RESPONSE OF *Bacillus subtilis* IN CLONAL COLONY COMPETITION

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RESPONSE OF *Bacillus subtilis* IN CLONAL COLONY COMPETITION

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TINDAK BALAS *Bacillus subtilis* DALAM PERSAINGAN KOLONI KLONAL

ABSTRAK

Persaingan adalah perkara biasa yang berlaku dalam persekitaran semula jadi kerana keterbatasan sumber dan ruang. Persaingan jangka panjang akhirnya akan membawa kepada koeksistensi yang stabil. Penyelidikan ini bertujuan untuk mengkaji dan meengesahkan kesan persaingan koloni klon dalam *Bacillus subtilis*. Ini termasuk keadaan fisiologi, status kebolehhidupan dan tindak balas transkriptom sel. Apabila koloni *Bacillus subtilis* ditumbuh berdekatan, pertumbuhan berlaku sehingga saiz tertentu dengan depan yang nyata dan menghasilkan jurang yang jelas di antara mereka. Pertumbuhan kelihatan terbantut di bahagian berdepanan, sementara bahagian yang tidak menghadap koloni lain terus tumbuh ke arah luar dan memperluaskan morfologi koloni. Berdasarkan pewarnaan kebolehhidupan, majoriti sel di bahagian berdepanan koloni berusia 72 jam mati atau mengalami perubahan pada membran sel mereka. Sel bakteria dari bahagian berdepanan diperhati melalui mikroskop cahaya mengalami sporulasi sementara sel di bahagian pertumbuhan arah luar tidak terjejas. Mikroskopi elektron pancaran menunjukkan bahawa sel di bahagian berdepanan kebanyakannya adalah endospora sementara sel di bahagian yang tumbuh ke arah luar kebanyakannya sel vegetatif yang membahagi secara aktif dengan badan nukleoplasma yang memanjang. Analisis transkriptomik perbandingan menunjukkan bahawa majoriti gen yang dikawal atur naik dalam sel di bahagian berdepanan adalah berkaitan dengan tekanan oksidatif dan nutrien. Tindak balas adaptif yang jelas dan sangat dikawal atur naik ini menunjukkan bahawa sel

mengalami pelbagai tekanan. Tekanan yang disebabkan oleh kekurangan nutrien nampaknya meningkatkan ekspresi gen yang terlibat dalam metabolisme asid amino atau karbohidrat. Sebaliknya, sel di bahagian koloni yang tumbuh ke arah luar sedang dalam proses peralihan ke sporulasi. Gen yang dikawal atur naik dalam sel ini dapat diklasifikasikan kepada beberapa kumpulan. Sebilangan besar gen yang dikawal atur naik adalah berkaitan dengan "kanibalisme". Pengumpulan faktor pembunuhan kanibalisme Skf dan Sdp mempengaruhi sel *B. subtilis* di bahagian berdepanan dan berpotensi menyebabkan pelepasan nutrien ke persekitaran. Sementara itu, sel di bahagian yang berkembang beralih dari fasa eksponen ke pertumbuhan pegun dan mekanisme gerak balas tekanan sampul sel diaktifkan. Gen yang berkaitan dengan tindak balas tekanan sel sampul, iaitu oxdC, ybfO dan liaD, didapati diekspresikan secara berlebihan dan signifikan. Dalam kesimpulan, *B. subtilis* akan bertindak balas terhadap kehadiran koloni klon dalam jarak yang dekat dengan menghalang pengembangan koloni klon. Femonena ini adalah disebabkan oleh pengumpulan pelbagai molekul perencatan di antara koloni klon yang berada dalam, jarak yang berdekatan. Akhirnya kesan perencatan bersama koloni klon menyebabkan sel yang terjejas mempunyai kitaran sel yang dipercepatkan. Kajian ini mencadangkan bahawa kehadiran molekul isyarat aktif yang mengawal pertumbuhan bakteria boleh digunakan untuk merawat penyakit bakteria berjangkit dengan tepat.

RESPONSE OF *Bacillus subtilis* IN CLONAL COLONY COMPETITION

ABSTRACT

Competition is a common occurring in the natural environment due to the limitation of resources and spaces. Long term competition will eventually lead to stable coexistence. This research aims to investigate and establish the effect of clonal colony competition in *Bacillus subtilis*. This includes the physiological condition, viability status and transcriptomic response of the cell. When *B. subtilis* colonies are grown close together, growth occurs to a certain size with a distinct front, leaving a gap between them. Growth appeared to be arrested on the fronting side, while the side that did not face any other colony continued to grow outward and extend the morphology of the colony. Based on viability staining. the majority of the cells on the fronting sides of a 72-hour colony were either dead or undergoing changes to their cell membranes. Bacterial cells from the fronting side were observed to be sporulating by light microscopy, while cells on the outward growing side were unaffected. Transmission electron microscopy revealed that the cells on the fronting sides were mostly endospores, while the cells on the outward growing sides were mostly actively dividing vegetative cells with elongated nucleoplasmic body. A comparative transcriptomic analysis showed that the majority of the up-regulated genes in the cells on the front side are related to oxidative and nutrient stress. These distinct and highly up-regulated adaptive responses indicated that the cells were subjected to a variety of stress. The stress caused by nutrient depletion appeared to increase the expression of genes involved in either amino acid or carbohydrate metabolism. In contrast, cells on the colony's outward growing side were in the

