aspA	5.585 up	5.600 down	tricarboxylic acid cycle, aspartate metabolic process
atpG	6.190 up	6.797 down	ATP biosynthetic process, ion transport
cbl	6.729 down	18.907 up	regulation of transcription, DNA-dependent
cysA	6.543 down	10.947 up	sulfate transport
cysH	5.171 down	5.385 up	cysteine biosynthetic process, oxidation reduction
cysJ	4.564 down	13.037 up	sulfate assimilation, cysteine biosynthetic process
dhaM	6.593 up	4.254 down	transport, phosphorylation
frdB	7.654 up	6.243 down	electron transport chain, oxidation reduction, tricarboxylic acid cycle
gatY	5.356 up	4.948 down	glycolysis
gatZ	5.818 up	5.023 down	galactitol metabolic process
glnP	4.261 down	6.110 up	amino acid transport
groL	4.144 up	7.077 down	protein folding
iraD	4.363 down	4.561 up	cellular response to stress
malK	4.598 up	5.182 down	maltose transport
menC	4.315 up	4.945 down	menaquinone biosynthetic process
metB	5.158 down	16.083 up	methionine biosynthetic process
nmpC	9.490 up	19.046 down	silent gene, outer membrane porin
nuoE	5.125 up	5.931 down	oxidation reduction
ompF	5.288 up	6.548 down	transport
pgi	5.374 up	6.964 down	gluconeogenesis, glycolysis
priB	7.254 up	13.990 down	DNA replication, synthesis of RNA primer
psiE	7.062 down	7.571 up	cellular response to phosphate starvation
pta	4.056 up	7.288 down	metabolic process
ptsG	4.960 up	9.362 down	phosphoenolpyruvate-dependent sugar phosphotransferase system, glucose transport
pykF	6.378 up	10.902 down	glycolysis
rplA	8.749 up	7.597 down	regulation of translation
rplI	4.913 up	5.814 down	translation
rplK	6.514 up	6.831 down	translation
rplR	4.770 up	5.897 down	translation
rpmD	4.015 up	4.087 down	translation
rpsA	5.972 up	10.952 down	translation
rpsB	5.421 up	6.251 down	translation
rpsF	4.169 up	8.939 down	translation
rpsR	7.347 up	10.801 down	translation
sbp	11.093 down	46.015 up	sulfate transport

Table 5.5. Representative determinants selected under both long term and short term tolerance inducing conditions. (cont'd)

Gene Symbol	AAC/AA	AA/R	Gene Ontology Biological Process
tauA	**7.203 down**	14.049 up	transport
tauB	**4.130 down**	7.769 up	taurine transport, transport
tktA	4.642 up	**5.448 down**	metabolic process
trpE	**6.765 down**	11.331 up	tryptophan biosynthetic process
trpL	**5.329 down**	43.922 up	tryptophan biosynthetic process
tsf	5.229 up	**6.551 down**	translational elongation
yciW	**7.230 down**	11.089 up	predicted oxidoreductase
yeeE	**5.188 down**	**4.935 down**	putative transport protein
yjiM	4.165 up	**7.579 down**	unknown function

5.3.3. Putative tolerance genes

Apart from the screening of putative tolerance genes according to the differential expression levels recorded under several tolerance inducing conditions, we also examined the level of expression of selected stress response genes under the test conditions in order to investigate the relative contribution of active stress response in tolerance development. A total of 118 genes were chosen on the basis of their established roles in alleviating the bactericidal effects of antibiotics and other protection functions (Table 5.6). Among these 118 genes, 49 (42%) were up-regulated under RDM-AA, i.e. the majority of stress response genes were expressed at a level lower than that of RDM. Under the condition of RDM-AAC, however, the number of up-regulated genes increased to 67 (57%) despite the fact that this culture medium was further depleted of carbon source. Consistent with this finding as well as that observed among the genes selected on the basis of differential expression level, the number of up-regulated genes under the condition of RDM-C was as high as 81 or 69% of the total. These results also indicated that the active gene expression status observed under the condition of RDM-AAC was partly attributed to the enhanced expression of specific genes under RDM-C, as many as 24 genes whose expression was down-regulated under RDM-AA but up-regulated under RDM-C ended up with an up-regulated status upon simultaneous depletion of amino acids and glucose. To further identify stress genes from this group that might confer tolerance during starvation, we searched for those which were down regulated under the condition of RDM-C but up-regulated under RDM-AA or RDM-AAC. A total of six and eight genes were respectively found to fall into this category under the two said conditions, among them were two genes for which expression was down-regulated under RDM-C but up-regulated under the two tolerance inducing conditions: *grxA* and *bolA* (Table 5.6). All the other genes whose expression was active during starvation of carbon source alone were regarded as starvation response genes with little role on tolerance development. Examination of the gene expression patterns of various stress response genes showed that SOS response and DNA repair genes were actively expressed under all starvation conditions. For example, 13 out of 17 selected genes in this category were up-regulated under RDM-AAC. However, activities of molecular chaperones, efflux pumps, quorum-sensing signaling components and oxidative stress defense enzymes were not particularly enhanced under the test conditions. Interestingly, expression of known toxin-antitoxin modules

as well as a number of stress response regulators was apparently more active under the condition of RDM-AAC than that of RDM-AA.

It should be noted, however, that all selected genes in this group exhibited less than four-fold difference in gene expression level when compared to that recorded for RDM. In order to assess the credibility of these data, we assessed the reproducibility of results obtained from three separate experiments and found that the expression level of 8 genes were up-regulated in three independent experiments under the both tolerance inducing conditions, whereas 13 genes were consistently down-regulated (*trxB*, *osmB*, *marR*, *rpoD*, *dacA*, *dnaJ*, *fur*, *groS*, *gshA*, *katE*, *mutS*, *spoT* and *uvrC*) (Table 5.6). The consistently up-regulated genes include *fnr* and *sodB*, both oxidative stress defence genes, *mdh* and *glpD*, both involved in sugar metabolism, *lexA*, *recN* and *sulA*, all involved in alleviating the detrimental effects of DNA damages, and finally the *cstA* gene, which played a role in regulating carbon starvation responses. In addition, there were also 5 genes (*mscS*, *sodA*, *cpxR*, *mutL* and *mutS*) whose expression level was repeatedly up-regulated under RDM-AA yet consistently down-regulated under RDM-AAC. For one of these 5 genes, *mscS*, the expression level under RDM-C was also reduced in all experiments.

Table 5.6. Expression analysis of putative stress tolerance genes under nutrient-limiting conditions. A total of 118 genes selected on the basis of their reported functional roles on antibiotic or multiple stress protection were subjected to expression analysis under specific starvation conditions (RDM-C, RDM-AA or RDM-AAC vs RDM control). Relative expression changes in all independent experiments were included.

		Average fold difference vs RDM			Relative expression (vs RDM) across three replicates								
Process	**Gene**	**-C**	**-AA**	**-AAC**	**C1**	**C2**	**C3**	**AA1**	**AA2**	**AA3**	**AAC1**	**AAC2**	**AAC3**
Stringent response	*relA*	1.934 up	1.843 up	1.773 up	5.281 up	1.024 down	1.007 up	10.448 up	1.378 up	1.416 down	3.679 up	1.029 down	1.993 down
	spoT	1.288 up	2.296 down	1.203 up	1.058 down	1.032 down	1.205 down	5.673 down	1.095 down	1.754 down	1.857 down	1.159 down	1.403 down
	dksA	1.006 down	1.085 down	1.150 down	1.023 down	1.041 up	1.560 up	2.487 up	1.052 down	1.408 up	1.255 up	none	1.713 down
	gppA	2.153 up	1.270 down	1.456 up	4.826 up	1.045 up	1.075 up	1.135 down	1.216 up	2.402 down	1.392 up	1.009 down	1.331 down
Sigma factors and regulators	*rpoS*	1.323 up	2.383 up	1.193 down	3.433 up	1.153 down	1.261 down	10.130 up	1.319 up	2.378 up	1.936 up	1.057 down	5.209 down
	rssB	1.229 up	1.046 up	1.101 down	1.015 up	1.035 down	1.053 down	1.714 up	1.088 down	1.094 down	1.285 up	1.004 down	1.806 down
	iraP	1.462 down	1.415 down	3.186 down	3.792 down	1.020 up	2.934 up	1.103 down	1.451 down	3.240 up	6.173 down	1.099 down	3.634 down
	iraD	1.248 up	4.561 up	1.045 up	1.555 down	1.042 down	1.873 up	33.212 up	1.339 up	4.717 up	2.795 down	1.018 down	1.535 up
	rpoE	1.222 down	1.424 up	1.016 down	4.281 down	1.048 up	1.340 up	3.548 up	1.803 up	1.311 up	1.358 down	1.048 up	1.060 down
	rpoD	1.537 up	1.883 down	1.284 down	2.389 up	1.017 up	1.868 up	1.531 down	1.036 down	1.280 down	1.635 down	1.006 down	1.218 down
	rpoH	1.126 up	1.988 up	1.068 down	1.882 up	1.103 down	1.091 down	8.058 up	1.096 up	2.879 up	1.107 up	1.057 down	1.727 down
	rpoN	1.348 down	1.896 down	1.408 down	1.375 down	1.066 up	1.138 down	1.149 down	1.327 up	1.731 down	1.322 down	1.054 up	2.484 down
	rpoF	1.385 up	1.159 down	1.122 up	1.142 down	1.100 down	1.314 up	2.340 down	1.120 up	1.117 up	1.870 down	1.020 down	1.366 up
	fecI	1.760 up	1.266 down	1.129 down	1.046 up	1.044 up	1.683 up	3.284 down	1.125 up	1.740 up	9.974 down	1.025 down	1.778 up
Oxidative stress defense	*dps*	1.499 up	1.115 up	1.346 up	4.746 up	1.083 down	1.300 up	11.170 up	1.029 up	1.725 down	6.067 up	1.065 down	3.441 down
	sodA	2.097 up	1.127 down	1.706 down	5.162 up	1.630 up	2.049 up	2.327 up	1.514 up	1.286 up	1.405 down	1.084 down	1.659 down
	sodB	1.392 down	1.554 down	1.818 up	1.558 down	1.003 up	1 669 up	2.485 up	1.043 up	1.121 up	2.243 up	1.232 up	2.037 up
	sodC	2.226 up	1.266 up	2.124 up	3.271 up	1.081 up	1.583 up	1.859 up	1.048 down	1.042 down	4.305 up	1.024 down	1.758 down

Table 5.6. Expression analysis of putative stress tolerance genes under nutrient-limiting conditions. (cont'd)

		Average fold difference vs RDM			Relative expression (vs RDM) across three replicates								
	Gene	-C	-AA	-AAC	C1	C2	C3	AA1	AA2	AA3	AAC1	AAC2	AAC3
Oxidative stress defense (cont'd)	*katE*	3.442 up	1.366 down	1.049 down	8.065 up	1.047 down	2.969 up	1.132 down	1.166 down	1.288 down	1.054 down	1.024 down	1.259 down
	katG	1.232 up	1.781 down	1.542 up	4.470 up	1.139 down	1.762 down	4.564 up	1.173 down	4.976 down	7.253 up	1.111 down	4.278 down
	ahpC	1.059 down	1.944 down	1.672 down	2.244 up	1.267 down	1.050 down	2.566 up	1.148 down	2.070 down	1.102 up	1.364 down	3.042 down
	ahpF	1.405 up	1.511 down	1.354 up	2.162 up	1.084 up	1.067 down	1.044 up	1.446 up	3.986 down	2.398 up	1.083 up	2.548 down
	iscR	2.052 up	6.338 up	3.251 up	7.079 up	1.023 down	1.057 down	14.579 up	1.154 up	18.25 5 up	5.561 up	1.035 down	3.259 up
	iscS	1.556 up	2.077 up	1.225 up	2.274 up	1.142 up	1.789 up	4.717 up	1.177 up	4.645 up	1.215 up	1.152 down	1.422 up
	gor	1.181 up	1.775 down	1.172 up	2 053 up	1.092 up	1.001 up	1.192 up	1.182 up	1.695 down	2.457 up	1.030 down	1.665 down
	grxA	1.877 down	1.598 up	2.107 up	14.85 down	1.042 down	1.116 down	2.224 up	1.028 down	1.617 up	2.225 up	1.053 up	1 239 up
	grxB	1.398 up	1.148 down	1.036 up	2.083 up	1.249 up	1.891 up	2.807 up	1.168 up	1.171 down	2.156 up	1.152 up	1.720 down
	trxA	1.607 down	2.045 down	1.746 down	1.395 down	1.076 down	1.245 down	1.470 up	1.380 up	1.464 down	1.055 down	1.165 down	3.489 down
	trxB	1.281 up	1.830 down	3.685 down	1.939 up	1.097 up	1.181 down	1.036 down	1.071 down	2.731 down	8.270 down	1.022 down	7.993 down
	yggX	1.269 down	1.051 down	1.363 down	2.628 down	1.096 up	1.421 up	1.294 up	1.060 down	1.634 up	1.597 down	1.072 up	1.984 down
	sufA	2.308 up	1.613 up	1.046 down	4 720 up	1.038 up	1.056 up	1.683 up	1.048 up	1.028 down	2.228 down	1.066 down	1.631 down
	sufB	2.928 up	1.190 up	1.494 up	6.087 up	1.013 up	1.056 down	2.179 up	1.261 down	1.445 down	1.198 up	1.059 up	1.031 up
	oxyR	2.049 up	2.384 up	2.309 up	6.743 up	1.032 up	1.039 down	14.786 up	1.170 up	1.309 up	10.671 up	1.059 up	1.251 down
	oxyS	2.252 down	1.711 down	1.242 up	13.12 down	1.084 up	1.033 up	1.796 down	1.159 up	1.295 up	1.244 up	1.007 up	1.468 up
	fnr	1.411 up	1.933 up	1.678 up	2.040 up	1.120 up	1.734 down	3.422 up	1.135 up	2.241 up	1.841 up	1.228 up	1.146 up
	fur	1.557 down	1.661 down	2.210 down	3.156 down	1.008 up	1.252 up	1.527 down	1.118 down	1.056 down	3.107 down	1.187 down	1.645 down
SoS response and DNA repair	*lexA*	2.430 up	3.918 up	3.486 up	4.158 up	1.161 up	1.034 up	16.812 up	1.331 up	2.511 up	9.506 up	1.086 up	1.354 up
	recA	1.167 up	1.135 up	1.235 down	1.637 up	1.227 up	1.334 up	3.567 up	1.155 up	1 633 up	1.043 up	1.106 down	2.245 down
	recB	1.837 up	1.330 down	2.356 up	1.895 up	1.027 up	1.261 up	2.107 down	1.034 up	1.669 down	2.737 up	1.075 down	1.199 up
	recC	1.484 up	1.034 up	1.091 up	2.714 up	1.093 down	1.050 down	1.517 up	1.110 up	1.245 down	1.220 up	1.283 down	1.275 down

