STUDY ON THE RELATIONSHIP BETWEEN SULFATE REDUCTION PATHWAYS AND DORMANCY OF *Microbulbifer aggregans* (CCB-MM1)

DIYANA BINTI TARMIZI

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by

DIYANA BINTI TARMIZI

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LIST OF SYMBOLS AND ABBREVIATIONS

%	percent
°C	degree Celcius
&	and
x g	relative centrifugal force
X	time (s)
µg	microgram
µl	microlitre
µM	micromolar
A	absorbance
ADP	adenosine diphosphate
AMP	adenosine monophosphate
ASOI	ATP synthase Operon I
ASOII	ATP synthase Operon II
ASW	artificial seawater
ATP	adenosine triphosphate
BLAST	Basic Local Alignment Search Tool
bp	base pair
$CaCl_2$	calcium chloride
CCB-MM1	*Microbulbifer aggregans*
Cm	chloramphenicol
Cm^r	chloramphenicol resistance
conc.	concentration
DAP	2,6-diaminopimelic acid

dH_2O	distilled water
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
e^-	electron
EDTA	ethylenediamine tetraacetic acid
FW	formula weight
g	gram
h	hour
H-ASWM	high nutrient artificial seawater medium
H_2S	hydrogen sulfide
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HS^-	sulfhydryl ion
k	kilo
KAc	potassium acetate
kb	kilobase
kDa	kilodalton
l	litre
LB	Luria Bertani
LM	Luria marine
M	molar
$MgSO_4$	magnesium sulfate
mg	milligram
min	minute
ml	millilitre
mM	millimolar

$NADP^+$	Nicotinamide adenine dinucleotide phosphate ion
NADPH	nicotinamide adenine dinucleotide phosphate hydrogen
NaOH	sodium hydroxide
ng	nanogram
nm	nanometre
OD	optical density
PAP	adenosine 3',5'-diphosphate
PAPS	3'-phosphoadenosine 5’-phosphosulfate
PCR	polymerase chain reaction
pH	potential hydrogen
pmf	proton motive force
PP_i	inorganic pyrophosphate
RNA	ribonucleic acid
RNA-seq	RNA-sequencing
rpm	revolutions per minute
RT	room temperature
RT-PCR	real-time polymerase chain reaction
SDS	sodium dodecyl (lauryl) sulfate
SO_3^{2-}	sulfite ion
SO_4^{2-}	sulfate ion
TAE	Tris base-acetic acid-EDTA
V	voltage
WT	wild-type

KAJIAN MENGENAI HUBUNGAN ANTARA LALUAN-LALUAN PENURUNAN SULFAT DAN KEADAAN DORMAN *Microbulbifer aggregans* (CCB-MM1)

ABSTRAK

Keadaan dorman adalah satu cara untuk bakteria terus hidup dalam keadaan-keadaan yang tidak digemari seperti kelaparan nutrien, suhu dan kekeringan yang sangat tinggi, dan kehadiran toksin dalam alam sekitar. Walaupun pengaturan genetik dan metabolik keadaan dorman untuk patogen-patogen telah difahami dengan baik, pengaturan metabolik untuk bakteria-bakteria marin memasuki keadaan dorman hampir tidak ada dikaji. *Microbulbifer aggregans* spesies CCB-MM1 yang telah diasingkan daripada sedimen muara Hutan Paya Bakau Matang, Perak, Malaysia memiliki kitaran sel rod-kokus di mana sel kokus adalah bentuk dormannya. Tambahan lagi, ia menunjukkan pengagregatan sel sebelum merubah morfologi sel ke kokus dan mempunyai keupayaan untuk mengurai polisakarida seperti kanji. Analisis penjujukan RNA (RNA-seq) CCB-MM1 mendedahkan yang *atpD* daripada Operon II ATP synthase (ASOII) bersama dengan gen "sulfite reductase" laluan penurunan sulfat bukan secara asimilasi, *asrA* telah diekspres dengan tingginya sedangkan gen "sulfite reductase" laluan penurunan sulfat secara asimilasi, *cysI* telah ditahan (daripada diekspreskan) dalam keadaan dorman. Berdasarkan maklumat tersebut, kajian ini telah dijalankan bertujuan untuk menyiasat hubungan antara laluan-laluan penurunan sulfat dan keadaan dorman CCB-MM1. Mutan-mutan gangguan *atpD*, *asrA*, dan *cysI* telah dibina dan dikulturkan di dalam empat medium yang berbeza iaitu

cairan terubah suai 0.1 % medium air laut buatan nutrient tinggi (H-ASWM) dengan $MgSO_4$ dan tanpa $MgSO_4$ dan air laut buatan (ASW) terubah suai dengan $MgSO_4$ dan tanpa $MgSO_4$. Secara amnya, keputusan-keputusan menunjukkan yang mereka memerlukan $MgSO_4$ untuk memasuki keadaan dorman dengan lebih cepat apabila nutrien-nutrien yang ada tidak mencukupi untuk menyokong keadaan vegetatif mereka. Dalam kalangan mereka, hanya mutan gangguan *cysI* menunjukkan perubahan morfologi sel yang berbeza daripada jenis liar (WT). Mutan gangguan *cysI* telah menunjukkan ketidakseragaman saiz sel rod dalam pra-kultur dan ia telah gagal memasuki keadaan dorman dalam tempoh pengeraman di dalam cairan terubah suai 0.1 % H-ASWM dengan $MgSO_4$ dan tanpa $MgSO_4$. Hal-hal ini telah mendorong kepada pengkulturan mutan ini di dalam cairan H-ASWM (medium yang kaya dengan nutrien) dengan kepekatan sistina (cysteine), satu produk akhir laluan penurunan sulfat secara asimilasi, yang berbeza (julat: 0 hingga 10 μg/ml). Meningkatkan kepekatan sistina telah membantu mutan gangguan *cysI* memulihkan tumbesarannya. Walaupun begitu, *cysI* diatur turun ketika keadaan dorman. Oleh yang demikian, pembabitan negatif laluan penurunan sulfat secara asimilasi berkemungkinan merupakan salah satu pengaturan metabolik yang bertanggungjawab untuk keadaan dorman CCB-MM1.

STUDY ON THE RELATIONSHIP BETWEEN SULFATE REDUCTION PATHWAYS AND DORMANCY OF *Microbulbifer aggregans* (CCB-MM1)

ABSTRACT

Dormancy is a way for bacteria to survive in unfavourable conditions such as nutrient starvation, very high temperature and desiccation, and toxin presence in the environment. Although genetic and metabolic regulations of dormancy for pathogens are well understood, metabolic regulation for marine bacteria entering dormancy is scarcely studied. *Microbulbifer aggregans* sp. CCB-MM1 isolated from estuarine sediment of Matang Mangrove Forest, Perak, Malaysia, possesses rod-coccus cell cycle in which coccus cell is its dormant form. Furthermore, it shows cell aggregation before changing cell morphology to coccus and has ability to degrade polysaccharide such as starch. RNA-sequencing (RNA-seq) analysis of CCB-MM1 revealed that *atpD* from ATP synthase Operon II (ASOII) together with sulfite reductase gene of dissimilative sulfate reduction pathway, *asrA* were highly expressed whereas sulfite reductase gene of assimilative sulfate reduction pathway, *cysI* was suppressed in dormant state. Based on that information, this study was conducted with the aim to investigate the relationship between sulfate reduction pathways and dormancy of CCB-MM1. The *atpD*, *asrA*, and *cysI* disruption mutants were constructed and cultured in four different media which were modified 0.1 % high nutrient artificial seawater medium (H-ASWM) broth with and without $MgSO_4$ and modified artificial seawater (ASW) with and without $MgSO_4$. Generally, results showed that they need $MgSO_4$ to enter dormancy faster when available nutrients were insufficient to support

their vegetative states. Among them, only *cysI* disruption mutant showed different cell morphological change from wild-type (WT). The *cysI* disruption mutant showed non-uniform size of rod cells in pre-culture and it failed to enter dormancy within incubation period in modified 0.1% H-ASWM broth with and without $MgSO_4$. These lead to culturing this mutant in H-ASWM broth (nutrient rich medium) with different concentrations of cysteine, a final product of assimilative sulfate reduction pathway (range: 0 to 10 μg/ml). Increase cysteine concentration helped *cysI* disruption mutant recovered its cell growth. Nevertheless, *cysI* is downregulated during dormancy. Thus, negative involvement of assimilative sulfate reduction pathway may be one of metabolic regulation pathways responsible for dormancy of CCB-MM1.