process of transitioning to sporulation. The up-regulated genes in these cells can be classified into a few groups. The majority of the up-regulated genes were associated with “cannibalism”. The accumulation of cannibalism killing factors Skf and Sdp affected *B. subtilis* cells mutually on the fronting sides, potentially causing nutrient release into the environment. Meanwhile, cells at the expanding side transitioned from exponential to stationary growth phases and their cell envelope stress response mechanism was activated. Genes, related to cell envelope stress response, namely *oxdC, ybfO* and *liaD*, were found to be significantly over-expressed. In conclusion, *B. subtilis* will react to the presence of clonal colonies in close proximity by inhibiting the expansion of clonal colonies. This is achieved by the accumulation of various inhibitory molecules in between clonal colonies in close proximity. Eventually mutual inhibitory effects of clonal colonies causes affected cells to have an accelerated cell cycle. This study suggested that the presence of active signalling molecules that control bacterial growth could be used to precisely treat infectious bacterial disease.

CHAPTER 1

INTRODUCTION

1.1 General Introduction

Microbes compete for survival in diverse and naturally mixed environments. They can coexist with, or dominate, other organisms when competing for the same pool of resources. They also exhibit social behaviour and establish cooperative or competitive biological interactions in the niches they occupy. In addition to biological interactions, abiotic influences such as temperature, humidity, salinity, and available nutrients also affect the composition of the community. They can use a variety of mechanisms as strategies to acquire nutrients as the latter become depleted by the growing population.

An ecosystem has many complex relationships occurring within it. Multispecies and multicellular interactions are an integral part of its dynamic and the most obvious relations to explore. In nature, the microbes are more likely to grow in multispecies communities than in monoculture. Interactions among members are essential for the growth as well as maintenance of the community and may include interspecies communications involving signalling molecules or physical contact. Some species may utilize the same or related signal molecules to exchange communication and modulate their behaviour accordingly in response to signals from other species. The ability of a bacterial cell to distinguish neighbouring cells enables them to cooperate or compete with one another. The creation of biofilm, which allows microbes of different species to be enclosed as a complex group, is an example of this cooperative action.

When bacteria compete with unrelated or distantly related strains, they produce soluble diffusible factors which are used during intercellular competition to inhibit the growth of other microbial competitors (Destoumieux-Garzón et al., 2002). Examples of these soluble factors are bacteriocides such as bacteriocins and antibiotics. It has been suggested that antibiotics at sub-inhibitory concentrations are used for cooperative interactions such as inter- and intra-species signalling (Davies et al., 2006). They can therefore modulate their behaviour in response to the variations of population heterogeneity and density in their environment.

Nealson (1978) observed luminescence in the marine bacterium *Vibrio fischeri* that could be induced at low density by growing in the spent medium of a high-density culture. This phenomenon was termed as "autoinduction" and this observation on bacterial social signalling resulted in the discovery of the cell-to-cell communication mechanism known as quorum sensing (Fuqua et al., 1994). The "autoinducer" was identified as an acylated homoserine lactone (acyl-HSL) molecule. It was later found that quorum sensing can be countered or thwarted by quorum quenching and quorum inhibitors (Uroz et al., 2005a; Dong et al., 2001). These diffusible signalling molecules are secreted to coordinate activities in the bacterial communities.

There are other bacterial growth inhibitory mechanisms but these involve physical contact between adjoining cells instead of diffusable signaling molecules. Aoki et al. (2005) observed that certain strains of *Escherichia coli* contained a bacterial growth-inhibition system that uses direct cell-to-cell contact. The growth state of the inhibitory cell and the state of expression of the target cell both influenced the inhibition. Contact-dependent inhibitor A (CdiA), a large cell-surface protein, and CdiB, a two-partner secretion family member, were both necessary for

the growth inhibition to occur. Another type of growth inhibitory mechanism is the type VI secretion system (T6SS), which is structurally similar to the puncturing device of bacteriophages (Nudleman et al., 2005). T6SS delivers bacteriolytic effectors to the target cells by breaching the peptidoglycan layer which forms the major structural constituent of the bacterial cell wall (Russell et al., 2014). This protein secretion system mediates interactions between a broad range of Gram-negative bacterial species such as *Pseudomonas*, *Erwinia* and *Salmonella*. In *Pseudomonas aeruginosa,* T6SS delivers two effector proteins, Tse1 and Tse3, to the periplasm of recipient cells (Benz et al., 2012).

A bacterial colony that has stopped growing on an agar surface will prevent a neighbouring colony from reaching full size (Hochberg and Folkman, 1972). The interaction between two proximately located bacterial colonies can be mutually beneficial or antagonistic in nature. Most will grow to a certain size with a distinct front and a distance between them. Growth seems to be arrested at the fronting side, while the side that does not face another colony continues to expand outward and extend the size.

The Gram-positive bacterium *Bacillus subtilis* strain 168 is used as the model for this study. This rod-shaped bacterium is a popular model to investigate physiological and genetic phenomena in Gram-positive bacteria. It has been widely studied due to its genetic amenability and ability to undergo sporulation, a typical form of cell development. Another important interest is its ability to produce high levels of small metabolites and enzymes at the industrial scale. The whole-genome sequence of *Bacillus subtilis* strain 168 (GenBank assembly accession number GCA_000009045.1) has also been made public and this facilitates analysis of its physiological characteristics and potentials (Blount, 2015; Minogue et al., 2014).