Table 5.6. Expression analysis of putative stress tolerance genes under nutrient-limiting conditions. (cont'd)

		Average fold difference vs RDM			Relative expression (vs RDM) across three replicates								
	Gene	-C	-AA	-AAC	C1	C2	C3	AA1	AA2	AA3	AAC1	AAC2	AAC3
SOS response and DNA repair (cont'd)	*recD*	1 739 up	1.265 down	1 706 up	1.032 down	1.031 down	1.534 up	1.813 down	1.345 down	1.140 down	1 297 up	1.047 down	none
	recN	2.522 up	2.425 up	2.263 up	2.915 up	1.168 up	1.292 up	6.471 up	1.406 up	1.037 up	4.324 up	1.016 up	1.379 up
	uvrA	2.450 up	1 130 up	1.425 up	6.558 up	1.035 up	1.321 up	1.302 down	1.002 down	1.483 down	1.070 up	1.010 down	1.299 down
	uvrB	1.677 up	1.566 up	1.396 up	1.906 up	1.072 up	1.028 down	3.241 up	1.001 down	1.301 up	2.525 up	1.016 up	2.121 down
	uvrC	1.743 down	2.756 down	1.735 down	3.847 down	1.052 down	1.307 down	3.963 down	1.351 down	2.025 down	2.879 down	1.104 down	2.122 down
	mutS	2.029 up	1.968 down	1.093 up	2.550 up	1.046 up	1.155 down	3.288 down	1.026 down	1.748 down	1.274 down	1.115 down	1.870 down
	mutL	1.685 up	3.279 up	1.277 down	9 377 up	1.071 up	1.140 up	11.275 up	3.547 up	1.524 up	1.036 down	1.464 down	1.044 down
	mutH	1.041 up	1.452 up	1.435 up	3.367 down	1.098 up	1.111 up	1.216 up	1.153 up	1.465 up	2.494 down	1.111 up	1.669 up
	umuD	1.207 down	2.570 up	1.001 down	1.372 down	1.094 down	1.072 up	18.033 up	1.048 up	1.527 up	1.509 up	1.109 down	1.090 up
	umuC	1 016 up	1.403 up	1.335 up	2.091 down	1.057 down	1.072 up	2.166 up	1.210 down	1.167 up	1.826 down	1.045 down	1.252 up
	dinB	1.069 up	1.877 up	2 250 up	3.501 down	1.024 down	1.115 up	1.275 up	1.029 up	1.501 up	1.561 up	1.004 down	2.053 up
	dinA	1.110 up	1.419 up	1.496 up	1.378 down	1.009 down	1.528 up	1.462 up	1.158 up	1.465 up	1.141 up	1.087 down	2.289 up
	sulA	1.596 up	4.974 up	2.550 up	1.743 up	1.146 up	1.419 up	16.243 up	2.015 up	5.582 up	5.259 up	1.135 up	1.266 up
Molecular chaperones	*dnaK*	1.362 up	5.896 down	1.499 down	3.589 up	1.292 up	1.236 up	2.764 down	1.253 down	12.50 down	1.727 up	1.057 down	6.918 down
	dnaJ	1.508 up	3.888 down	1.474 down	2.831 up	1.117 up	1.044 up	3.986 down	1.204 down	4.447 down	1.137 down	1.052 down	3.160 down
	grpE	1.709 up	2.230 down	2.960 down	2.956 up	1.393 up	1.712 up	1.244 up	1.161 down	3.236 down	2.943 down	1.113 down	5.457 down
	groS	1.032 down	12.43 down	7.563 down	1.930 up	1.026 up	1.259 down	13.288 down	1.330 down	29.69 down	10.675 down	1.543 down	22.177 down
	groL	1.613 up	7.077 down	1.707 down	5.090 up	1.199 up	2.114 up	2.945 down	1.074 up	23.86 down	1.583 up	1.038 down	6.412 down
	degP	2.002 up	1.978 up	1.414 up	2.392 up	1.258 up	1.047 up	7.719 up	1.093 up	1.166 up	2.302 up	1.019 down	1.938 down
	hslO	2.389 up	1.423 up	1.314 up	7.954 up	1.021 up	1.024 up	2 376 up	1.022 up	1.028 down	1.488 up	1.036 down	1.950 down
	htpG	3.055 up	3.343 down	1.123 down	6.000 up	1.223 up	2.827 up	3.710 down	1.255 down	5.042 down	1.239 up	1.001 down	2.704 down
	ibpA	1.045 up	2.064 down	1.163 up	1.264 up	1.077 up	1.282 down	2.625 down	1.217 up	1.145 down	1.417 down	1.031 up	1.503 up

Table 5.6. Expression analysis of putative stress tolerance genes under nutrient-limiting conditions. (cont'd)

		Average fold difference vs RDM			Relative expression (vs RDM) across three replicates								
	Gene	-C	-AA	-AAC	C1	C2	C3	AA1	AA2	AA3	AAC1	AAC2	AAC3
Osmotic stress	*proP*	1.944 up	1.528 up	1.697 down	4 405 up	1.064 down	1.082 down	6.067 up	1.246 up	1.411 down	2.736 down	1.130 down	4.608 down
	osmB	1.551 up	1.466 down	1.034 up	1.734 down	1.188 down	5.003 up	2.038 down	1.937 down	1.259 down	1.119 down	1.097 down	1.712 down
	mscL	1.508 down	1.384 down	1.336 down	2.418 down	1.071 up	1.060 down	1.320 up	1.046 up	1.151 up	1.054 down	1.010 down	1.744 down
	mscS	3.267 down	1.168 up	2.868 down	5.899 down	1.188 down	3.692 down	2.834 up	1.025 up	2.585 up	4.402 down	1.169 down	5.008 down
Drug efflux	*acrA*	1.177 up	1.583 down	1.734 down	1.741 up	1.043 up	1.562 down	1.234 up	1.404 up	3.465 down	1.821 down	1.026 up	4.948 down
	acrB	1.092 down	2.806 down	1.101 down	1.009 down	1.073 down	1.152 down	3.086 down	1.213 down	2.259 down	1.049 up	1.049 down	1.587 down
	tolC	1.129 down	2.138 down	1.503 down	1.254 up	1.056 up	1.299 up	1.147 down	1.203 down	1.836 down	1.018 down	1.003 up	2.288 down
	marR	1.578 down	1.465 down	1.418 down	3.930 down	1.077 down	1.494 down	2.227 down	1.546 down	2.280 down	5.948 down	1.123 down	1.950 down
	marA	1.408 down	1.363 down	1.564 down	4.781 down	1.007 down	1.669 down	2.388 down	1.009 up	2.315 down	7.252 down	1.010 up	1.812 down
	marB	1.224 up	1.048 up	1.026 down	1.380 up	1.294 up	1.203 down	1.605 up	1.649 up	1.138 down	1.027 down	1.229 up	1.052 up
Carbon starvation	*cstA*	10.34 up	2.514 up	13.39 up	64.61 8 up	1.386 up	6.813 up	20.823 up	1.026 up	none	99.461 up	1.252 up	9.238 up
Quorum sensing	*luxS*	1.006 down	1.862 down	1.565 down	1.413 up	1.027 up	1.072 up	1.390 up	1.028 down	1.649 down	1.045 up	1.026 down	3.948 down
	sdiA	1.331 down	1.330 up	1.136 down	3.160 down	1.091 down	1.464 down	1.033 down	1.219 down	2.089 up	2.841 down	1.002 down	1.014 down
	lsrR	2.104 up	1.371 up	3.031 up	2.520 up	1.068 up	1.760 up	2.278 up	1.029 up	1.191 down	10.393 up	1.121 up	1.251 up
Toxin and Antitoxins	*mazE*	1.066 down	1.391 down	1.131 up	1.307 down	1.058 up	1.051 down	1.111 down	1.094 up	1.560 down	1.584 up	1.014 down	1.560 down
	mazF	1.438 up	1.026 down	1.083 up	1.099 up	1.014 up	1.207 down	1.048 up	1.045 up	1.529 down	1.048 up	1.013 down	2.080 down
	relB	1.041 up	1.256 up	1.335 up	1.193 down	1.078 down	1.392 down	4.323 up	1.391 down	1.001 up	3.291 up	1.044 down	1.713 down
	relE	1.429 down	1.073 down	1.030 up	1.593 down	1.020 up	1.535 down	2.402 up	1.247 down	1.437 up	1.625 up	1.028 up	1.614 down
	hipB	2.169 down	1.627 down	1.316 up	6.681 down	1.058 down	2.560 down	1.709 down	1.448 down	1.694 down	1.778 up	1.015 down	1.407 down
	hipA	1.019 up	1.083 down	1.268 up	2.184 down	1.111 down	1.714 down	1.791 down	1.283 down	1.480 down	1.015 up	1.153 down	1.863 down

Table 5.6. Expression analysis of putative stress tolerance genes under nutrient-limiting conditions. (cont'd)

		Average fold difference vs RDM			Relative expression (vs RDM) across three replicates								
	Gene	-C	-AA	-AAC	C1	C2	C3	AA1	AA2	AA3	AAC1	AAC2	AAC3
Toxin and Antitoxins (cont'd)	*mqsR*	1.232 up	2.905 up	2.145 up	1.239 down	1.014 up	1.256 down	4.964 up	1.215 up	2.145 up	2.786 up	1.087 down	1.292 up
Two component systems	*cpxR*	1.679 down	1.320 down	1.254 down	2.090 down	1.014 up	1.243 down	1.237 up	1.180 up	1.490 up	1.395 down	1.045 down	1.211 down
	cpxA	1.143 up	1.434 down	1.055 down	1.522 up	1.091 up	1.157 down	1.625 up	1.119 up	1.408 down	1.409 up	1.004 up	1.343 down
	arcA	1.451 down	1.154 down	1.960 down	1.449 up	1.164 down	1.880 down	4.397 up	1.072 up	1.390 down	1.137 up	1.281 down	6.035 down
	arcB	1.238 up	1.236 down	1.711 up	2.135 up	1.032 up	1.021 up	1.415 up	1.236 up	2.736 down	3.644 up	1.030 down	1.447 down
	creB	1.062 down	1.284 down	1.105 down	2.301 down	1.017 up	1.039 up	1.674 down	1.060 down	1.097 down	3.154 down	1.118 down	1.138 up
	rcsB	1.452 down	1.838 down	1.776 down	1.403 down	1.133 down	1.250 down	1.314 up	1.317 down	1.292 down	1.714 down	1.228 down	2.403 down
	rcsC	1.048 down	1.238 down	1.001 up	2.752 down	1.054 down	1.101 down	2.752 down	1.035 up	1.099 up	3.760 down	1.026 down	1.010 up
Metabolism	*apaH*	1.834 up	1.498 down	1.202 up	1.860 up	1.043 up	1.150 up	1.822 down	1.083 down	1.142 down	1.842 down	1.006 up	1.408 down
	surA	1.214 up	2.410 down	1.044 up	2.804 up	1.014 down	1.019 down	1.153 down	1.031 up	3.114 down	1.501 up	1.011 down	1.629 down
	ubiG	1.010 up	1.120 down	1.865 down	1.723 up	1.059 down	2.476 down	2.089 up	1.078 down	1.454 down	2.035 down	1.092 down	4.471 down
	speA	2.647 up	1.571 down	1.373 down	7.323 up	1.134 up	1.688 up	1.120 up	1.112 up	3.687 down	1.068 down	1.008 up	3.992 down
	mrcB	1.060 down	2.617 down	1.158 up	1.492 down	1.049 up	1.328 up	4.309 down	1.011 down	1.043 up	1.264 down	1.021 up	1.939 up
	dacA	1.073 down	2.514 down	2.766 down	1.529 up	1.009 down	1.604 down	2.267 down	1.067 down	2.875 down	4.051 down	1.123 down	4.135 down
	mgsA	1.086 up	1.690 down	1.351 up	1.308 down	1.218 up	1.874 up	1.037 up	1.545 down	1.252 down	1.666 up	1.260 up	1.003 up
	gshA	1.140 down	3.601 down	2.594 down	1.005 down	1.114 down	1.190 down	3.639 down	1.252 down	2.773 down	4.416 down	1.172 down	4.392 down
	ubiF	2.120 up	1.966 up	1.923 up	4.571 up	1.026 down	1.117 down	5.835 up	1.046 up	1.117 up	5.066 up	1.158 down	1.790 down
	plsB	2.221 up	1.641 down	1.179 up	2.883 up	1.046 up	1.873 up	1.426 down	1.278 up	1.960 down	1.150 up	1.020 up	1.406 down
	glpD	2.011 up	1.631 up	4.939 up	1.414 up	1.077 up	1.727 up	1.680 up	1.188 up	2.511 up	4.961 up	1.045 up	7.770 up
	phoU	2.392 up	1.362 up	1.692 up	1.261 up	1.039 up	6.430 up	1.529 up	1.172 up	1.986 up	1.236 up	1.112 up	1.379 up
	acnB	5.334 up	1.795 up	1.122 up	18.70 4 up	1.283 up	3.540 up	4.376 up	1.280 down	2.052 up	1.743 up	1.149 down	1.698 down

Table 5.6. Expression analysis of putative stress tolerance genes under nutrient-limiting conditions. (cont'd)