CHAPTER 1

INTRODUCTION

1.1 Background of research

Fluctuations in environment such as temperature, humidity, and nutrient availability, force the bacteria to have quick adaptations to survive. A good strategy is needed to maintain their population in the environment. Dormancy is a possible fitness trait that bacteria will adopt in those conditions. "Any rest period or reversible interruption of the phenotypic development of an organism" (Sussman & Douthit, 1973) becomes the reference in observing the occurrence of dormancy in bacteria. It is an interesting topic that captured microbiologists' attention earlier because of its involvement in human diseases such as anthrax by *Bacillus anthracis* (Coffin *et al.*, 2015; Dragon & Rennie, 1995), cholera by *Vibrio cholerae* (Almagro-Moreno *et al.*, 2015; Colwell *et al.*, 1985; Emch & Ali, 2001; Jesudason *et al.*, 2000), and tuberculosis by *Mycobacterium tuberculosis* (Gengenbacher & Kaufmann, 2012; Wayne, 1994). For environmental microbiologists, they are attracted on how the environmental bacteria perform dormancy to survive in unfavourable environment. In order to achieve successful life survival strategy, bacteria need to complete these 3 stages of dormancy which are initiation (stresses from the environment induce cells with active metabolism to form dormant cells), resting (dormant cells), and resuscitation (dormant cells revert to metabolically active cells) (Lennon & Jones, 2011). Widely acknowledged forms of dormant cells are spores and persisters (Dworkin & Shah, 2010).

Elucidating the mechanism of bacterial dormancy provides a good insight for issues such as elimination of human pathogens and maintenance of microbial

community in environment. Upon receiving signals from environment, there must be a regulation in molecular level that can manipulate metabolic pathways such as lipid, amino acids, and sulfur metabolisms of the cell accordingly. Generally, several sigma factors, global regulators, and toxin/antitoxin (TA) systems or modules responsible for the bacterial dormancy. In human pathogen, for example *M. tuberculosis*, sigma(H) upregulated expression of several sulfur metabolism genes such as *cysA1, cysT, cysW, cysM, and cysN* when the pathogen was exposed to oxidative stress (Mehra & Kaushal, 2009). This metabolism was studied for its role in maintaining pathogenicity and survival of *M. tuberculosis* in the host cell (Hatzios & Bertozzi, 2011; Mehra & Kaushal, 2009). Marine bacteria might have distinct dormant mechanisms as TA systems were not found in bacteria isolated from marine environment (Lennon & Jones, 2011).

This study focused on mangrove (a type of marine environment), in which the bacteria in estuarine sediment specifically experience inconsistent temperature and salinity due to periodically inundation by sea or fresh water, very low oxygen level (except the surface layer of sediment), and anthropogenic activities (Holguin *et al.*, 2001; Thatoi *et al.*, 2013). *Microbulbifer aggregans*, designated CCB-MM1 (subject of this study) was isolated from estuarine sediment of Matang Mangrove Forest, Perak, Malaysia, shows rod-coccus cell cycle and cell aggregation before entering stationary phase (Moh *et al.*, 2017a). The rod-coccus cell cycle is a feature of marine bacteria belonging to genus *Microbulbifer* that are recognised as polysaccharide degrading bacteria. Nishijima and co-researchers reported that coccus cells (resulted from consecutive division and probably concurrent fragmentation of cells) of *M. variabilis* and *M. epialgicus* were able to survive up to 14 months on 1/10 MA plates. Conclusion was made that the coccus cell of those species is a resting form (Nishijima *et al.*, 2009).

Therefore, Furusawa and co-researchers carried out the RNA-sequencing (RNA-seq) analysis on CCB-MM1 as the first step to clarify the molecular mechanism of its resting form. They discovered that important metabolisms of vegetative cells such as cellular respiration, Krebs cycle, and ATP production were downregulated for coccus cells of CCB-MM1 (unpublished data). Both works indicated that the coccus cells of the genus *Microbulbifer* could be characterised as dormant cells. Thus, CCB-MM1 was selected for dormancy investigation due to possible role as a polysaccharide decomposer in mangrove next to its uniqueness which is cell aggregation (Moh *et al.*, 2017a).

The dormant mechanism for marine bacteria is unclear as study on metabolic regulation for marine bacteria to enter dormancy is almost none. To understand metabolic regulation in dormancy of CCB-MM1, RNA-seq analysis on the dormant cells of CCB-MM1 was checked. Two sets of ATP synthases (Operon I and II) were discovered in the genome and genes involving ATP synthase Operon II (ASOII) were upregulated in the dormant state. Besides that, both assimilative and dissimilative sulfate reduction pathways were found in the genome. Dissimilative sulfite reductase gene, *asrA* was also simultaneously upregulated in the dormant state. Generally, it was known that the dissimilative sulfate reduction pathway requires one ATP molecule.

We hypothesised that ASOII is a source of ATP to the dissimilative sulfate reduction pathway. In turn, energy generated from the pathway is useful for entering dormancy. On the other hand, assimilative sulfate reduction pathway might be negatively involved in dormancy because of assimilative sulfite reductase gene, *cysI* was downregulated in dormant state. To confirm these hypotheses, gene disruption mutants that are related to these pathways were constructed and cell morphological changes of the mutants towards dormancy were observed.

1.2 Objectives

The main objective of this study is to investigate the relationship between sulfate reduction pathways and dormancy of CCB-MM1. To achieve the main objective, the study is divided into 2 objectives:

1. To construct gene disruption mutants involving ATP synthase Operon II (ASOII) and dissimilative and assimilative sulfate reduction pathways.

2. To investigate the effect of sulfate onto wild-type (WT) and gene disruption mutants of CCB-MM1 in entering dormancy using specific media.

CHAPTER 2

LITERATURE REVIEW

2.1 Genus *Microbulbifer*

Genus *Microbulbifer* was first proposed in 1997 with the discovery of *Microbulbifer hydrolyticus* in marine pulp mill effluent. Blebs and vesicles were noticed around the cell surface. Thus, *Microbulbifer* was derived from *micro* means small, *bulbus* means onion or bulb, and suffix *–fer* means carrying or bearing; together *Microbulbifer* means small bearer of bulbs. The description for the cells are rod, Gram-negative, strictly aerobic, and oxidase and catalase positive. *Microbulbifer hydrolyticus* also can grow with the presence of sugars, fatty acids, and amino acids but sea salt-based medium is a must for growth (Gonzalez *et al.*, 1997).