The motivation for this study came from the observation that when two *Bacillus subtilis* colonies are inoculated adjacent to each other on a rich nutrient agar plate, they grow and form a distinct interface at the colony fronts without a complete merger. There has been a significant amount of information gathered regarding cell-to-cell interactions from Gram-negative bacteria, yet there are major gaps in our understanding of the molecular events in the Gram-positive group. Studies on *Paenibacillus dendritiformis* showed that it produces and secretes a lethal factor which acts as an inhibiting agent when two neighbouring sibling colonies are simultaneously inoculated and grown on the surface of a rich agar (Be'er et al., 2009b). It was proposed that self-killing of clonemates is a strategy to reduce overcrowding and avoid nutrient depletion in order to allow the population to spread. However, the molecular events occurring within the cells were not studied.

1.2 Research Objectives

The objective of this study is to observe the physiology of the cells at the two opposing sides of the colonies and understand the mechanism of growth inhibition. Understanding of the mechanism(s) involved in this mutual inhibition may lead to novel pathways responsible for such a display of clonal colony inhibition.

This will be accomplished with the following specific objectives:

1) To perform comparative observation of clonal colonies facing each other versus non-fronting outward growing side.

2) To investigate the viability of cells facing clonal colony competition

3) To determine mechanisms of inhibition using transcriptomic approach

CHAPTER 2

LITERATURE REVIEW

2.1 Social Behaviour of Bacteria

Aristotle once said “Man is by nature a social animal; an individual who is unsocial naturally and not accidentally is either beneath our notice or more than human”. Social behaviour is a focus of study for biologist for a long time. However, social behaviours are not limited to humans. Animal and plants too exhibit social behaviours, not only among themselves but also with others. Typical social behaviours in the animal kingdom includes primate social hierarchies, pheromone-based signalling, male competition for mating partners. Recent studies also revealed that plants distinguish between self and non-self and react accordingly (Dudley and File, 2007). It is evident that social interaction is pervasive among living organisms, including bacteria.

Prokaryote (*Bacteria* and *Archaea*) are commonly viewed as single-celled organism, but prokaryotes also display social behaviours (Claessen et al., 2014). Social lifestyle may vary widely across species due to unique evolution pathways but certain tell-tale sign remains common, including cell-cell adhesion, division of labour and intercellular cooperation (Lyons and Kolter, 2015). It is fascinating that these behaviours are found in creatures that lack neurons or nephrons.

Prokaryotes are simple creatures in terms of structure and morphology and lack of cell differentiation. However, prokaryotes can still display social behaviours that resembles those in multicellular organisms with multicellularity (van Vliet and Ackermann, 2015). Multicellularity is achieved by differential expression of a same set of genes in response to different microenvironment, leading to different

phenotype observation in a clonal population of cells. Stochastic fluctuation during gene regulation can also result in random cellular variability (Veening et al., 2008).

Multicellularity is beneficial towards a bacterial population. Division of labour allowed specialised cell types to work together, coordinated by intercellular communications. This develops complex group behaviours that is synchronised, allowing the population to improve efficiency and function more efficiently, ensuring better survival (Aguilar et al., 2015).

Social behaviour can be classified into four classes according to their effects: benefit, altruism, selfishness, and spite (Hamilton, 1964; Hamilton et al., 1971; Hamilton, 1970). Mutualism is an interaction when both the performer and recipient benefited. Selfishness is when the performer benefited from the interaction while the recipient is harmed. On the other hand, altruism is the opposite of selfishness as the recipient are benefited but the actors do not get any benefit or even may be harmed. Although spiteful interaction is rare in nature but it has been discovered where both performer and recipient are harmed in such interaction (BASHEY et al., 2012). These classifications should be made based of the reproductive success over a lifetime. For example, a selflessness or altruistic behaviour may not bring immediate benefits for the performer, but long-term benefits may outweigh the short-term cost, making it a mutualistic behaviour. Hence, these classifications can be complicated as long-term fitness consequences of a behaviour is difficult to measure. Besides, a single behaviour may have multiple outcomes. What we observed maybe just a snapshot of a part of a lifecycle and interactions (Trivers, 1985). Therefore, sometime a behaviour is defined by their short-term effects since long term effects may not be certain yet.

2.2 Competition and Cooperation in Bacteria

Diversity of microbes dictates that microbe typically lives in an environment that is surrounded by different strains and species. They have to compete for limited resources and space (Hibbing et al., 2010). Therefore, microbes have developed many phenotypes to compete and eventually displace other species. However, this competition behaviour slowly changed into cooperative behaviour that eventually led to stable coexistence of microbes, even with genetically distinct strains (Ghoul and Mitri, 2016). Nonetheless, competition provides selection force that led to different evolution of different species or strains according to their ecological conditions.

Bacterial cooperation and competition often also start with a shared public goods that are finite in nature (Frank, 2019). These public goods are secreted compound that require energy and time to produce. Given the finite nature of public goods, bacterial cell will either compete for the resources or cooperate to maximise the resource. However, there is always a risk of cheater cells that siphon off this common pool of public good but do not contribute to the general population (Hamilton, 1964). As cheater cell do not need to invest anything in this competition, they may grow over time and dominate over other member of the population.