		Average fold difference vs RDM			Relative expression (vs RDM) across three replicates								
	Gene	-C	-AA	-AAC	C1	C2	C3	AA1	AA2	AA3	AAC1	AAC2	AAC3
Metabolism (cont'd)	*icdA*	2.032 up	1.288 down	1.234 up	3.427 up	1.631 up	2.923 up	3.124 up	1.034 down	1.069 down	1.792 up	1.018 down	1.152 down
	sucB	6.361 up	1.372 down	1.492 up	13.64 6 up	1.793 up	10.45 6 up	1.447 up	1.226 down	1.597 down	2.424 up	1.187 down	1.087 down
	mdh	4.969 up	1.099 up	2.196 up	9.432 up	2.192 up	9.334 up	4.810 up	1.221 up	1.109 up	3.762 up	1.084 up	1.368 up
General stress regulators	*hns*	1.543 down	1.266 down	1.195 down	1.455 up	1.201 down	1.366 down	2.937 up	1.159 down	1.221 up	1.366 up	1.218 down	1.499 down
	fis	3.863 down	3.530 down	3.664 down	15.76 down	1.167 down	2.405 down	7.161 down	1.060 down	2.676 down	27.241 down	1.250 down	2.185 down
	bolA	1.005 down	1.428 up	1.676 up	1.093 down	1.085 up	1.274 up	4.972 up	1.002 down	1.310 up	6.166 up	1.019 down	2.052 down
	ygfA	1.113 up	1.059 down	1.204 up	1.300 up	1.033 down	1.043 up	1.249 down	1.002 up	1.455 up	2.229 down	1.057 down	1.550 up
	yigB	1.857 up	2.134 down	1.037 up	7.871 up	1.039 up	1.023 down	1.644 down	1.012 up	2.288 down	1.224 up	1.129 down	1.984 down
	yhbO	2.762 up	1.088 up	1.538 up	7.061 up	1.003 up	3.225 up	2.695 up	1.265 up	1.391 up	2.811 up	1.018 down	2.400 up
	uspA	1.675 up	1.257 up	1.911 up	5.186 up	1.159 up	1.488 up	7.364 up	2.531 up	1.015 up	5.146 up	1.088 up	1.119 down
	uspB	2.468 up	2.600 up	4.053 up	8.358 up	1.017 down	1.407 up	35.789 up	1.200 up	1.063 down	37.480 up	1.094 down	1.174 up
	uspC	2.388 down	1.550 down	1.231 down	5.297 down	1.017 up	1.664 down	1.085 down	1.230 down	1.405 down	1.095 up	1.029 up	1.774 down
	uspD	1.102 up	1.707 up	1.295 up	1.410 up	1.078 up	1.704 down	7.391 up	1.238 up	1.024 down	4.013 up	1.064 up	2.639 down
	uspE	1.197 up	1.802 down	1.195 up	1.957 up	1.025 up	1.168 up	2.494 up	1.563 down	3.427 down	3.301 up	1.031 up	2.134 down
	uspG	2.140 up	1.257 up	3.052 up	2.589 up	1.065 up	1.608 up	3.719 up	1.694 down	1.026 down	4.480 up	1.082 up	1.606 up
Acid stress	*yqgB*	2.359 up	3.072 down	3.526 down	13.43 up	1.034 down	1.110 up	2.166 down	1.103 up	10.98 down	4.266 down	1.184 down	11.548 down

5.3.4. Gene deletion studies

Stress response genes as well as those which exhibited a significantly different expression level under tolerance inducing conditions were shortlisted for evaluation of their relative contribution towards tolerance development through analysis of the effect of gene knockout. In a preliminary attempt to identify the genetic determinants responsible for induction of the specific tolerance phenotypes that we observed under starvation conditions, we selected a panel of putative tolerance genes which had previously been implicated in tolerance formation, and tested whether deletion of these genetic components would undermine the ability of bacteria to develop antibiotic tolerance under conditions known to induce formation of differential phenotypes. In particular we included a Δ*relA*Δ*spoT* double knockout mutant, which was defective in producing the stringent response, to determine if the tolerance induction potential of amino acids depletion and the regulatory effects of nucleotides on tolerance formation were mediated through this major starvation response pathway. As shown in Figure 5.2 and 5.3, all the six gene knockout mutants tested displayed tolerance phenotypes which differed from each other as well as from that of the wild-type strain in terms of induction criteria, drug specificity and the size of the resultant tolerant population that emerged under specific test conditions.

The Δ*relA*Δ*spoT* double knockout mutant, which was defective in producing the stringent response, was found to exhibit pleiotropic effects on tolerance formation. Firstly, this mutant displayed a reduced capability to produce sustainable ofloxacin tolerance under two conditions (MOPS and RDM minus amino acids and nucleobases) which were known to induce prolonged tolerance to this drug in its wild-type counterpart (CF1943, Figure 5.2). Secondly, the phenomenon of nucleobase-dependent amino acid induction of ampicillin tolerance was not observable in this mutant background, as the proportion of the test population that became tolerant to ampicillin was similar regardless of availability of nucleobases in the growth medium. Thirdly, the level of prolonged ampicillin tolerance was reduced in a medium in which both amino acids and glucose were simultaneously depleted. However, such effect was not observable in MOPS base. It should also be noted that, in the absence of antibiotics, the survival rate of this mutant was significantly lower than that of the wild-type strains when nucleobases were supplemented in an amino acids–depleted medium (Figure 5.2). However, this mutant exhibited prominent

growth when nucleobases were absent in the amino acids depletion medium. On the other hand, the Δ*relA*Δ*spoT* mutant was not capable of surviving for 48 h in a medium depleted of amino acids and ammonium salts, hence we were not able to examine if the suppressive effect of nucleobases on the ampicillin tolerance phenotypes inducible by a lack of amino acids and ammonium salts was observable in the absence of stringent response. We confirmed that the test populations of this strain did not harbor RNA polymerase suppressor mutants under all treatment conditions as no viable colonies were recoverable in minimal glucose agar; hence all phenotypic characteristics of this strain could be attributed to the lack of ppGpp production.

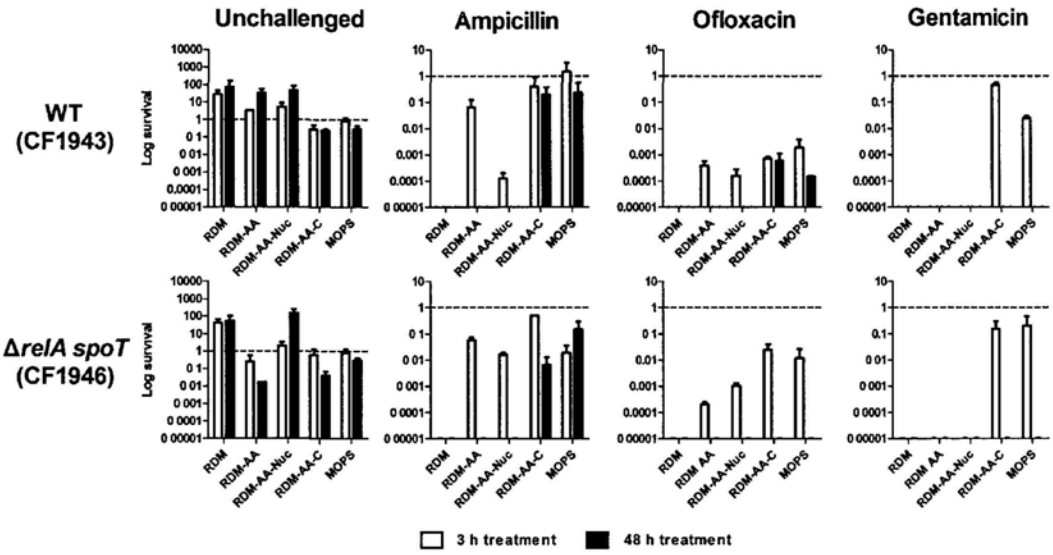


Figure 5.2. Assessment of the relative role of stringent response in nutrient-depletion mediated drug tolerance. Exponentially growing CF1943 (WT, ppGpp+) and CF1946 (*ΔrelAΔspoT* , ppGpp^0) populations were pre-incubated in RDM (RDM), RDM without amino acids (RDM-AA), RDM without amino acids and nucleobases (RDM-AA-Nuc), RDM without amino acids and carbon source (RDM-AA-C), or MOPS base (MOPS), followed by antibiotic challenge and evaluation of survival rate.

Another key finding in this preliminary gene knockout test is that the Δ*recA* mutant failed to develop ofloxacin tolerance under all conditions including stationary-phase, yet production of ampicillin and gentamicin tolerance was generally not affected, except under specific conditions such as overnight culture or a medium in which amino acids alone was depleted, where development of sustainable gentamicin tolerance and transient ampicillin tolerance was affected respectively (Figure 5.3). Since the RecA protein plays a role in regulating the SOS response by promoting the self-cleavage of the LexA protein, which represses the SOS response genes, we postulated that a mutation which rendered the LexA protein non-cleavable would exhibit the same tolerance phenotype as that of the Δ*recA* mutant. This idea was tested by assessing the phenotypes of *lexA3* mutant, which expressed a non-cleavable LexA protein, under known tolerance inducing conditions. The ability of this strain to develop ofloxacin tolerance was drastically suppressed in the same manner as that observed in the Δ*recA* mutant, except that a small portion of the stationary-phase organisms could survive the entire course of drug treatment (Figure 5.4). Also resembling the phenotypes of the Δ*recA* mutant is that development of tolerance to ampicillin and gentamicin was not affected in the *lexA3* mutant, confirming the role of SOS response genes in fluoroquinolone-specific tolerance induction. In view of the importance of the SOS response in development of fluoroquinolone tolerance, we further tested the effect of the *lexA51* mutation, which resulted in expression of a defective LexA protein that failed to suppress expression of the SOS gene cluster. In the absence of the LexA suppressor function, SOS response genes are expected to be constitutively expressed, potentially eliciting tolerance in the absence of starvation signals. It was observed that prolonged tolerance to all the three test drugs was detectable under non-inducing condition (RDM as induction medium) (Figure 5.4). Despite the small size of the sustainable tolerant population, this finding indicated that the killing rate was drastically reduced in the *lexA51* mutant at the late stage of the treatment course. However, development of fluoroquinolone tolerance in this mutant was affected under starvation and stationary-phase conditions, during which the level of ofloxacin tolerance was lower than that of a strain in which LexA-mediated regulatory functions were normal.

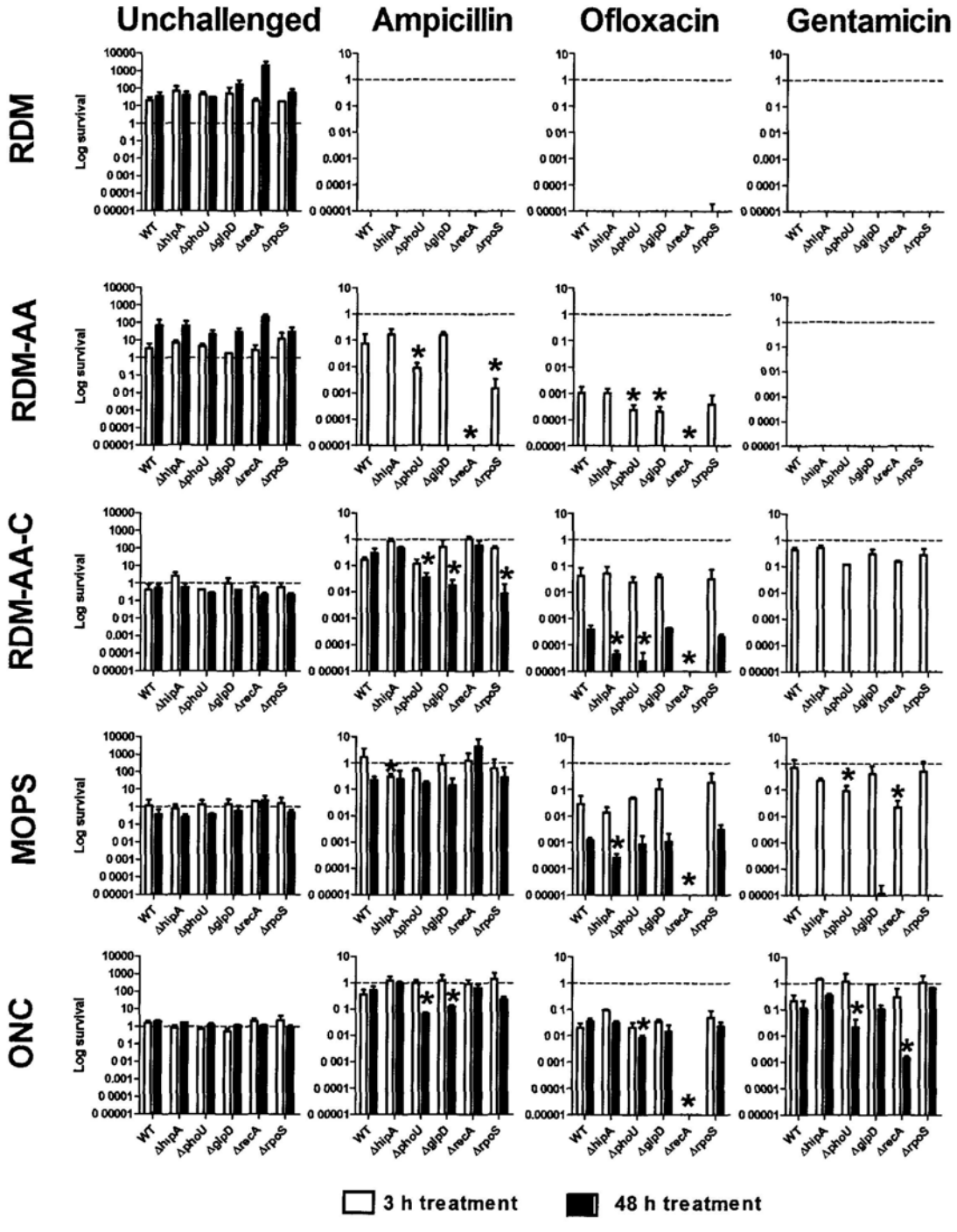

Unchallenged
Ampicillin
Ofloxacin
Gentamicin
RDM
RDM-AA
RDM-AA-C
MOPS
ONC
Log survival
WT
ΔhipA
ΔphoU
ΔglpD
ΔrecA
ΔrpoS
3 h treatment
48 h treatment

Figure 5.3. Effects of stress response regulators and putative antibiotic tolerance genes on nutrient starvation-induced drug tolerance. BW25113 (WT) and its isogenic gene deletion mutants (Δ*hipA*, Δ*phoU*, Δ*glpD*, Δ*recA* and Δ*rpoS*) were either grown to log-phase and pre-incubated for 2 h in different tolerance-inducing medium backgrounds including RDM (RDM), RDM minus amino acids (RDM-AA), RDM minus amino acids and carbon source (RDM-AA-C), MOPS base (MOPS), or grown overnight to the stationary-phase (ONC) prior to antibiotic treatment and assessment of population survival. An asterisk (*) denotes mutants which exhibited a 4-fold or more reduction in the tolerant population size as compared to the wild-type.