The genus is known as polysaccharide degrading bacteria. *M. mangrovi* DD-13^{T} can degrade and use agar and alginate as carbon source (Imran *et al.*, 2017), more than 10 different polysaccharides can be hydrolysed by *M. mangrovi* including agar, alginate, chitin, cellulose, laminarin, pectin, pullulan, starch, carrageenan, xylan, and *β*-glucan (Vashist *et al.*, 2013), *Microbulbifer* sp. CMC-5 is capable of degrading and using agar, alginate, xylan, carrageenan, cellulose, and chitin (Jonnadula *et al.*, 2009), and *Microbulbifer* sp.6532A can degrade *Undaria pinnatifida* thallus fragments into single cell detritus (Wakabayashi *et al.*, 2012). These results indicated that the genus is polysaccharide decomposer in marine ecosystems. Moreover, enzymes secreted by *Microbulbifer* members such as agarase (Su *et al.*, 2017), α-amylase (Lee *et al.*, 2015), and ι-carrageenase (Hatada *et al.*, 2011) were characterised by researchers. Members of genus *Microbulbifer* recorded up until this writing comprised of 24 species, as listed in Table 2.1.

Table 2.1 *Microbulbifer* sp.

Species	Isolation source	Polysaccharide (s) that got degraded	Reference
M. hydrolyticus	Marine pulp mill effluent enrichment cultures	Cellulose, chitin, gelatin, starch, and xylan	Gonzalez *et al.*, 1997
M. arenaceous	Inside of a red sandstone found at coastal area	Chitin, gelatin, and starch	Tanaka *et al.*, 2003
M. salipaludis	Salt marsh	Starch and xylan	Yoon *et al.*, 2003a
M. elongatus	Coastal area	Alginic acid, cellulose, chitin, and starch	Yoon *et al.*, 2003b
M. maritimus	Intertidal sediment	Gelatin	Yoon *et al.*, 2004
M. celer	Marine solar saltern	Not stated	Yoon *et al.*, 2007
M. halophilus	Saline soil sample	Gelatin	Tang *et al.*, 2008
M. agarilyticus & *M. thermotolerans*	*M. agarilyticus*- deep-sea bacterial mat *M. thermotolerans*- deep-sea sediment	Both strains degrade agar, chitin, gelatin, starch, and xylan	Miyazaki *et al.*, 2008
M. variabilis & *M. epialgicus*	*M. variabilis*- surfaces of green, brown and red algae, a cyanobacterium, and seagrass *M. epialgicus*- surface of a green alga	Both strains degrade gelatin and starch	Nishijima *et al.*, 2009
M. donghaiensis	Marine sediment	Gelatin and starch	Wang *et al.*, 2009
M. chitinilyticus & *M. okinawensis*	Mangrove mud	Both strains degrade chitin	Baba *et al.*, 2011
M. taiwanensis	Coastal soil	Not stated	Kämpfer *et al.*, 2012
M. marinus & *M. yueqingensis*	Marine sediment	*M. marinus*- starch *M. yueqingensis*- Not stated	Zhang *et al.*, 2012
M. gwangyangensis & *M. pacificus*	*M. gwangyangensis*- sediment of a tidal flat *M. pacificus*- a marine sponge	Both strains degrade starch	Jeong *et al.*, 2013
M. mangrovi	Water sample from mangrove	Agar, alginate, cellulose, chitin, laminarin, pectin, pullulan, starch, carrageenan, xylan, and β-glucan	Vashist *et al.*, 2013
M. rhizosphaerae	Rhizosphere of *Arthrocnemum macrostachyum*	Not stated	Camacho *et al.*, 2016
M. echini	Gut of a purple sea urchin (*Heliocidaris crassispina*)	Gelatin and starch	Lee *et al.*, 2017
M. aestuariivivens	Tidal flat sediment	Gelatin	Park *et al.*, 2017
M. aggregans	Estuarine sediment	Starch	Moh *et al.*, 2017a

Genus *Microbulbifer* also has a unique property which is rod-coccus cell cycle. The first report of the rod-coccus cell cycle in genus *Microbulbifer* was from *M. variabilis* and *M. epialgicus* (Nishijima *et al.*, 2009). Since then, the cycle was also reported possessed by newly isolated species of *Microbulbifer*. Previously, *M. elongatus* (Yoon *et al.*, 2003b) and *M. thermotolerans* (Miyazaki *et al.*, 2008) were reported to have the coccus cells but no further report of them having the rod-coccus cell cycle. The rod-coccus cell cycle is associated with growth phase; rod cells during exponential phase, coccus cells during stationary phase, coccus cells revert to rod cells when inoculating them into fresh medium, and so the cycle repeats (Nishijima *et al.*, 2009). Conclusion was made that coccus cells of *M. variabilis* and *M. epialgicus* are a resting form since they were able to resuscitate even had been on 1/10 MA plates for 14 months (Nishijima *et al.*, 2009).

2.2 *Microbulbifer aggregans* (CCB-MM1)

Microbulbifer aggregans (CCB-MM1) isolated from the estuarine sediment of Matang Mangrove Forest, Perak Darul Ridzuan, Malaysia (4.85228 N, 100.55777 E) was first described by Moh and colleagues (Moh *et al.*, 2017a). Morphology of CCB-MM1 strain is rod cells and colonies formed on MA are white, circular, and raised with entire edges. The cells are negative Gram stained, strictly aerobic, halophilic, and non-motile. The bacterium exhibits the rod-coccus cell cycle in association with growth phase, identical to the finding frodim *M. variabilis* and *M. epialgicus* (Nishijima *et al.*, 2009). Interestingly, CCB-MM1 performed cell aggregation also when it is ready to enter stationary phase. Length of the rod cells is 1.3-2.5 μm and

width is 0.3 μm while diameter of the coccus cells is 0.6 μm. It is catalase and oxidase positive and has starch degradation ability (Moh *et al.*, 2017a).

Growth requirements are as followed, temperature range is 15-45 ºC with optimum at 30 ºC, sodium chloride (NaCl) concentration range (salinity range) is 0.5-8 % (w/v) with optimum at 2-3% (w/v), and pH range is 6.0-9.0 with optimum at pH 7.0. Major cellular fatty acids of CCB-MM1 strain are iso-$C_{17:1}$ ω9c (31.45% (31.45%)) and iso-$C_{15:0}$ (21.36%) and total polar lipid profile showed the presence of phosphatidylglycerol, phosphatidylethanolamine, phosphoaminolipid, two unidentified lipids, an unidentified glycolipid, an unidentified aminolipid, and an unidentified phospholipid. Ubiquinone Q-8 is the important respiratory quinone for CCB-MM1 (>67%) (Moh *et al.*, 2017a).

Based on API ZYM test, the strain showed activities of alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, and N-acetyl-b-glucosaminidase. Biolog profile presented that it can oxidise dextrin, D-mannose, D-galactose, 3-methyl glucose, L-rhamnose, L-galactonic acid lactone, glucuronamide, and α-keto-glutaric acid. CCB-MM1 strain also resistant to kanamycin and streptomycin (Moh *et al.*, 2017a). Genome properties of CCB-MM1 are as followed. It possesses one circular chromosome and no plasmid. The chromosomal size is 3,864,326 bp and contains 58.85% of G+C content. The complete genome reveals 3313 ORFs (2030 of them can be categorised to functional prediction and 2563 of them can be categorised to COG functional groups), 79 tRNA, 12 rRNA and 1 tmRNA genes. It also consists of 71 genes encode for carbohydrate-active enzymes (Moh *et al.*, 2017b).