2.2.1 Reason for Competition

Competition is when an isogenic or homogenic population of microbes compete for a limited pool of resources. Competition interaction is widespread and prevalent in ecosystem. Genomic studies revealed that 25% of Gram-negative bacteria possess gene coding for the type VI secretion system (T6SS) (Boyer et al., 2009). Most actinomycetes devote 5-10% of their gene to the secretion of secondary metabolites, including antibiotics and other compounds (Nett et al., 2009). The extent

of competition can be assessed by building and stimulating metabolic models, using sequence data. Freilich et al. (2011) is one of the first to use this approach to study microbe's interaction during coexistence. They have predicted in a mixed culture of bacteria; competition is abundant with only a handful of positive interaction. Coculture experiments using bacterial isolates from tree-holes have confirmed these trends (Fiegna et al., 2015).

Competition is postulated to be favoured under three conditions: (i) overlapping metabolic niches and resources need, (ii) spatial mixing of cells of different strains where nutrients and secretion is mixed, and (iii) resources is a limiting factor in relation to the population of microbes (Ghoul and Mitri, 2016).

Environmental factors play a significant role to fulfil these conditions in order for competition interaction to occur. Environment with complex nutrient structures with multiple resources or niches may have reduce competition selection in the population, vice versa. However, resource ratio theory also dictates that while one resource may be abundant but other resource may act as limiting factor (Thomas E. Miller et al., 2005). Besides, phylogenetic of bacteria in a community may also play a role in determining the resource niche (Mitri and Foster, 2013). Distantly related species may tend to coexist together with minimal or positive effect due to difference in resource needs (Hardin, 1960). However, this does not rule out the possibility of lateral metabolic gene transfer leading to eventual overlapping of metabolic niche (Niehus et al., 2015; Shapiro et al., 2012).

Meanwhile, spatial mixing depends on multiple factors including nutrient abundance and mechanical aspect of the environment. Investigation carried out using *Pseudomonas aeruginosa* have demonstrated that high nutrient situation influence

spatial structuring of colonies (Mitri et al., 2016). However, it is interesting to note that regardless of nutrient situation, spatial mixing of bacteria population will lead to the loss of diversity over time, suggesting competition taking place as nutrients deplete over time. Mechanical aspects of the environment such as fluid dynamics and surface mechanics influences spatial organisation of bacteria colonies (Persat et al., 2015). For instance, Cardinale (2011) have demonstrated that a mixture of algae can cooperate to remove nitrate from stream water only if the water flow is heterogenous. Competitive exclusion will occur if the water flow is uniform (Cardinale, 2011).

Lastly, cell density may act as a trigger for competitive phenotype. As cell density increases, bacterial cell experience increasing physiological stress. This physiological stress includes lack of nutrients due to increasing cell population or cellular damage due to secretion of bacteriocins by competitive strains. Physiological stress act as signals to tell bacterial cell to respond by regulating competitive phenotypes to ensure survival (Cornforth and Foster, 2013; LeRoux et al., 2015b). For example, if *Pseudomonas aeruginosa* detects antibiotic, it will be triggered to form biofilm for protection (Oliveira et al., 2015). It can also trigger counterattacks utilising its T6SS when a neighbouring cell is killed (LeRoux et al., 2015a). Similar respond can also be found in *B. subtilis* that triggers lethal compound secretion if a *Bacillus simplex* biofilm is detected in close proximity (Rosenberg et al., 2016). As in *B. subtilis*, many soil bacteria can also respond to neighbouring colony by altering competitive behaviours via regulating antibiotic production (Kelsic et al., 2015; Abrudan et al., 2015).

2.2.2 Explorative Competition

Explorative competition is a passive competition where other bacteria is not actively targeted during the competition. Instead, a strain of bacteria rapidly exploits the environment resources to gain an upper hand over other strain (Hibbing et al., 2010). It can either increase nutrient uptake to outcompete other strains in the population or secrete extracellular compound to harvest environmental resources more efficiently. Either way of competition does not pose harm to competing strains directly.

For instance, facultative anaerobes *Saccharomyces cerevisiae* and *E. coli* are capable of fermentative metabolism in an anaerobic environment using alternative electron receptor (Unden and Bongaerts, 1997; Merico et al., 2007). However, once oxygen is present, fermentative *Saccharomyces cerevisiae* and *E. coli* are able to change into oxidative metabolism instantly (Yasid et al., 2016; Otterstedt et al., 2004). This led to instant elevation of growth rate, but a low yield, but it allows the strain outcompete other bacteria strain that respond slower to the presence of oxygen, by a more robust nutrient uptake (Pfeiffer et al., 2001; MacLean and Gudelj, 2006).

Secreted product that increases resource harvesting efficiency includes digestive enzymes and siderophores. *B. subtilis* also secrete at least seven different exoprotease, two major protease (subtilisn and neutral metalloprotease E), and five minor proteases (bacillopeptidase F, Mpr, Epr Npr and Vpr) (Pero and Sloma, 1993). Low level of nutrient availability triggers these exoprotease synthesis to degrade of a variety of molecules such as lipids, glutathione, phytic acid and extracellular nucleic acids in the environment, making them accessible for cells, providing them with a competitive edge (Hata et al., 2001; Tjalsma et al., 2004; Msadek, 1999).