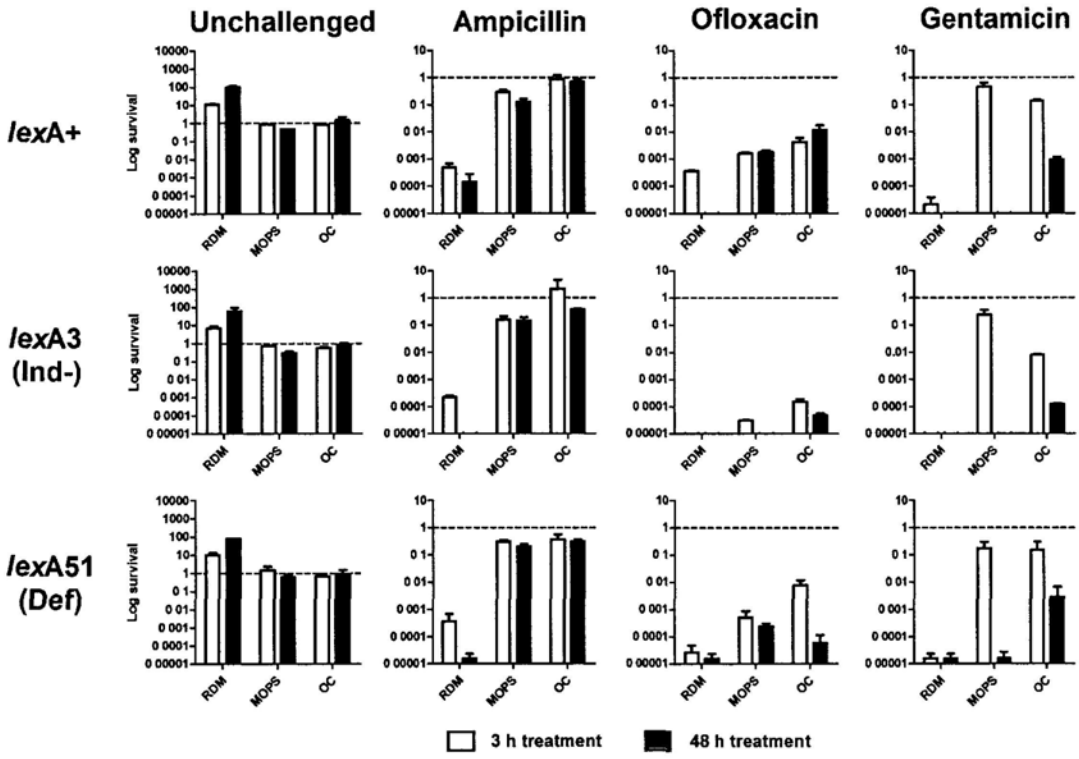
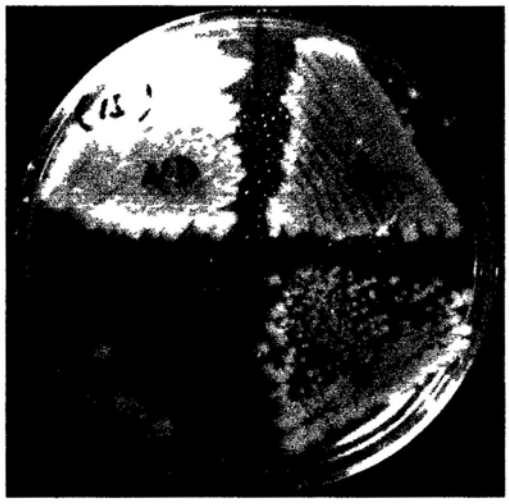


Figure 5.4. Assessment of relative contribution of SOS response in starvation-mediated antibiotic tolerance. RDM-grown *Escherichia coli* K-12 *lexA* variants (*lexA*+: wild-type with functional SOS response, *lexA3*: isogenic strain with non-inducible SOS response, *lexA51*: isogenic strain with constitutive SOS gene expression) were subjected to tolerance induction by 2-h exposure to MOPS (MOPS) or growth to stationary-phase in RDM (OC), followed by assessment of survival against the bactericidal effects of antibiotics. An RDM-incubated control (RDM) was included to account for starvation-independent, strain-specific variation in tolerance phenotypes.

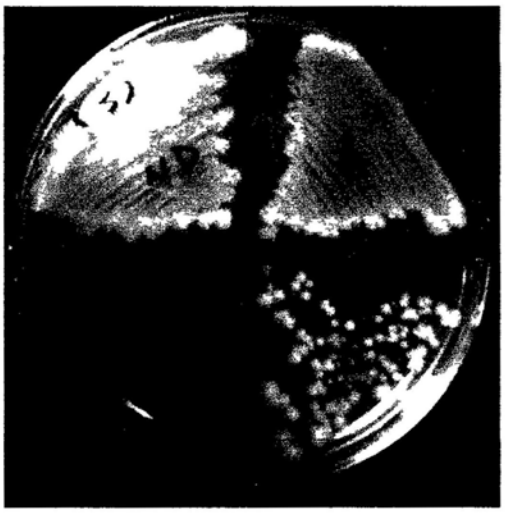
Apart from observed phenotypic changes in the Δ*recA* and Δ*relA*Δ*spoT* mutants, deletion of the *rpoS* gene was also found to consistently result in a slight defect in formation of ampicillin tolerance (Figure 5.3). However, development of ofloxacin and gentamicin tolerance was not affected in the Δ*rpoS* mutant. On the other hand, deletion of several other genes which were suggested to be key regulators of antibiotic tolerance, including *phoU*, *glpD* and *hipA*, was found to produce detectable effects on antibiotic tolerance induction in a condition and drug-specific manner (Figure 5.3). Comparative analysis of these findings showed that the lack of a cellular function could result in defect in formation of condition-specific tolerance without affecting the phenotypes of overnight culture. For example, tolerance to ofloxacin was not sustainable in the Δ*hipA* mutant under the conditions of RDM-AAC or MOPS, however, phenotypic tolerance in stationary-phase population of this strain was not altered.

In order to probe the regulatory and functional basis of starvation-mediated antibiotic tolerance and assess whether stress protection mechanisms can help withstand the bactericidal activities of antibiotics, the effects of gene knockout of selected stress response determinants as well as those which exhibited differential expression patterns under the condition of RDM-AAC were studied by use of a quick screening approach (see Methods and Figure 5.5). This assay condition was chosen because it has previously been shown to induce sustainable tolerance to both β-lactams and fluoroquinolones, as well as short term tolerance to aminoglycosides (Figure 2.1). As shown in Table 5.7, which tabulates the test genes according to their known biological functions and the gene knockout effects, including those which had previously been subjected to preliminary assessment (Figure 5.3), a wide spectrum of stress protection factors were found to be indispensable for tolerance formation. Among the 100 test genes, a total of 36 were found to have a significant impact (approximately 10-fold or more reduction) on phenotypic tolerance upon deletion. Consistent with the microarray findings, which showed that protein synthesis was more active when both amino acids and carbon source were simultaneously depleted from the growth medium, gene knockout was found to affect long-term tolerance predominantly. Development of tolerance to ampicillin and ofloxacin could be inhibited either independently or simultaneously upon knockout of specific determinant. Among the genes for which deletion had an effect, tolerance to

ampicillin and ofloxacin was suppressed in 16 and 28 cases respectively. For genes which exhibited drug-specific effect, abolishment of DNA repair and toxin-antitoxin functions (involving the *mfd*, *recA*, *hipA* and *relE* genes respectively) was found to affect ofloxacin tolerance specifically. For 10 genes (*dksA*, *relA, spoT*, *dnaJ*, *proP*, *acrA*, *rcsB*, *rcsC*, *phoU*, *fis*), deletion resulted in significant reduction in tolerance to both drugs. Gentamicin tolerance was rarely affected upon deletion of the above list of genes, except in the case of *yqgB*, for which knockout had led to abolishment of even short term tolerance to ofloxacin and gentamicin. Interestingly, ampicillin tolerance was not affected upon deletion of this gene. Several categories of cellular functions were found to play a negligible role in tolerance formation, as none of the deletion mutants in these groups exhibited an altered phenotype. These include the carbon starvation and known quorum sensing genes. An overall feature of these gene knockout results is that genetic constituents of similar or complementing cellular functions often display markedly different roles in tolerance formation. For example, AcrA, a component of the TolC-AcrAB efflux pump complex, was shown to play at least an indirect role in development of tolerance to both ampicillin and ofloxacin, however, deletion of *acrB* gene, which encoded another functional component of this complex, did not affect the test phenotypes. On the other hand, there are several clusters of functionally related genes for which defect in tolerance formation could be observed upon deletion of each of the components in the group. These include the *dksA*, *gppA*, *relA* and *spoT* which are involved in stringent response and transcriptional regulation, *dnaJ* and *dnaK* which encode known molecular chaperones, the two component systems *arcAB* and *rcsBC*, as well as the stress regulators *uspA* and *uspE*. In addition, deletion effects on tolerance formation were observed for both osmotic stress genes (*osmB* and *proP*).



Wild-type



Δ*mdf*
(>=10-fold reduction)

Δ*yqgB*
(>= 100-fold reduction)

Figure 5.5. A semi-quantitative plating assay used to screen for putative tolerance determinants. Knockout mutants were subjected to amino acids and carbon starvation, followed by antibiotic treatment (ND: untreated control, A: 100 μg/ml ampicillin, O: 0.75 μg/ml ofloxacin and G: 6.25 μg/ml gentamicin) and plating of 10 μl of the cell suspension on LB agar. The examples as shown above are mutants which exhibited different degrees of reduction in ofloxacin tolerance relative to the wild-type. Mutants which exhibited reduced tolerance by this rapid assay were re-confirmed by the regular plate-count method (Fung *et al.*, 2010).

Table 5.7. Effect of selected genetic determinants on starvation-induced drug tolerance. A total of 100 genes shortlisted according to results of microarray analysis (underlined) or their known biological functions were selected for analysis of the effect of gene deletion on drug tolerance development under concomitant amino acids and carbon starvation. Genetic determinants which exhibited a 10-fold or more reduction in survival (i.e. reduced tolerance) in the test antibiotics (labeled with ↓) relative to the wild-type were marked in green.

		Tolerance Under RDM-AA-C					
		Amp		Ofx		Gen	
Process	**Gene**	**3 h**	**48 h**	**3 h**	**48 h**	**3 h**	**48 h**
Stringent response (4)	*dksA*		↓		↓		na
	gppA		↓				na
	relAspoT		↓		↓		na
Sigma factors and regulators (7)	*fecI*		↓				na
	iraD						na
	rpoD				↓		na
	rpoF						na
	rpoN						na
	rpoS		↓				na
	rssB						na
Oxidative stress defense (13)	*ahpC*						na
	ahpF				↓		na
	dps				↓		na
	grxA						na
	iscR						na
	katE						na
	katG						na
	oxyR						na
	rof						na
	sodA		↓				na
	sodB						na
	sodC						na
	trxA				↓		na

Table 5.7. Effect of selected genetic determinants on starvation-induced drug tolerance. (cont'd)

		Tolerance Under RDM-AA-C					
		Amp		Ofx		Gen	
Process	**Gene**	**3 h**	**48 h**	**3 h**	**48 h**	**3 h**	**48 h**
SoS response and DNA repair (4)	*lexA*						na
	mfd				↓		na
	recA				↓		na
	sulA						na
Molecular chaperones (8)	*cpxP*						na
	degP						na
	dnaJ		↓		↓		na
	dnaK			↓	↓		na
	groL						na
	hslO						na
	htpG						na
	ibpA						na
Osmotic stress (2)	*osmB*		↓				na
	proP		↓		↓		na
Drug efflux (5)	*acrA*		↓		↓		na
	acrB						na
	marA						na
	marR						na
	tolC						na
Carbon starvation (3)	*cstA*						na
	psiE						na
	yjiY						na
Quorum sensing (3)	*lsrR*						na
	luxS						na
	sdiA						na
Toxin-Antitoxins (4)	*hipA*				↓		na
	mazF						na
	mqsR						na
	relE				↓		na

Table 5.7. Effect of selected genetic determinants on starvation-induced drug tolerance. (cont'd)

		Tolerance Under RDM-AA-C					
		Amp		Ofx		Gen	
Process	**Gene**	**3 h**	**48 h**	**3 h**	**48 h**	**3 h**	**48 h**
Two component systems (6)	*arcA*				↓		na
	arcB				↓		na
	cpxA						na
	cpxR						na
	rcsB		↓		↓		na
	rcsC		↓		↓		na
Metabolism (15)	*aceB*						na
	apaH				↓		na
	atpG						na
	fdoG						na
	fdoI						na
	glpD		↓				na
	gshA						na
	livK						na
	mgsA						na
	phoU		↓		↓		na
	pstS						na
	sucB				↓		na
	surA				↓		na
	ubiF						na
	yjiW				↓		na
General stress regulators (11)	*bolA*						na
	fis		↓		↓		na
	hns						na
	uspA				↓		na
	uspB						na
	uspC						na
	uspD						na
	uspE				↓		na
	uspG						na
	ygfA		↓				na
	yigB						na
Acid stress (1)	*yqgB*			↓	↓	↓	na

Table 5.7. Effect of selected genetic determinants on starvation-induced drug tolerance. (cont'd)

		Tolerance Under RDM-AA-C					
		Amp		Ofx		Gen	
Process	**Gene**	**3 h**	**48 h**	**3 h**	**48 h**	**3 h**	**48 h**
Nucleobase analogue protection (1)	*yiiM*						na
Unknown function (13)	*cyaY*						na
	xni						na
	yaaX						na
	ybhQ						na
	yciW						na
	yeeE						na
	yfeW						na
	yhfG						na
	yhfZ				↓		na
	yigI						na
	yihM						na
	yjfN						na
	yjiM						na

5.3.5. Role of antibiotic uptake in starvation-mediated tolerance

Since genetic elements which encode multidrug efflux system were identified in our phenotypic screens (Table 5.7), we further tested whether drug tolerance was partially due to reduced antibiotic uptake in tolerant cells. Direct measurement of antibiotic entry in RDM or MOPS incubated cells using tritium-labeled penicillin-G, norfloxacin or gentamicin demonstrated that the intracellular concentrations of all three classes of bactericidal antibiotics were similar in both drug-sensitive RDM-based and drug-tolerant MOPS-based populations; occasionally the accumulated drug level was even higher in MOPS-based cells, this phenomenon is especially apparent in penicillin-G and gentamicin assays (Figure 5.6, middle). Since such finding suggested that reduced drug permeation had minimal contribution to the observed starvation-induced tolerance phenotypes, we hypothesized that antibiotic molecules which had already entered starving organisms could irreversibly trigger cellular lethality if the organisms were not triggered into development of a tolerance mode.