2.3 Bacterial dormancy

Dormancy was defined as "any rest period or reversible interruption of the phenotypic development of an organism" (Sussman & Douthit, 1973). It is a reversible state of survival in which metabolism of bacteria cells become inactive in response to stresses from undesired environmental conditions such as lacking of nutrients, very high temperature and desiccation, and presence of toxins (Dworkin & Shah, 2010; Guppy & Withers, 1999; van Vliet, 2015). Bacteria must complete these three stages for a successful life survival strategy. They are initiation (cells with active metabolism are induced by stresses from the environment to form dormant cells), resting (cells are in dormant state), and resuscitation (dormant cells return to metabolically active cells (Lennon & Jones, 2011). In dormancy, energy is required for maintenance and survival but not as high as for active vegetative cells. Motility, macromolecules turnover, osmotic pressure and intracellular pH regulation, and keeping energized membrane for ATP synthesis are among non-growth functions supported by maintenance energy (van Bodegom, 2007) while survival energy is needed for repairing macromolecules experiencing damage. Survival energy is very important to ensure the viability of dormant cells so that resuscitation of them could be successful (Johnson *et al.*, 2007; Price & Sowers, 2004). Here, two acknowledged forms of dormancy in bacteria are discussed such as the spores and persisters.

Sporulation occurs in *Bacillus* and *Clostridium* species (Setlow, 2007). Generally, sporulation is induced by nutrient starvation. Deprivation of nutrients in the environment such as carbon, nitrogen, or phosphorus stimulated spores formation of *Bacillus subtilis* (Piggot & Hilbert, 2004). Phosphorylation of the Spo0A protein (an activator and also a repressor of gene expression) initiates the process. A large mother cell and a smaller forespore are produced as a result of unequal cell division. Itbegins

with growth of mother cell's plasma membrane circling the forespore that leads to engulfment, and then the latter is being enveloped by two apposed membranes. Then, a thick peptidoglycan cortex between the outer and inner forespore membranes are synthesised followed by forespore protoplast modification in term of a large volume reduction and water content and a drop in forespore pH. The water content of forespore protoplast is further reduced when the forespore takes up a large amount of pyridine-2,6-dicarboxylic acid [dipicolinic acid (DPA)] that comes from mother cell. Coat proteins synthesised in the mother cell also form a complex proteinaceous coat on the outer surface of the spore to complete the spore formation. Exosporium, a large external balloon-like layer is added in some species. Finally, the lysis of mother cell freeing the spores into the environment (Setlow, 2007). Four sigma factors are important in sporulation; σ^F and σ^G in forespore and σ^E and σ^K in mother cell (Saujet *et al.*, 2013). Spore morphogenesis in *Clostridium acetobutylicum* and *Clostridium perfringens* was studied to figure out the importance of having sporulation sigma factors. The *sigF* and *sigG mutants* of both Clostridia and *sigE* mutant of *C. acetobutylicum* could not have resistant spores while sporulation was severely defective in *sigE* and *sigK* mutants of *C. perfringens* (Harry *et al.*, 2009; Jones *et al.*, 2011; Li & McClane, 2010; Tracy *et al.*, 2011). Sporulation involves TA system also. *SpoIISA-SpoIISB* that is involved in sporulation of *B. subtilis*, is synthesised in the mother cell. In *C. difficile*, a different TA system which is *mazF-mazE* operon expression is controlled by σ^E (Rothenbacher *et al.*, 2012).

Figure 2.1 shows structure of dormant spore of *Bacillus* species. The outmost layer is exosporium, a balloon-like structure in which made up of proteins and carbohydrate, available on some species spores only such as *Bacillus anthracis*. Its function is still unknown. More than 40 different proteins, almost all being spore-

specific, made up the proteinaceous coat layer (Kim *et al.*, 2006). The coat is useful to keep the spores safe from predatory eukaryotic microbes and reactive chemicals (Klobutcher *et al.*, 2006; Nicholson *et al.*, 2000; Setlow, 2006)**.** The underlying outer membrane is needed in formation of spore but in mature spores, it cannot be guarantee to be a permeability barrier. Peptidoglycan (same structure with the peptidoglycan in growing cells) is the main component of cortex. But, the peptidoglycan of spore cortex showed two modifications which are muramic acid-δ-lactam (MAL) and muramic acid linked only to alanine. Peptidoglycan also becomes the main component for germ cell wall, the outgrowing spore's cell wall. Both the cortex and germ cell wall are important for preservation of spore inner membrane's integrity. The low permeability of inner membrane to small molecules is the key to protect the cell especially the DNA from damaging chemicals (Cortezzo & Setlow, 2005; Nicholson *et al.*, 2005; Setlow, 2006; Westphal *et al.*, 2003). The innermost layer is the core in which the spore DNA, RNA and most enzymes settle in. Spore resistance is contributed by the low core water content (25–50% of wet weight relying on the species), the high amount of Ca-DPA (25% of core dry weight) and the α/β-type small, acid-soluble spore proteins for DNA saturation (Driks, 2002; Loshon *et al.*, 1999; Nicholson *et al.*, 2005; Setlow, 1995; Setlow, 2006; Setlow et *al.*, 2006).

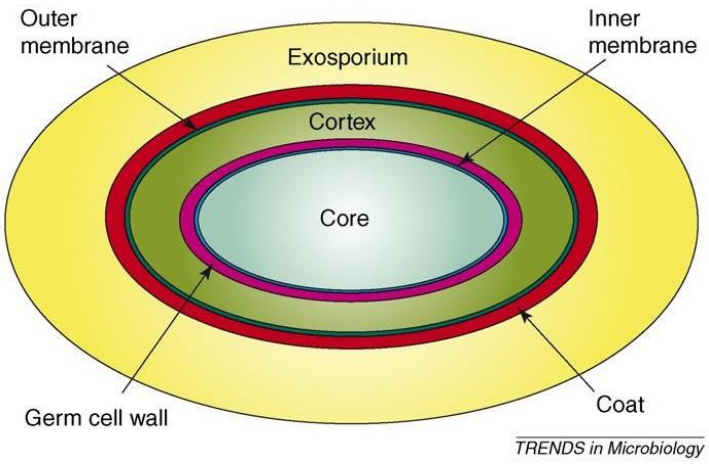

Figure 2.1 Structure of spore of *Bacillus* species

Note. Reprinted from "I will survive: DNA protection in bacterial spores", by P. Setlow, 2007, *TRENDS in Microbiology*, 15(4), p. 173. Copyright 2007 by Elsevier Ltd.

Minimal spore DNA damage is important so that survival of the spores can be up to hundred years and maybe longer. Thus, two mechanisms, protection and repair are there for the spore DNA. Throughout dormancy, the former option is suitable than the latter to avoid possible mutagenesis. DNA repair can be done after spore germinated and outgrowth of spore starts. Ca-DPA contributes to resistance of spore DNA towards wet and dry heat, desiccation and hydrogen peroxide, but not to UV radiation (Douki *et al.*, 2005; Setlow *et al.*, 2006). Spore DNA gets the most protection from α/β-type SASP. Spores of *Bacillus* and *Clostridium* species and their close relatives contain high level of those proteins, around 5-10% of total core protein while spores of *B. megaterium* and *B. subtilis* (and possibly other species) contain enough α/β-type SASP for spore DNA saturation (Driks, 2002; Nicholson *et al.*, 2005; Setlow, 1994; Setlow, 1995; Setlow, 2006). Lacking of both α/β-type SASP and Ca-DPA lead to DNA damage consequently viability loss during sporulation (Setlow, 2006).