Siderophores (Greek: iron bearer) are low molecular mass iron chelating compound that help in the scavenging of iron from the environment (Neilands, 1993). Iron limitation introduced by rapid oxidation of Fe^{2+} to Fe^{3+} under aerobic conditions and the low solubility of Fe^{3+} at neutral pH make siderophores an essential compound to allow bacteria access of iron from the environment. During iron starvation situation, both *B. subtilis* and *E. coli* secretes siderophore bacillibactin and enterobactin under the control of *fur* regulon (ferric uptake regulation) (Baichoo et al., 2002; Neilands, 1993). Siderophores bind to insoluble Fe^{3+} to allow transport through cell membrane. Hence, allowing more efficient scavenging of iron from the environment.

These secreted compounds are costly to produce, requiring energy and time. However, they are considered "public goods" as it is secreted outside of the cell. This brings up another approach to competition. Nearby strains can utilise these secreted compounds to their own benefits, while themselves reduce or stop secretion, thereby shifting cost of production to other organisms (Khan et al., 2006). Strains that do not produce siderophores but maintain siderophores utilisation ability cans also benefit from the public pool of secreted siderophores (West and Buckling, 2003). Therefore, these cells are referred as "cheaters" (Rainey and Rainey, 2003). The caveat to this strategy is that co-operators need to be present to allow cheaters to leech on. Siderophores and quorum sensing molecules are some examples of compounds prone to cheater cells (Cordero et al., 2012; Diggle et al., 2007). However, one can also see this relationship as an altruistic cooperation. Take siderophores as an example, the presence of siderophores in a population increases iron access to the entire community by simply acting as metal buffer that increases the availability of soluble metal in the ecosystem (Gerringa et al., 2000; Sunda et al., 2005). Besides,

siderophores presence also increases the overall wellbeing of the community by acting as protection against metal toxicity (O'Brien et al., 2014).

Beside nutrients, strains also compete for space in the natural environment. Like plants that compete for sunlight, bacteria also compete for prime locations within an ecological niche for preferential access of resources (Kim et al., 2014). To ensure stable positioning at favourable sites in the environment, competing strains will have to colonise rapidly and aggregate to form a community. Simultaneously, competing strains need to drive out or kill other competing strains of bacteria either by inhibiting biofilm formation or secreting bacteriocins (Bucci et al., 2011). Formation of biofilm is essential as it provides protection to the community from antimicrobials, predators and other environmental threats, Yin et al. (2019). Biofilm also promotes swarming and eliminates competing strains. A classic example is *E. coli* production of surfactant and EPS that inhibit biofilm formation of *Staphylococcus aureus* and *Pseudomonas aeruginosa* upon entry into biofilm (Rendueles et al., 2011; Valle et al., 2006). *P. aeruginosa* also have a similar mechanism known as “surface blanketing” where it rapidly colonises a surface by swarming and biofilm formation, while simultaneously inhibiting biofilm formation by *Agrobacterium tumefaciens* (An et al., 2006).

Colonisation and biofilm formation is assisted by a wide array of molecules. Rhamnolipids is a biosurfactant that is essential for dispersing biofilm, thus preventing biofilm colonisation of competing strains and promotes swarming (Wood et al., 2018; Caiazza et al., 2005). Adhesins helps bacteria anchor to a surface, preventing displacement by competing strains (Schluter et al., 2015). Meanwhile, exopolysaccharides provide structure and protection while driving expansion of biofilm and simultaneously suffocate and starve competitors (Gupta et al., 2019;

Xavier and Foster, 2007). Fruiting body is another interesting bacterial development within biofilms. Fruiting bodies are aerial projections that rise above the biofilm. Fruiting bodies is the preferred site of sporulation as the positioning of fruiting bodies helps in dispersion of mature spores. *Myxococcus xanthus* and wild type *B. subtilis* are two examples of bacteria that form fruiting body (Branda et al., 2001; Muñoz-Dorado et al., 2016).

2.2.3 Active Competition

Active competition occurs when one strain of bacteria produces antagonistic factors and actively seeks out and harm other stains of competing bacteria (Hibbing et al., 2010). These antagonistic factors are mostly specialised metabolites termed secondary metabolites as they usually do not participate in the primary metabolism of bacteria and only produced during late growth stages (Davies, 2013). Secondary metabolites may not be essential to the growth of bacteria but is necessary for a bacterium to persist in the environment or under competitive stress (Price-Whelan et al., 2006). However, it is interesting to note that secondary metabolites not only participate in interference competition but also explorative competition. Siderophores are considered as secondary metabolites (Neilands, 1993).

An example of this strategy is antimicrobial compound production. Antimicrobial compounds can either be strain specific or a broad-spectrum peptides and antibiotic. Some may also target genetically identical individuals in a population. Some researchers have proposed that sublethal concentration of bacteriocin may help the community in term signalling and cooperation (Yim et al., 2007; Goh et al., 2002). However, studies have demonstrated that antibiotics mostly served its contemporary role as a weapon to mediate competition (Chao and Levin, 1981).

Although these antimicrobial compounds did not qualify as signalling molecules, it may act as a chemical cue, prompting and influencing a variety of cell functions and developments (Shank and Kolter, 2009). For example, antimicrobial compound can cause potassium leakage and triggers biofilm formation in *B. subtilis* via the upregulation of extracellular matrix component genes (López et al., 2009a). In *Pseudomonas aeruginosa*, a small redox-active antimicrobials phenazine pyocyanin, can affect gene expression in several bacteria species (Dietrich et al., 2008).