To test this hypothesis, a rescue experiment was performed to assess the degree of viability of MOPS-resuspended organisms if they were first subjected to drug exposure, followed by resuspension in drug-free RDM. Detectable cell death in the second phase of this experiment, when extracellular antibiotic molecules were removed, indicated that antibiotics which had entered the intracellular compartment of bacteria could automatically induce cell death (Figure 5.6, bottom), and that antibiotic tolerance might be attributed to protection against cellular damages caused by intracellular drug molecules. Due to various technical limitations such as potential drug leakage during the washing and re-suspension steps, the actual rate of killing might have been underestimated in this assay (Figure 5.6, bottom). Nevertheless, continuous killing was still observable in both MOPS and RDM treated organisms upon re-suspension in drug-free RDM (Figure 5.6, bottom, dotted lines). In addition, the rate of killing elicited by intracellular drug molecules was highly similar (approximately 1-log reduction) in both conditions, indicating that the initial drug accumulation level was similar regardless of the environmental nutrition status. However, we also observed that the bactericidal effects diminished gradually in the drug-free environment, suggesting that continuous drug entry is required to potentiate the killing process if complete eradication of the entire bacterial population is desired.

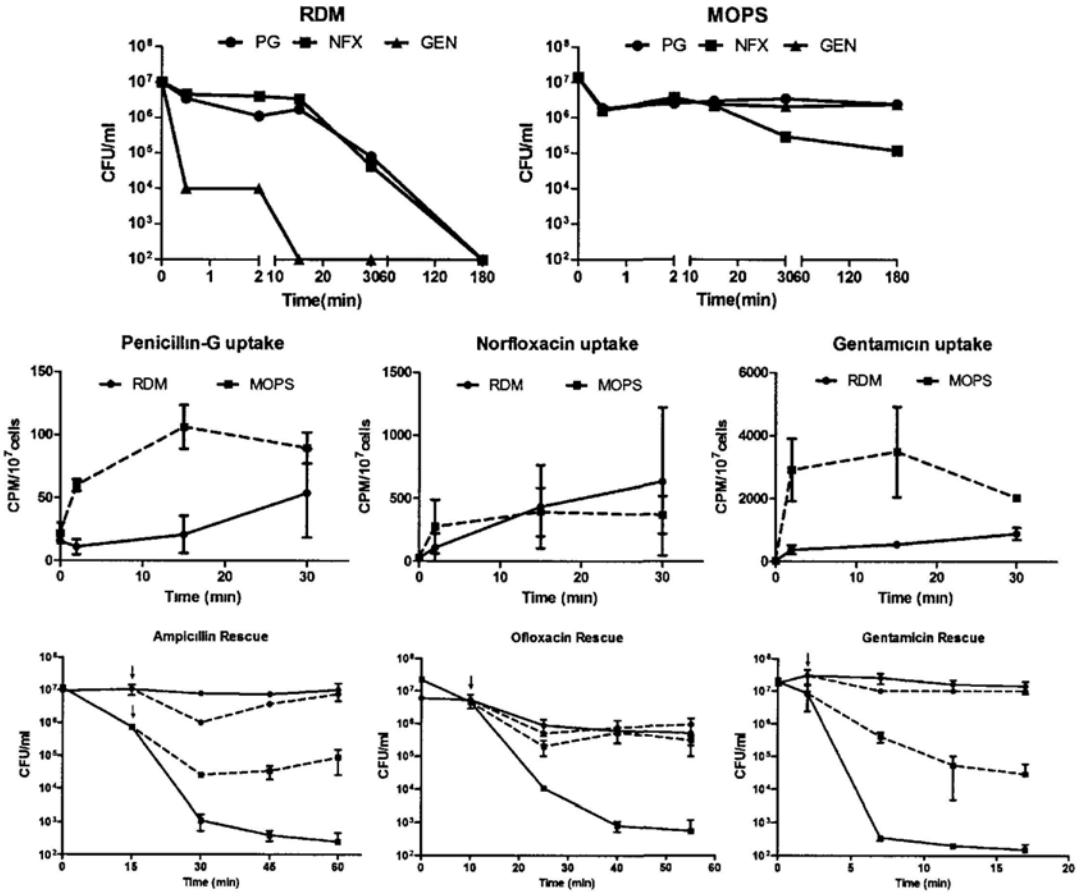


Figure 5.6. Antibiotic permeation in starvation-induced antibiotic tolerant cells. RDM-grown log-phase cells were subjected to starvation induction in MOPS base along with the RDM-incubated controls for 2 h and subsequently monitored for their viability against antibiotic treatment (PG, penicillin-G; NFX, norfloxacin; GEN, gentamicin) at 25X MIC concentrations (top panel) as well as their drug entry status using the corresponding [3H]-labeled derivatives (middle panel). The post-drug uptake effects on survival of antibiotic sensitive or tolerant organisms were assayed by means of a 'rescue'or two-step killing experiment (bottom panel) in which RDM (squares) or MOPS (circles) resuspended log-phase cells were subjected to brief antibiotic treatment followed by re-constitution in drug-free RDM (dotted line) or continuous incubation in the presence of antibiotics (solid line). An arrow denotes the time of switching.

5.3.6. Effect of hydroxyl radical scavengers on antibiotic-mediated killing

In view of the putative role of oxidative stress defense genes in tolerance formation as suggested by the deletion mutant studies as well as recent findings that neutralization of deleterious hydroxyl radicals might serve as a major drug tolerance mechanism (Kohanski *et al.*, 2007), we proceeded to verify the role of hydroxyl radical damage in antibiotic-induced killing. In this experiment we tested whether supplementation of hydroxyl radical scavenger thiourea could result in development of tolerance against antibiotic in a non-inducing background (RDM). Interestingly, the effect of 150mM thiourea had no effect on preventing antibiotic-killing at 25X MIC (data not shown), which is the standard antibiotic concentration utilized in our tolerance assays. Since the protective effect of scavenger, if any, was only expected to partially counteract the deleterious effects of antibiotics, we further tested the effect of thiourea using a relatively low dose (5X MIC) of antibiotics (150mM thiourea with 20 μg/ml ampicillin, 0.125 μg/ml ofloxacin or 1.25 μg/ml gentamicin) which was comparable to that of the previous study (150 mM thiourea with 5 μg/ml ampicillin, 0.25 μg/ml norfloxacin or 5 μg/ml kanamycin) (Kohanski *et al.*, 2007). Again, protection by hydroxyl radical scavenger was only barely detectable for the first 3 h of treatment (Figure 5.7). Our results suggested that hydroxyl free radical damage may not be the major killing mechanism triggered by antibiotics under our assay conditions. It should be noted that the previous study by Kohanski *et al.* (Kohanski *et al.*, 2007) utilized a much higher aeration rate (e.g. 25 ml culture in 250 ml flasks at 300 rpm agitation) which produced a higher respiration rate and hence potential for generation of free radicals. The impact of growth conditions on bactericidal efficiency, which is expected to correlate inversely with the level of antibiotic tolerance, is not further investigated.

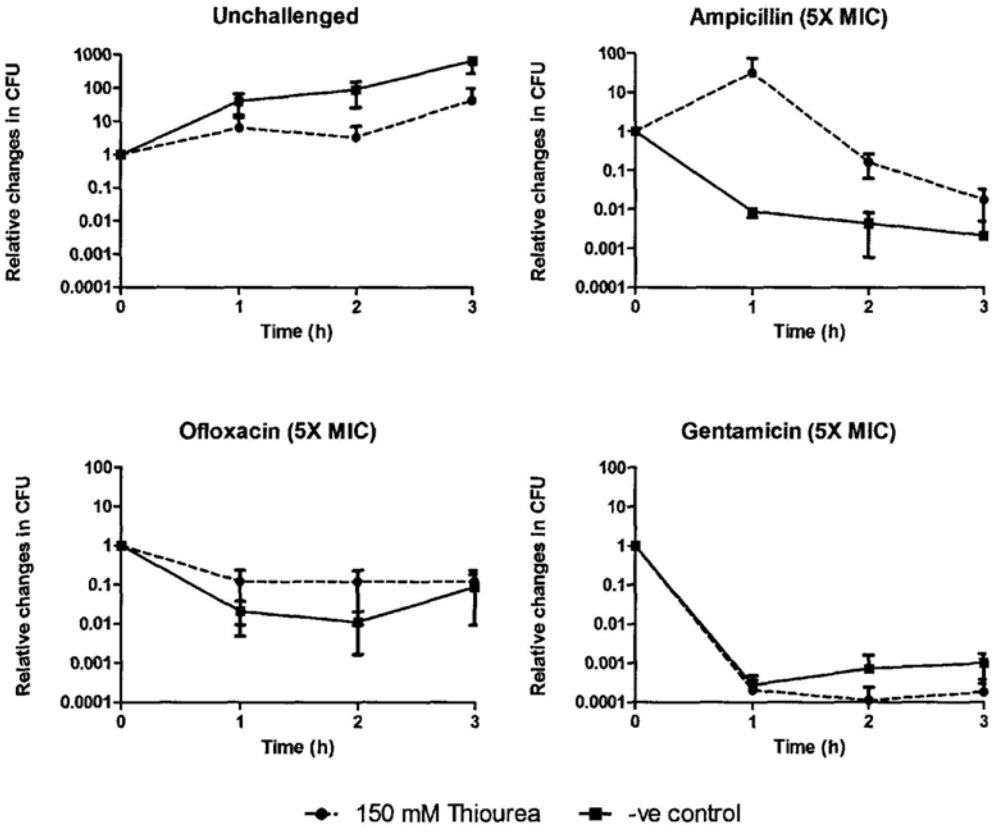


Figure 5.7 Protection of hydroxyl radical scavenger against antibiotic lethality. RDM-grown log-phase cells were challenged with 5X MICs of ampicillin (20 μg/ml), ofloxacin (0.125 μg/ml) or gentamicin (1.25 μg/ml) with (dotted line) or without (solid line) the presence of 150 mM of the free radical scavenger thiourea.

5.3.7. Role of protein synthesis on tolerance development

We verified the concept that active cellular mechanisms might be involved in tolerance formation during nutrient starvation by assessing the effects of tetracycline, a protein synthesis inhibitor, on the potential of MOPS base to induce and maintain tolerance upon corruption of the bacterial protein translation machinery. To avoid potential development of tolerance to tetracycline itself, which would otherwise prevent us from accurately assessing its effects on tolerance to other drugs, we added tetracycline to RDM-grown log-phase culture and continued incubation at 37°C for 2 h prior to switching the culture medium to a MOPS base which also contained the agent, followed by treatment with the three test drugs and assessment of population survival. The presence of tetracycline was found to confer a significant yet incomplete inhibitory effect on MOPS-induced long term tolerance to ampicillin as well as short term tolerance to gentamicin (Figure 5.8). However, formation of ofloxacin tolerance was not affected by prior inhibition of protein synthesis. We also noticed that this inhibitory effect was not observed if tetracycline was added only after switching to MOPS instead of 2 h prior to switching, indicating that bacteria might also develop tolerance to tetracycline during starvation. In view of this finding, we speculated that the inhibitory effect of this protein synthesis inhibitor might have been suppressed when the cells became tolerant; hence the detrimental effect of corrupting protein synthesis on MOPS-induced tolerance development might have been underestimated.

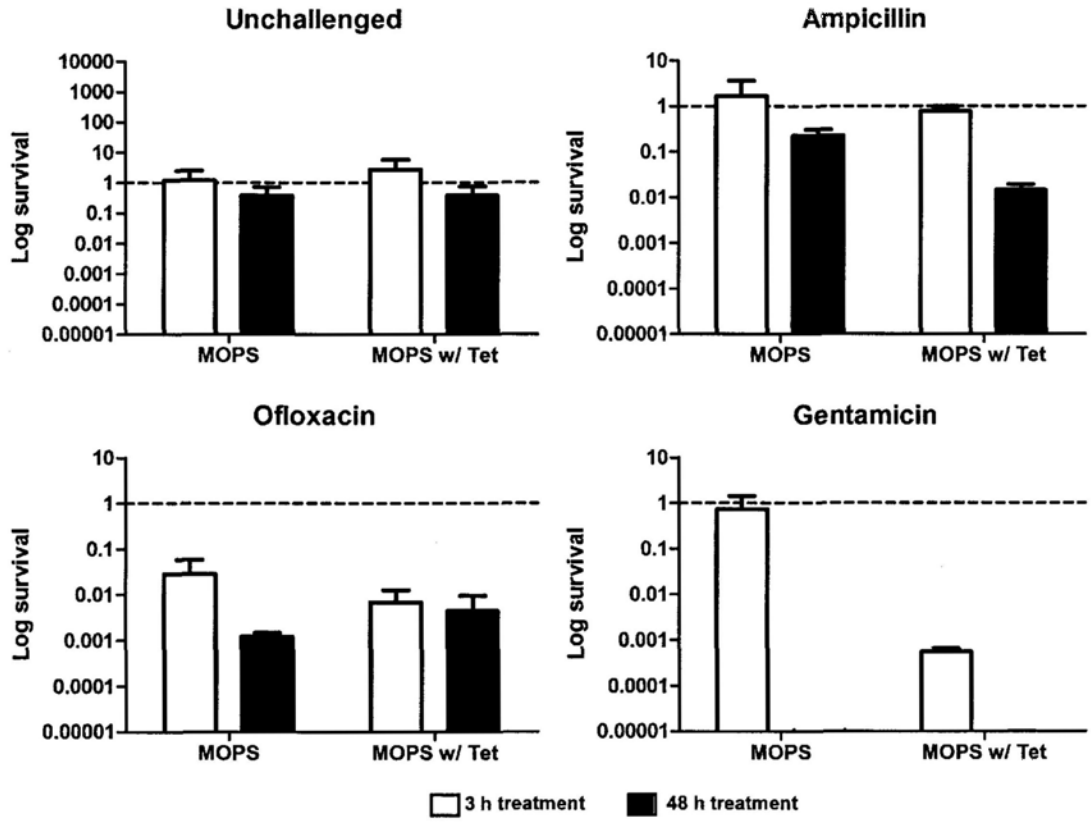


Figure 5.8 Inhibition of MOPS-induced antibiotic tolerance by protein synthesis inhibitors. Tetracycline (4 μg/ml) was added to a RDM-grown log-phase culture, incubated at 37°C for 2 h and then reconstituted in MOPS basal medium containing the same concentration of tetracycline (MOPS w/Tet) before being subjected to drug treatment and assessment of tolerance. A parallel control without tetracycline (MOPS) was included for comparison.