Persister is a fraction of cells population that survives killing by antibiotics. Examples are *Escherichia coli*, *Pseudomonas aeruginosa*, *Mycobacterium tuberculosis*, *Salmonella enterica* subsp. *enterica* serovar Typhimurium and *Staphylococcus aureus* (Harms *et al.*, 2016; Helaine & Kugelberg, 2014; Michiels, *et al.*, 2016). This tolerance is due to inhibition of processes that promote growth such as synthesis of cell wall or translation that always be the target of antibiotics; not because of specific mutation that form antibiotic resistant cells (Dworkin & Shah, 2010; Fisher *et al.*, 2017). In *E. coli*, TA system also involved for its persistence such as RelB–RelE, DinJ–YafQ, MazF–MazE and HipA–HipB (Lennon & Jones, 2011). Global regulators such as DksA, DnaKJ, HupAB, and IhfAB are also involved in persistence of cells (Hansen *et al.*, 2008). The human pathogen, *M. tuberculosis* can be in dormant state for more than 40 years in order to maintain the pathogenesis (Barry *et al.*, 2009; Downing *et al.*, 2005; Kana *et al.*, 2008). It was reported that it has more than 80 TA systems (Ramage *et al.*, 2009) and sulfur metabolism contributes to the persistence in the host cell where induction of sigma(H) controls transcription of several genes related to sulfur metabolism such as *cysA1*, *cysT*, *cysW*, *cysM*, and *cysN* (Mehra & Kaushal, 2009). Biphasic killing curve, generated from exposure of bacterial culture in log phase to a lethal dose of antibiotics showed majority of cells dead during the first phase of killing and after second phase, the viable and culturable cells left are termed as persisters which is not more than 1% of the original population. Higher numbers of persisters are actually harvested from stationary phase culture (Ayrapetyan *et al.*, 2018). Other than exposure to antibiotics and stationary phase, formation of persisters can be from nutrient limitation, transition of carbon source, sub-optimal pH, oxidative stress, macrophages, indole, and damage to DNA (Amato *et al.*, 2013;

Bernier and Surette, 2013; Dörr *et al.*, 2010; Helaine & Kugelberg, 2014; Vega *et al.*, 2012; Wu, *et al.*, 2012).

Several studies has been done in searching for key initiators for bacteria to exit dormancy. Those are nutrients presence (Dworkin & Shah, 2010), stochastic germination (Epstein, 2009), and cell wall muropeptides (Shah *et al.*, 2008). Observing nutrients availability is a strategy for dormant cells to reinitiate metabolism. Presence of nutrients always supports the regrowth of bacteria but it is useless if there is other threat that can kill vegetative cells such as presence of antimicrobial in high concentration (Dworkin & Shah, 2010). On the other hand, stochastic germination is when random individual cells 'wake up' to exit dormancy without sensing the environment first (Epstein, 2009). The *B. subtilis*, *E. coli*, and *M. smegmatis* exit the dormancy using this strategy (Balaban *et al.*, 2004; Buerger *et al.*, 2012; Sturm & Dworkin, 2015). The fate of the germinated cells will then depend on the environment; if good for regrowth, they survive and begin a new population but if the conditions are still bad for them, they die (Epstein, 2009; van Vliet, 2015). Then, a deduction was made, there is possibility that the growing bacteria after stochastic germination will stimulate the neighbouring dormant cells to germinate. If this is true, searching for germination inducer that will be utilised by the growing bacteria is needed (Epstein, 2009). A study of *B. subtilis* spores revealed that peptidoglycan-derived muropeptides were the inducer of germination (Shah *et al.*, 2008). Peptidoglycan or murein, a component of bacterial cell wall is composed of repetition of *N*-acetylmuramic acid and (MurNAc)-*N*-acetylglucosamine (GlcNAc) subunits, which are cross-linked by either short peptide bridges (L-Ala-D-Glu-meso-DAP-D-Ala) for Gram-negative bacteria or short peptide bridges (L-Ala-D-Gln-L-Lys-D-Ala) for Gram-positive bacteria (Dworkin & Shah, 2010). Polymer of peptidoglycan that has been digested by

enzymes produced peptidoglycan fragments that are known as muropeptides (Boudreau *et al.*, 2012). The released muropeptides by growing bacteria to the environment cue other dormant cells that growth-permissive conditions are present (Shah *et al.*, 2008).

2.4 Sulfate reduction pathways

Sulfur cycle that proceeds by oxidation-reduction reactions produced a constant flux of oxidised and reduced states of sulfur compounds. It is one of the biogeochemical cycles, the transformations of an element that are catalysed by either biological or chemical agents (or both) (Leustek, 2002; Madigan *et al.*, 2012).

Sulfate (SO_4^{2-}) with the +6 valence state is the most oxidation form of sulfur exists in the aerobic atmosphere of the Earth. It is also an important anion in seawater. Other oxidation states of sulfur like sulfite (SO_3^{2-}; +4), elemental sulfur (S^0; 0), and hydrogen sulphide (H_2S; -2) can be found in anaerobic or volcanic environment, and within living cells (Canfield *et al.*, 2005; Leustek, 2002; Madigan *et al.*, 2012). Most of organisms chose SO_4^{2-} as sulfur source and it is ranked second after phosphate for soluble oxyanion abundance in the bacterial cell (Silver & Walderhaug, 1992). Sulfur is present in cysteine, methionine, and cellular cofactors such as biotin, coenzyme A, S-adenosylmethionine, thiamine, glutathione, lipoic acid, and iron-sulfur clusters (Scott *et al.*, 2007). As part of protein, it also involves in structure, regulation, and catalysis of the protein and as a component in tripeptide glutathione and certain proteins such as thioredoxin, glutaredoxin, and protein disulfide isomerase, it can act as a main buffer for cellular redox (Leustek, 2002).

Sulfur metabolism is one of cell metabolisms being studied in successful human pathogen *M. tuberculosis*. Its persistence in conditions of phagosomal environment such as oxidative stress and nutrient limitation is indebted to sulfate assimilation pathway producing reduced sulfur metabolites (Hatzios & Bertozzi, 2011). This lead to interest of investigating sulfate reduction pathways in dormancy of CCB-MM1.

There are two pathways for reducing sulfate which are assimilative sulfate reduction and dissimilative sulfate reduction. Assimilative sulfate reduction pathway plays role in producing organic sulfur compounds like cysteine, methionine, etc. that are needed by plants, fungi, yeasts, and bacteria (Madigan *et al.*, 2012). In contrast, dissimilative sulfate reduction pathway that used to be limited to sulfate-reducing bacteria and archaea is for anaerobic respiration (Goldhaber, 2003). Sulfates, sulfonates, and sulfate esters are strong acids that prefer to be in ions state at physiological pH. Thus, an active transport system is needed as passive diffusion is not an option to transport them into the cell. Based on a review done by Aguilar-Barajas *et al.*, 2011, sulfate permeases from different families as shown in Table 2.2 are the responsible transporters to take up the sulfate into the bacterial cell. Sulfate is an oxyanion (an anion containing oxygen) that structurally related to molybdate, tungstate, selenate and chromate. Thus, it can also be taken up into the bacterial cell by the ModABC molybdate transport system (Aguilar-Barajas *et al.*, 2011). Once the sulfate is inside the cell, there will be reduction of sulfate depending on the need of the cell, either for energy production using dissimilative sulfate reduction pathway or cysteine synthesis using assimilative sulfate reduction pathway (Madigan *et. al*, 2012). Sulfur metabolism is as shown in Figure 2.2.