Production of antimicrobial compound usually is a coordinated effort in a population to ensure that enough compounds is produced to effectively inhibit competitors. Basal, sub-inhibitory production of antimicrobial compound can lead to physiological tolerance in target species which in turn poses harm towards producer cells (Munita and Arias, 2016; Zhao et al., 2020). Therefore, microbial population utilise quorum sensing to ensure that once microbial compound is produced, it packs enough punch to kill and also prevent tolerance development. Antimicrobial compounds are only produced when the population reaches a certain threshold, allowing producers to release a lethal pulse of antimicrobial compounds (Doekes et al., 2019; Kareb and Aïder, 2020).

Since antimicrobial compounds depends on quorum sensing, that give rise to another strategy of interference by disrupting signalling process of cells in a population. Although the effect of quorum sensing disruption towards competitive advantage is not fully demonstrated, investigations have demonstrated that bacteria can produce and secrete compounds to muddle with quorum sensing. For example, protobacteria that utilise acyl-homoserine lactones (AHLs) as signal molecules can easily be disrupted by secretion of lactonases, acylases and oxidoreductases (Uroz et al., 2005b; Dong et al., 2007). These three classes of enzymes are commonly found

in complex environments, such as soil and termite hindguts, in a concentration high enough to disrupt quorum sensing. It has been demonstrated that synthetic signalling molecules are rapidly degraded in these samples (Wang and Leadbetter, 2005). However, the observation is lost when these samples are sterilised. Interestingly, *Variovorax paradoxus* take in AHL and degrade it internally and utilise the breakdown product as carbon and nitrogen source (Leadbetter and Greenberg, 2000).

Beside destroying signalling molecules, some bacteria also use signalling antagonism to disrupt quorum sensing. Auto-inducer 2 (AI-2) quorum sensing signals is utilised by a wide array of bacteria including bacteria such as *E. coli* and *Salmonella typhimurium* (Taga and Bassler, 2003). Both these enteric bacteria can produce and consume AI-2 signalling molecules, regulated by AI-2 levels (Taga, 2007; Taga et al., 2001). When *E. coli* was cultured together with *Vibrio harveyi* or *V. cholerae,* disruption of AI-2 controlled functions of all the organisms occurred (Xavier and Bassler, 2005). However, the exact function of these antagonistic relations in term of competition is still unclear.

Gram-positive organisms utilise short signalling peptides that are ribosome produced and modified post transcription as the main quorum sensing mechanism (Lyon and Novick, 2004). For example, the virulence factor of *Staphylococcus aureus* is regulated by thiolactone-based auto-inducing peptides (AIPs) (Ji et al., 1997). These AIPs classify species of *S. aureus* into four group (Jarraud et al., 2000). It has been discovered that AIP of one class inhibits sensing ability of another class via quorum sensing disruption (Geisinger et al., 2008). Besides, peptide-based sensing is also vulnerable to signal degradation. This is demonstrated in *Streptococcus gordonii* degradation of competence signalling peptide (CSP) of *S.*

mutans during competition within the human oral cavity as CSP control the production of bacteriocin inhibitory to *S. gordonii* (Wang and Kuramitsu, 2005).

2.2.4 Consequences of Competition Over Time

Competition will eventually lead to the local reduction in diversity and increase in ecological stability (Allesina and Levine, 2011; Coyte et al., 2015). The "local" in this context refers to the scale which cells have fitness effects on each other. This phenomenon may occur in a number of ways and eventually end up in three possible outcomes. Firstly, less competitive strain will be driven out while the others remain dominant in the community (Louca and Doebeli, 2016; Hardin, 1960). Next, different strains may continue to coexist by specialising in different metabolic niches and utilise different resource type. Lastly, different strains may eventually split into different spatial niches and occupy different location in an environment.

The tree-hole microbial community evolution experiment is a great demonstration of niche differentiation in term of resources (Fiegna et al., 2015). In this experiment, different species of bacteria initially competes for resources. However, the surviving species eventually coevolves and diverged in resources use. Species in the community evolved to use each other's waste product leading to overall increase in productivity. Therefore, we can see that different species can create and exploit the limited resources within the niche even when no new niche is present. With niche differentiation, competition strength is reduced and therefore slowly neutralised, which may even lead to symbiotic relationship and overall increase in community production.

Coexistence vie spatial separation is common on solid or semi-solid surfaces such as mucus, soil leaf surface or agar surface that allows simultaneous existence of

difference spatial niche. Similar to resource differentiation, the spatial differentiation also starts from a homogenous population of cells that compete against each other and slowly differentiate into different spatial separation as they slowly expand outwards (Mitri et al., 2016; Hallatschek et al., 2007).

Beside the three well established outcomes, there are some scenarios that are postulated recently. Firstly, stable coexistence between different strains within a same niche may be established due to an explorative relationship. The Black Queen Hypothesis propose that within a homogenous population where public goods are essential, if only one species is left to produce said public goods, it will continue to produce to avoid extinction, even if in benefits the competitors (Morris, 2015; Morris et al., 2012). Similar phenomenon is observed in an intraspecies co-operators and cheaters interaction (Sanchez and Gore, 2013). Siderophores production in marine bacteria is an excellent example of this phenomenon (Cordero et al., 2012). The same equilibrium is also observed in a cyclic rock-paper-scissor interaction, typical between antibiotic producers, resistance cells and sensitive cells (Narisawa et al., 2008; Czárán et al., 2002). This interaction may seem diverse and ecologically stable but their stability is always in limbo. This is because producers may choose to become selfish and evolve to make secretion that are less accessible by cheaters, leading to elimination of competitors (Morris et al., 2014). On the other hand, cheaters may evolve to produce secretion that may benefit producers, leading to cooperative behaviours (Estrela et al., 2016; Sachs and Hollowell, 2012).