5.4. Summary

Molecular mechanisms conferring drug tolerance in starvation-induced cells were investigated by various functional and molecular approaches. Comparative transcriptomic analysis of tolerant and non-tolerant organisms which had been subjected to induction by selected nutrition recipes revealed several characteristic features of gene expression profiles in tolerant bacteria. Such data suggest that the key cellular changes underlying the development of transient antibiotic tolerance (e.g. 3-h tolerance inducible by amino acids depletion alone) involve extensive decline in cellular metabolism and biosynthetic activities. In fact more than 50% of all down-regulated genes were found to be controlled by multiple central metabolic pathways as well as the up-regulated stringent response regulators *relA*, *spoT* and *dksA*. For development of sustainable tolerance, which was exemplified by the phenotypic characteristics of bacteria subjected to starvation of both amino acids and carbon, the underlying physiological change is suggestive of a metabolically active state which involves activation of multiple stress protection systems, including heat shock, oxidative stress defense, DNA repair and acid stress protection.

Detailed deletion analysis on the role of established tolerance determinants, namely *hipA*, *phoU*, *glpD*, *rpoS*, *recA*, *relA* and *spoT* under multiple tolerance-inducing conditions revealed that the effect of these tolerance genes are condition and/or drug specific, suggesting the existence of complex regulatory network governing the development of starvation-induced drug tolerance. Follow-up deletion studies on shortlisted putative tolerance determinants identified 36 tolerance genes, representing a diverse range of stress protection functions. These include novel determinants such as *yqgB* and *yhfZ*. In addition, more than 90% (34 out of 36) of the selected genes affected only sustainable tolerance rather than short-term tolerance, thus further confirming that transient and prolonged tolerance are regulated by functionally different mechanisms. The fact that only one gene (*yqgB*) was found to play a role in gentamicin tolerance is somewhat consistent with results of tolerance inhibition assay using translation inhibitor, which indicated that development of gentamicin tolerance was dependent on active translation of some unknown regulators. Further drug permeation assay using radiolabeled antibiotics, as

well as tolerance induction assay using free radical scavenger suggested that impedance of antibiotic entry and neutralization of deleterious hydroxyl radicals were in each case insufficient to account for the starvation-activated drug tolerance phenotypes.

Chapter 6. Discussion

6.1. Overview of starvation-activated tolerance regulatory network

This study established a functional link between bacterial starvation responses and development of antibiotic tolerance. Our data suggested the existence of complex cellular mechanisms that regulated bacterial physiology according to the nutritional status in the environment, and effectively exerted sensitive control over the strength and specificity of antibiotic tolerance induction. The major regulatory strategies are summarized as follows. Firstly, deprivation of amino acids, alone or in combination with limitation of other compounds, can trigger both primary and synergistic tolerance responses, producing an optimal ratio of actively growing and dormant populations which best enhances population survival under the prevailing environmental condition; secondly, nucleobases confer either positive or negative regulatory effects on the development of the basal phenotypes depending on the triggering factors, thus fine-tuning the core responses; thirdly, several nutrient classes including carbon and nitrogen sources, either individually or in combination with each other, can selectively suppress tolerance triggered by depletion of other nutrients, thereby imposing sophisticated negative control on tolerance development. Amino acids are nevertheless the key regulator, as their presence inhibits formation of most phenotypes. The differential, interdependent, counter-interactive and summative features of tolerance induction indicate that bacteria rely heavily on nutrient sensing pathways to activate matching metabolic adaptation and stress protection mechanisms, which in turn produce differential antibiotic tolerance phenotypes.

6.2. Effect of adaptive metabolism

Present phenotypic data reflect to some extent the result of interplay between the multiple physiological effects exerted by changing levels and composition of endogenous intermediate metabolites during the dynamic process of bacterial adaptation to transient starvation of different nutrients. Such interactive effects on growth rate, drug target metabolism, and production of signaling molecules that regulate stress responses render it impossible to determine the exact role of starvation-induced adaptive mechanisms in tolerance formation. Nevertheless,

analysis of the differential phenotypes concerning the availability of nucleobases suggests that they may play a role in enhancing the mRNA synthetic ability and thereby active production of stress protection proteins, which have been compromised as a result of repression and slow induction of purine and pyrimidine nucleotide biosynthesis under various test conditions (Neuhard and Nygaard, 1987). In fact the intrinsic ability of the test organisms to de-repress biosynthetic pathways that replenish the depleted ingredients often fails to compensate for the induction effects elicited by the lack of specific nutrients, especially under amino acids depletion conditions. It is envisaged that the regulatory effects of adaptive metabolism in tolerance development diminish upon prolonged starvation, giving way to active stress defence which then plays a key role in conferring sustainable tolerance.

6.3. Progressive tolerance development in response to starvation signals

Apart from the spontaneous starvation response, serial analysis of the phenotypic and resuscitation characteristics of starvation-inducible tolerant populations confirmed that cellular responses produced under nutrient limiting conditions were responsible for stepwise transformation of a non-tolerant bacterial population into diverse population structures including organisms which exhibited sustainable multidrug tolerance, and a distinct subpopulation of persisters which only slowly recuperated to the non-tolerant mode even when nutrients became available. These findings provide novel evidence which suggests that cellular mechanisms supporting persistence of antibiotic tolerance in nutrient-rich environment are induced progressively during a prolonged starvation period. However, the basis of discrepancy between the level of persisters detectable under starvation and stationary-phase conditions indicates that environmental factors other than nutrition availability also play a role in persister formation. The nature of such factors entails further investigation. Revelation of the origin of multidrug tolerance and the basis of its maintenance beyond the starvation phase also provided an ideal setting for identification of the key determinants of nutrition sensing and stress protection mechanisms involved in governing antibiotic susceptibility in bacteria. Preliminary results of our gene knockout experiments confirm the presence of independent yet overlapping genetic pathways regulating the drug specificity, sustainability and reversibility of antibiotic tolerance. Since emergence of tolerance must precede that

of persisters, we expect that specific cellular mechanisms inducible during prolonged starvation must play a role in the concomitant development of sustainable tolerance and subpopulation persisters. However, the specific knockout effect of certain determinants, such as *ubiF* which affected formation of persisters but not the preceding sustainable tolerance phenotypes, suggests that tolerance induction mechanisms are more redundant, and that some of these mechanisms do not necessarily drive persister formation. All in all, our data help confine the key regulators of bacterial antibiotic tolerance and persistence to those involved in energy production and components of the stringent and SOS responses.

6.4. Induction of antibiotic tolerance by increasing population densities

In addition to the starvation-mediated protection effects, this study provides evidence on the existence of high cell density-mediated cellular mechanisms which may be activated to confer intrinsic multidrug tolerance once a growing bacterial population reached a threshold cell density of 10^8 cells per ml. Results of phenotypic and supplementation experiments indicated that this phenomenon was only partially explainable by changing physical and physiological parameters that resulted in impeded solute permeation of nutrients or elicitation of starvation responses under these conditions. Apart from such physical constraints, it was demonstrated that the high cell density phenotypes were closely linked to the ability of exponentially growing organisms to produce and export tolerance signals which could markedly reduce their own antibiotic susceptibility even under nutrition-rich conditions. Evidence of cell density-dependent modulation of cellular activities has been well documented (Bassler and Losick, 2006; Blango and Mulvey, 2009; Camilli and Bassler, 2006; Jayaraman and Wood, 2008), which notably involves growth inhibition (Aoki *et al.*, 2009; Carbonell *et al.*, 2002) that may lead to the reduction of bactericidal activities of antibiotics. Our finding that quorum sensing-like signals can induce tolerance formation in *Escherichia coli* under high cell density conditions is contradictory to those of several previous studies, which suggested that tolerance development was not quorum sensing-dependent (Lewis, 2007; Moker *et al.*, 2010). We speculate that the failure to identify such signals in those previous works was due to the fact that signaling molecules are produced and detectable only in a high density population with an active growth status, disappearing or re-consumed in an autoinducer (AI-2)-like sensing and processing mechanism (Xavier and Bassler,

2005b; Yang *et al.*, 2006) when the population enters the stationary-phase or a protracted starvation phase. This idea is reinforced by the observations that spent medium recovered from late log-phase and artificially adjusted high density populations exhibited tolerance induction potential when tested against non-tolerant cells, yet those derived from stationary-phase or MOPS-reconstituted high density cultures only produced nutrient-sensitive tolerance responses which were presumably mediated by starvation stress (Figure 4.5). Taken together, we speculate that generation and accumulation of tolerance-inducible molecules to an effective level requires two prerequisite conditions: high cell density and sufficient supply of specific nutrients, which may be utilized for synthesis of such signals. Since this type of stress response appears to confer tolerance at a time when the population encounters relatively little environmental stress such as nutrition starvation, it may be regarded as a preparatory mode of physiological changes that confer phenotypic resistance to the imminent onset of detrimental factors, which are expected to be readily triggered under a high cell density condition. This view is supported by the observation that non-tolerant cells were responsive to the high cell density signals and hence expected to possess receptors to the high cell density signals. On the basis of drug tolerance profiles and sustainability of the phenotypes observed, we believe that this preparatory response is functionally different from those elicited spontaneously by starvation stress, and contributes significantly to the phenotypic characteristics of late log-phase populations. Beyond this point, nutrients become limiting and bacteria are expected to activate starvation responses that play a more decisive role in tolerance maintenance.

At this stage, more work is warranted before we could achieve the identification of the true nature of the putative tolerance-inducing quorum sensing signals produced by a high cell density *Escherichia coli* population. A recent study reported that quorum sensing signals were produced at later stages of growth with the effect of mediating antibiotic-tolerant persister formation in *Pseudomonas aeruginosa* (Moker *et al.*, 2010), for which spent medium of stationary-phase population was also found to contain such signals. This finding therefore contradicts our observation that in *Escherichia coli*, tolerance-inducing quorum sensing signals were produced only during exponential growth when nutrients were abundant. In fact our data are more relevant to an earlier report which demonstrated that *Escherichia coli* exhibited a cell

density-sensitive mode of growth rate regulation independent of nutrient abundance (Yang *et al.*, 2006), thereby mediating entry into stationary-phase despite availability of excessive nutrients. Consistent with what we found, the cell density-dependent reduction in growth rate as observed in this previous study was not affected by a defect in producing AI-2, the only known quorum sensing molecule in *Escherichia coli*. Whether the cellular mechanism underlying growth rate reduction in late log-phase is responsible for tolerance formation remains to be proven; we have also demonstrated, however, that specific bacteriostatic agents could trigger transient tolerance (Figure 2.2). Our results also suggested that spent medium of high cell density culture also inhibited cellular growth, albeit only in the presence of excess nutrients (Figure 4.5). At present, we cannot explain why bacterial growth was supported by such spent medium but inhibited when excess nutrients were present.

Integrating all available information to date, we tentatively conclude that bacteria produce and secrete an inhibitory molecule, presumably a utilizable substrate recoverable during stationary-phase, over a narrow range of cell density. It should be stressed that inhibitory molecules are not detectable even in spent medium of bacterial culture with 10^8 cells per ml density, when the medium was tested against log-phase cells, yet a characteristic tolerance profile was observable in a culture of this cell concentration. Neither phenotypic tolerance itself nor tolerance induction potential was detectable in populations of 10^7 cells per ml and the corresponding spent medium, respectively, suggesting that 10^8 cells per ml was a threshold tolerance-inducible density. This threshold was also observable in biofilm-derived planktonic cells of *Candida albicans*, including strains defective in resistance development (Perumal *et al.*, 2007). Tolerance induction signals in microbial culture are not readily detectable as they are not effective in mediating tolerance development at low concentration, whereas production of such signals apparently slows down or is grounded to a halt when nutrients become limiting, presumably giving way to starvation-mediated tolerance response. The narrow window of high cell density-mediated, starvation-independent tolerance may be considered as a hallmark of bacterial self-regulated insurance system in which inhibitory signals are produced only during exponential growth and become optimally effective when the population reaches a threshold size, thereby striking a balance between growth and early defence. Future work should therefore focus on identifying the nature of high

density-dependent signals and the corresponding mechanisms that they regulate using conditions illustrated in this study. Currently, complex adaptive or transitional metabolic activities are known to occur when bacteria enter stationary-phase, often releasing small molecules such as nucleobases into the surrounding medium (Kell and Young, 2000; Rinas *et al.*, 1995). Acetate is another intermediate metabolite which is excreted in large quantities during the late log-phase of bacterial growth in batch culture, potentially causing growth inhibition (Arnold *et al.*, 2001); yet our finding confirmed that it could not mediate tolerance formation. Physiological changes mediated by high cell density signals may partially resemble those regulated by autoinducers, especially cellular functions associated with those of drug targets as well as those concerning protection against drug-induced damages (Kayama *et al.*, 2009). In view of the resemblance of production and metabolism characteristics between the putative tolerance induction signals revealed by this work and known autoinducers, which are produced during exponential growth and internalized during the stationary-phase, future work should also focus on determining if the cell density-dependent tolerance induction phenomenon as described in this study involves acyl-homoserine lactone-based molecules. One approach is to test whether the high density signals of *Escherichia coli* can mediate expression of bioluminescence in *Vibrio harveyi* (Xavier and Bassler, 2005a). Perhaps more comprehensive examination of the effects of such signals on assayable physiological functions of bacteria can help reveal their true identities.