Table 2.2 Sulfate transporters for bacteria

Transporter	Family	TC number[a]	Organism(s)	References
Sulfate-thiosulfate permease (CysPTWA)	Sulfate/tungstate uptake transporter SulT	3.A.1.6	*Salmonella typhimurium*	Ohta *et al.*, (1971)
			Escherichia coli	Sirko *et al.*, (1990)
			Mycobacterium tuberculosis	Wooff *et al.*, (2002)
			Synechococcus elongatus	Laudenbach and Grossman (1991)
SulP	Sulfate permease SulP	2.A.53	*Burkholderia cenocepacia*	Farmer and Thomas (2004)
			Acidithiobacillus ferrooxidans	Valdés *et al.*, (2003)
			Mycobacterium tuberculosis	Zolotarev *et al.*, (2008)
CysP/(PiT)	Inorganic phosphate transporter PiT	2.A.20	*Bacillus subtilis*	Mansilla and de Mendoza (2000)
CysZ	Putative sulfate transporter	9.B.7	*Escherichia coli*	Parra *et al.*, (1983)
CysZ	Putative 4-toluene sulfonate uptake permease (TSUP)	9.A.29	*Corynebacterium glutamicum*	Rückert *et al.*, (2005)

[a] According to the Transport Classification Database (TCDB)

Note. Reprinted from "Bacterial transport of sulfate, molybdate, and related oxyanions", by E. Aguilar-Barajas *et al.*, 2011, *Biometals*, 24(4), p. 689. Copyright 2011 by Springer Science+Business Media, LLC.

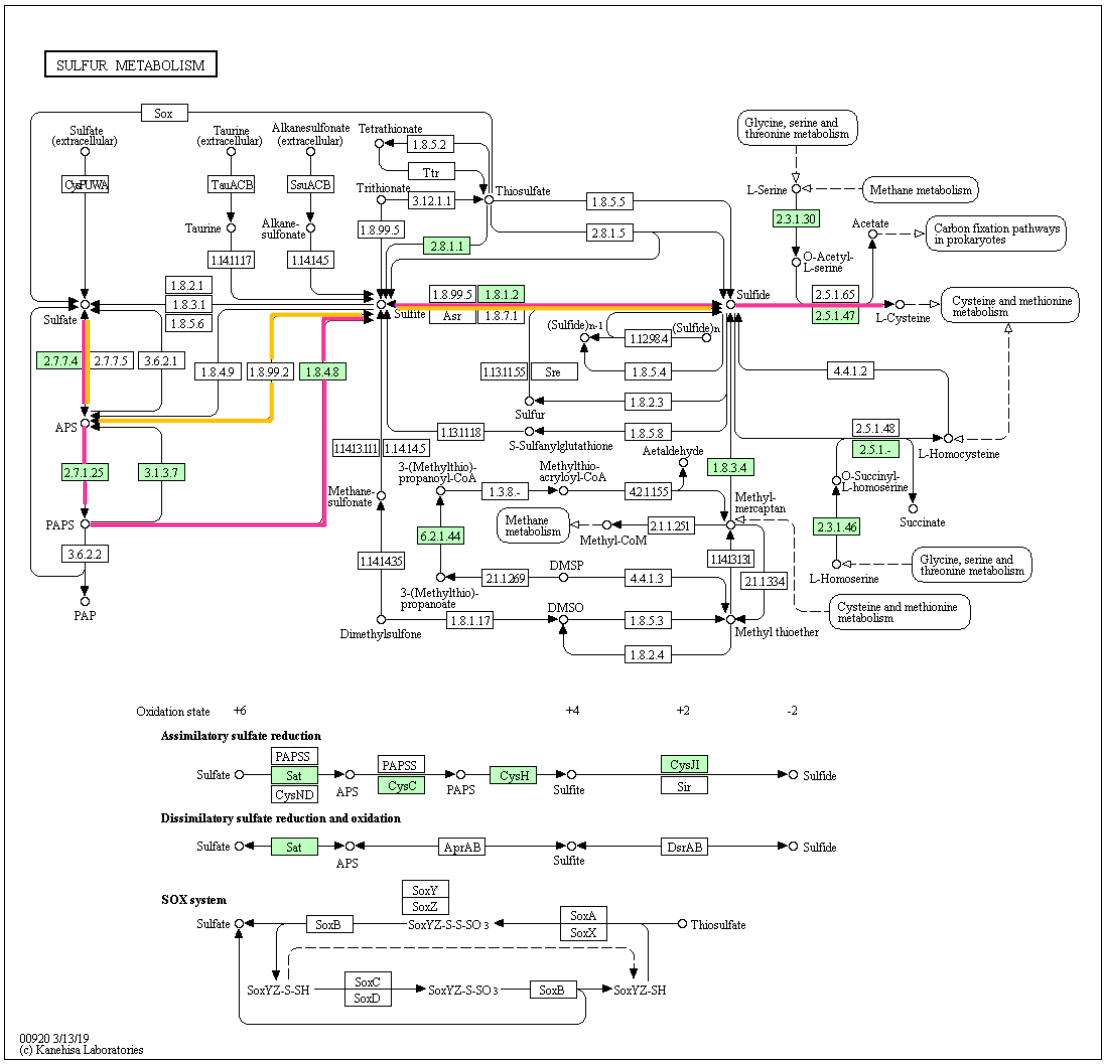

Figure 2.2 Sulfur metabolism of *Microbulbifer aggregans* (CCB-MM1). The highlighted enzymes mean they are present in CCB-MM1. Pink line indicated assimilative sulfate reduction pathway whereas orange line indicated dissimilative sulfate reduction pathway

Note. Reprinted from "Sulfur metabolism - Microbulbifer aggregans", by Kanehisa Laboratories, (2019, 13, 3). Retrieved from https://www.genome.jp/kegg-bin/show_pathway?micc00920

Before sulfate can be reduced, it needs to be activated first since it is metabolically inert. The activation requires ATP so the ATP sulfurylase (EC 2.7.7.4) catalyses the activation. The bond between alpha and beta phosphates of ATP is hydrolysed by the enzyme, continue with sulfate addition to the alpha phosphate in which producing adenosine 5'-phosphosulfate (APS) (Figure 2.3) (Leustek, 2002;

Madigan *et al.*, 2012). The phosphoric acid-sulfuric acid anhydride bond of the APS stores energy that permits the sulfate to undergo either two pathways, assimilative or dissimilative sulfate reduction (Leustek, 2002).

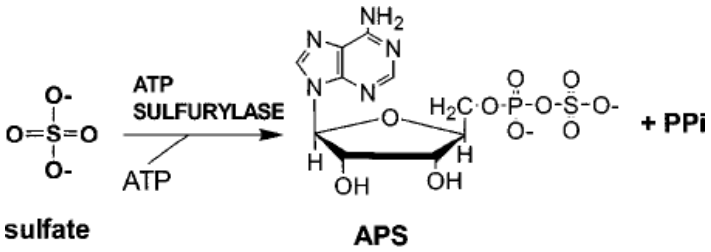

Figure 2.3 Sulfate activation before reduction takes place in which APS is formed

Note. Reprinted from "Sulfate metabolism", by T. Leustek, 2002, *The arabidopsis book*, 1, e0017, p. 6. Copyright 2002 by American Society of Plant Biologists.