Strains that are in competition may maintain aggressive phenotypes by ramping up and diversifying in an arms race (Kinkel et al., 2011). This is an evolution process rather than a result of competition, which may eventually lead to anyone of the outcomes mentioned above. However, this arms race is largely

favoured when strains are spatially differentiated (Czárán et al., 2002; Bucci et al., 2011; Biernaskie et al., 2013). Environmental conditions and the nature of the competitive phenotypes are essential to maintain the stability and diversity of this dynamic interaction. For example, phenotypes that need more resources to maintain arms race maybe more vulnerable to elimination (Chao and Levin, 1981; Borenstein et al., 2015). Soil bacteria is found to limbo between aggressive phenotype such as antimicrobial production and efficient growth mode depending on environmental conditions such as population density (Conlin et al., 2014). The delicate balance between the two states is essential as there is a trade-off once they select one mode over the other (Schlatter and Kinkel, 2015).

Lastly, there is a chance where warfare between two strains may ended up being neutralised by other community member within the population. One such example is antibiotic antagonism among a population of antibiotic producers (Abrudan et al., 2015). A theoretical model has demonstrated that this may lead to a delicate balance where different antibiotic production species cancels out each other's effect, allowing diversity to be maintained (Kelsic et al., 2015). However, this equilibrium is expected to only last for a short period as strains evolve over time in order to gain competitive advantage.

Generally, a general reduction in diversity that result in the ecological stability is the expected outcome of competition interactions. The long-term effect of competitive interaction also depends on the selection pressure from various environmental factors (Ghoul and Mitri, 2016). Multiple outcome of competition may coexist stably in different niches within the same environment (Thompson, 2005).

2.3 Cooperation

Cooperation is one of the many core aspects in bacterial social behaviours (Crespi, 2001). All cooperation interactions involve one individual acting to benefit one or more other individuals, which may or may not provide benefit toward the former. Different strains communicate and cooperate with each other to perform various activities to enhance survivability. Cooperation mediates a wide array of activities such as dispersal, foraging, construction of biofilms, reproduction, chemical warfare, and signalling. The importance of communication and cooperation is demonstrated in *Pseudomonas aeruginosa* where 6 to 10% of its gene is controlled by cell-cell signalling (Schuster et al., 2003).

Similar to competitive behaviour, cooperative behaviour also begins with the production of public goods. As mentioned before public goods is vulnerable to cheaters which is problematic for co-operators as they are metabolically costly to produce (West et al., 2006). So, why should an individual carry out cooperative behaviour that are costly for them while other benefits? This is a difficult behaviour to explain as they go against the Darwinian idea of survival of the fittest (Hamilton, 1964; Hamilton, 1963). Cheaters benefits for other co-operators and may gain a competitive edge over co-operators, which may eventually lead to invasion and elimination of co-operators. Tragedy of the common's theory dictate that cooperation maybe beneficial towards everyone but selfish nature of individual organism may push for short term benefits, rendering such cooperation the be highly unstable. Therefore, it is important to address the issue of cheaters that do not produce public goods but receive benefit from producers. Siderophores production in *Pseudomonas aeruginosa* provides a good model for such conflict (Griffin et al., 2004). Siderophores production is beneficial when iron is a limiting factor, demonstrated by

the fact that wild type strains outgrow mutant strains with defects in siderophore production. However, siderophore is metabolically costly to produce. Therefore, in an iron rich environment, mutant strains will outgrow wild type strains. It was observed that in a mixed culture, mutant strains gain benefits from siderophores produced by wild type without paying the cost, slowly increase in frequency and eventually outcompetes wild type (Griffin et al., 2004). This scenario also applies to a wide array of public goods aside from siderophores. Thus, the fundamental problem that need to be addressed is, what makes such cooperative behaviour stable in the face of cheaters that might arise via migration or mutation (West and Buckling, 2003).

It is important to distinguish whether the benefits is enjoyed by all individuals of the population (whole group trait) or all individuals except the producers (others only trait) (Pepper, 2000). These two scenarios demand a different set of explanation. Public goods production is a classic example of whole group trait as the population enjoys the benefits of the shard pool together. Another classical example is when a population lowers growth rate but increase growth yield in an attempt efficiently and economically consume common resources (Pfeiffer et al., 2001; Kreft, 2004). Others only trait describe a scenario where the co-operators sacrifice themselves in order to benefit others. Cellular slime mould *Dictyostelium discoideum* and *Myxococcus xanthus* bacteria formation of fruiting bodies is one classical example where some cells sacrifice themselves to form non-viable stalks in order help dispersal of sporulating cells (Bonner, 2015; Strassmann et al., 2000; Velicer et al., 2000). Autolysis of cells to provide nutrition, bacteriocins and genetic material to the population is also a form of others only trait (Webb et al., 2003; Riley and Gordon, 1999).