6.5. The starvation induced transcriptome

Previous studies on drug tolerance mechanisms usually involved manipulation of a single, undefined working condition in which multiple essential nutrients were concomitantly limiting (Hansen *et al.*, 2008; Li and Zhang, 2007). In contrast, the fact that progressive and differential drug tolerance phenotypes were observable in this work suggests that the use of defined nutrient starvation recipes (Chapter 2) can provide a unique platform for elucidation of tolerance mechanisms and their corresponding determinants. Therefore, transcriptomic profiling analysis of cells under weak (e.g. 3-h survival inducible by amino acids deprivation alone) tolerance-inducing conditions could effectively reveal a metabolically inactive state featuring down-regulation of stable RNA expression, energy generation and translation machinery, as well as up-regulation of different amino acids uptake and

biosynthesis genes, which is consistent with features of a physiological state typical of cells under stringent control (Magnusson *et al.*, 2005; Potrykus and Cashel, 2008). However, further deprivation of carbon sources from this amino acids-starved condition, which formed the basis of prolonged antibiotic tolerance (e.g. 48hr survival), was found to result in active induction of specific biosynthetic and metabolic functions. This tolerance state was therefore characterized by markedly enhanced expression of genes encoding the translation apparatus and energy production enzymes (Figure 5.1). The idea that active defense is involved in tolerance development is also supported by results of gene expression analysis on selected tolerance and stress response determinants, which demonstrated that expression of multiple stress responses and protection systems including oxidative stress, DNA repair, chaperones, osmotic stress and a number of general stress regulators were all up-regulated during starvation (Table 5.6). Taken together, these preliminary results suggest that bacteria undergo a unique physiological adaptation process which governs the development of both transient and prolonged tolerance modes through cellular sensing of environmental starvation signals.

6.6. The relative role of stringent response in tolerance development

Being one of the key cellular systems that mediate stress protection in bacteria and activated in an amino acids-limiting environment through induced synthesis of the alarmone guanosine 3',5'-bispyrophosphate (ppGpp) (Potrykus and Cashel, 2008), the stringent response may best account for the nutrient-dependent drug susceptibility phenotypes observable in this study. Given its inhibitory effects on the synthesis of macromolecules such as DNA, phospholipids and cell wall peptidoglycan (Magnusson *et al.*, 2005; Srivatsan and Wang, 2008), initiation of the stringent response in the absence of amino acids is therefore highly consistent with the onset of ampicillin and ofloxacin tolerance under such condition. This idea may further explain the observation that nucleobases were required to elicit amino acids starvation-induced ampicillin tolerance (Figure 2.1, C_1i and iii). The results of our gene knockout experiments, however, suggest an alternative story, because deletion of the genetic determinants responsible for producing the stringent response had mixed effects on the differential tolerance phenotypes observed in the wild-type strains (Figure 5.2). Firstly, ofloxacin tolerance was not sustainable in this mutant, suggesting that induction of the stringent response was nevertheless required for

formation of tolerance to fluoroquinolones. The fact that tolerance to ampicillin and gentamicin could still be produced in the absence of stringent response, however, suggested that its effect was drug target specific. Secondly, the nucleobase dependency in formation of transient ampicillin tolerance when amino acids were depleted from the growth medium was relieved in the ppGpp-null background, suggesting either that stringent response did not play an important role in formation of ampicillin tolerance under amino acids limiting conditions, or that failure to produce stringent response resulted in de-repression of an unidentified mechanism which could then mediate formation of primary or transient tolerance to ampicillin in the absence of both amino acids and nucleobases. These results were in agreement with those of Hansen *et al* (Hansen *et al.*, 2008), which showed that deletion of *dksA*, determinant of a ppGpp-dependent modulator of RNA polymerase, resulted in significant defect in tolerance formation.

The ppGpp-null mutant exhibited enhanced growth fitness in an amino acid-limiting background if nucleobases were further depleted (Figure 5.2). It has previously been suggested that a lack of stringent response would undermine the ability of a bacterial population to switch to a defence mode at the expense of continued synthesis of macromolecules during nutrient starvation, thereby enabling the ppGpp-null mutant to grow at a normal or even enhanced rate under transient nutrient limiting conditions (Gummesson *et al.*, 2009; Potrykus and Cashel, 2008). Evidence gathered in this study shows that nucleobases have a significant negative impact on bacterial growth fitness under specific nutritional stress; whether such effect is mediated through complex, stringent response-independent growth capacity / stress response regulatory pathways entails further investigation. Nevertheless, the intriguing findings regarding the multiple effects of nucleobases on growth rate and tolerance development under different genetic and nutritional backgrounds shed light on our limited knowledge on the role of ppGpp in eliciting antibiotic tolerance in response to nutrient starvation (Rodionov and Ishiguro, 1995; Rodionov *et al.*, 1995).

The role of stringent response in development of sustainable antibiotic tolerance was also analyzed in the context of its functional relationship with the stationary-phase sigma factor RpoS, which is known to be activated during limitation of carbon and nitrogen sources, as well as phosphate, and play a role in regulating

cellular metabolism during nutrient starvation (Peterson *et al.*, 2005) and up-regulating transcription of genes involved in stress protection (Weber *et al.*, 2005). Firstly, carbon starvation was shown to enhance ppGpp production (Potrykus and Cashel, 2008), which might in turn activate RpoS-dependent responses (Klauck *et al.*, 2007; Magnusson *et al.*, 2005). Secondly, our data clearly demonstrated that distinct but overlapping physiological changes were elicited when amino acids and glucose were individually or concomitantly depleted, yet deletion of the RpoS and stringent response determinant exerted differential effects on the development of prolonged tolerance. These findings indicate that each of these two major stress response systems play a partial and non-identical role in formation and maintenance of antibiotic tolerance.

6.7. Tolerance induction specificity of SOS response

The finding that RecA function was essential for ofloxacin-specific tolerance was consistent with its role in mediating SOS response-regulated DNA repair and therefore alleviation of the damaging effects of fluoroquinolones ((Drlica *et al.*, 2008), Figure 5.3). This role is further confirmed by the finding that the phenotype exhibited by the *lexA3* mutant, in which the SOS response is constantly repressed ((Fernandez De Henestrosa *et al.*, 2000), Figure 5.4), were similar to that of the *recA* mutant. However, the fact that the *lexA51* strain, which constitutively expresses the SOS response, was not able to withstand ofloxacin treatment under non-inducing condition suggests that SOS response has to act in concert with other cellular mechanisms in tolerance development. The precise role of SOS response in fluoroquinolone tolerance warrants further investigation. We speculate that un-regulated expression of SOS genes may actually produce pleiotropic effects on diverse cellular defense functions, such as a compromised efficiency in translation, thereby potentially leading to reduced protection against ofloxacin-mediated damages. Another unresolved issue is the ambiguous relationship between nutrient limitation and activation of SOS response. Currently there is little evidence which illustrates that SOS response can be spontaneously turned on upon nutrient depletion. The possibility that prolonged starvation could result in reduced integrity of genetic materials and hence elicitation of SOS responses needs to be investigated. Our data also suggest a need to examine the possible relationship between the stringent and SOS response in tolerance formation, as both Δ*relA*Δ*spoT* and Δ*recA* mutants were

defective in exhibiting prolonged tolerance to ofloxacin but not ampicillin (Figure 5.2 and 5.3).

6.8. Other tolerance determinants

Among the candidate genes selected according to their established biological functions or expression characteristics in our microarray experiments, a repertoire of putative tolerance determinants (36 out of 100) have been identified through rapid phenotypic screening (Table 5.7). Apart from the aforementioned stress defense regulators such as *relA/spoT* (stringent response) (Magnusson *et al.*, 2005; Potrykus and Cashel, 2008; Srivatsan and Wang, 2008), *rpoS* (stationary-phase sigma factor) (Hengge-Aronis, 2002) and *lexA/recA* (SOS response) (Janion, 2008), a number of such determinants have previously been reported to play a role in mediating drug tolerance; these include heat shock proteins (*dnaK* and *dnaJ*) (Lund, 2001; Yura and Nakahigashi, 1999), toxin-antitoxin modules (*hipA* and *relE*) (Gerdes *et al.*, 2005; Van Melderen and Saavedra De Bast, 2009), metabolic regulators (*apaH*, *glpD*, *phoU*, *sucB* and *ygfA*) (Hansen *et al.*, 2008; Li and Zhang, 2007; Ma *et al.*, 2009; Spoering *et al.*, 2006) as well as upstream regulators of multiple cellular functions (*fis* and *surA*) (Hansen *et al.*, 2008). Although the underlying basis by which the products of these putative tolerance genes mediate drug tolerance is still largely unclear, it has been speculated that these cellular components act by protecting the cell against drug lethality, and that a variety of mechanisms were involved in the process. These include drug target inhibition (Lewis, 2007), metabolic modulation (Li and Zhang, 2007; Zhang, 2007), DNA repair (Debbia *et al.*, 2001; Dorr *et al.*, 2010) as well as energy generation supporting survival during the drug-tolerant state (Ma *et al.*, 2009).

A number of tolerance determinants which were identified in our study have never been shown to play a role in tolerance formation. These include genes whose products play a role in oxidative stress defense (*ahpF*, *dps*, *sodA* and *trxA*), DNA repair (*mfd*), osmotic stress response and protection (*osmB* and *proP*), drug efflux (*acrA*), general stress response regulation (*uspA* and *uspE*), acid stress protection (*yqgB*); in addition, this group also include known members of the two component systems (*arcA*, *arcB*, *rcsB* and *rcsC*) and one gene (*yhfZ*) with unknown function. Among these candidate tolerance determinants, *ahpF* and *sodA* are known to encode

the F52a subunit of the alkyl hydroperoxide reductase subunit and manganese-containing superoxide dismutase, which play a functional role in detoxification of intracellular hydrogen peroxide (Poole, 2005) and superoxide free radical (Fridovich, 1995) respectively. On the other hand, *trxA* is known to code for the synthesis of thioredoxin, which is responsible for maintenance of cellular redox homeostasis (Aslund and Beckwith, 1999; Gleason and Holmgren, 1988); likewise, the Dps protein is produced in abundance in stationary-phase and presumably involved in protection against oxidative stresses through serving as a DNA replication checkpoint (Almiron *et al.*, 1992; Chodavarapu *et al.*, 2008). The fact that a number of genetic components which encode diverse functions in oxidative stress defense were simultaneously found to play a role in tolerance development indicates that such function is indeed one of the actively expressed tolerance mechanisms during nutrient starvation.

Importantly, several miscellaneous tolerance determinants which were identified in the deletion studies exhibited related functions. These include the Mfd transcription-repair coupler which recognizes stalled RNA polymerases caused by DNA lesions and recruits the excision repair components to the site of damage (Roberts and Park, 2004), and the UspA and UspE proteins which were found to confer protection against DNA damaging agents (Kvint *et al.*, 2003). The fact that oxidative defense and DNA protection/repair functions are both found to be involved in antibiotic tolerance is consistent with several recent findings on antibiotic mediated cell death, which suggested that the killing process was triggered by accumulation of hydroxyl free radicals (Dwyer *et al.*, 2007; Kohanski *et al.*, 2007). However, our experimental data regarding the effect of supplementation of hydroxyl free radical scavenger suggested that alleviation of oxidative stress only produced a weak tolerance phenotype under non-inducing conditions (Figure 5.7). This finding indicates that neutralization of free radicals only plays a minor role in the process of drug tolerance development. Data regarding the drug specificity of *recA*-dependent tolerance as discussed above also seem to complement these results: the fact that tolerance to fluoroquinolones requires both oxidative stress protection and DNA repair suggests that each of these protection mechanisms is by itself not sufficient to produce tolerance. This idea is compatible with findings regarding the weak tolerance induction effect of the *lexA51* strain (Figure 5.4). It is also tempting to

speculate that *yhfZ*, a gene with hitherto unknown function but found to affect formation of the prolonged ofloxacin tolerance phenotype upon gene deletion, may also fall into this category. At this stage the tentative conclusion is that these protection systems likely confer partial protection by scavenging free radicals induced by antibiotics (e.g. *ahpF* and *sodA*), as well as aiding in the repair of damaged DNA and proteins (e.g. *trxA, dps* and *mfd*). This idea corroborates with a recent finding which suggested that multiple antibiotics could elicit both free radical-dependent and independent damages (Wang *et al.*, 2010).

Identification of the multidrug efflux pump subunit *acrA* (Nikaido and Zgurskaya, 2001) as a tolerance determinant suggests that temporary phenotypic reduction in drug susceptibility could be caused by reduced accumulation of antibiotic molecules as a result of active extrusion of such molecules. However, drug permeation assay using radiolabeled antibiotics revealed similar intracellular drug concentrations in both non-tolerant and starvation-induced tolerant cells (Figure 5.6), implying that tolerance is not likely to be caused by active antibiotic efflux. One explanation for such discrepancy is that the AcrA protein may have unidentified roles on the regulation of antibiotic tolerance; alternatively, disruption of this periplasmic protein may result in specific alteration of the cell envelope, facilitating antibiotic entry and hence reduction in tolerance level even during starvation. The same theory may be applicable to explaining the fact that deletion of each of the two genes coding for membrane-localized osmotic stress response proteins, *osmB* and *proP*, could affect development of sustainable tolerance to ampicillin and ofloxacin (Table 5.7). The gene *osm*B is known to encode a membrane lipoprotein inducible by stationary-phase and osmotic shock (Jung *et al.*, 1990). Its physiological function is not clear but has been suggested to involve regulation of cellular growth under high osmolarity (Jung *et al.*, 1989). On the other hand, ProP is an osmosensitive transporter which facilitates uptake of compatible solutes such as proline when bacteria are exposed to high extracellular solute concentrations (Wood *et al.*, 2001). While the theory of disruption of membrane-based antibiotic barrier may provide an explanation of the observed phenotypes, it still falls short of accounting for the unaltered gentamicin tolerance phenotypes as exhibited by these mutants (Table 5.7). Another plausible explanation is that mechanisms of osmotolerance can indirectly promote tolerance to other stresses by an ill-defined mechanism, as in the case where high osmolality

triggered thermotolerance independent of the heat shock proteins in *Salmonella typhimurium* (Wood *et al.*, 2001). Furthermore, these membrane-located regulators are commonly associated with both ampicillin and fluoroquinolone tolerance (Table 5.7); such observation supports the idea that predominant tolerance mechanisms may exist for specific antibiotic classes despite the multitude of factors affecting tolerance development. Nevertheless, carefully designed experiments, such as measurement of specific drug target activities regulated by these genetic determinants, would allow validation of such concept.