In assimilative sulfate reduction pathway, reduction of sulfate utilises 8 electrons to form sulfide (Leustek, 2002). APS is the substrate for APS kinase (CysC; EC 2.7.1.25) in which phosphorylation of 3'OH position of APS occurred and 3'-phosphoadenosine 5'-phosphosulfate (PAPS) is produced. An ATP molecule is utilised in this reaction. Next, reduction of PAPS into sulfite (SO^{2-}_3) yielding a by-product of adenosine 3',5'-diphosphate (PAP). Then, NADPH-sulfite reductase (EC 1.8.1.2), encoded by operon cysJIH reduces sulfite ion to sulfhydryl ion, (HS-). Two subunits α and β make up the enzyme. *cysJ* encodes subunit α involves FAD whereas *cysI* encodes subunit β involves an iron-sulfur centre and a siroheme prosthetic group (analogous to siroheme-dependent nitrite reductases). Cystein is produced when sulfide reacts with O-acetylserine (OAS) with OAS thiol-lyase (EC 4.2.99.8) as a catalyst: O-acetylserine (OAS) + S^{2-} → L-cysteine + acetate. Acetylation of serine with acetylCoA catalysed by serine acetyltransferase (EC 2.3.1.30) formed OAS: serine + acetylCoA → OAS + CoA (Leustek, 2002). Cysteine is important forseveral

occasions such as a precursor of methionine, biotin, coenzyme A and coenzyme M, thiamine, lipoic acid, involvement in the biogenesis of [Fe – S] clusters, present in the several enzymes' catalytic site, helps folding and assembly of protein with disulfide bonds formation, makes up protein such as thioredoxin or glutathione that mainly shield cells from oxidative stress, as nutritional supplement, and as a pharmaceutical (antidote) or drugs' precursor (Guédon & Martin-Verstraete, 2006).

In dissimilative sulfate reduction pathway, APS reductase (EC 1.8.4.9) reduced SO_4^{2-} in APS directly to sulfite (SO_3^{2-}) in which used 2 electrons and released AMP. Then, SO_3^{2-} is reduced to hydrogen sulphide (H_2S) by sulfite reductase (EC 1.8.7.1) that consumed 6 electrons. The H_2S produced is excreted out of cell. High concentration of sulfhydryl ion (HS^-) produced is quite reactive and toxic to the cell (Sekowska *et al.*, 2000). The purpose of this reaction is to generate energy. Sulfate acts as an electron acceptor. Electron transport reactions caused a proton motive force (pmf) in which encourage ATPase to synthesise ATP. While reduction reactions take place in the cytoplasm, the electron transport chain for dissimilatory sulfate reduction takes place in periplasmic proteins (Goldhaber, 2003). Molecular hydrogen, contributed either from the external environment or by the organic compounds' oxidation such as lactate is required by hydrogenase. Cytochrome c3 is the main electron carrier. Eight electrons are accepted by cytochrome c3 from a hydrogenase that is located in periplasm and are transferred to a second cytochrome complex (membrane-associated protein complex) known as Hmc. The Hmc responsibles to transport the electrons across the membrane of cytoplasm and to cater them to APS and sulfite reductase that produces sulfite and sulfide, respectively (Goldhaber, 2003; Madigan *et al.*, 2012). Figure 2.4 shows summarisation of both assimilative and dissimilative reduction pathways.

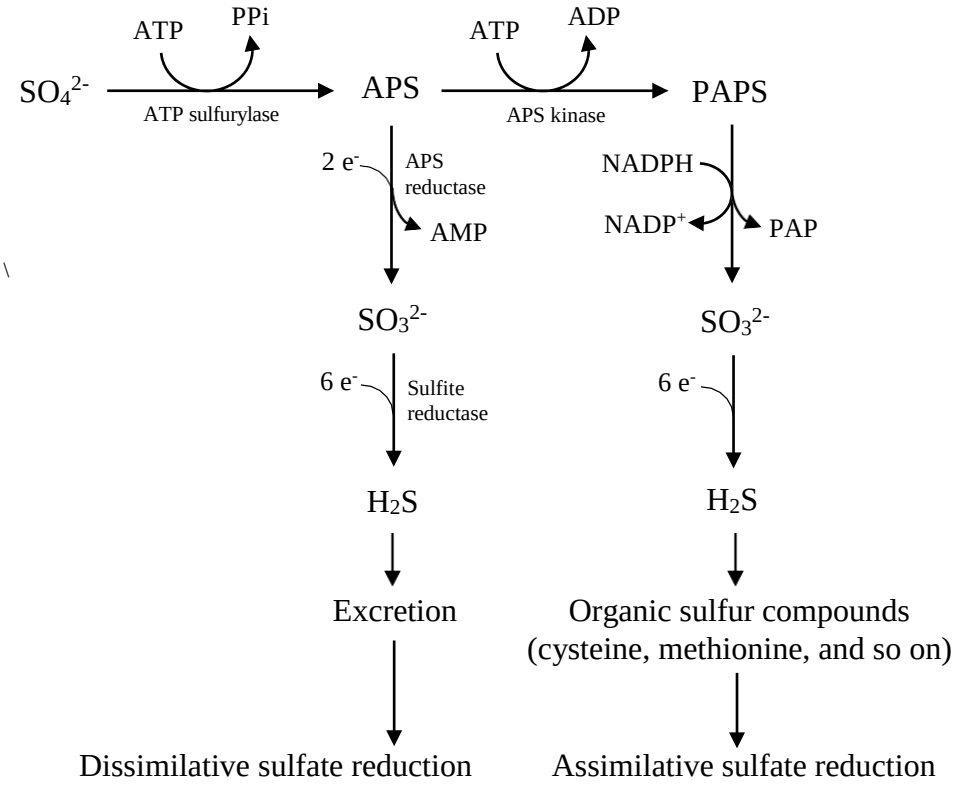

Figure 2.4 Schemes of dissimilative and assimilative sulfate reduction

Note. Reprinted from "*Brock Biology of Microorganisms*" (p. 415), by M. T. Madigan *et al.*, 2012, Pearson. Copyright 2012 by Pearson Benjamin Cummings.

2.5 ATP synthase

ATP synthase is a large protein complex (~500 kDa) that is also known as F0F1-ATP synthase, F0F1-ATPase or ATPase for short (Madigan *et al.*, 2012; Yoshida *et al.*, 2001). It is located in the cristae and the inner membrane of mitochondria, the thylakoid membrane of chloroplasts, and the bacterial plasma membrane. Although the location is different, the structure and procedure of ATP synthesis is same except that transmembrane movement of H^+ ions occur in chloroplasts due to excited electrons by light energy (Devenish *et al.*, 2008). Here, the discussion is focusing on the bacterial ATP synthase. ATP synthase catalyses cellular ATP production from

ADP and inorganic phosphate (Pi) and the reaction is reversible. ATP is the major energy currency for the biological processes. ATP synthesis is driven by pmf, the force that is generated due to protons (H^+) movement downhill a gradient of electrochemical potential across membrane ($\Delta\mu_{H+}$) (Madigan *et al.*, 2012; Yoshida *et al.*, 2001).