The rationale of social cooperation can be classified into two categories: direct and indirect fitness benefits. The first category provides a direct fitness benefit to the performer of cooperation where the benefits outweigh the cost of cooperation (Sachs et al., 2004). In this case all parties are mutually benefited. This usually happened when the performer has a shared interest in the cooperative behaviours, where cooperating will allow mutual benefits. For instance, in cooperative breeding species, larger community size brings larger overall benefits and allow better survival. In this case, it would be beneficial for an individual to tend to offspring aside to their own to ensure the overall community size (Kokko et al., 2001). This kind of cooperation may also be enforced via a mechanism that reward the co-operators or punish the cheaters (Trivers, 1971; Frank, 2003).

Indirect benefits happens when cooperation does not bring immediate benefits as it improving the fitness of other individual that carry the cooperative gene (Hamilton, 1964). In this case, why should an individual cell pay the price for the benefits of others? Indirect benefits commonly occur when two individuals carry a gene identical by descent, making them genealogical relatives (kin). Hence, this cooperation is termed kin selection. Genetically related individuals may help each other reproduce to pass down the gene to the next generation, providing benefit to the offspring indirectly. There are two mechanism that allows such cooperation to happen: kin discrimination and limited dispersal (Hamilton, 1964). Kin discrimination happens when cooperation favours relatives. Limited dispersal is achieved by regulating population viscosity, to keep relatives in close proximity allowing cooperation to be achieved easily. Indirect fitness can also achieve in nonrelatives that share the same cooperative gene. One can view this as a form of kin discrimination because cooperative behaviour is directed towards phenotype markers

unique to a single gene or a group of tightly related gene (Dawkins and Davis, 2017; Jansen and van Baalen, 2006).

Complication arises when cooperative behaviour can be explained by direct or indirect benefits. A whole group trait such as siderophore production can be viewed as direct or indirect benefits. In this case, the relative importance of the direct or indirect benefits dictates whether the relationship is mutualistic or altruistic. In contrast, others only traits only make sense if viewed as providing indirect fitness benefits.

2.3.1 Kin Selection

Generally, kin selection describes how relatives help each other reproduce to gain indirect fitness benefits (Smith, 1964). Kin selection can be classified into a narrow term where interactions in limited to individuals that share common ancestry and therefore genetically related. The broader term includes interaction between individuals that carry a common gene of interest, either vie ancestry or other mechanisms. The two classification is used to describe a behaviour based on whether the kinship and relatedness is established based on average genetic similarity over the genome or only at the particular location of behaviour being observed. Hamilton (1975) argues that general inclusive fitness should be distinguished from kinship effects, and thus prefers the narrower definition of kin selection. However, the broader term is preferably used by researchers nowadays as kinship is generally the sensible reason for indirect fitness benefit.

2.3.2 Mutual Benefit

Mutualism is traditionally defined as a social behaviour that have fitness benefits on both the actor and the recipient (Hamilton, 1964; LEHMANN and KELLER, 2006). The term cooperation and mutualism are sometime used interchangeably but this may cause confusions as mutualism is generally used to refer to specific interspecies cooperation (Brown, 1983; Herre et al., 1999; FOSTER and WENSELEERS, 2006). The two terms describe two different ideas. Cooperation describes a simple mutually beneficial social behaviour between an actor and recipient which generally explains direct benefits. This does not explain the possibility of indirect benefits where such interaction may bring harm in short term but benefits in long term (West et al., 2006). On the other hand, interspecific mutualism describes a bigger picture of the impact of each party on each other. While it is easy to explain how mutually beneficial interaction evolve but interspecific mutualism is a complex issue to address. Hence, the term mutual benefit is a more suitable description of a behaviour that is generally beneficial to both actor and recipient. Smith (1982) used the term mutual benefit to describe cooperation via interaction between kin or cooperating individuals that result in mutual benefits. Smith and Szathmary (1997) also used the term mutual benefit to distinguish synergistic cooperation benefits from enforced cooperation.

2.3.3 Altruism

Generally, altruism is defined as a selfless act where the actor did not gain benefit or may even pay cost to benefit recipients. Altruism should be defined based on long-term consequences and absolute fitness benefit. For example, if a cooperative behaviour is costly in short term but beneficial in the future, it should be considered mutually beneficial instead of altruistic.

Reciprocal altruism can happens between nonrelatives, where individuals take turn helping each other by preferentially helping those that have helped them in the past (Trivers, 1971). However, reciprocal altruism cannot be considered truly altruistic as it provides a direct fitness advantage to cooperating. Individuals pay the cost of cooperation now to gain benefit if the long term. Therefore, it can be considered mutually beneficial (Hamilton and Hamilton, 1996).

Altruism was later defined based on the fitness of the actor relative to the recipient in its group (Wilson, 1975; Colwell, 1981). An interaction can be described as weakly altruistic if it decreases the fitness of the actor relative to the other member of a group. Scenario such as public good production where the actor pays the cost but everyone gains fitness benefit including the actors can be considered altruistic. Hence, this is often called whole-group or group beneficial traits (Pepper, 2000; Dugatkin et al., 2003; Dugatkin et al., 2005). However, whole-group trait can be considered altruistic or mutually beneficial depending on the relative cost and benefits of the behaviour, as well as population structure (Pepper, 2000; Rousset, 2013).

There is a problem with defining altruism relative to the local group instead of the whole population. Natural selection selects for a whole population but not a arbitrarily defined group of individuals (Grafen et al., 1984). Another perspective of this problem is examining altruism relative to the group will ignore any benefits of the behaviour equally spread throughout the group. Traits that provide benefit to the whole population are therefore defined as altruistic because the benefits are ignored.