Compared to beta-lactams and fluoroquinolones, the molecular basis of aminoglycoside tolerance remains elusive, presumably due to the ill-defined mechanism of bactericidal activity triggered by this class of antibiotics. There are currently two plausible explanations for aminoglycoside tolerance: the first one is through inhibition of drug targets (e.g. translation machinery) by metabolic regulators such as HipA (Keren *et al.*, 2004b), or potentially through neutralization of antibiotic-induced free radical damage (Kohanski *et al.*, 2008). Our observations that free radical scavenger had no noticeable protection effect against gentamicin (Figure 5.7), and that none of the ROS defense genes tested had significant impact on gentamicin tolerance under all conditions tested (Table 5.7), argue against the free radical protection theory. However, both transient and sustainable tolerance to this drug are at least conditionally dependent on the SOS response as well as the metabolic down-regulator PhoU (Figure 5.3), suggesting that both mechanisms are essential in protection against aminoglycoside-induced damages. Another interesting feature regarding gentamicin tolerance is that even transient tolerance to this antibiotic is heavily dependent on active protein synthesis (Figure 5.8). This finding is consistent with the observation that deletion of the *yqgB* gene exerted a pronounced suppressive effect on gentamicin tolerance (Table 5.7). It should be noted that the YqgB protein has recently been found to play a role in acid resistance, and is among a group of low molecular weight proteins (16 to 50 amino acids in length) with ill-defined functions (Hobbs *et al.*, 2010). We speculate that active production of specific small proteins constitutes the functional basis of aminoglycoside tolerance.

Our results also showed that specific two-component systems *arcAB* (Bekker *et al.*,

2010) and *rcsBC* (Majdalani and Gottesman, 2005) were involved in tolerance formation (Table 5.7), suggesting that nutrient-deprivation may also impose passive physiological perturbations such as increase in oxidative stress as a result of reduced growth rate (Nystrom, 2004), followed by activation of specific stress defense mechanisms. Regardless of their upstream induction cues, which await further experimental evidence, the established roles of these two-component systems correlated well with those of the stress protection determinants which were concomitantly identified in the same experiments. The oxygen sensor *arcAB* (Bekker *et al.*, 2010) has been found to play a role in oxidative stress resistance under aerobic growth conditions, presumably by complex metabolic regulation (Loui *et al.*, 2009). Since the role of *arcA*-mediated regulation of Dps in oxidative stress resistance in *Haemophilus influenzae* has been disputed by the same study, whether such conclusion can be applied to our starvation and tolerance models awaits further investigation. The *rcsBC* phosphorelay, which are sensitive to membrane stresses and known to control capsule synthesis and biofilm development (Majdalani and Gottesman, 2005), has also been found to activate RpoS-regulated genes including the membrane protein OsmB in an RpoS-independent manner (Boulanger *et al.*, 2005). These functional properties thus enabled the *rcsBC* phosphorelay system to mediate tolerance to both ampicillin and ofloxacin, and explained the phenotypic discrepancy between OsmB and the ArcAB systems, which was shown to affect ampicillin or ofloxacin tolerance alone upon gene deletion (Table 5.7).

6.9. Functional relationship between signaling and protection systems

Despite the fact that a large number of tolerance determinants were identified by deletion studies, how they are subjected to regulation by starvation signals is largely unknown. Given the diversity of stress response and protection systems involved in the development of antibiotic tolerance, the upstream induction processes of these components are believed to comprise both redundant and specific pathways. As mentioned, starvation signals such as ppGpp (stringent response) are expected to mediate re-programming of the RNA polymerase activities, leading to repression of central metabolic activities (Magnusson *et al.*, 2005; Potrykus and Cashel, 2008), global activation of multiple stress defense mechanisms in concert with alternative sigma factors RpoS (osmotic and oxidative stresses), RpoH (heat stress) and RpoE (periplasmic stress) (Magnusson *et al.*, 2005), or up-regulation of additional stress

protection proteins such as the USPs (universal stress proteins) (Kvint *et al.*, 2003). On the other hand, RpoS itself is a general stress regulator subjected to multiple regulation including starvation (Klauck *et al.*, 2007; Peterson *et al.*, 2005), controlling a number of stress adaptation genes such as *osmB*, *osmC* and *dps* (Weber *et al.*, 2005), some of which were identified to play a role in our tolerance assays. Similarly, the RNA polymerase binding protein DksA has been recently found to activate *uspA* through a ppGpp-independent mechanism (Magnusson *et al.*, 2007).

6.10. Tolerance induction effects of antibiotics

In recent years, an intriguing finding regarding the development of antibiotic tolerance is that antibiotics can by itself induce partial development of its own tolerance (Dorr *et al.*, 2009; Miller *et al.*, 2004). This phenomenon was observable in both beta-lactams (Miller *et al.*, 2004) and fluoroquinolones (Dorr *et al.*, 2009), for which sub-lethal antibiotic concentrations was found to confer tolerance to higher concentrations of the same drug in a SOS-dependent manner. Although the functional relationship between starvation stress signals and SOS response remains to be proven, the SOS-dependency of starvation-mediated phenotypes as demonstrated in this work suggests the possibility that such phenotypes were partly attributable to drug induction.

6.11. Distinctive survival strategies featuring transient and prolonged drug tolerance

In summary, results of integrated and global analysis of physiological changes in bacteria subjected to starvation of amino acids and carbon sources, through the microarray and gene knockout approaches, suggested that only a small fraction of starvation responses are responsible for tolerance induction. In fact there is little correlation between the gene expression and deletion data: genes which exhibited significant changes in expression level during tolerance inducing conditions did not necessarily have impact on antibiotic tolerance phenotypes upon deletion, indicating that the majority of differentially expressed genes only played a role in mediating physiological adaptation to nutrient limitation without interfering the antibiotic-induced bactericidal process. Nevertheless, the microarray data, especially those of selected stress protection determinants, suggested that active synthesis of gene products occurred during limitation of carbon source alone or with amino acids, but not amino acids alone, for which our microarray data showed that shutdown of

antibiotic targets rather than active defence was more prevalent, presumably leading to transient tolerance.

Mechanisms of starvation-activated antibiotic tolerance have been largely attributed to reduction in cellular growth and metabolism in face of nutrient depletion (Eng *et al.*, 1991; Gilbert *et al.*, 1990). While it is logical to deduce that down-regulation of central metabolic activities can contribute to reduced efficacy of antimicrobial actions, such protection strategies were shown to be insufficient to insure long term antibiotic survival especially if the stressful conditions persist. Evidence supporting this idea was obtained from experiments using various bacteriostatic agents (Figure 2.2), as well as transcriptomic studies, which showed that organisms subjected to a more severe starvation stress actually switched to a more metabolically active state (Figure 5.1). Active mechanisms which enable protection against various environmental stresses have been well-documented (Giuliodori *et al.*, 2007; Imlay, 2008; Yura and Nakahigashi, 1999). Activation of such cellular mechanisms is undoubtedly advantageous for population survival under fluctuating environment conditions. In terms of physiological features, our data showed that these defense mechanisms allowed rapid transitions between vegetative growth and the stationary or dormant mode. Such view is further supported by the fact that most bacterial cells retain basal metabolic activities during an extended stationary-phase (Kolter *et al.*, 1993; Nystrom, 2004).

6.12. An elaborate model of bacterial multidrug tolerance

Data presented in this thesis facilitate the establishment of a new functional model of bacterial antibiotic tolerance. By means of condition-based tolerance induction assays, environmental nutrition status and population density were identified as two major parameters that regulate antibiotic susceptibility and hence development of tolerance with distinctive phenotypic features (Chapters 2 to 4). Such finding is consistent with the view that nutrient deprivation and high population densities represent the types of environmental stress or perturbation commonly encountered by members of multidrug-tolerant bacterial communities such as those recoverable in biofilms (Costerton *et al.*, 1999; Høiby *et al.*, 2010; Lewis, 2008; Perumal *et al.*, 2007) or batch-cultivated stationary-phase populations (Keren *et al.*, 2004a; Levin and Rozen, 2006; Lewis, 2007). According to our tolerance model (Figure 6.1), the

nature and duration of starvation stress constitute the induction basis of the strength and drug specificity of phenotypic tolerance, whereas increasing cell density is accompanied by activation of a hitherto-undefined quorum sensing-like mechanism and increasing physical constraints that impede solute diffusion, resulting in reduced susceptibility to multiple antibiotics (Figure 6.1). At the initial stage of nutrition starvation or under situations where amino acids become limiting, ppGpp-/ RpoS mediated down-regulation of genes responsible for metabolic or biosynthesis activities occurs, eliciting emergence of transient tolerance (Chapter 5). As starvation stress persists or becomes more severe, a wide variety of specific stress protection determinants are activated (Tables 5.6 and 5.7), producing sustainable tolerance to beta-lactams and fluoroquinolones, as well as antibiotic persisters which remain transiently tolerant even when nutrients are replenished to the growth medium (Figures 2.1 and 3.1).

Essential components of the active stress defense network include the SOS response, without which fluoroquinolone tolerance cannot be produced (Figure 5.4), and the putative acid stress survival protein, YqgB, which is important for both fluoroquinolone and aminoglycoside tolerance (Table 5.7). Since YqgB belongs to a class of abundant yet mostly uncharacterized low molecular weight proteins (Hobbs *et al.*, 2010), this finding suggests a need to investigate the functional role of such proteins in tolerance regulation. To summarise, this antibiotic tolerance model takes into account two seemingly incompatible theories on stress protection: the active and energy-dependent mechanisms (Ma *et al.*, 2009; Powell and Young, 1991) and the shut-down oriented responses (Keren *et al.*, 2004b; Li and Zhang, 2007; Schumacher *et al.*, 2009). Such model (Figure 6.1) shall serve as a framework for further characterization of the functional components of bacterial antibiotic tolerance mechanisms.

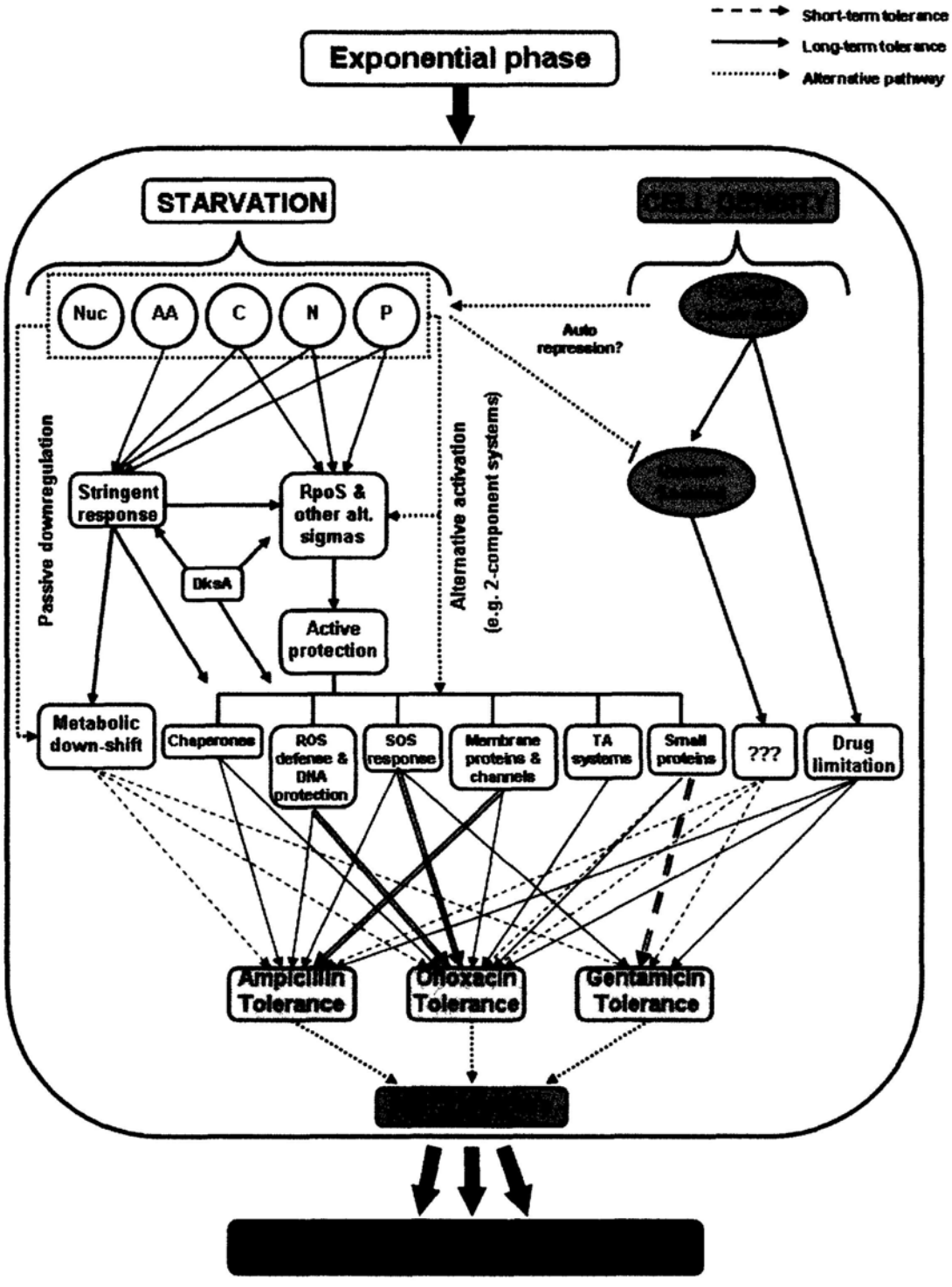

Short-term tolerance
Long-term tolerance
Alternative pathway
Exponential phase
STARVATION
Nuc
AA
C
N
P
Auto repression?
Passive downregulation
Alternative activation
(e.g. 2-component systems)
Stringent response
RpoS & other alt. sigmas
DksA
Active protection
Metabolic down-shift
Chaperones
ROS defense & DNA protection
SOS response
Membrane proteins & channels
TA systems
Small proteins
???
Drug limitation
Ampicillin Tolerance
Ofloxacin Tolerance
Gentamicin Tolerance

Figure 6.1. An elaborate model of phenotypic antibiotic tolerance. Non-tolerant log-phase cells may develop into stationary-phase, multidrug tolerant (MDT) cells in response to two major sets of induction signals. Starvation signals (yellow) trigger (i) metabolic down-regulation which reduces bactericidal effects of antibiotics, resulting in short-term tolerance, or (ii) activation of a repertoire of stress protection mechanisms that confer more sustainable phenotypes, depending on the strength of starvation signals. Putative major tolerance mechanisms pertinent to tolerance of each antibiotic class are indicated by double-lined arrows. Extended nutrient limitation further triggers the formation of persister cells, which presumably arise from existing tolerant populations. Furthermore, increasing population density (purple) introduces physical constraints that result in local drug / nutrient limitation effects, which may partially account for prolonged drug tolerance in high cell density populations. Increasing cell density is also accompanied by activation of a hitherto un-defined quorum sensing-like mechanism, which induces short-term multidrug tolerance. Rapid increase in cell density may also lead to rapid consumption of nutrients and further elicits starvation-mediated tolerance.

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