Two components build up the ATPase which are F1 (Fraction 1), a multiprotein cytoplasmic complex that executes the chemical function (ATP synthes~~is~~) and F0 (read as 'ef oh'), a membrane-integrated component that executes ion-translocating function (Figure 2.5). Both F1 and F0 are rotary motors in which F1 is an ATP-driven motor while F0 is a proton-driven motor. Five types of polypeptides which are α_3, β_3, γ, ε, and δ made up the F1 (Devenish *et al.*, 2008; Madigan *et al.*, 2012; Yoshida *et al.*, 2001). A cylinder of $(\alpha\beta)_3$ resulted from alternate arrangement of three α-subunits and three β-subunits around the coiled-coil structure of the γ-subunit. The side of γ subunit attached to the ε subunit and together they attached to the F0. F0 is made up of a_1, b_2 and $c_{10\text{-}14}$ subunits (Devenish *et al.*, 2008). F0 c-subunits that are arranged like a ring is a rotor (Yoshida *et al.*, 2001). The central portion ($F1\gamma\varepsilon$–$F0c_{10–14}$) termed as rotor, rotates in proportion to the surrounding portion ($F1\alpha_3\beta_3\delta$–$F0ab_2$), the stator. Large magnitude of $\Delta\mu_{H+}$ causes downhill proton flow through F0 and rotates the F0 rotor in which consequently rotates the γε-subunits of F1. ATP synthesis occurs as the result of rotary motion of the γ that alternates the β-subunit structure **(**Yoshida *et al.*, 2001**).**

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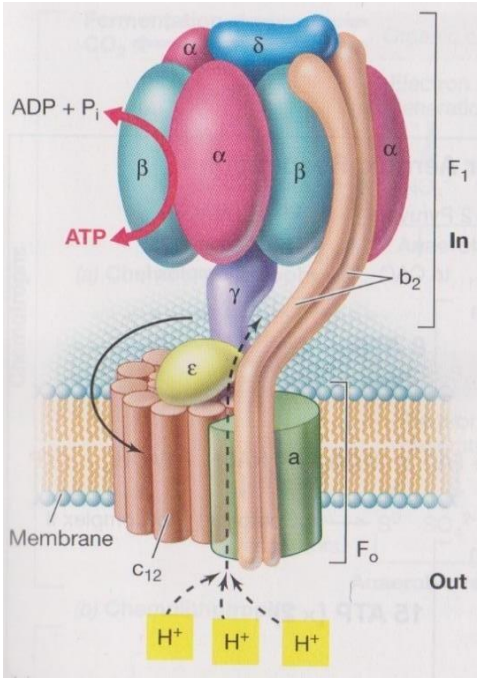

Figure 2.5 Structure of ATP synthase. F1 comprised of α_3, β_3, γ, ε, and δ while F0 comprised of a_1, b_2, and $c_{10\text{-}14}$ subunits

Note. Reprinted from "*Brock Biology of Microorganisms*" (p. 133), by M. T. Madigan *et al.*, 2012, Pearson. Copyright 2012 by Pearson Benjamin Cummings.

CHAPTER 3

MATERIALS AND METHODS

3.1 Overview of methodology

In this chapter, details of the materials and methods used to carry out all experiments related to this study are described. Figure 3.1 shows the overview of methodology.

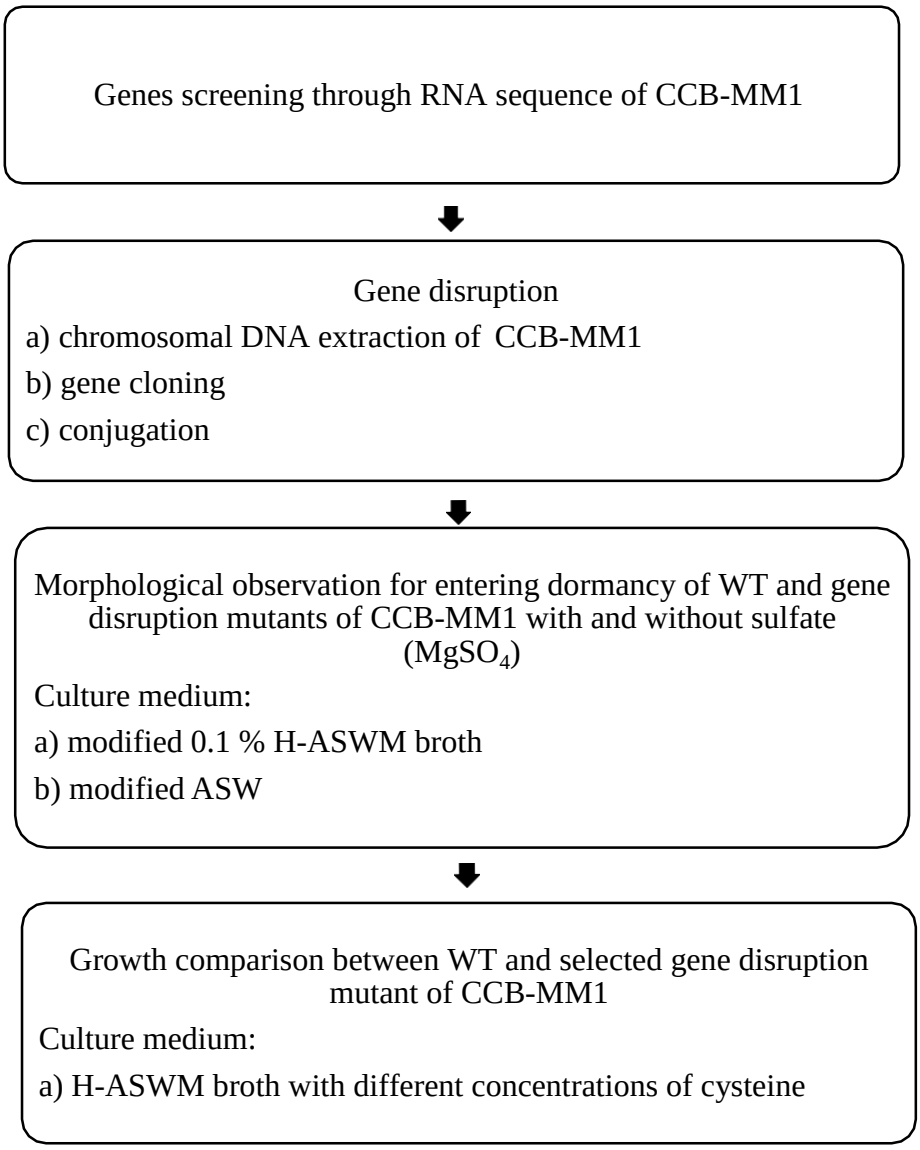

Figure 3.1 Overview of methodology

3.2 Materials

3.2.1 Equipment and apparatuses

Equipment and apparatuses used throughout this study are listed in Table 3.1

Table 3.1 List of equipment and apparatuses

Equipment or apparatus	Brand
AccuBlock™ Digital Dry Bath	Labnet International
Beaker (1000 ml)	Schott-DURAN
Laboratory glass bottle with PP screw cap	
Centrifuge 5810 R	Eppendorf
Centrifuge 5417 R	
Eppendorf™ Innova™ 44R Incubator Shaker	
Eppendorf Research® Plus (pipettes)	
Thermomixer comfort	
Erlenmeyer flasks, narrow-neck (100 and 250ml)	Pyrex
Flake ice maker	Hoshizaki
Gel Doc XR + Gel Documentation System	Bio Rad, USA
Mini-Sub Cell GT Horizontal Electrophoresis System and PowerPac Basic Power Supply	
Incubator	Memmert & Binder
JB1603-L-C Caratbalance	Mettler Toledo
JL1502-G Goldbalance	
S20 SevenEasy™ pH	
Laminar Flow Cabinet AHC 4DI	Esco
Measuring cylinders (100, 500, & 1000 ml)	Vitlab
Nanodrop 2000	Thermo Scientific
Revco™ General-Purpose Refrigerators	
Revco® Value Plus Ultra Low Temp Freezer	
Olympus BX51 Upright Fluorescence Microscope	Olympus
UV-1800 Shimadzu Spectrophotometer	Shimadzu
Veriti™ 96-Well Thermal Cycler	Applied Biosystems
Vertical Autoclave Hi-Clave HV-110	Hirayama
Vortex Mixer SA8	Stuart